

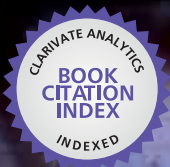


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# Abiotic Stress in Plants

## Mechanisms and Adaptations

*Edited by Arun Shanker and B. Venkateswarlu*



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# **ABIOTIC STRESS IN PLANTS – MECHANISMS AND ADAPTATIONS**

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Edited by **Arun Kumar Shanker** and  
**B. Venkateswarlu**

## Abiotic Stress in Plants - Mechanisms and Adaptations

<http://dx.doi.org/10.5772/895>

Edited by Arun Shanker and B. Venkateswarlu

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First published in Croatia, 2011 by INTECH d.o.o.

eBook (PDF) Published by IN TECH d.o.o.

Place and year of publication of eBook (PDF): Rijeka, 2019. IntechOpen is the global imprint of IN TECH d.o.o.

Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from [orders@intechopen.com](mailto:orders@intechopen.com)

Abiotic Stress in Plants - Mechanisms and Adaptations

Edited by Arun Shanker and B. Venkateswarlu

p. cm.

ISBN 978-953-307-394-1

eBook (PDF) ISBN 978-953-51-4431-1

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# Meet the editors



Arun K. Shanker is a Senior Scientist (Plant Physiology) in the Indian Council of Agricultural Research (ICAR) at the Central Research Institute for Dryland Agriculture in Hyderabad, India. He earned his Bachelor's and Master's degrees in Agriculture and Crop Physiology at the Tamil Nadu Agricultural University, Coimbatore. He is working with the ICAR as a full time researcher since 1993 and has earned his Advanced degree in Crop Physiology while in service. His main area of research has been Cr-plant interactions and various other abiotic stresses in crops and plants. Presently he is embarking on systems biology approach to study the mechanism of abiotic stress tolerance in crops involving in silico approaches to gene finding and data mining to identify and validate candidate genes for multiple abiotic stress tolerance in the family Poacea.



Dr. Venkateswarlu holds a Doctorate in Soil microbiology and is currently the Director of Central Research Institute for Dryland Agriculture (CRIDA), Hyderabad, India. He is leading the national research programs of the Indian Council of Agricultural Research (ICAR) on rainfed agriculture and climate change. His research experience is mainly on quantifying the impacts of abiotic stresses of biological nitrogen fixation (BNF) in legumes and understanding plant-microbe interactions in arid and semi arid ecosystems. Dr. Venkateswarlu published over 150 research papers on abiotic stress management and crop nutrition in drylands and authored six books. He has participated in several academic visits to US, UK, Canada, Australia and China and received many national awards for his work on rainfed farming and abiotic stress management. He is a member of the National Academy of Agricultural Sciences of India and serving on key committees set up by the Government in the areas of drought management, rainfed farming and climate change.





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## Preface

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World population is growing at an alarming rate and is anticipated to reach about six billion by the end of the year 2050. On the other hand, agricultural productivity is not increasing at a required rate to keep up with the food demand. The reasons for this are water shortages, depleting soil fertility and mainly various abiotic stresses. Therefore, minimizing these losses is a major area of concern for all nations to cope with the increasing food requirements. Stress is defined as any environmental variable, which can induce a potentially injurious strain in plants. The concept of optimal growth conditions is a fundamental principle in biology. Since living organisms cannot control environmental conditions, they have evolved two major strategies for surviving adverse environmental conditions i.e. stress avoidance or stress tolerance. The avoidance mechanism is most obvious in warm blooded animals that simply move away from the region of stressful stimuli. Plants lack this response mechanism, which is mobility; hence they have evolved intricate biochemical, molecular and genetic mechanisms to avoid stress. For example, they alter life cycle in such a way that a stress sensitive growth period is before or after the advent of the stressful environmental condition. On the other hand, tolerance mechanisms mainly involve biochemical and metabolic means which are in turn regulated by genes. All the abiotic stresses have profound influence on ecological and agricultural systems. Water stress is the predominant stress among all the abiotic stresses which causes enormous loss in production of crops, more so because water stress is usually accompanied by other stresses like salinity, high temperature and nutrient deficiencies. In addition, the impact of global climate change on crop production has emerged as a major research priority during the past decade. Several forecasts for coming decades project increase in atmospheric CO<sub>2</sub> and temperature, changes in precipitation resulting in more frequent droughts and floods, widespread runoff leading to leaching of soil nutrients and reduction in fresh-water availability. Each one of the abiotic stress conditions in singularity or in combination requires a set of specific acclimation response, tailored to the definite needs of the plant, and that a combination of two or more different stresses might require a response that is also equally specific. Experimental evidence indicates that it is not adequate to study each of the individual stresses separately and that the stress combination should be regarded as a new state of abiotic stress in plants that requires a new defense or acclimation response.

This book is broadly divided into sections on the stresses, their mechanisms and tolerance, genetics and adaptation. The book focuses on the mechanistic aspects in addition to referring to some adaptation features. Furthermore, tools to study abiotic stresses such as chlorophyll and fluorescence are highlighted in one of the chapters of the book. Of special significance is the comprehensive state of the art understanding of plant response to heavy metals. The fast pace at which developments and novel findings that are recently taking place in the cutting edge areas of molecular biology and basic genetics, have reinforced and augmented the efficiency of science outputs in dealing with plant abiotic stresses. We have moved in to the next phase in science, i.e. 'post-genomics era'. The book addresses the role of the new area of plant sciences namely "plant metabolomics" in abiotic stress which essentially is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind under stress. The emerging area of epigenetics, which is the study of changes produced in gene expression caused by mechanisms other than changes in the underlying DNA sequence and its role in abiotic stress is emphasized in this book in the context of the role of chromatin regulators.

This multi authored edited compilation attempts to put forth a comprehensive picture in a systems approach wherein mechanistic and adaptation aspects of abiotic stress will be dealt with. The chief objective of the book hence is to deliver state of the art information for comprehending the nature of abiotic stress in plants. We attempt here to present a judicious mixture of outlooks so as to interest workers in all areas of plant sciences. We trust that the information covered in this book will be useful in building strategies to counter abiotic stress in plants.

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# **Part 1**

## **Abiotic Stresses**



# Imaging of Chlorophyll a Fluorescence: A Tool to Study Abiotic Stress in Plants

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## 1. Introduction

Chlorophyll (Chl) fluorescence is a tool which is widely used to examine photosynthetic performance in algae and plants. It is a non-invasive analysis that permits to assess photosynthetic performance in vivo (Baker, 2008; Baker & Rosenqvist, 2004; Chaerle & Van Der Straeten, 2001; Woo et al. 2008). Chl fluorescence analysis is widely used to estimate photosystem II (PSII) activity, which is an important target of abiotic stresses (Balachandran et al., 1994; Baker et al., 1983; Briantais et al., 1996; Calatayud et al., 2008; Chaerle & Van Der Straeten, 2000; Ehlert & Hinch, 2008; Gilmore & Govindjee, 1999; Guidi et al., 2007; Guidi & Degl'Innocenti, 2008; Hogewoning & Harbinson, 2007; Krause, 1988; Lichtenthaler et al., 2007; Massacci et al., 2008; Osmond et al., 1999; Scholes & Rolfe, 1996; Strand & Oquist, 1985).

It is known as the energy absorbed by Chl molecules must be dissipated into three mechanisms, namely internal conversion, fluorescence and photochemistry (Butler, 1978). All of these downward processes competitively contribute to the decay of the Chl excited state and, consequently, an increase in the rate of one of these processes would increase its share of the decay process and lower the fluorescence yield. Typically, all processes that lower the Chl fluorescence yield are defined with the term *quenching*.

Kaustky and co-workers (1960) were the first which observed changes in yield of Chl fluorescence. These researchers found that transferring a leaf from the dark into the light, an increase in Chl fluorescence yield occurred. This increase has been explained with the reduction of electron acceptors of the PSII and, in particular, plastoquinone  $Q_A$ : once PSII light harvesting system (LCHII) absorbs light and the charge separation occurs,  $Q_A$  accepts electron and it is not able to accept another electron until it has been passed the first one onto the subsequent carrier, namely plastoquinone  $Q_B$ . During this time the reaction centers are said to be *closed*. The presence of closed reaction centers determines a reduction in the efficiency of PSII photochemistry and, consequently, an increase in the Chl fluorescence yield.

Transferring the leaf from the dark into light, PSII reaction centers are progressively closed, but, following this time, Chl fluorescence level typically decreases again and this phenomenon is due to two types of quenching mechanisms. The presence of light induced the activation of enzymes involved in  $CO_2$  assimilation and the stomatal aperture that determines that electrons are transferred away PSII. This induced the so-called *photochemical quenching*,  $q_P$ . At the same time, there is an increase in the conversion of light energy into

heat related to the *non-photochemical quenching*,  $q_{NP}$ . This non-photochemical quenching  $q_{NP}$ , can be divided into three components. The major and most rapid component in algae and plants is the pH- or energy-dependent component,  $q_E$ . A second component,  $q_T$ , relaxes within minutes and is due to the phenomenon of state transition, the uncoupling of LHCII from PSII. The third component of  $q_{NP}$  shows the slowest relaxation and is the least defined. It is related to photoinhibition of photosynthesis and is therefore called  $q_I$ .

To evaluate Chl fluorescence quenching coefficients during illumination we must determine minimal and maximal fluorescence yields after dark adaptation,  $F_0$  and  $F_m$  respectively. This is important because these values serve as references for the evaluation of the photochemical and non-photochemical quenching coefficients in an illuminated leaf by using the *saturation pulse method*. The concept on the basis of this method is extremely simple: at any give state of illumination,  $Q_A$  can be fully reduced by a saturation pulse of light, such that photochemical quenching is completely suppressed. During the saturation pulse, a maximal fluorescence  $F_m'$  is achieved which generally shows value lower than the dark reference values ( $F_m$ ). With the assumption that non-photochemical quenching does not change during a short saturation pulse, the reduction of  $F_m$  is a measure of non-photochemical quenching.

In **Figure 1** the calculation of Chl fluorescence parameters by using the saturation pulse method is reported. The photochemical quenching coefficient  $q_P$  is measured as

$$q_P = (F_m' - F_t) / (F_m' - F_0') \quad (1)$$

where  $F_m'$  is the maximum Chl fluorescence yield in light conditions,  $F_t$  is the steady-state Chl fluorescence immediately prior to the flash. For determination of  $F_0'$  in the light state, the leaf has to be transiently darkened and it has to be assured that  $Q_A$  is quickly and fully oxidized, before there is a substantial relaxation of non-photochemical quenching. In order to enhance of oxidation of the intersystem electron transport chain, far-red light is applied that selectively excited PSII. Usually the alternative expression of this quenching coefficient is used and it is  $(1 - q_P)$ , i.e. the proportion of centers that are closed and it is termed *excitation pressure* on PSII (Maxwell & Johnson, 2000).

An other useful fluorescence parameter derived from saturation pulse method is the efficiency of PSII photochemistry, which is calculated as:

$$\Phi_{PSII} = (F_m' - F_t) / F_m' \quad (2)$$

This parameter has also termed  $\Delta F / F_m'$  or, in fluorescence imaging technique,  $F_q' / F_m'$  and it is very similar to the  $q_P$  coefficient even if its significance is somewhat different. The  $\Phi_{PSII}$  is the proportion of absorbed light energy being used in photochemistry, whilst  $q_P$  gives an indication of the proportion of the PSII reaction centers that are open. A parameter strictly related with both  $q_P$  and  $\Phi_{PSII}$  is the ratio  $F_v / F_m$  determined as:

$$F_v / F_m = (F_m - F_0) / F_m \quad (3)$$

This third parameter is determined in dark adapted leaves and it is a measure of the maximum efficiency of PSII when all centers are open. This ratio is a sensitive indicator of plant photosynthetic performance because of it has an optimal values of about 0.83 in leaves of healthy plants of most species (Bjorkman & Demmig, 1987). An other useful parameter which describes energy dissipation is  $F_v' / F_m'$ , an estimate of the PSII quantum efficiency if all PSII reaction centers are in the open state. It is calculated as reported in equation 4:



$$F_v'/F_m' = (F_m' - F_0')/F_m' \quad (4)$$

Since  $\Phi_{\text{PSII}}$  is the quantum yield of PSII photochemistry, it can be used to determine linear electron transport rate (ETR) as described by Genty et al., (1989):

$$\text{ETR} = \Phi_{\text{PSII}} \times \text{PPFD} \times 0.5 \quad (5)$$

where PPFD (photosynthetic photon flux density) is the absorbed light and 0.5 is a factor that accounts for the partitioning of energy between PSII and PSI.

The excess of excitation energy which is not used for photochemistry can be de-excited by thermal dissipation processes. Non-photochemical quenching of Chl fluorescence is an important parameter that gives indication of the non-radiative energy dissipation in the light-harvesting antenna of PSII. This parameter is extremely important taking into account that the level of excitation energy in the antenna can be regulated to prevent over-reduction of the electron transfer chain and protect PSII from photodamage. Non-photochemical quenching coefficient is calculated as:

$$q_{\text{NP}} = (F_m - F_m') / (F_m - F_0') \quad (6)$$

In some circumstances  $F_0'$  determination is difficult, e.g. in the field when a leaf cannot be transiently darkened. In this case, another parameter can be used to describe non-photochemical energy dissipation NPQ (Schreiber & Bilger, 1993), which does not require the knowledge of  $F_0'$ . The parameter NPQ is derived from Stern-Volmer equation and its determination implies the assumption of the existence of traps for nonradiative energy dissipation, like zeaxanthin, in the antenna pigment matrix (Butler, 1978). NPQ is calculated as reported in equation 7 (Bilger & Bjorkman, 1990):

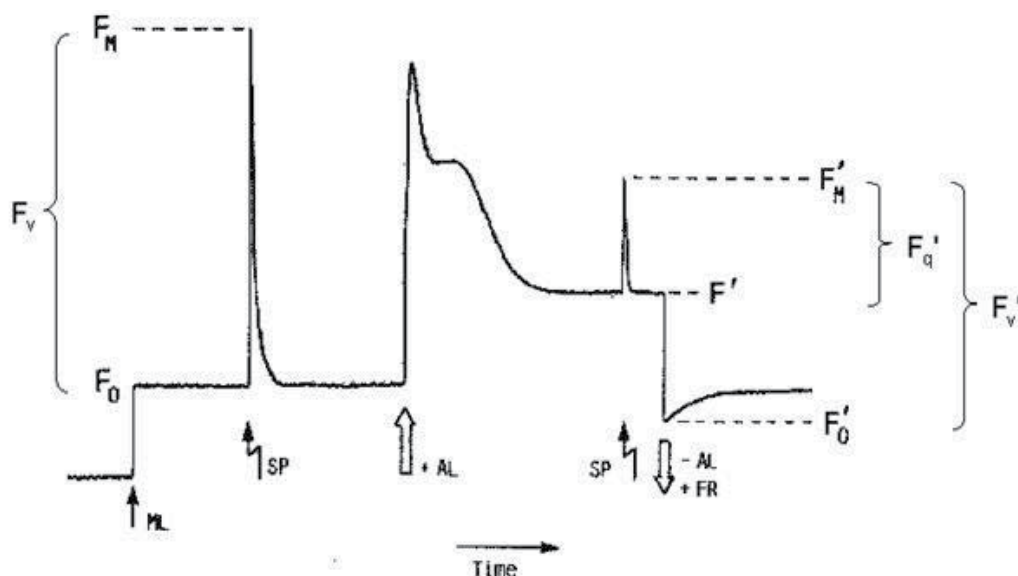


Fig. 1. Measurement of chlorophyll fluorescence by the saturation pulse method (adapted from Van Kooten & Snell, 1990).

$$\text{NPQ} = (F_m - F_m') / F_m' \quad (7)$$

NPQ is linearly related to heat dissipation and varies on a scale from 0 until infinity even if in a typical plants value ranges between 0.5 and 3.5 at light saturation level.

Chl fluorescence analysis gives a measure of the photosynthetic rate and for this reason it is extremely useful. Really, Chl fluorescence gives information about the efficiency of PSII photochemistry that, in laboratory conditions, is strictly correlated with CO<sub>2</sub> photoassimilation (Edwards & Baker, 1993; Genty et al., 1989). Under field conditions, this correlation is lost because other processes compete with CO<sub>2</sub> assimilation such as photorespiration, nitrogen metabolism and Mehler reaction (Fryer et al., 1998). In addition to, a complication derives to heterogeneity between samples. To calculate ETR we assume that the light absorbs by PSII is constant, but it is not true. Even if there are some limitations, Chl fluorescence can give a good, rapid and non invasive measurements of changes in PSII photochemistry and then also the possibility to evaluate the effects of abiotic stresses on PSII performance.

## 2. Chl fluorescence imaging

The evolution of Chl fluorescence analysis is represented by Chl fluorescence imaging which can be useful applied into two general areas: the study of heterogeneity on leaf lamina and the screening of a large numbers of samples. This technique has been widely applied in the past during induction of photosynthesis (Bro et al., 1996; Oxborough & Baker, 1997), with changes in carbohydrate translocation (Meng et al., 2001), in response to drought (Meyer & Genty, 1999; West et al., 2005), chilling (Hogewoning & Harbinson, 2007), ozone pollution (Guidi et al., 2007; Guidi & Degl'Innocenti, 2008; Leipner et al., 2001), wounding (Quilliam et al., 2006), high light (Zuluaga et al., 2008) and infection with fungi (Guidi et al., 2007; Meyer et al., 2001; Scharte et al., 2005; Scholes & Rolfe, 1996; Schwarbrick et al., 2006) or virus (Perez-Bueno et al., 2006). With Chl fluorescence imaging is possible to detect an analysis of stress-induced changes in fluorescence emission at very early stage of stress. In addition to, Chl fluorescence imaging technique represents a useful screening tool for crop yield improvement.

The most essential new information provided by Chl fluorescence imaging relates to the detection of lateral heterogeneities of fluorescence parameters which reflect physiological heterogeneities. It is well known that even physiologically healthy leaves are "patchy" with respect to stomatal opening. Furthermore, stress induced limitations, which eventually will lead to damage, are not evenly distributed over the whole leaf area. Fluorescence imaging may serve as a convenient tool for early detection of such stress induced damage. The main difference between the conventional fluorometer and the imaging fluorometer is the possibility of parallel assessment of several samples under identical conditions.

For example we treated plants of *Phaseolus vulgaris* (cv. Cannellino) with a single pulse of ozone (O<sub>3</sub>) (150 nL L<sup>-1</sup>for 5 h) and evidenced upon leaf lamina and evident heterogeneity in some Chl fluorescence parameter as compared to control exposed to charcoal filtered air for the same period (Guidi & Degl'Innocenti, *data not published*) (**Figure 2**).

It is know as in plants exposed to chilling stress, photosynthetic enzymes may be inactivated or degraded and photodamage to PSII may happen, reducing photosynthesis (Dai et al., 2007; Feng & Cao, 2005; Flexas et al. 1999). The reduction in photosynthetic CO<sub>2</sub> assimilation may lead to accumulation of excess energy especially at high irradiance and consequently to

photoinhibition (Feng & Cao, 2005; Hovenden & Warren, 1998). In variegated leaves of *Calathea makoyana* the effect of chilling (5° and 10°C for 1-7 d) on PSII efficiency was studied in order to understand the causes of chilling-induced photoinhibition (Hogewoning & Harbinson, 2007). The individual leaves were divided into a shaded zone and two illuminated, chilled zones. Chilling up to 7 d in the dark did not influence PSII efficiency whereas chilling in the light caused severe photoinhibition. Data obtained from Chl fluorescence imaging were confirmed by visual appearance of symptoms which were evident in the portion of leaves chilled and illuminated. Obtained results showed that photoinhibition was due to a secondary effect in the unchilled leaf tip (sink limitation) as revealed by starch accumulation data. Instead it was a direct effect of chilling and irradiance in the chilled illuminated zones.

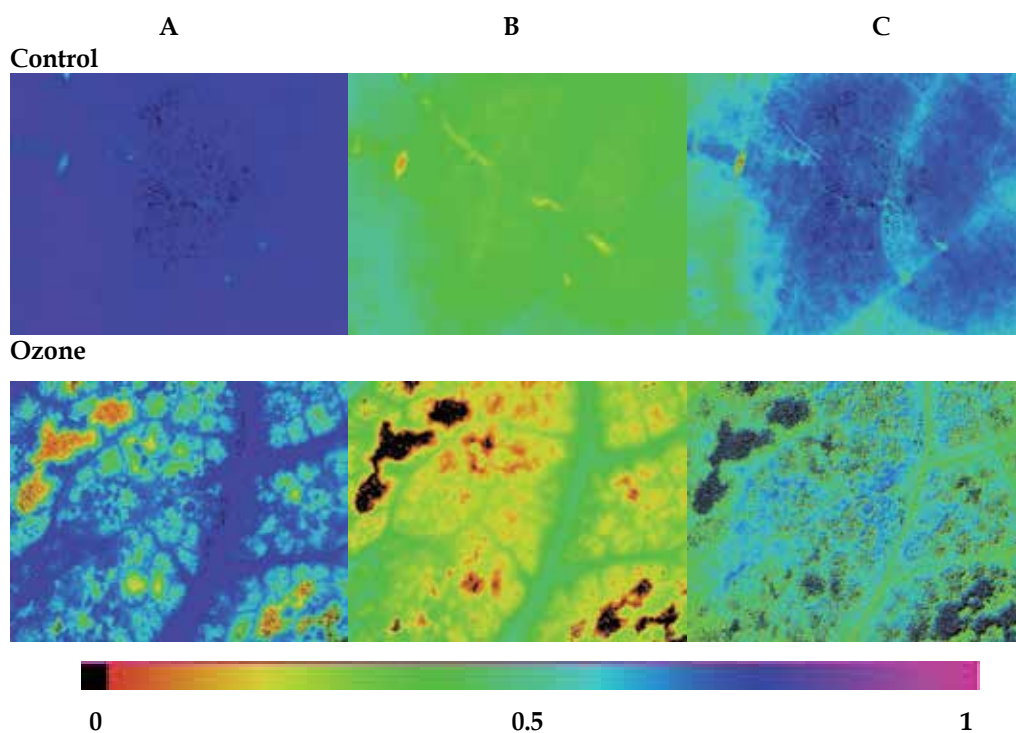


Fig. 2. Chl fluorescence imaging of  $F_v/F_m$  (A),  $\Phi_{PSII}$  (B) and non-photochemical quenching (C) in leaves of *P. vulgaris* cv. Cannellino exposed for 5 h at an  $O_3$  concentration of 150 nL L<sup>-1</sup> (Ozone) or 2 nL L<sup>-1</sup> (Control). All images are normalised to the false colour bar provided. The analyses of  $F_v/F_m$  were carried out on dark-adapted leaves, while  $\Phi_{PSII}$  and  $q_{NP}$  at a light intensity of 500  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . The pixel value display is based on a false-colour scale ranging from black (0.00 to 0.040) via red, yellow, green, blue to purple (ending at 1.00) (from Guidi & Degl'Innocenti, data not published).

Calatayud et al. (2008) studied the effects of two nutrient solution temperatures (10° and 22°C) during the flowering of *Rosa x hybrida* by using Chl fluorescence imaging. The obtained results showed as the nutrient solution temperatures of 10°C induced an increase

in  $\Phi_{\text{PSII}}$  parameters indicating that the majority of photons absorbed by PSII were used in photochemistry and that PSII centers were maintained in an oxidized state.

Water stress is another important abiotic stress that induces reduction of growth and yield of plants. For this reason the development of drought-tolerance is an important target of the researchers. The effects of drought on photosynthetic process have been extensively studied in many plant species and the possible mechanisms involved in the responses have been suggested (Cornic & Fresneau, 2002; Flexas et al., 2002, 2004; Grassi & Magnani, 2005; Long & Bernacchi, 2003). Masacci et al. (2008) took Chl fluorescence images from leaves of *Gossypium hirsutum* to study the spatial pattern of PSII efficiency and non-photochemical quenching parameters. They found that under low and moderate light intensity, the onset of drought stress caused an increase in the operating quantum efficiency of PSII ( $\Phi_{\text{PSII}}$ ) which indicated increased photorespiration since photosynthesis was hardly affected by water shortage. The increase in  $\Phi_{\text{PSII}}$  was caused by an increase in  $F_v'/F_m'$  and by a decrease in non-photochemical quenching. Chl fluorescence imaging showed a low spatial heterogeneity of  $\Phi_{\text{PSII}}$ . The authors concluded that the increase in photorespiration rate in plants during the water stress can be seen as an acclimation process to avoid an over-excitation of PSII under more severe drought conditions.

Qing-Ming et al., (2008) used Chl fluorescence imaging analysis to detect the effects of drought stress and elevated  $\text{CO}_2$  concentration ( $780 \mu\text{mol mol}^{-1}$ ) in cucumber seedlings. They found that electron transport rate and the light saturation level declined significantly with drought stress aggravation in both  $\text{CO}_2$  concentrations. Drought stress decreased maximal photosynthetic ETR and subsequently decreased the capacity of preventing photodamage. At the same time, elevated  $\text{CO}_2$  concentration increased the light saturation level significantly, irrespective of the water conditions. Elevated  $\text{CO}_2$  concentration can alleviate drought stress-induced photoinhibitory damage by improving saturating photosynthetically active radiation.

Sommerville et al. (2010) examined the different spatial response in photosynthesis with drought in two species with contrasting hydraulic architecture. The authors hypothesized that areole regions near primary nerves would show a smaller decline in the maximum efficiency of PSII photochemistry with drought compared with regions between secondary nerves and that the difference between areole regions would be smaller in phyllodes with higher primary nerve density. Indeed, the phyllodes of *Acacia floribunda* were found to have both greater primary nerve density and show greater spatial homogeneity in photosynthetic function with drought compared with the phyllodes of *Acacia pycnantha*. *A. floribunda* phyllodes also maintained function of the photosynthetic apparatus with drought for longer and recovered more swiftly from drought than *A. pycnantha*.

Drought is a type of stress which can induce heterogeneity in leaf photosynthesis that probably occurs when dehydration is rapid as in the case of drought experiments performed on potted plants by withholding water. Using Chl fluorescence imaging, Flexas et al. (2006) showed in herbaceous species that exogenous ABA did not induce patchy stomatal closure even when stomatal conductance dropped to much lower values lower than  $0.05 \text{ mol m}^{-2} \text{ s}^{-1}$ .

Even the quality and quantity of light intensity notable influence the photosynthetic apparatus and functioning. Generally, sun- and shade leaves differ in the composition of leaf pigment, electron carriers on thylakoids membranes, structure of the chloroplast and photosynthetic rate (Anderson et al., 1995; Boardman, 1977; Lichtenthaler, 1981, 1984; Lichtenthaler et al., 2007; Takahashi & Badger, 2010). Lichtenthaler et al. (2007) studied the differential pigment composition and photosynthetic activity of sun and shade leaves of

deciduous (*Acer pseudoplatanus*, *Fagus sylvatica*, *Tilia cordata*) and coniferous (*Abies alba*) trees by using Chl fluorescence imaging analysis. This tool not only provided the possibility to screen the differences in photosynthetic CO<sub>2</sub> assimilation rate between sun and shade leaves, but in addition permitted detection and quantification of the large gradient in photosynthetic rate across the leaf area existing in sun and shade leaves.

Chl fluorescence analysis is used also to characterized photosynthetic process in transgenic plants such as tomato (*Lycopersicon esculentum*) cv. Micro-Tom transformed with the *Arabidopsis thaliana* MYB75/PAP1 (PRODUCTION OD ANTHOCYANIN PIGMENT 1) gene (Zuluaga et al., 2008). This gene encodes for a well known transcription factor, which is involved in anthocyanin production and is modulated by light and sucrose. The presence of a higher constitutive level of anthocyanin pigments in transgenic plants could give them some advantage, in terms of adaptation and defence against environmental stresses. To test this hypothesis, a high light experiment was carried out exposing wild type and transgenic tomato plants to a strong light irradiance for about ten days and monitoring the respective phenotypic and physiological changes. The light intensity used was very high and likely not similar to normal environmental conditions (at least for such a prolonged period). Chlorophyll fluorescence imaging on control and stressed leaves from both genotypes suggest that, in transgenic leaves, the apparent tolerance to photoinhibition was probably not due to an increased capacity for PSII to repair, but reflected instead the ability of these leaves to protect their photosynthetic apparatus.

Certainly among abiotic stress the pollutants can alter the physiology and biochemistry of plants. Ozone is an air pollutant that induces reduction in growth and yield of plants species. The major target of the O<sub>3</sub> effects is represented by photosynthetic process and many works have been reported as this pollutant can impair CO<sub>2</sub> assimilation rate. Plant response depends also on the dose (concentration x time). In fact, it can distinguish chronic exposure to O<sub>3</sub> from acute one. It is termed chronic exposure the long-term exposure at concentration < 100 nL L<sup>-1</sup> whereas the acute O<sub>3</sub> exposure is generally defined as exposure to a high level of O<sub>3</sub> concentration (> 100 nL L<sup>-1</sup>) for a short period of time, typically on the order of hours (Kangasjarvi et al., 2005). Chen et al. (2009) studied the effects of acute (400 nL L<sup>-1</sup>, 6 h) and chronic (90 nL L<sup>-1</sup>, 8 h d<sup>-1</sup>, 28 d) O<sub>3</sub> concentration on photosynthetic process of soybean plants. Although both acute and chronic O<sub>3</sub> treatment resulted in a similar overall photosynthetic impairment compared to the controls, the fluorescence imaging analysis revealed that the physiological mechanisms underlying the decreases differed. In the acute O<sub>3</sub> treatments over the chronic one there was a greater spatial heterogeneity related to several bases. The higher O<sub>3</sub> concentration typically induced oxidative stress and the hypersensitive response within a matter of hours leading to programmed cellular death (PCD). By the end of chronic O<sub>3</sub> treatment, control leaves showed an increase in spatial heterogeneity of photosynthesis linked to the process of natural senescence. Clearly, in this study it has been demonstrated as Chl fluorescence imaging represents a useful tool to study also mechanisms on the basis of plants responses to abiotic stress such as O<sub>3</sub> pollution.

Guidi et al. (2007) used Chl fluorescence analysis to study the effects of an acute O<sub>3</sub> treatment (150 nL L<sup>-1</sup> for 5 h) or artificial inoculation with a pathogen (*Pleiochaeta setosa*) on photosynthesis of *Lupinus albus*. The aim of the work was to compare the perturbations in photosynthesis induced by an abiotic or biotic stress. In addition to, in the work were compared results obtained by conventional Chl fluorescence analysis and the technique of Chl fluorescence imaging. Image analysis of F<sub>v</sub>/F<sub>m</sub> showed a different response in plants

subjected to ozone or inoculated with *P. setosa*. Indeed, in ozonated leaves fluorescence yield was lower in leaf veins than in the mesophyll with the exception of the necrotic areas where no fluorescence signals could be detected. This suggests that the leaf area close to the veins were more sensitive to ozone. The parameter  $\Phi_{\text{PSII}}$  decreased significantly in both infected and ozonated leaves, but image analysis provides more information than the conventional fluorometer. In fact, until 48 h after ozone treatment or fungal inoculation,  $\Phi_{\text{PSII}}$  tended to decrease, especially in the infected leaves. Afterwards, a distinct stimulation of photosynthesis was observed in the area surrounding the visible lesions induced by the fungus. This did not occur in the ozonated leaves, as suggested also by the higher values of qP (data not shown). This phenomenon was not observed using the conventional fluorometer which recorded a similar reduction in this parameter in both ozonated and inoculated leaves.

In an other work Guidi and Degl'Innocenti (2008) studied the response to photoinhibition and subsequent recovery in plants of *Phaseolus vulgaris* (cv. Pinto) exposed to charcoal-filtered air or to an acute  $\text{O}_3$  exposure ( $150 \text{ nL L}^{-1}$  for 3 or 5 h). Susceptibility to photoinhibition in bean leaves was determined as changes in the  $F_v/F_m$  ratio and the images of the ratio are reported in **Figure 3**. Initial values of  $F_v/F_m$  were 0.796, 0.784 and 0.741 for plants maintained in charcoal-filtered air, or treated with a single exposure to  $\text{O}_3$  for 3 h, or for 5 h, respectively. The results indicate that treatment with  $\text{O}_3$  for 5 h induced a slight photoinhibition. The exposure of control plants (charcoal-filtered air for 5 h) at a light intensity of  $1000 \mu\text{mol m}^{-2}\text{s}^{-1}$  resulted in a significant reduction in  $F_v/F_m$  ( $P < 0.01$ ) (Fig. 2b), while plants treated with  $\text{O}_3$  for 3 h showed an increased tolerance to photoinhibition with less reduction in  $F_v/F_m$  (Fig. 2f). Plants treated with  $\text{O}_3$  for 5 h and then exposed to high light showed a reduction in  $F_v/F_m$  ratio values similar to those recorded in control plants (Fig. 2i and l). However, while control plants or treated with  $\text{O}_3$  for 3 h recovered their initial value 24 h after photoinhibition treatment, plants treated with  $\text{O}_3$  for 5 h did not show the same ability to recover. In these plants the values of the  $F_v/F_m$  ratio did not recover and, 48 h after photoinhibition leaves showed visible symptoms of damage over the entire surfaces which precluded further analysis. At the same time, severe wilting did not permit chlorophyll fluorescence imaging.

Most of the abiotic stresses induce in plants an oxidative damage of the cell structure and consequently a loss in the cellular activities. Chloroplast represents the organelle which possesses pigments that absorb light and drive redox reactions of thylakoids but also the site in the cell where  $\text{O}_2$  is evolved from water. Clearly, it represents an organelle such as mitochondria, in which the formation of reactive oxygen species (ROS) can occur. On the other hand, chloroplasts are able to produce strong oxidants associated with PSII which are responsible for the splitting of  $\text{H}_2\text{O}$  molecules, but they can also oxidize pigments, proteins and lipid of the thylakoid membranes as well. This characteristic makes the chloroplast a major stress sensor in green plants (Biswal & Biswal 1999). Even the separation charge and the electron transport rate associated represent another important factor that makes chloroplast sensitive to stress. Using image analysis tools Aldea et al. (2006) observed a statistical relationship between ROS and reductions in photosynthetic efficiency ( $\Phi_{\text{PSII}}$ ) in leaves damaged simultaneously by  $\text{O}_3$  ( $80 \text{ nL L}^{-1}$  for 8 h) and viral infection (soybean mosaic virus). The author by using Chl fluorescence analysis overlapped spatial maps of  $\Phi_{\text{PSII}}$  and ROS and found that areas with depressed  $\Phi_{\text{PSII}}$  corresponded to areas of high ROS concentration.

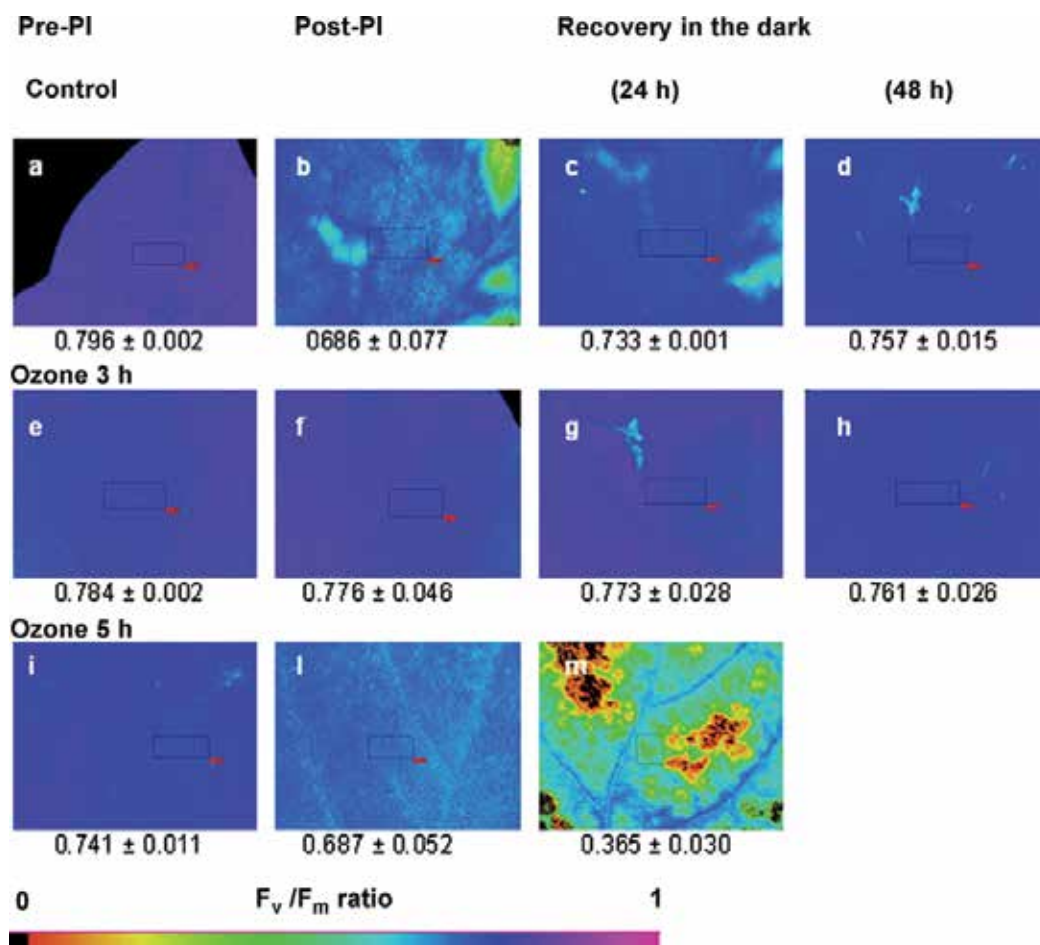


Fig. 3. Representative fluorescence images of the  $F_v/F_m$  ratio in leaves of *Phaseolus vulgaris* L. cultivar Pinto after a single exposure to  $O_3$  (150 ppb) for 3 h (Ozone 3 h; e-h) or 5 h (Ozone 5 h; i-m) or exposed to charcoal-filtered air (control, a-d) (Pre-PI). The images correspond to different measurement times: after charcoal-filtered air or  $O_3$  exposure (a, e and i), after photoinhibitory treatment for 5 h (b, f and l), after recovery in the dark for 24 h (c, g and m) or for 48 h (d and h). All images are normalised to the false colour bar provided. The analyses of  $F_v/F_m$  were carried out on dark-adapted leaves. The pixel value display is based on a false-colour scale ranging from black (0.00 to 0.040) via red, yellow, green, blue to purple (ending at 1.00) (from Guidi & Degl'Innocenti, 2008).

Wounding is another common abiotic stress which induces a spatial and temporal complex series of responses in plants. In fact, wounding induced by herbivore or mechanical damage determines localized cell death, loss of water and solutes from cut surface which provides a point of entry of bacterial and fungal pathogens and disrupts vascular system. Many responses can be activated following wounding such as defense and repair mechanisms which require a high metabolic demand upon wounded region. These responses determine

the synthesis of new molecules and then energy and carbon skeleton. An interesting work reported the study of the spatial and temporal changes in source-sink relationships which occur in mechanically wounded leaves of *Arabidopsis thaliana* (Quilliam et al., 2006). When the Chl fluorescence imaging analyses was made immediately after wounding there was a localized reduction in the steady-state of  $\Phi_{\text{PSII}}$  in cells adjacent to the wound margin and this suggests that these cells were damaged. No changes in  $F_v/F_m$  ratio were observed. Twenty-four hours after wounding, cells proximal to the wound margin showed a rapid induction of  $\Phi_{\text{PSII}}$  upon illumination whilst cells more distal to the wound margin exhibited a much slower induction of  $\Phi_{\text{PSII}}$  and a large increase of NPQ. The obtained results indicate of an increase in sink strength in the vicinity of the wound.

Chl fluorescence imaging has been used also for particular studies such as the characterization of a mutants with altered leaf morphology that are useful as markers for the study of genetic systems and for probing the leaf differentiation process. In a study carried out by Fambrini et al (2010) a mutant with deficient greening and altered development of the leaf mesophyll appeared in an inbred line of sunflower (*Helianthus annuus* L.). The mutation, named *mesophyll cell defective1 (mcd1)*, has pleiotropic effects and it is inherited as a monogenic recessive. The structure and tissue organization of *mcd1* leaves are disrupted. A deficient accumulation of photosynthetic pigments characterizes both cotyledons and leaves of the mutant. In *mcd1* leaves, Chl fluorescence imaging evidences a spatial heterogeneity of leaf photosynthetic performance. Little black points, which correspond to PSII maximum efficiency ( $F_v/F_m$ ) values close to zero, characterize the *mcd1* leaves. Similarly, the light adapted quantum efficiency ( $\Phi_{\text{PSII}}$ ) values show a homogeneous distribution over wild type leaf lamina, while the damaged areas in *mcd1* leaves, represented by yellow zones, are prominent (**Figure 4**).

In conclusion, the loss of function of the *MCD1* gene in *Helianthus annuus* is correlated with a variegated leaf phenotype characterized by a localized destruction of mesophyll morphogenesis and defeat of PSII activity.

Another interesting application of Chl fluorescence imaging is represented by its used to analyze the generation of action potentials in irritated *Dionaea muscipula* traps to determine the 'site effect' of the electrical signal-induced inhibition of photosynthesis (Pavlovic et al. 2011). Irritation of trigger hairs and subsequent generation of action potentials resulted in a decrease in the effective photochemical quantum yield of photosystem II ( $\Phi_{\text{PSII}}$ ) and the rate of net photosynthesis (**Figure 5**).

During the first seconds of irritation, increased excitation pressure in PSII was the major contributor to the decreased  $\Phi_{\text{PSII}}$ . Within 1 min, NPQ released the excitation pressure at PSII. All the data presented in this work indicate that the main primary target of the electrical signal induced inhibition of photosynthesis is the dark reaction, whereas the inhibition of electron transport is only a consequence of reduced carboxylation efficiency. In addition, the study also provides valuable data confirming the hypothesis that chlorophyll a fluorescence is under electrochemical control.

Chl fluorescence imaging combined with thermal imaging has been used also for monitoring and screening plant population (Chaerle et al., 2006). Rapid screening for stomatal responses can be achieved by thermal imaging, while, combined with fluorescence imaging to study photosynthesis, can potentially be used to derive leaf water use efficiency as a screening parameter.



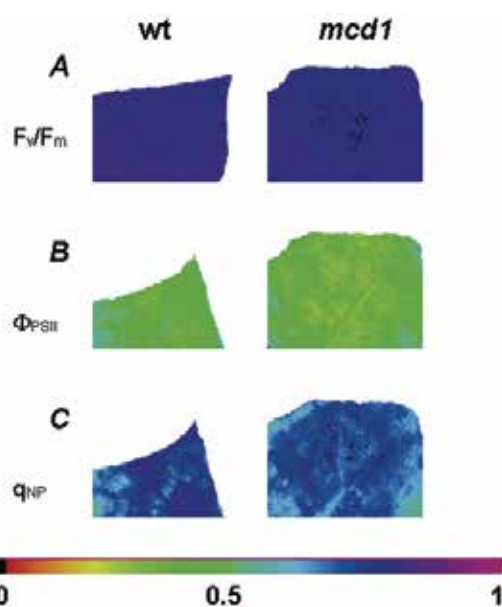


Fig. 4. Analysis of chlorophyll fluorescence parameters in wild type (wt) and *mesophyll cell defective1* (*mcd1*) mutant plants of sunflower (*Helianthus annuus* L.). A–C: Fluorescence images of the maximum efficiency of PSII ( $F_v/F_m$ ; A), the proportion of absorbed light, which is utilized for photosynthetic electron transport ( $\Phi_{PSII}$ ; B), and the nonphotochemical quenching coefficient ( $q_{NP}$ ; C), in representative leaves from wild type (left column) and *mcd1* mutant (right column), are shown (from Fambrini et al., 2010).

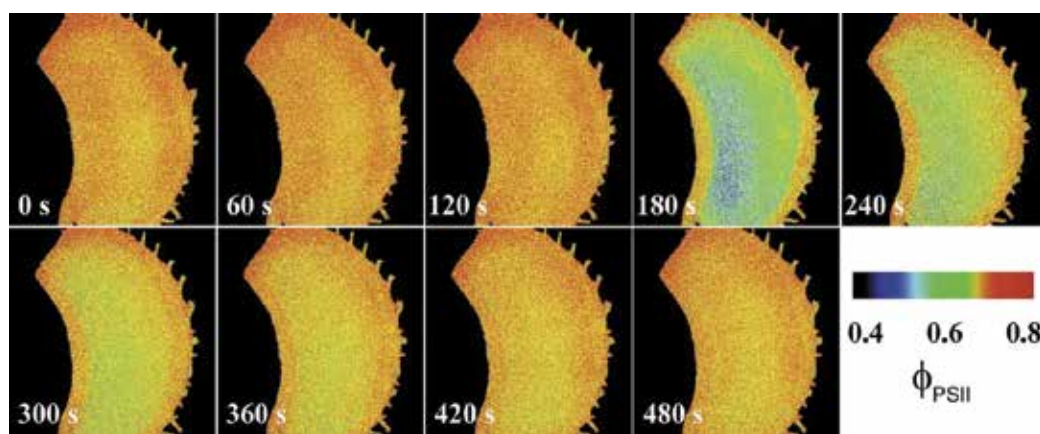


Fig. 5. Spatiotemporal changes of effective photochemical quantum yield of PSII ( $\Phi_{PSII}$ ) in a *D. muscipula* closed trap assessed by chlorophyll fluorescence imaging. The trap was irritated by a thin wire between 162 s and 177 s (image obtained from Pavlovic et al. 2011).

Although Chl fluorescence fluorometers have been developed to measure chlorophyll fluorescence from green tissues, which are high in chlorophyll content, the extraordinary sensitivity of current instruments enables measurements in non-green plant tissues that have relatively low chlorophyll content. This includes many types of ripening fruit that during development degrade the chloroplasts (including chlorophyll) that are contained in the fruit skin. Even non-green fruit that are highly colored (*e.g.*, apples, tomatoes), contain active chloroplasts that yield a chlorophyll fluorescence signal of sufficient strength that it can be used as a probe of photosynthetic activity in the fruit skin (DeEll et al., 1995). In food technology, Chl fluorescence imaging can provide a rapid and non-invasive, post-harvest evaluation of the quality of fruits and vegetables (DeEll et al., 1995; DeEll & Toivonen, 2000). Nedbal & Withmarsh (2004) reported an interesting article on this topic. By applying fluorescence imaging on individual fruit before any symptoms of bitter pit were apparent, lower fluorescence was shown to be associated with bitter pit development in apples in selective cases (Lotze et al., 2006). The authors showed that, using averaged cumulative distribution functions (CDFs) of pitted and non-pitted fruit classes, it was possible to show a difference between these classes with fluorescence imaging. Results of pre-harvest imaging on apples to identify fruit with bitter pit potential at harvest showed that pitted fruit were correctly classified (75–100%). However, misclassification of non-pitted fruit (50% and less) with fluorescence imaging is still too high to be of any commercial.

Obenland & Neipp (2005) used Chl fluorescence analysis in green lemons (*Citrus uniuon*) 30 minutes after immersion of the fruit into 55°C water for 5 minutes to determine if this methodology could be used to identify areas of hot water-induced rind injury before the appearance of visible symptoms. Fluorescence was variable in intensity over the surface of the rind with defined areas of enhanced fluorescence being present that corresponded in shape and location with visible injury that later developed during 24 hours of storage. The authors concluded that imaging of Chl fluorescence has potential as a means to identify areas of incipient rind injury in citrus to facilitate study of the causal mechanisms of postharvest rind disorders. On the other hand, previously Nedbal et al. (2000) demonstrated the potential for using rapid imaging of Chl fluorescence in post-harvest fruit to develop an automated device that can identify and remove poor quality fruit long before visible damage appears.

Meyerhoff & Pfündel (2008) used Chl fluorescence imaging to detect the presence of functioning PSII in fruits of strawberries. From obtained results authors concluded that it is unclear if photosynthesis in strawberry fruits is capable to support seed development.

Chl fluorescence imaging can be conveniently used to study the functioning of PSII in leaves and permits to detect the heterogeneity of photosynthesis which is particularly evident in stressed leaves. However, it has been reported as it can be conveniently used also for particular application such as the study of fruit quality in postharvest. For these reasons Chl fluorescence imaging represents an important and useful tool in ecophysiological and post harvest studies that permits to detect the effects of abiotic stress even at early stages and before the visual appearance of symptoms of damage.

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# Salinity Stress and Salt Tolerance

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## 1. Introduction

Salinity is one of the most serious factors limiting the productivity of agricultural crops, with adverse effects on germination, plant vigour and crop yield (R Munns & Tester, 2008). Salinization affects many irrigated areas mainly due to the use of brackish water. Worldwide, more than 45 million hectares of irrigated land have been damaged by salt, and 1.5 million hectares are taken out of production each year as a result of high salinity levels in the soil (R Munns & Tester, 2008). High salinity affects plants in several ways: water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion, genotoxicity (Hasegawa, Bressan, Zhu, & Bohnert, 2000; R. Munns, 2002; Zhu, 2007). Together, these effects reduce plant growth, development and survival.

During the onset and development of salt stress within a plant, all the major processes such as photosynthesis, protein synthesis and energy and lipid metabolism are affected (Parida & Das, 2005). During initial exposure to salinity, plants experience water stress, which in turn reduces leaf expansion. The osmotic effects of salinity stress can be observed immediately after salt application and are believed to continue for the duration of exposure, resulting in inhibited cell expansion and cell division, as well as stomatal closure (T. J. Flowers, 2004; R. Munns, 2002). During long-term exposure to salinity, plants experience ionic stress, which can lead to premature senescence of adult leaves, and thus a reduction in the photosynthetic area available to support continued growth (Cramer & Nowak, 1992). In fact, excess sodium and more importantly chloride has the potential to affect plant enzymes and cause cell swelling, resulting in reduced energy production and other physiological changes (Larcher 1980). Ionic stress results in premature senescence of older leaves and in toxicity symptoms (chlorosis, necrosis) in mature leaves due to high  $\text{Na}^+$  which affects plants by disrupting protein synthesis and interfering with enzyme activity (Hasegawa, Bressan, Zhu, & Bohnert, 2000; R. Munns, 2002; R Munns & Termaat, 1986). Many plants have evolved several mechanisms either to exclude salt from their cells or to tolerate its presence within the cells. In this chapter, we mainly discuss about soil salinity, its effects on plants and tolerance mechanisms which permit the plants to withstand stress, with particular emphasis on ion homeostasis,  $\text{Na}^+$  exclusion and tissue tolerance. Moreover we give a synthetic overview of the two major approaches that have been used to improve stress tolerance: exploitation of natural genetic variations and generation of transgenic plants with novel genes or altered expression levels of the existing genes. A fundamental biological understanding and knowledge of the effects of salt stress on plants is necessary to provide additional

information for the dissection of the plant response to salinity and try to find future applications for ameliorating the impact of salinity on plants, improving the performance of species important to human health and agricultural sustainability.

## 2. Soil salinity

The earliest written account of salt lands dates back to 2400 BC and was recorded in the Tigris-Euphrates alluvial plains of Iraq (Russel, Kadry, & Hanna, 1965). Salt-affected lands occur in practically all climatic regions, from the humid tropics to the polar regions. Saline soils can be found at different altitudes, from below sea level (e.g. around the Dead Sea) to mountains rising above 5000 meters, such as the Tibetan Plateau or the Rocky Mountains. Furthermore, the occurrence of saline soils is not limited to desert conditions (Singh & Chatrath, 2001). All soils contain salts, and all irrigation waters, whether from canals or underground pumping, including those considered of very good quality, contain some dissolved salts. In fact, salts are a common and necessary component of soil, and many salts (e.g. nitrates and potassium) are essential plant nutrients. Salts originate from mineral weathering, inorganic fertilizers, soil amendments (e.g. gypsum, composts and manures), and irrigation waters (Kotuby-Amacher, Koenig, & Kitchen, 2000). In particular, the process of soil salinization is dramatically exacerbated and accelerated by crop irrigation. The overall effect of irrigation in the context of salinity is that it “imports” large quantities of new salts to the soil that were not there before (R Munns, Goyal, & Passioura, 2004). Actually, about 2% of the lands farmed by dry-land agriculture, and more than 45 million hectares of irrigated land (at least 20% of total irrigated acreage) have been already damaged by salt (Lauchli, James, Huang, McCully, & Munns, 2008) (Fig. 1).

Mediterranean regions are currently experiencing increasing salt stress problems resulting from seawater intrusion into aquifers and irrigation with brackish water (Rana & Katerji, 2000). While an important cause of salinity in Australian continent is the deposition of oceanic salts carried in wind and rain (R Munns & Tester, 2008). An additional, important source of

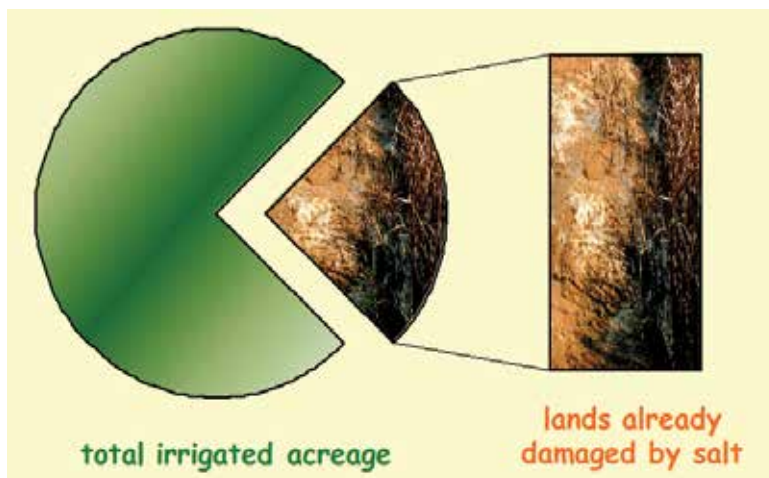


Fig. 1. Percentage of irrigated lands damaged by salinity

salts in many landscape soils comes from ice melters used on roads and sidewalks. The addition of virtually any soluble material will increase soil salinity (Singh & Chatrath, 2001). Among the various sources of soil salinity, irrigation combined with poor drainage is the most serious, because it represents losses of once productive agricultural land (Zhu, 2007). The irrigation water contains calcium ( $\text{Ca}^{2+}$ ), magnesium ( $\text{Mg}^{2+}$ ), and sodium ( $\text{Na}^+$ ). When the water evaporates,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  often precipitate into carbonates, leaving  $\text{Na}^+$  dominant in the soil (Serrano, Culianz-Macia, & Moreno, 1999). As a result  $\text{Na}^+$  concentrations often exceed those of most macronutrients by one or two orders of magnitude, and by even more in the case of micronutrients. High concentrations of  $\text{Na}^+$  in the soil solution may depress nutrient-ion activities and produce extreme ratios of  $\text{Na}^+/\text{Ca}^{2+}$  or  $\text{Na}^+/\text{K}^+$  (Grattana & Grieveb, 1999). Increases in cations and their salts,  $\text{NaCl}$  in particular, in the soil generates external osmotic potential that can prevent or reduce the influx of water into the root. The resulting water deficit is similar to drought conditions and additionally compounded by the presence of  $\text{Na}^+$  ions (Bohnert, 2007).

Improper management of salinity may lead to soil sodicity, damaging soil structure. In particular, the action of  $\text{Na}^+$  ions, when they occupy the cation exchange complex of clay particles, cause soil aggregates to break down, increase bulk density, make the soil more compact and decrease total porosity, thereby hampering soil aeration. As a result, plants in saline soils not only suffer from high  $\text{Na}^+$  levels, but are also affected by some degree of hypoxia (Singh & Chatrath, 2001; Tisdale, Nelson, & Beaton, 1993).

According to the USDA salinity laboratory, saline soil can be defined as soil having an electrical conductivity of solution extracted from the water-saturated soil paste ECe (Electrical Conductivity of the extract) of  $4 \text{ dS m}^{-1}$  (decisiemens per meter), where  $4 \text{ dS m}^{-1} \approx 40 \text{ mM NaCl}$  or more (Chinnusamy, Jagendorf, & Zhu, 2005; Kotuby-Amacher, Koenig, & Kitchen, 2000).

Soil type and environmental factors, such as vapour, pressure deficit, radiation and temperature may further alter salt tolerance (Chinnusamy, Jagendorf, & Zhu, 2005). In fields, in fact, the salt levels fluctuate seasonally and spatially, and variation will occur due to the circumstances influencing each particular plant (Estes, 2002). In addition, the continuous use of same soil for growing vegetables results in an increase of salinization.

### 3. Effects of salinity on plants

Soil salinity is a major factor that limits the yield of agricultural crops, jeopardizing the capacity of agriculture to sustain the burgeoning human population increase (T. J. Flowers, 2004; R Munns & Tester, 2008; Parida & Das, 2005).

At low salt concentrations, yields are mildly affected or not affected at all (Maggio, Hasegawa, Bressan, Consiglio, & Joly, 2001). As the concentrations increase, the yields move towards zero, since most plants, glycophytes, including most crop plants, will not grow in high concentrations of salt and are severely inhibited or even killed by 100-200 mM  $\text{NaCl}$ . The reason is that they have evolved under conditions of low soil salinity and do not display salt tolerance (R Munns & Termaat, 1986). On the contrary halophytes can survive salinity in excess of 300-400 mM. Halophytes are known to have a capability of growth on salinized soils of coastal and arid regions due to specific mechanisms of salt tolerance developed during their phylogenetic adaptation. Depending on their salt-tolerating capacity, these plants can be either obligate and characterized by low morphological and taxonomical diversity with relative growth rates increasing up to 50% sea water or facultative and found

in less saline habitats along the border between saline and non-saline upland and characterized by broader physiological diversity which enables them to cope with saline and non-saline conditions (Parida & Das, 2005). Measurements of ion contents in plants under salt stress revealed that halophytes accumulate salts whereas glycophytes tend to exclude the salts (Zhu, 2007).

High salinity affects plants in two main ways: high concentrations of salts in the soil disturb the capacity of roots to extract water, and high concentrations of salts within the plant itself can be toxic, resulting in an inhibition of many physiological and biochemical processes such as nutrient uptake and assimilation (Hasegawa, Bressan, Zhu, & Bohnert, 2000; R. Munns, 2002; R Munns, Schachtman, & Condon, 1995; R Munns & Tester, 2008). Together, these effects reduce plant growth, development and survival. A two-phase model describing the osmotic and ionic effects of salt stress was proposed by Munns (1995) (Fig. 2).

Plants sensitive or tolerant to salinity differ in the rate at which salt reaches toxic levels in leaves. Timescale is days or weeks or months, depending on the species and the salinity level. During Phase 1, growth of both type of plants is reduced because of the osmotic effect of the saline solution outside the roots. During Phase 2, old leaves in the sensitive plant die and reduce the photosynthetic capacity of the plant. This exerts an additional effect on growth.

In the first, osmotic phase, which starts immediately after the salt concentration around the roots increases to a threshold level making it harder for the roots to extract water, the rate of shoot growth falls significantly. An immediate response to this effect, which also mitigates ion flux to the shoot, is stomatal closure. However, because of the water potential difference between the atmosphere and leaf cells and the need for carbon fixation, this is an untenable long-term strategy of tolerance (Hasegawa et al., 2000). Shoot growth is more sensitive than root growth to salt- induced osmotic stress probably because a reduction in the leaf area development relative to root growth would decrease the water use by the plant, thus allowing it to conserve soil moisture and prevent salt concentration in the soil (R Munns & Tester, 2008). Reduction in shoot growth due to salinity is commonly expressed by a reduced leaf area and stunted shoots (A. Läuchli & Epstein, 1990). The growth inhibition of leaves sensitive to salt stress appears to be also a consequence of inhibition by salt of symplastic xylem loading of  $\text{Ca}^{2+}$  in the root (A. Läuchli & Grattan, 2007). Final leaf size depends on both cell division and cell elongation. Leaf initiation, which is governed by cell

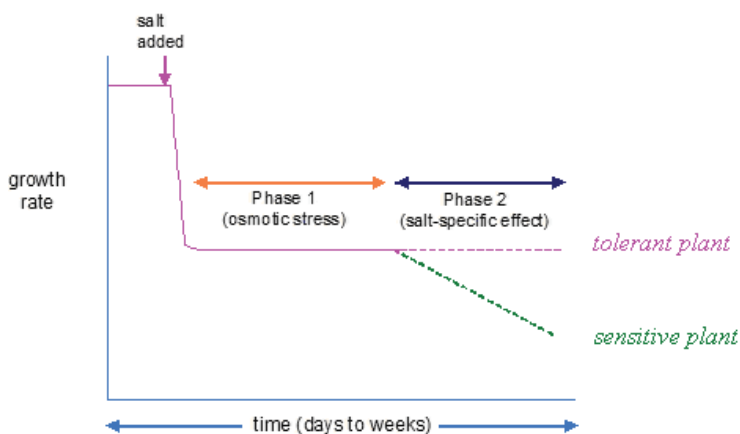


Fig. 2. Scheme of the two-phase growth response to salinity. Adapted from Munns (1995).

division, was shown to be unaffected by salt stress in sugar beet, but leaf extension was found to be a salt-sensitive process (Papp, Ball, & Terry, 1983), depending on  $\text{Ca}^{2+}$  status. Moreover the salt-induced inhibition of the uptake of important mineral nutrients, such as  $\text{K}^+$  and  $\text{Ca}^{2+}$ , further reduces root cell growth (Larcher, 1980) and, in particular, compromises root tips expansion (Fig. 3). Apical region of roots grown under salinity (Fig. 3 C, D) show extensive vacuolization and lack of typical organization of apical tissue. A slight plasmolysis due to a lack of continuity and adherence between cells is present with a tendency to the arrest of growth and differentiation. Otherwise, control plants root tips (Fig. 3 A, B) are characterized by densely packed tissues with only small intercellular spaces.

The second phase, ion specific, corresponds to the accumulation of ions, in particular  $\text{Na}^+$ , in the leaf blade, where  $\text{Na}^+$  accumulates after being deposited in the transpiration stream, rather than in the roots (R. Munns, 2002).  $\text{Na}^+$  accumulation turns out to be toxic especially in old leaves, which are no longer expanding and so no longer diluting the salt arriving in them as young growing leaves do. If the rate at which they die is greater than the rate at which new leaves are produced, the photosynthetic capacity of the plant will no longer be able to supply the carbohydrate requirement of the young leaves, which further reduces their growth rate (R. Munns & Tester, 2008). In photosynthetic tissues, in fact,  $\text{Na}^+$  accumulation affects photosynthetic components such as enzymes, chlorophylls, and carotenoids (Davenport, James, Zakrisson-Plogander, Tester, & Munns, 2005). The derived reduction in photosynthetic rate in the salt sensitive plants can increase also the production of reactive oxygen species (ROS). Normally, ROS are rapidly removed by antioxidative mechanisms, but this removal can be impaired by salt stress (Allan & Fluhr, 1997; Foyer & Noctor, 2003). ROS signalling has been shown to be an integral part of acclimation response to salinity. ROS play, in fact, a dual

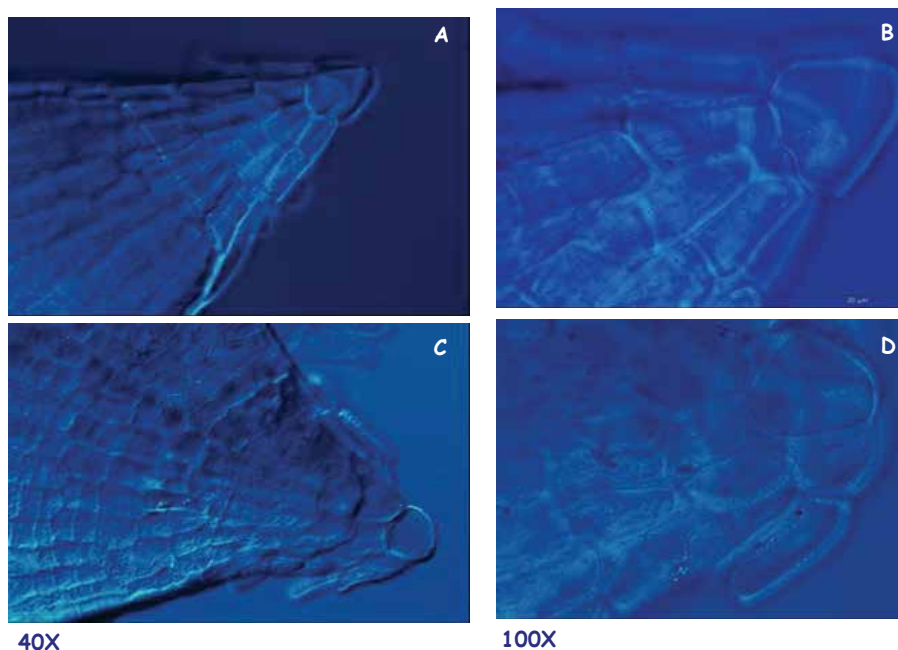


Fig. 3. Root tips of durum wheat grown in absence (A, B) or presence of 100 mM NaCl (C, D) observed by DIC microscopy (P. Carillo, D. Parisi, E. Maximova, unpublished data).

role in the response of plants to abiotic stresses functioning as toxic by-products of stress metabolism, as well as important signal transduction molecules integrated in the networks of stress response pathway mediated by calcium, hormone and protein phosphorylation (Miller, Suzuki, Ciftci-Yilmaz, & Mittler, 2010).

ABA plays an important role in the response of plants to salinity and ABA-deficient mutants perform poorly under salinity stress (Xiong, Gong, Rock, Subramanian, Guo, Xu, et al., 2001). Salt stress signalling through  $\text{Ca}^{2+}$  and ABA mediate the expression of the late embryogenesis-abundant (LEA)-type genes including the dehydration-responsive element (DRE)/C-repeat (CRT) class of stress-responsive genes Cor. The activation of LEA-type genes may actually represent damage repair pathways (Xiong, Schumaker, & Zhu, 2002).

Salt and osmotic stress regulation of Lea gene expression is mediated by both ABA dependent and independent signalling pathways. Both the pathways use  $\text{Ca}^{2+}$  signalling to induce Lea gene expression during salinity. It has been shown that ABA-dependent and -independent transcription factors may also cross talk to each other in a synergistic way to amplify the response and improve stress tolerance (Shinozaki & Yamaguchi-Shinozaki, 2000).

#### 4. Salt tolerance

The mechanisms of genetic control of salt tolerance in plants have not yet fully understood because of its complexity. There are in fact several genes controlling salinity tolerance in the different species whose effect interacts strongly with environmental conditions. Thus, genetic variation can only be demonstrated indirectly, by measuring the responses of different genotypes. Probably the most suitable response to measure is growth or yield, especially at moderate salinities (Allen, Chambers, & Stine, 1994). Salt tolerance, in fact, can be usually assessed as the percent biomass production in saline versus control conditions over a prolonged period of time (this usually correlates with yield) or in terms of survival, which is quite appropriate for perennial species (R. Munns, 2002).

Salt tolerance may vary considerably with genetic traits. A plant species' tolerance for salinity will be overridden by a sudden exposure to salinity, even if the species is a halophyte (Albert, 1975). Different adaptive mechanisms may be involved in gradual acclimation to salinity in contrast to adjustment to a sudden shock. The sensitivity to salinity of a given species may change during ontogeny. Salinity tolerances may increase or decrease depending on the plant species and/or environmental factors. For some species, salt sensitivity may be greatest at germination, whereas for other species, sensitivity may increase during reproduction (Howat, 2000; Marschner, 1986).

Plants have evolved several mechanisms to acclimatize to salinity. It is possible to distinguish three types of plant response or tolerance: a) the tolerance to osmotic stress, b) the  $\text{Na}^+$  exclusion from leaf blades and c) tissue tolerance (R Munns & Tester, 2008).

##### 4.1 Osmotic tolerance

The growth of salt-stressed plants is mostly limited by the osmotic effect of salinity, irrespective of their capacity to exclude salt, that results in reduced growth rates and stomatal conductance (Fricke *et al.* 2004; James *et al.* 2008). In fact, osmotic tolerance involves the plant's ability to tolerate the drought aspect of salinity stress and to maintain leaf expansion and stomatal conductance (Rajendran, Tester, & Roy, 2009). It was demonstrated in a study of genetic variation in tolerance to osmotic stress on 50 international durum

varieties and landraces that there is a positive relationship between stomatal conductance and relative growth rate in salt treated plants and that higher stomatal conductance is related to higher CO<sub>2</sub> assimilation rate (R.A. James, von Caemmerer, Condon, Zwart, & Munns, 2008). But if the accumulation of salts overcomes the toxic concentrations, the old leaves die (usually old expanded leaves) and the young leaves, no more supported by the export of photosynthates, undergo a reduction of growth and new leaves production. For this reason increased osmotic tolerance involves an increased ability to continue production and growth of new and greater leaves, and higher stomatal conductance. The resulting increased leaf area would benefit only plants that have sufficient soil water, such as in irrigated food production systems where a supply of water is ensured, but could be undesirable in water-limited systems (R Munns & Tester, 2008). At the end, while the mechanisms involved in osmotic tolerance related to stomatal conductance, water availability and therefore to photosynthetic capacity to sustain carbon skeletons production to meet the cell's energy demands for growth have not been completely unraveled, it has been demonstrated that the plant's response to the osmotic stress is independent of nutrient levels in the growth medium (Hu, Burucs, von Tucher, & Schmidhalter, 2007).

#### 4.2 Na<sup>+</sup> exclusion

In the majority of plant species grown under salinity, Na<sup>+</sup> appears to reach a toxic concentration before Cl<sup>-</sup> does, and so most studies have concentrated on Na<sup>+</sup> exclusion and the control of Na<sup>+</sup> transport within the plant (R Munns & Tester, 2008). Therefore, another essential mechanism of tolerance involves the ability to reduce the ionic stress on the plant by minimizing the amount of Na<sup>+</sup> that accumulates in the cytosol of cells, particularly those in the transpiring leaves. This process, as well as tissue tolerance, involves up- and down-regulation of the expression of specific ion channels and transporters, allowing the control of Na<sup>+</sup> transport throughout the plant (R Munns & Tester, 2008; Rajendran, Tester, & Roy, 2009). Na<sup>+</sup> exclusion from leaves is associated with salt tolerance in cereal crops including rice, durum wheat, bread wheat and barley (Richard A. James, Blake, Byrt, & Munns, 2011). Exclusion of Na<sup>+</sup> from the leaves is due to low net Na<sup>+</sup> uptake by cells in the root cortex and the tight control of net loading of the xylem by parenchyma cells in the stele (Davenport, James, Zakrisson-Plogander, Tester, & Munns, 2005). Na<sup>+</sup> exclusion by roots ensures that Na<sup>+</sup> does not accumulate to toxic concentrations within leaf blades. A failure in Na<sup>+</sup> exclusion manifests its toxic effect after days or weeks, depending on the species, and causes premature death of older leaves (R Munns & Tester, 2008).

An efficient cytosolic Na<sup>+</sup> exclusion is also got through operation of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiports that move potentially harmful ions from cytosol into large, internally acidic, tonoplast-bound vacuoles. These ions, in turn, act as an osmoticum within the vacuole, which then maintain water flow into the cell, thus allowing plants to grow in soils containing high salinity. Antiports use the proton-motive force generated by vacuolar H<sup>+</sup>-translocating enzymes, H<sup>+</sup>-adenosine triphosphatase (ATPase) and H<sup>+</sup>-inorganic pyrophosphatase (PPiase), to couple downhill movement of H<sup>+</sup> (down its electrochemical potential) with uphill movement of Na<sup>+</sup> (against its electrochemical potential) “. AtNHX1 is the Na<sup>+</sup>/H<sup>+</sup> antiporter, localized to the tonoplast, predicted to be involved in the control of vacuolar osmotic potential in Arabidopsis (Apse, Aharon, Snedden, & Blumwald, 1999).

Durum wheat is a salt-sensitive species and germination and seedling stages are the most critical phases for plant growth under salinity (Flagella, Trono, Pompa, Di Fonzo, & Pastore, 2006). Its sensitivity to salt stress is higher than bread wheat, due to a poor ability to exclude

Na<sup>+</sup> from the leaf blades, and a lack of the K<sup>+</sup>/Na<sup>+</sup> discrimination character displayed by bread wheat (Gorham, Hardy, Jones, Joppa, & Law, 1987; Lauchli, James, Huang, McCully, & Munns, 2008). However, a novel source of Na<sup>+</sup> exclusion has been found in an unusual durum wheat genotype named Line 149. Genetic analysis has shown that line 149 contains two major genes for Na<sup>+</sup> exclusion, named Nax1 and Nax2 (Rana Munns, Rebetzke, Husain, James, & Hare, 2003). The proteins encoded by the Nax1 and Nax2 genes are shown to increase retrieval of Na<sup>+</sup> from the xylem in roots, thereby reducing shoot Na<sup>+</sup> accumulation. In particular the Nax1 gene confers a reduced rate of transport of Na<sup>+</sup> from root to shoot and retention of Na<sup>+</sup> in the leaf sheath, thus giving a higher sheath-to-blade Na<sup>+</sup> concentration ratio. The second gene, Nax2, also confers a lower rate of transport of Na<sup>+</sup> from root to shoot and has a higher rate of K<sup>+</sup> transport, resulting in enhanced K<sup>+</sup> versus Na<sup>+</sup> discrimination in the leaf (R. James, Davenport, & Munns, 2006). The mechanism of Na<sup>+</sup> exclusion allows the plant to avoid or postpone the problem related to ion toxicity, but if Na<sup>+</sup> exclusion is not compensated for by the uptake of K<sup>+</sup>, it determines a greater demand for organic solutes for osmotic adjustment. The synthesis of organic solutes jeopardizes the energy balance of the plant. Thus, the plant must cope ion toxicity on the one hand, and turgor loss on the other (R Munns & Tester, 2008).

The knowledge on how Na<sup>+</sup> is sensed is still very limited in most cellular systems. Theoretically, Na<sup>+</sup> can be sensed either before or after entering the cell, or both. Extracellular Na<sup>+</sup> may be sensed by a membrane receptor, whereas intracellular Na<sup>+</sup> may be sensed either by membrane proteins or by any of the many Na<sup>+</sup>-sensitive enzymes in the cytoplasm. In spite of the molecular identity of Na<sup>+</sup> sensor(s) remaining elusive, the plasma-membrane Na<sup>+</sup>/H<sup>+</sup> antiporter SALT OVERLY SENSITIVE1 (SOS1) is a possible candidate (Silva & Gerós, 2009). In fact, in Arabidopsis, ion homeostasis is mediated mainly by the SOS signal pathway (Yang et al. 2009). SOS proteins are sensor for calcium signal that turn on the machinery for Na<sup>+</sup> export and K<sup>+</sup>/Na<sup>+</sup> discrimination (Zhu, 2007). In particular, SOS1, encoding a plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter, plays a critical role in Na<sup>+</sup> extrusion and in controlling long-distance Na<sup>+</sup> transport from the root to shoot (Shi, Ishitani, Kim, & Zhu, 2000; Shi, Quintero, Pardo, & Zhu, 2002). This antiporter forms one component in a mechanism based on sensing of the salt stress that involves an increase of cytosolic [Ca<sup>2+</sup>], protein interactions and reversible phosphorylation with SOS1 acting in concert with other two proteins known as SOS2 and SOS3 (Oh, Lee, Bressan, Yun, & Bohnert, 2010) (Fig. 4).

Both the protein kinase SOS2 and its associated calcium-sensor subunit SOS3 are required for the posttranslational activation of SOS1 Na<sup>+</sup>/H<sup>+</sup> exchange activity in Arabidopsis (Qiu, Guo, Dietrich, Schumaker, & Zhu, 2002; Quintero, Martinez-Atienza, Villalta, Jiang, Kim, Ali, et al., 2011), and in rice (Martínez-Atienza, Jiang, Garcíadeblas, Mendoza, Zhu, Pardo, et al., 2007). In yeast, co-expression of SOS1, SOS2, and SOS3 increases the salt tolerance of transformed yeast cells much more than expression of one or two SOS proteins (Quintero, Ohta, Shi, Zhu, & Pardo, 2002), suggesting that the full activity of SOS1 depends on the SOS2/SOS3 complex. Recently, SOS4 and SOS5 have also been characterized. SOS4 encodes a pyridoxal (PL) kinase that is involved in the biosynthesis of pyridoxal-5-phosphate (PLP), an active form of vitamin B6. SOS5 has been shown to be a putative cell surface adhesion protein that is required for normal cell expansion. Under salt stress, the normal growth and expansion of a plant cell becomes even more important and SOS5 helps in the maintenance of cell wall integrity and architecture (Mahajan, Pandey, & Tuteja, 2008).



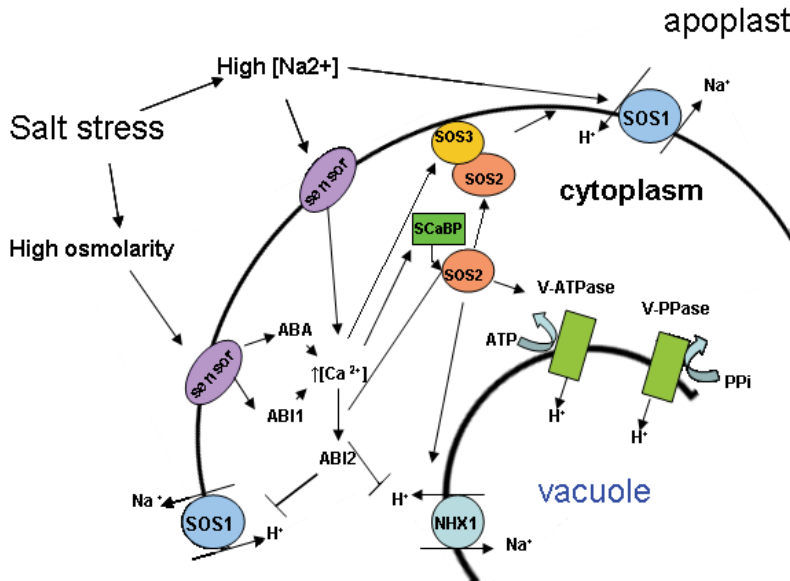


Fig. 4. Signalling pathways responsible for Na<sup>+</sup> extrusion in Arabidopsis under salt stress. Excess Na<sup>+</sup> and high osmolarity are separately sensed by unknown sensors at the plasma membrane level, which then induce an increase in cytosolic [Ca<sup>2+</sup>]. This increase is sensed by SOS3 which activates SOS2. The activated SOS3-SOS2 protein complex phosphorylates SOS1, the plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter, resulting in the efflux of Na<sup>+</sup> ions. SOS2 can regulate NHX1 antiport activity and V-H<sup>+</sup>-ATPase activity independently of SOS3, possibly by SOS3-like Ca<sup>2+</sup>-binding proteins (SCaBP) that target it to the tonoplast. Salt stress can also induce the accumulation of ABA, which, by means of ABI1 and ABI2, can negatively regulate SOS2 or SOS1 and NHX1. Adapted from Silva & Gerós (2009).

### 4.3 Tissue tolerance

The third mechanism, tissue tolerance entails an increase of survival of old leaves. It requires compartmentalization of Na<sup>+</sup> and Cl<sup>-</sup> at the cellular and intracellular level to avoid toxic concentrations within the cytoplasm, especially in mesophyll cells in the leaf (R Munns & Tester, 2008) and synthesis and accumulation of compatible solutes within the cytoplasm. Compatible solutes play a role in plant osmotolerance by various ways, protecting enzymes from denaturation, stabilising membrane or macromolecules or playing adaptive roles in mediating osmotic adjustment (Ashraf & Foolad, 2007). The function of the compatible solutes is not limited to osmotic balance. Compatible solutes are typically hydrophilic, and may be able to replace water at the surface of proteins or membranes, thus acting as low molecular weight chaperones (Hasegawa, Bressan, Zhu, & Bohnert, 2000). These solutes also function to protect cellular structures through scavenging ROS (Hasegawa et al., 2000; Zhu, 2001). Compatible solutes are small molecules, water soluble and uniformly neutral with respect to the perturbation of cellular functions, even when present at high concentrations (Sakamoto & Murata, 2002; Yancey, Clark, Hand, Bowlus, & Somero, 1982). They comprise nitrogen containing compounds such as amino acids, amines and betaines, but also organic acids, sugars and polyols (Mansour, 2000) (Fig. 5).

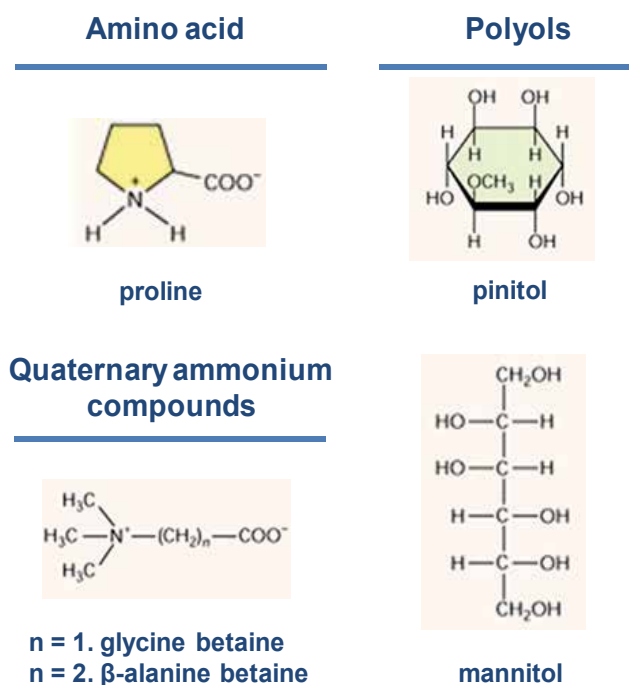


Fig. 5. Chemical structures of some important compatible compounds.

Among the best known compatible solutes, proline and glycine betaine (GB) have been reported to increase greatly under salt and drought stresses (R. Munns, 2002; Sakamoto & Murata, 2002) and constitute the major metabolites found in durum wheat under salt stress, as in other Poaceae (Ashraf & Foolad, 2007; Carillo, Mastrodonato, Nacca, & Fuggi, 2005; Sairam & Tyagi, 2004). In many halophytes, proline and/or glycine betaine concentrations in leaves contribute to the osmotic pressure in the cell as a whole (T. J. Flowers, Troke, & Yeo, 1977). In glycophytes, their concentrations are much lower but if partitioned exclusively to the cytoplasm, they could generate a significant osmotic pressure and then balance the vacuolar osmotic potential. In durum wheat seedlings, proline can contribute for more than 39% of the osmotic adjustment in the cytoplasmic compartments of old leaves, while the contribution of GB can account for up to 16% of the osmotic balance in younger tissues, independently of nitrogen nutrition, unlike proline (Carillo et al. 2008).

Proline (Fig. 5) is a proteinogenic amino acid with an exceptional conformational rigidity, essential for primary metabolism, which normally accumulates in large quantities in response to drought or salinity stress (Ashraf & Foolad, 2007; Carillo, Mastrodonato, Nacca, Parisi, Verlotta, & Fuggi, 2008; Hasegawa, Bressan, Zhu, & Bohnert, 2000; Szabados & Savouré, 2010). Its accumulation normally occurs in the cytosol where it contributes substantially to the cytoplasmic osmotic adjustment (Ketchum, Warren, Klima, Lopezgutierrez, & Nabors, 1991).

Proline accumulation is due primarily to de novo synthesis associated with decreased oxidation and utilization, but increased transport processes are also likely involved (Aubert, Hennion, Bouchereau, Gout, Bligny, & Dorne, 1999; Flagella, Trono, Pompa, Di Fonzo, & Pastore, 2006; Kishor, Hong, Miao, Hu, & Verma, 1995). Proline accumulation occurs rapidly after the onset of stress and this supports the hypothesis that this accumulation is initially a

reaction to salt stress and not a plant response associated with tolerance (Carillo, Mastrolonardo, Nacca, Parisi, Verlotta, & Fuggi, 2008; de Lacerda, Cambraia, Oliva, Ruiz, & Prisco, 2003). In addition to its role as an osmolyte for osmotic adjustment, proline contributes to stabilizing sub-cellular structures (e.g. membranes and proteins), scavenging free radicals, and buffering cellular redox potential under stress conditions. It may also function as a protein compatible hydrotrope (Srinivas & Balasubramanian, 1995), alleviating cytoplasmic acidosis, and maintaining appropriate NADP<sup>+</sup>/NADPH ratios compatible with metabolism (Hare & Cress, 1997). Also, rapid breakdown of proline upon relief of stress may provide sufficient reducing agents that support mitochondrial oxidative phosphorylation and generation of ATP for recovery from stress and repairing of stress-induced damages (Carillo, Mastrolonardo, Nacca, Parisi, Verlotta, & Fuggi, 2008; Hare & Cress, 1997). Furthermore, proline is known to induce expression of salt stress responsive genes, which possess proline responsive elements (e.g. PRE, ACTCAT) in their promoters (Ashraf & Foolad, 2007; Chinnusamy, Jagendorf, & Zhu, 2005).

GB (Fig. 5) is an amphoteric compound that is electrically neutral over a wide range of physiological pH values and is extremely soluble in water despite a non-polar hydrocarbon moiety that consists of three methyl groups. The molecular features of GB allow it to not only act as an osmoregulator but also to interact with both hydrophilic and hydrophobic domains of macromolecules, such as protein complexes (Sakamoto and Murata 2002; Chen and Murata 2008) and enzymes, stabilising their structures and activities, and maintaining the integrity of membranes against the damaging effects of excessive salt, cold, heat and freezing (Rhodes and Hanson 1993; Gorham 1995; Sulpice *et al.* 1998; Sakamoto and Murata 2002). It has also been suggested that GB plays a role as a scavenger of ROS generated during these various stresses (Papageorgiou and Murata 1995; Ashraf and Foolad 2007). A comparison of near-isogenic maize lines with contrasting GB accumulation showed that lines that were homozygous for the *Bet1* (GB accumulation) gene had a 10–20% higher biomass under saline conditions (Saneoka *et al.* 1995; Munns and Tester 2008). However, while proline is probably the most widely distributed osmolyte accumulated in plants (Delauney and Verma 1993; Szabados and Savoure 2010), the occurrence of GB seems to be restricted to several halophytes and a few crop plants (Weretilnyk *et al.* 1989). Metabolic engineering of GB biosynthesis by the insertion of foreign genes from plants or microbes in plants not naturally accumulating it improved their tolerance to salt, drought and extreme temperature stresses, despite the very low amounts of GB accumulated by these plants (Sakamoto and Murata 2002; Sulpice *et al.* 2003; Quan *et al.* 2004; Chen and Murata 2008; Ashraf and Akram 2009).

#### 4.4 Engineering salt tolerance in plants

Several efforts have been undertaken to enhance the salt tolerance of economically important plants by traditional plant breeding as well as by biotechnological approaches. Traditional breeding programs trying to improve abiotic stress tolerance have had some success, but are progressing relatively slow being limited by the polygenic inheritance (Silva & Gerós, 2009). In fact, the susceptibility or tolerance to salinity in plants is a coordinated action of multiple stress responsive genes, which also cross talk with other components of stress signal transduction pathways. Therefore, the inconsistent results obtained employing traditional approaches may be ascribed to the difficulty to identify genomic regions controlling resistance in “Perennial” quantitative trait locus (QTL) end to the long time (also twenty years) required for their introgression by breeding.

Molecular marker technology has developed rapidly over the last decade, with the development of high-throughput genotyping methods that have made it possible to analyze the QTL's responsible for tolerance. The identification of these regions is fundamental for helping the selection efficiency in breeding programs and mapping the major genes controlling salt tolerance in order to operate genetic manipulations using the real candidate genes rather than non-specific abiotic responsive genes. Many studies have focused on mapping QTLs for salt tolerance-related traits in rice because of its requirement for irrigation for maximum yield, its sensitivity to salinity and its relatively small genome (T.J. Flowers, Koyama, Flowers, Sudhakar, Singh, & Yeo, 2000). Better results have been obtained at the seedling stage, while the expression and relationship of QTLs detected in different developmental stages are more difficult to study and fully understand at tillering and reproductive stages (Alam, Sazzadur Rahman, Seraj, Thomson, Ismail, Tumimbang-Raiz, et al., 2011; Prasad, Bagali, Hittalmani, & Shaahidhar, 2000). Recently, QTLs related to antioxidant content and the response of tomato antioxidants to salt-stress have also been identified. Although these QTLs may be useful for the development of higher antioxidant tomato cultivars, whether or not a direct correlation between antioxidant levels and salinity tolerance exists is more difficult to prove (Frery, Gol, Keles, Okmen, Pinar, Sigva, et al., 2010). Contrary to the notion that multiple traits introduced by breeding into crop plants are needed to implement salt-tolerant plants, one of the main strategies for improving plant salt tolerance has been through the overexpression of single genes that are either induced by stress and/or have been shown to be required for normal levels of tolerance. Transgenic plants overexpressing the genes participating in the synthesis or accumulation of osmoprotectants that function for osmotic adjustment, such as proline (Kishor, Hong, Miao, Hu, & Verma, 1995), glycinebetaine (Holmström, Somersalo, Mandal, Palva, & Welin, 2000) or other osmolytes show increased salt tolerance. Other genes that encode enzymes that are involved in oxidative protection, such as glutathione S-transferase, peroxidase, superoxide dismutase, ascorbate peroxidases, and glutathione reductases, can also be modified to improve plant salt tolerance (Yang, Chen, Zhou, Yin, Li, Xin, et al., 2009). Overexpression of regulatory genes in signalling pathways, such as transcription factors (DREB/CBF) and protein kinases (MAPK, CDPK) also increases plant salt tolerance (Chen, Ren, Zhong, Jiang, & Li, 2010). The overexpression of the vacuolar  $\text{Na}^+/\text{H}^+$  antiporter has shown to improve salinity tolerance in several plants (Silva & Gerós, 2009). The first evidence showed that the overexpression of AtNHX1 in Arabidopsis plants promoted sustained growth and development in soil watered with up to 200 mM NaCl (Apse, Aharon, Snedden, & Blumwald, 1999), although recent evidences report that transgenic Arabidopsis do not show a significantly improved salt tolerance as compared to that of control plants (Yang, et al., 2009). In addition, transgenic tomato plants overexpressing AtNHX1 were able to grow, flower and produce fruit in the presence of 200 mM NaCl (H.-X. Zhang, Hodson, Williams, & Blumwald, 2001; H. Zhang & Blumwald, 2001). Also, transgenic tobacco plants overexpressing GhNHX1 from cotton and transgenic rice overexpressing the  $\text{Na}^+/\text{H}^+$  antiporter gene clone from OsNHX1 exhibited higher salt tolerance (Fukuda, Nakamura, Tagiri, Tanaka, Miyao, Hirochika, et al., 2004; Wu, Yang, Meng, & Zheng, 2004). Overexpression of AtNHX1 in *Petunia hybrida* enhanced salt and drought tolerance in this plant, which accumulated more  $\text{Na}^+$ ,  $\text{K}^+$ , and proline in their leaf tissue than that of the WT *Petunia* plants, maintaining high water contents and high ratio of  $\text{K}^+/\text{Na}^+$  (Xu, Hong, Luo, & Xia, 2009). By introgressing *Nax* genes from *Triticum monococcum* into hexaploid bread wheat (*Triticum aestivum*), the leaf blade  $\text{Na}^+$  concentration was reduced by 60% and the

proportion of Na<sup>+</sup> stored in leaf sheaths was increased. The results indicate that *Nax* genes have the potential to improve the salt tolerance of bread wheat (Richard A. James, Blake, Byrt, & Munns, 2011). The increased expression in tomato and rice of *Arabidopsis* Arginine Vasopressin 1 (AVP1), encoding a vacuolar pyrophosphatase acting as a proton pump on the vacuolar membrane, enhanced sequestering of ions and sugars into the vacuole, reducing water potential and resulting in increased salt tolerance when compared to wild-type plants (Pasapula, Shen, Kuppu, Paez-Valencia, Mendoza, Hou, et al., 2011). Furthermore, overexpression of genes encoding Late Embryogenesis Abundant (LEA) proteins, which accumulate to high levels during seed development, such as the barley HVA1 (Xu et al., 1996) and wheat dehydrin DHN-5 (Brini et al., 2007), can enhance plant salt tolerance, although their function is obscure.

## 5. Conclusions

Salinity is a significant problem affecting agriculture worldwide and is predicted to become a larger problem in the coming decades (<http://www.fao.org/ag/agl/agll/spush/>). The detrimental effects of high salinity on plants can be observed at the whole-plant level in terms of plant death and/or decrease in productivity (Parida & Das, 2005). Some plant species are clearly more flexible than others in their requirements for survival in salty environments. An understanding of how single cell responses to salt are coordinated with organismal and whole-plant responses to maintain an optimal balance between salt uptake and compartmentation is fundamental to our knowledge of how plants successfully adapt to salt stress. (Volkmar, Hu, & Steppuhn, 1998). Use of both genetic manipulation and traditional breeding approaches will be required to unravel the mechanisms involved in salinity tolerance and to develop salt-tolerant cultivars better able to cope with the increasing soil salinity constraints (Rajendran, Tester, & Roy, 2009). It is important to underline that transgenic technology is certainly useful for aiding the search for the cellular mechanisms that increase tolerance, but the complexity of the traits is likely to mean that the road to engineering such tolerance into sensitive species will be long (T. J. Flowers, 2004). Anyway, there are a number of reasons for optimism. These include recent developments in the area of plant molecular biology, among which in particular the complete sequencing of model plant genomes, the production of T-DNA insertional lines of *Arabidopsis* for gene tagging and the availability of microarray analysis tools which offer advantages and solutions to the complex intriguing questions of salt resistance and tolerance (Hussain, Chandrasekhar, Hazara, & Sultan, 2008).

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# Abiotic Stress in Harvested Fruits and Vegetables

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## 1. Introduction

Harvested fruits and vegetables can be potentially exposed to numerous abiotic stresses during production, handling, storage and distribution (Hodges, 2003). Some of these stresses can be minor in nature, resulting in no quality loss or, in some cases, in quality improvement (Hodges et al., 2005) during distribution. However, when the abiotic stress is moderate or severe, quality losses almost always are incurred at market (Toivonen, 2003a&b). As a consequence it is important to understand the nature and sources for abiotic stresses that affect fruits and vegetables. In addition, with improved understanding, options for better management or resistance become available (Toivonen, 2003b; Toivonen, 2005). One of the challenges facing fruit and vegetable production globally is that regional climate regimes are becoming more unpredictable from year to year. Hence understanding of effects of field abiotic stresses (e.g. drought, extreme temperatures, light and salinity) on postharvest stress susceptibility will become more important since postharvest stresses limit the storage and shelf life potential of fruits and vegetables (Toivonen, 2005). It is the intent of this chapter to first describe the nature of pre- and post-harvest abiotic stress events, delve into their importance for product quality and marketing and then explore the technologies available to begin managing the sensitivity of fruits and vegetables to stresses they encounter in the handling and distribution chain.

## 2. Major preharvest stresses which influence postharvest abiotic stress response

Abiotic stresses occurring during production can either be the primary cause (direct) for disorders that exhibit themselves during postharvest handling and storage practices or they can influence the susceptibility of a fruit or vegetable to postharvest conditions that cause abiotic stresses resulting in disorders (indirect) (Ferguson et al., 1999). It is important to characterize the relationship between preharvest abiotic stresses occurring during production and postharvest abiotic stresses that the fruit or vegetable is exposed after harvest and during storage and distribution, since the solution to these different problems will be best resolved by focusing on preharvest or postharvest abiotic stress amelioration, respectively. Moderate levels of preharvest stress can potentially work towards enhancing stress resistance of a fruit or vegetable through up-regulating genes and pathways which

renders the tissues cross-tolerant to many stresses (Lesham & Kuiper, 1996; Bowler & Fluhr, 2000) which may occur subsequently in postharvest handling, storage and distribution.

## 2.1 Drought

The occurrence of drought conditions during production of fruit and vegetable crops is becoming more frequent with climate change patterns (Whitmore, 2000). While much work has been devoted to understanding of drought effects on production and productivity of these crops (Whitmore, 2000), there is limited published literature on the effects of pre-harvest water stress on responses to postharvest stresses and hence on subsequent quality and shelf life. However, the existing literature provides some insight which may lead to better understanding and perhaps also encourage future research.

Water stress during the production phase of some fruits and vegetables may affect their physiology and morphology in such a manner as to influence susceptibility to weight loss in storage. There have been both positive effects reported for field water deficits (stress) in tree fruits and root vegetables. In the case of peaches, it has been shown that lower levels of irrigation results in higher density of fruit surface trichomes and consequent lower weight losses in storage (Crisosto et al., 1994). In addition, two studies have shown that deficit irrigation of apples and pears could reduce water loss of these fruit in subsequent storage (Kilili et al., 1996; Lopez et al., 2011) and this was attributed to reduction in skin permeance of the deficit irrigated fruit (Kilili et al., 1996). Presumably, fruit grown under moderate water stresses imposed by deficit irrigation practices adapt by developing a less water permeable cuticle. In terms of understanding that water deficits can have negative effects on postharvest stress susceptibility, irrigation of apples has been shown to enhance apple size which was associated with lower to water losses during storage (Veličković, 1994). This observation highlights a main concern about using deficit irrigation, which is the reduced size of fruit from such treatments (Lopez et al., 2011). Size of fruit is important, since larger fruit have lower surface area to volume ratios, which confers lower relative water loss (Shibairo et al., 1997) Another negative affect associated with water deficits is the case of root vegetables, such as carrot, where preharvest water stress (watering to 25-75% of soil water field capacity) can weaken the cells, resulting in higher membrane leakage (i.e. cell damage) and consequently greater weight loss in storage (Shibairo et al., 1998b).

Timing of a water stress event can also be very important in determining response to postharvest abiotic stress response. One example is that 'Kensington' mango fruit (*Mangifera indica* L.) will be significantly more susceptible to postharvest chilling injury with exposure to water stress during the cell expansion phase of growth as opposed to being exposed to the stress during cell division or at a time near to harvest maturity (Léchaudel and Joas, 2007). Therefore it is critical to avoid water stress until the fruit has reached maximum size in order to minimize incidence of chilling-induced injury in storage. Water stress, particularly at the tuber forming stage, can also lead to a higher susceptibility of potatoes to postharvest development of black spot disorder (Hamouz et al., 2011). Black spot disorder is correlated primarily to susceptibility of cells in the potatoes to undergo decompartmentation in response to mechanical stress (i.e. bruising) (Stevens and Davelaar, 1997).

## 2.2 Plant nutrition

There is limited literature regarding the effects of crop nutrition on the susceptibility of fruits and vegetables to postharvest abiotic stress. There is one review dealing with the

effect of preharvest nutrition on postharvest physiology and disorders of fruits and vegetables (Sams and Conway, 2003), however most of the reviewed literature touches on nutrition effects on postharvest biotic stress effects (i.e. disease resistance).

Calcium nutrition during production has been well documented in regard to postharvest disorders of many fruits and some vegetables (Sams and Conway, 2003). Calcium is also been suggested as a putative signalling molecule involved in the development of cross-tolerance to abiotic stresses (Bowler and Fluhr, 2000). Therefore the role of preharvest calcium nutrition is postharvest stress resistance may be complex, and dependent on whether the fruit or vegetable is also exposed to environmental abiotic stresses.

Potassium nutrition has been shown to have a few important effects on postharvest abiotic stress susceptibility of vegetables. In carrots, deficiency in potassium is associated with greater weight loss (desiccation stress) in storage (Shibairo et al., 1998c). At levels below 1 mM potassium in the soil medium, weight loss was directly associated with increased membrane leakage (i.e. damaged cells) in the carrot tissues. Above 1 mM potassium, there were no significant differences in weight loss under standardized storage conditions (Shibairo et al., 1998c). Improved potassium nutrition has also been shown to reduce susceptibility of potatoes to internal bruising in response to mechanical stresses imposed during postharvest handling (Maier et al., 1986; McGarry et al., 1996).

Relatively high preharvest nitrogen is often associated with poor postharvest quality of many fruits and vegetables (Sams and Conway, 2003). In regards to affecting susceptibility to postharvest stress, applying higher than recommended levels of preharvest nitrogen for a specific crop have been linked to storage discoloration susceptibility in both cabbage and potato. In the case of cabbage, it appears that excessive nitrogen fertilization leads to high accumulations of zinc and aluminum and nitrate induced manganese deficiency (Berard et al., 1990). High nitrogen applications in the field resulted in increased incidence and severity of black midrib in cold storage, particularly for the susceptible cultivar, 'Safekeeper' (Berard, 1990). In the case of potatoes, black spot susceptibility (a consequence of bruising; Stevens and Davelaar, 1997) is influenced by nitrogen fertilization, particularly the balance of nitrogen applied in relation to levels of potassium applied (Horneburg and Wirsing, 1995). In contrast nitrogen deficiency or lower than recommended nitrogen application rates will most often results in increased vitamin C content in many fruits and vegetables (Lee & Kader, 2000). Vitamin C content has been tightly linked with storage life potential (Hodges et al., 2001), which is likely a consequence of the importance of this antioxidant nutrient in forestalling oxidative injury that leads to quality losses in storage (Noctor and Foyer, 1998).

### **2.3 Temperature extremes**

Susceptibility to high (heat injury inducing) or low (chill injury inducing) temperatures is known to be reduced by prior exposure of the sensitive fruit or vegetable to low ambient temperatures (Saltveit and Morris, 1990; Wang, 1990). However, if the preharvest temperature leads to chilling induced injury in the field, then susceptibility to postharvest chilling injury can be increased (Morris, 1954). Therefore, the level of the preharvest temperature extreme will be a determinant as to if the exposure will have positive or negative effects on postharvest stress sensitivity.

Extreme high temperatures can occur in the field and apple fruit exposed to direct sunlight can reach in excess of 40 °C (Ferguson et al., 1999). High temperatures during the late season

(leading up to harvest) can enhance susceptibility of apples to superficial scald which develops in storage (Bramlage and Weis, 1997). In contrast, the authors found that low temperatures in the preharvest interval could reduce susceptibility.

## 2.4 Salinity

Tomatoes grown under high salinity will produce smaller fruit with higher soluble solids (Mizrahi, 1982). Smaller fruit will have higher surface area to volume ratios, hence greater susceptibility to postharvest water loss (i.e. desiccation stress) (Shibairo et al., 1997). While there is no direct information in the literature to confirm that smaller tomato fruit from saline growing conditions would be subject to greater desiccation stress postharvest, firmness declines for tomatoes grown under 3 and 6 dS m<sup>-1</sup> salinity levels were increased by 50 to 130%, respectively, at two weeks holding at 20 °C compared with control fruit (Mizrahi et al., 1988).

## 2.5 Light

It would be considered logical to assume that effects of exposure to high light are difficult to dissociate from effects exposure to high temperatures. However, research has shown that low light (bagging of apples) in the preharvest interval reduced susceptibility of apples to developing superficial scald in cold storage (Barden and Bramlage, 1994) and, in contrast, high ambient temperatures resulted in increased susceptibility (Bramlage and Weis, 1997). Generally, only sun-exposed surfaces of susceptible cultivars of apples develop scald in storage (Ferguson et al., 1999).

In the case of ambient low light, when lettuce is grown under low light which is sub-optimal for photosynthetic activity, shelf life of fresh cut lettuce (i.e. lettuce subjected to mechanical stress) is much shorter than lettuce produced under optimal light conditions (Witkowska & Woltering, 2010). Tomato size is smaller when the crop is grown under ambient low light levels, such as in the early spring season in northern latitudes (Gruda, 2005) and since surface area to volume ratio is greater in smaller fruits, susceptibility to postharvest desiccation stress would increase (Shibairo et al., 1997). Low light also results in lower levels of ascorbate in many greenhouse-grown fruits and vegetables (Gruda, 2005), which would render them less fit to deal with postharvest stresses since ascorbate contents are general directly proportional to relative levels or stress tolerance (Noctor and Foyer, 1998).

## 3. Postharvest stresses during handling and storage

### 3.1 Temperature extremes

Postharvest temperature abuse during distribution is an ongoing challenge for many products, particularly those being shipped by air or ocean container (East et al., 2008). Breaks in cool chain temperatures can result in acceleration of climacteric ripening and softening (i.e. reduction in shelf potential) for apples harvested and stored at the pre-climacteric stage of maturity (East et al., 2008). However, those authors also found that temperature breaks had minimal effects on apples harvested and stored at post-climacteric stage of maturity. Fruit harvested at post-climacteric stages of maturity are not generally of concern to industry since those fruit have shorter storage and shelf life potential (Toivonen and Beveridge, 2005).

Chilling injury susceptibility is a significant issue for many crops derived from subtropical and tropical growing regions. Generally fruits, fruit vegetables (fruits which are consumed as

vegetables) and root and tuber crops are chilling sensitive (Kader, 2002; Toivonen, 2010). There is a significant literature pertaining to chilling sensitivity of crops and much effort has been devoted to better understanding of chilling injury mechanisms and approaches to ameliorating the disorder (Wang, 1993; Saltveit, 2003). While there are visible (surface pitting, internal browning) and textural (accelerated softening and development of mealiness) changes often associated with chilling injury (Wang, 1993; Saltveit, 2003), flavour generation capacity has been shown to be a sensitive early indicator of chilling stress effects (Maul et al., 2000).

Use of heat treatments has become popular for disinfestation and disinfection of fruits and vegetables (Lurie, 1998). Appropriately applied levels of heat treatment can also induce temperature tolerance to both high and low temperatures (Lurie, 1998). However, when excessively harsh heat treatments are applied, heat-induced damage can occur.

### 3.2 Low O<sub>2</sub> and high CO<sub>2</sub>

Managing postharvest handling and storage atmospheres to avoid low O<sub>2</sub> or high CO<sub>2</sub> stress is a constant concern and the problem is more severe when handling products in modified atmosphere (MA) packages as opposed to controlled atmosphere (CA) systems since temperature is often not as easily controlled in the MA packages as the produce moves through a distribution chain (Toivonen, 2003a; Toivonen et al., 2009). As such, the importance atmospheric stress in postharvest systems deserves significant discussion.

While low O<sub>2</sub> levels are well-known to induce stress-induced changes in metabolism and resultant metabolite accumulations (Kanellis et al., 2009), acute low oxygen injury is not expressed until the tissue is re-aerated (Biemelt et al., 1998) and a consequent uncontrolled oxygen burst (consisting of hydrogen peroxide and other radicals) occurs, resulting in lipid peroxidation, protein denaturation and membrane injury (Blokchina et al., 2001, 2003). Different fruits and vegetables have varying thresholds for low O<sub>2</sub> stress, dependent on anatomy, temperature, physiological age, presence of supplemental gases (e.g. CO<sub>2</sub>, CO, SO<sub>2</sub>, C<sub>2</sub>H<sub>4</sub>) and duration of exposure (Kader and Saltveit, 2003; Kanellis et al., 2009). Threshold limits for low O<sub>2</sub> tolerance can range from approximately 10 kPa partial pressure (for early potatoes and asparagus) down to approximately 1 kPa partial pressure (for mushroom, broccoli, and chipping potatoes), assuming that the produce is cooled to its recommended temperature when it is placed into that atmosphere (Kader and Saltveit, 2003). When a fruit or vegetable is converted to fresh-cut format, it generally becomes tolerant to lower levels of oxygen.

One of the most notable effects of high CO<sub>2</sub> levels in postharvest handling is to competitively inhibit ethylene binding and action hence delaying ripening in climacteric fruits (Kanellis et al., 2009). High CO<sub>2</sub> will directly inhibit succinate dehydrogenase, thus impairing the functioning of the tricarboxylic acid cycle and aerobic respiration. There are numerous physiological disorders that can be attributed to high CO<sub>2</sub> stress, including black heart of potatoes (Davis, 1926), brown heart or core in apples and pears (Meheriuk et al., 1994), surface bronzing in apples (Meheriuk et al., 1994) and brown stain of lettuce (Kader and Saltveit, 2003). High CO<sub>2</sub> can also modulate chilling stress, ethylene induced disorders and susceptibility to pathogenic attack (Kader and Saltveit, 2003).

### 3.3 Mechanical injury

There are two types of mechanical injury that can be incurred during harvest and handling of fruits and vegetables; 1) cuts or punctures, and 2) impacts leading to bruises. Cuts can

lead to transitory increases in respiration, ethylene production, phenolics production and cell deterioration near the site of the injury (Toivonen and DeEll, 2002 ; Toivonen et al., 2005). Several factors influence the severity and size of bruising sustained, including maturity, water potential, tissue or cellular orientation at the site of the injury, shape of the object imparting the bruising force, energy and angle of the impact, and temperature of the product (Miller, 2003).

Cut type injuries are most prevalent in fresh-cut fruit and vegetable products (Toivonen and DeEll, 2002). Severity of response to cutting is very much dependant on the tissue characteristics, maturity of the fruit or vegetable of interest, the coarseness or sharpness of the cutting implement used and the temperature at which the cutting is done (Toivonen and DeEll, 2002). Cut injuries occur during the harvest process of many fruits and vegetables and is more severe in machine-harvested as compared with hand-harvested product (Miller, 2003). Products such as tomatoes, pickling cucumber, snap beans, green peas, potatoes, garlic can sustain significant damage during machine harvest, whereas asparagus, Brussels sprouts, leafy greens and head lettuce are more tolerant (Miller, 2003). Cutting associated disorders are primarily visualized as cut-edge browning or blackening, but may also include yellowing in green tissues and whitening on carrots (Toivonen and DeEll, 2002).

Impact caused injuries are associated with loading of product for transport, events during transport (particularly when uneven or rough roads or lanes are encountered), during unloading and throughout packaging and processing lines (Miller, 2003). Impact bruising has been shown to induce significant levels of ethylene production in mature-green tomatoes, with the levels produced being concomitant with the number of impacts sustained (MacLeod et al., 1976). Both physically visible internal damage and slight declines in ascorbic acid content of the fruit were observed in response to the impact events imposed on the fruit. Impact bruising leads to development of internal black spots in potato tubers (Stevens and Vavelaar, 1997).

### 3.4 Desiccation

Loss of water leading to deterioration in fruit and vegetable tissues is a common issue for postharvest handling and distribution (Ben-Yehoshua and Rodov, 2003). In addition to wilting (Ben-Yehoshua and Rodov, 2003), water stress can lead to accelerated senescence or ripening which are expressed as softening of tissues, membrane deterioration and yellowing (Lurie et al., 1986; Burden et al., 1994). As mentioned previously, one of the main characteristics of a fruit or vegetable that defines susceptibility to water loss is surface area to volume ratio (Shibairo et al., 1997b). Burton (1982) described a range of surface area to volume ratios from as low as  $0.2 \text{ cm}^{-1}$  in winter cabbage or turnip to as high as  $50\text{-}100 \text{ cm}^{-1}$  in leafy vegetables, explaining differences in water loss characteristics in these very different types of vegetables. In beans, the density of hairs on the cuticle can modulate rates of water loss to some extent and damage to these hairs will lead to increased losses (Hoffman, 1967). In other fruits and vegetables, lenticel density or epicuticular wax thickness can modulate water loss (Ben-Yehoshua and Rodov, 2003).

The driving force for water loss is the vapour pressure deficit (vpd), which is the relationship that describes the difference in water activity of the fruit or vegetable and the water activity of the atmosphere surrounding it (Ben-Yehoshua and Rodov, 2003). The greater the vapour pressure deficit, the greater the water loss. Three postharvest handling principles are important in minimizing water loss of any fruit or vegetable; 1) warm product



loses water faster than cool product when placed into a cool room, hence the importance of rapid precooling before storage, 2) delays in cooling will lead to longer exposures to higher vapour pressure deficit conditions, hence timely cooling after harvest is of utmost importance, and 3) storing product at the coldest storage temperature and highest relative humidity possible will minimize water loss (Toivonen, 2010). Following these principles will result in the least desiccation stress for any fruit or vegetable.

## 4. Effects of abiotic stresses on plant metabolism

### 4.1 Metabolic changes Induced by stress

Heat stress induces metabolic changes associated with heat shock protein accumulations which are known to confer persistent levels of stress resistance in heat-exposed produce (Wang et al., 2004). Heat stress can also inhibit the production and accumulation of lycopene (Hall, 1964). The duration and temperature of exposure will determine if such an effect will occur, but tomato fruit exposed to 32 °C continuously will not turn red, remaining yellow even at full ripeness.

Chilling stress results in the accumulation of lipid peroxidation products, superoxide anions and hydrogen peroxide and also to losses in flavor volatile production in sensitive fruits and vegetables (Wang, 1993; McDonald et al., 1996; Maul et al., 2000). Chilling stress affects ethylene metabolism (Wang & Adams, 1982; Oberland et al., 2008), leading to accelerated softening. It also modifies cell wall metabolism, with resultant up-regulation of cell wall dismantling enzymes such as pectin methylesterase and endopolygalacturonase (Nilo et al., 2010).

There are two distinct phases for response to exposure to anaerobic or anoxic atmospheres. The first phase involves the metabolic shift induced by impairment of the mitochondrial electron transport chain by limitation of the primary electron acceptor, molecular O<sub>2</sub> (Hanhijärvi and Fagerstedt, 1994). The resultant impairment has been well-characterized as declines in ATP levels, pyruvate dehydroxylase activity and cytoplasmic pH (Kader and Saltveit, 2003). These conditions lead to an increase in pyruvate decarboxylase, alcohol dehydrogenase and lactate dehydrogenase activities (Kader and Saltveit, 2003). Anaerobic respiration is induced and there is an accumulation in acetaldehyde, ethanol, ethyl acetate and/or lactate (Purvis, 1997). In addition, the impairment of the mitochondrial electron transport chain results in electron leakage and consequent generation of superoxide anions and hydrogen peroxide in the cells which can be decomposed by existing cellular antioxidant systems (Blokhina et al., 2001). Ascorbate and glutathione levels can also increase during an anoxic or hypoxic event (Biemelt et al., 1998). Any or all of these changes are indicators of oxygen stress in fruit and vegetable tissues and affect quality attributes. However, actual injury to the tissue does not occur until the second phase, where the fruit or vegetable is returned to higher O<sub>2</sub> atmospheres. On return to aerobic atmospheres, rates of oxygen radical production in the impaired electron transport chain becomes accelerated, leading to great accumulations of superoxide anion, hydrogen peroxide and hydroxyl radical that cannot be fully decomposed by existing antioxidant protection systems and consequent lipid peroxidation and protein denaturation ensues, leading to membrane damage, enzymatic browning and cell death (Blokhina et al., 2001, 2003).

Kubo et al. (1990) found that different fruits and vegetables responded in different ways to high CO<sub>2</sub>. Those fruits and vegetables showing no change in O<sub>2</sub> uptake (respiration) in response to 60% CO<sub>2</sub> exposure did not show any change in ethylene production. Those

showing a decline in respiration generally also showed a decline in ethylene production, except banana (Kubo et al., 1990) and those which showed a significant increase in respiration also showed an increase in ethylene production (cucumber, eggplant, podded pea, spinach and iceberg lettuce). This suggests that these latter vegetables were more sensitive to CO<sub>2</sub> stress than the other fruits and vegetables tested. Mathooko et al. (1998) determined that CO<sub>2</sub> stress-induced ethylene biosynthesis was likely regulated at a post-transcriptional level and it involves the *de novo* synthesis of novel protein(s). They also identified that protein phosphorylation and dephosphorylation processes as being required in one or more of the steps leading to the induction of ACC synthase, which is the last enzyme in the ethylene biosynthesis pathway.

Initial response to wounding stress is characterized with a progressive accumulation of ACC synthase, ACC and ethylene production in tomatoes and this lasts for up to 2 hours, but if the cut tomatoes are held over longer durations, ethylene production declines (Kende and Boller, 1981). However, both ACC synthase and ACC continue to accumulate, indicating that there is a capacity to produce ethylene, but transformation of ACC to ethylene is blocked or inhibited (Kende and Boller, 1981). Wounding has also been shown to induce phenolic accumulation through up-regulation of phenylalanine ammonia lyase (PAL) (Ke and Saltveit, 1989). This up-regulation of PAL was associated with wound-induced ethylene production. Other metabolites increase in response to bruising or wounding stress, including isocoumarin in carrots (Lafuente et al., 1996), anthocyanins in midribs of red-pigmented lettuce (Ferrerres et al., 1997), methanethiol, allyl isothiocyanates and dimethyl disulfide in cabbage (Yano et al., 1986; Chin and Lindsay, 1993), six-carbon aldehydes and alcohols in cut peppers (Wu and Liou, 1986) and suberin in tomatoes and bean pods (Dean and Kolattukudy, 1976). Some of the metabolic shifts are mediated by stress response messengers (e.g. phenolics, suberin and isocoumarin accumulation) and others are a direct consequence of cellular disruption that occurs during wounding or bruising (e.g. methanethiol, allyl isothiocyanate and dimethyl sulphide accumulations).

Desiccation stress has been shown to cause some metabolic changes in fruits and vegetables. In carrots, desiccation stress at extreme handling conditions (i.e. at 13 °C) led to increases in osmotic potential, which are a function of free sugars in the roots (Shibairo et al., 2002). Increase in osmotic potential in response to water loss was most likely explained by increases in polysaccharide hydrolyzing enzymes activities in response to the stress. Hence, enzymes such as polygalacturonase and pectinesterase may increase in activity leading to loss of cell wall structure and concomitant increases in soluble sugars (Inari et al., 2002). This may explain at least a component of the loss of firmness that has been observed with carrots as they lose water (Shibairo et al., 2002). This hypothesis is borne out by results of work with cucumbers where water stress resulted in up-regulation of polygalacturonase activity, suggesting that water loss itself was not the only factor in causing softening of stressed fruit (Kubo et al., 2000). Another aspect of water stress is induction ethylene production (Kubo et al., 2000), which may explain why water stress leads to accelerated ripening in bananas (Burdon et al., 1994) and accelerated senescence in bell peppers (Lurie et al. 1986).

#### **4.2 Effects on quality**

Postharvest abiotic stressors can lead to numerous quality problems in fruits in vegetables, including scald, core and flesh browning of fruits, sweetening, pitting, water-soaking appearance, abnormal ripening, russeting and tissue softening (Hodges et al., 2005). Stress can also result in the losses of nutrient constituents in the fruit or vegetable, with vitamin C

loss being the most sensitive indicator of stress exposure (Noctor & Foyer 1998; Pignocchi & Foyer, 2003; Ioannidi et al., 2009).

#### **4.3 Mechanisms for abiotic stress response at the biochemical and molecular levels**

Response to all abiotic stresses can be acute and sub-acute in nature, where acute responses represent cases where cell death is a direct result and sub-acute responses represent cases where the stress leads to induction of adaptive changes in biochemical and gene expression (Toivonen, 2005). Many reactive oxygen species (ROS), particularly hydrogen peroxide, behave as signalling agents to trigger biochemical changes at the gene expression level (Jaspers & Kangasjärvi, 2010). In general, abiotic stressors will induce perturbations in the fruit or vegetable cellular homeostasis which will then result in the increased generation of ROS in the apoplast, mitochondria, peroxisomes, cytoplasm, chloroplasts and endoplasmic reticulum (Jaspers & Kangasjärvi, 2010). The ability of the cell to initially cope will depend largely on the endogenous free radical scavenging capacity (Mittler, 2002).

When free radical generation exceeds the endogenous scavenging capacity, the ROS interact with sensors, for which the full nature are not currently understood, that will initiate mitogen activated-protein kinase (MAPK) cascade reactions and also directly up-regulate transcription factors and calcium/calmodulin kinases (Mittler, 2002; Jaspers & Kangasjärvi, 2010). The MAPK cascade reaction will activate various transcription factors that enable *de novo* production of ROS, ROS scavenging systems, accumulation of heat shock proteins, and modulate NADPH supply in the cell (Mittler, 2002). Some of the MAPK cascade paths have been also shown to be linked specifically to ethylene production (Jaspers & Kangasjärvi, 2010), which is probably why ethylene production seems to be intrinsic to most stress responses. However, not all stressors produce identical response pathways, and so there is still a lot of work to be done in mapping of stress response networks (Jaspers & Kangasjärvi, 2010).

Chilling, bruising and cutting injuries all lead to increased activities of cell wall hydrolysing enzymes (polygalacturonase and pectinmethylesterase), which accounts for accelerated softening and abnormal ripening that occurs in response to those abiotic stresses (Van linden et al., 2008). Water stress will also lead to accelerated softening (Kubo et al., 2000), and that response has been associated with the induction of ethylene production in response to water stress. It is quite likely that the effect of all these stresses on up-regulating the cell wall hydrolysing enzymes and consequently accelerating softening is an ethylene-mediated response.

Accumulation of heat shock proteins (HSPs) which are mediated via transcription factor activation downstream of the MAPK cascade (Mittler, 2002) have been reported to enhance persistent levels of stress resistance in affected tissues (Sabehat et al., 1996). HSPs are thought to be an important factor for protein folding, assembly, translocation and degradation under normal, stress free conditions (Wang et al., 2004). HSPs have also been linked to stabilization of proteins and membranes, and enabling protein refolding under stress conditions. As a consequence HSPs are thought to have a pivotal function of protecting plant tissues against stress by maintaining cellular homeostasis.

## **5. Approaches to ameliorate abiotic stress sensitivity**

### **5.1 Treatments to enhance stress resistance**

Numerous postharvest treatments have been evaluated for enhancing abiotic stress resistance of fruits and vegetables (Toivonen, 2003b). Generally, temperature modulation

(including intermittent warming), extreme atmospheres (high O<sub>2</sub>, CO<sub>2</sub> and low O<sub>2</sub>), growth regulators, anti-transpirants, antioxidant dips, growth regulators, nitric oxide and ethanol have been tested (Toivonen, 2003b). Application of treatments in combination can often improve the stress resistance level to the fruit or vegetable (Toivonen, 2005 & 2009).

One treatment that has not been widely studied to date is the concept of gradual cooling to enhance resistance to chilling injury. Gradual cooling (2 °C per day) has been shown to reduce susceptibility of tomato to chilling injury, presumably by cooling slowly enough to allow the inherent stress resistance systems to develop before actual chilling temperatures were reached (Gálvez et al., 2010). The approach is perhaps more amenable to commercial practice than some others tested (i.e. intermittent warming).

While there has been significant effort placed on developing treatments to enhance postharvest abiotic stress resistance in fruits and vegetables, the efforts have only resulted in incremental success (Toivonen, 2004). This is largely due to the fact that stress response and resistance is a very complex matrix of processes and pathways, which are not fully understood at this point in time (Toivonen, 2005; Jaspers & Kangasjärvi, 2010). Hence, it is very difficult to design effective treatments to achieve the resistance required for any or all abiotic stresses that a fruit or vegetable may encounter during harvest, handling, storage and distribution.

Hot or warm water treatments have been shown to minimize cutting induced injuries in fresh-cut products (Lurie, 1998). Such treatments may also be used to control chilling injury via induction of heat shock proteins (Collins et al., 1995; Sabehat et al., 1996). Warm water treatment has also been shown to reduce sensitivity to irradiation in cut and packaged lettuce (Fan et al., 2003). Each product has a differing tolerance to temperature and so such treatments must be developed case by case for each vegetable or fruit of interest. The key is apply a sublethal temperature exposure, in order to enhance the adaptive response of the fruit or vegetable tissue (Saltveit, 2003).

Another approach is to apply conditioning treatments to enhance chill stress tolerance. This has been shown to provide enhanced chilling stress in several fruits and vegetables (Saltveit, 1991; Wang, 1993). It also has the advantage over heat treatments in that the risk of causing acute injury during treatment is very low.

Atmospheric treatments are quite often beneficial to controlling response to postharvest stresses. Modified or controlled atmospheres have been shown to help minimized chilling injury in a number of fruits and vegetables (Wang, 1993). However, it must be noted that in some cases, application of a controlled atmosphere led to detrimental effects on the fruit (e.g. asparagus, cucumber, limes, sweet bell peppers). Therefore, a case by case evaluation is required to determine if secondary stress is induced by the atmosphere treatments.

Growth regulator applications can also potentially enhance stress resistance, particularly for fruits and vegetables which are prone to show accelerated ripening or senescence in response to stress (Baldwin, 2003). Accelerated ripening or senescence is most often mediated by ethylene production in response to the stress. As consequence anti-ethylene products such as aminovinylglycine (AVG) and 1-methylcyclopropene (1-MCP) could enhance storage or shelf if ethylene production in response to stress was a main concern (Baldwin, 2003; Blankenship and Dole, 2003). Other growth hormones, such as methyl jasmonate (which promotes leaf senescence), can enhance chilling resistance in avocado, grapefruit, bell peppers and zucchini squash (Meir et al., 1996; Wang, 1994). Abscisic acid has been demonstrated to reduce chilling-induced injury in some crops (Wang, 1993). Other growth regulators have been suggested for use in preventing senescence in leafy vegetables (e.g. 2, 4-D), however their practical application is limited.

Quite often a single stress resistance enhancing treatment may not confer optimal resistance levels to all postharvest stresses encountered (Toivonen, 2003b). Therefore it may be productive to consider application of a combination of two or more stress tolerance enhancing treatments to achieve optimal levels of resistance (Toivonen, 2005 & 2009). There are several examples in the literature which show the added benefit of applying treatment combinations (Toivonen, 2009).

## 5.2 Germplasm selection

There have been some reports on the selection of germplasm and cultivars from breeding programs that will have greater postharvest stress resistance and hence better storage capability. Hodges et al. (2001) were able to show that a cultivar which was more susceptible to yellowing was so because of differences in balance of antioxidant systems in the tissues, which resulted in higher accumulations of ROS, particularly hydrogen peroxide. They hypothesized that the higher levels of ROS were directly causal of the yellowing of the chlorophyll in the spinach leaves. A similar relationship was found for a broccoli cultivar resistant to yellowing when compared with a cultivar highly susceptible to yellowing during storage (Toivonen & Sweeney, 1997). Apple cultivars which have resistance to developing browning in response to cutting have greater levels of apoplastic antioxidant enzymes and lower levels of ROS in the apoplast after cutting than cultivars with lower levels of antioxidant enzymes (Toivonen et al., 2003). While measures of antioxidant enzymes and ROS levels can be instructive to understand the basis for difference in stress tolerance in selections of a breeding population, they are too labour intensive to consider for incorporation as selection tools into a breeding program.

*In vitro* selection is an approach where plant cells of a fruit or vegetable of interest are tissue-cultured and exposed to a stressor, taking surviving cells to regenerate new plants having a superior level of stress resistance (Rai et al., 2011). This approach has been highly successful to regenerate germplasm of many crop plants which can be regenerated via tissue culture techniques. *In vitro* selection is much less expensive approach than molecular engineering and laboratories can easily be set up almost anywhere in the world having access to basic utilities, using inexpensive technology (Rai et al., 2011). The fact that the approach relies on the cells to survive in response to the applied stress means that the complexity of the stress response network is incorporated into the plants regenerated in this way.

## 5.3 Use of molecular probes for marker - assisted breeding

There are a large number of genes and proteins associated with stress tolerance in plants and so the best approach to identifying stress tolerant lines is apply the stress of interest and perform quantitative trait loci (QTL) analyses (Foolad, 1999). This approach may be used with intact plants and/or harvested plant parts, generally using the plant part which is of interest in the breeding improvement strategy (Foolad, 1999). The approach requires analysis of adaptive changes in QTL expression as opposed to constitutive expression (Collins et al., 2008). A stress protocol, to which target fruit or vegetable will be exposed, must be established to provide clear differentiation between resistant and susceptible lines. However, as stated earlier, the stress response is complex and so success using QTLs will require interdisciplinary effort, integrating biochemistry, gene mapping and phenotyping activities to allow reliable interpretation and successful use of adaptive QTLs for selecting for stress resistance (Collins et al., 2008).

#### 5.4 Molecular engineering

Molecular engineering for stress resistance in fruits and vegetables is limited due two major factors; 1) the complexity of the stress response network (Toivonen, 2005; Jaspers & Kangasjärvi, 2010) means that modulating the stress resistance with single gene insertions is unlikely, and 2) methods to successfully transform many important fruit and vegetable crops have yet to be developed (Rai et al., 2011). One area where there has been some advance is insertion of anti-freeze genes to protect against low temperature injury (Kole & Hall, 2008). However, the future advances will require a better basic knowledge of the stress response network and control points at the molecular level. This approach may be a helpful adjunct to adaptive QTL analysis, since insertion of a putatively important gene may alter the adaptive QTL profile and provide a probe to better understand the functional changes induced by a stress (Collins et al., 2008).

#### 5.5 Improved postharvest harvest and handling protocols

Most existing postharvest handling and storage procedures are generally not considered to be stress-inducing, except in cases where quality issues arise (Toivonen, 2009). However, there is increasing information in the research literature that suggests that there are significant stresses that could be modulated during the postharvest continuum. It is often difficult to avoid low temperature stress, since most produce is refrigerated as a necessary step to control spoilage and preserve food safety (Toivonen, 2009).

Simple modifications to postharvest handling systems can sometimes result in significant reduction in stress exposure and consequently result in storage and/or shelf life extension. One of the most successful strategies is the application of plastic film packaging or wraps to prevent desiccation, resulting in significant improvements and shelf life and quality of many fruits and vegetables (Ben-Yehoshua and Rodov, 2003). In many cases, modified atmosphere packaging is considered to largely control humidity around product and thus prevent moisture loss of fresh-cut and whole fruits and vegetables (Toivonen et al., 2009). Also, anti-transpiration coatings have been shown to be effective for maintaining quality through control of water loss (Baldwin, 2003). In regards to maintaining water content on the retail shelf, the application of misting systems can 'recharge' the vegetable and in so doing maintain quality over longer durations at less than ideal storage temperatures (Barth et al., 1990; Shibairo et al., 1998).

While rapid cooling is generally recommended to preserve quality, delayed or gradual cooling may be useful to chilling sensitive crops to allow them to acclimatize or adapt to storage and handling conditions. Delays in cooling of apples after harvest can be used to reduce the development of low temperature stress induced disorders in 'Honeycrisp' apples (DeLong et al., 2004). Similarly, delays in cooling can reduce CO<sub>2</sub>-induced internal browning in 'Braeburn' apples (Toivonen et al., 2003). Recently, slow cooling at a rate of 2 °C per day from 12 °C to 4 °C of tomatoes has shown promise to reduce chilling injury when stored at the lower temperature (Gálvez et al., 2010).

### 6. Conclusions

Abiotic stresses are significant determinants of quality and nutritional value of fruits and vegetables during harvest, handling, storage and distribution to consumer. Crop management can have a significant influence on susceptibility to stress. In addition, climate change has created additional environmental variables which may influence postharvest

stress susceptibility of fruits and vegetables. While breeding is underway for many crops to develop stress resistance that will allow them to adapt to climate change, it is not clear that breeding for stress resistance in the field will also extend stress resistance characteristics to the harvested portion. It is important understand the basis of molecular and biochemical response networks to various stresses encountered in the field and in the postharvest continuum to better evaluate the benefits that abiotic stress during production may yield for postharvest abiotic stresses.

The focus of effort should probably be on use of breeding or directed breeding to enhance stress tolerance in fruits and vegetables, as opposed to genetic engineering. This is because stress response networks are extremely complex and, as such, specific target transformations may be insufficient to confer significant increase in stress tolerance. An important aspect of the breeding and selection approach is that there must be stressors applied in reproducible ways to allow the breeder to identify expression of stress resistance since that characteristic is adaptive, rather than constitutive, in nature. The researcher must determine whether the adaptive stress response is best tested in the field, greenhouse or test tube.

In the context of postharvest handling treatments, there is some indication that such approaches may benefit in enhancing tolerance and thereby extending shelf and nutritional life of fruits and vegetables. However, again there is a limited amount of basic understanding to help guide the development of approaches to reliably confer useful levels of stress tolerance to stresses in general, and, more importantly, to specific stresses that a product is known to be subjected to during its normal distribution.

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# Towards Understanding Plant Response to Heavy Metal Stress

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## 1. Introduction

Metals like zinc, iron and copper are essential micronutrients required for a wide range of physiological processes in all plant organs for the activities of various metal-dependent enzymes and proteins. However, they can also be toxic at elevated levels. Metals like arsenic, mercury, cadmium and lead are nonessential and potentially highly toxic. Once the cytosolic metal concentration in plant turns out of control, phytotoxicity of heavy metal inhibits transpiration and photosynthesis, disturbs carbohydrate metabolism, and drives the secondary stresses like nutrition stress and oxidative stress, which collectively affect the plant development and growth (Krämer & Clemens, 2005).

Plants have developed a complex network of highly effective homeostatic mechanisms that serve to control the uptake, accumulation, trafficking, and detoxification of metals. Components of this network have been identified continuously, including metal transporters in charge of metal uptake and vacuolar transport; chelators involved in metal detoxification via buffering the cytosolic metal concentrations; and chaperones helping delivery and trafficking of metal ions (Clemens, 2001).

This chapter summarizes heavy metal stress and detoxification in plant. Special focus is given to metallothionein, yet vacuolar metal transporters, phytochelatins as well as certain organic acids, amino acids, and chaperones are also addressed with recent advances. Besides, heavy metal-induced oxidative stress and tolerance as an example of abiotic stress cross-talk will be discussed.

### 1.1 The vacuolar compartmentation mediated by transporter families CDF and Nramp

A balanced cytosolic metal concentration has to be maintained all the time via strict compartmentation and chelation. The plant vacuole is a main storage compartment site for heavy metals present in excess (Ernst et al., 1992). Nickel-hyperaccumulator plant *Alyssum serpyllifolium* keeps its 72% of the cellular Ni in the vacuole (Brooks et al., 1980). Analysis with leaves from barley grown at heavy metal-polluted environment showed that cadmium, molybdenum, and zinc are mainly subjected to vacuolar compartmentation (Brune et al., 1995). Study on *Phragmites australis* under zinc pollution revealed that most Zn was immobilized in the apoplast or sequestered into the vacuolar lumen (Jiang & Wang, 2008). The CDF (cation diffusion facilitator) transporters, once named as MTP for metal tolerance protein, are involved in mediating the cytoplasmic efflux of transition metal cations such as

Zn<sup>2+</sup>, Cd<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup> or Mn<sup>2+</sup>. In *S.cerevisiae*, two proteins COT1 and ZRC1 confer overexpression lines cobalt and zinc/cadmium tolerance respectively (Conklin et al., 1992; Kamizono et al., 1989), and both are localized to the vacuolar membrane, indicating a role in metal sequestration (Li & Kaplan, 1998). The identification of *Arabidopsis* ZAT/MTP1, a member of CDF family, provides the first information for a possible vacuolar zinc transporter in plant. AtMTP1 is localized to vacuolar membranes, and overexpression of the complete protein-coding domain of ZAT results in enhanced Zn resistance and strongly increased Zn content in the roots under high Zn exposure (Kobae et al., 2004; van der Zaal et al., 1999). The ectopic expression of poplar *PtdMTP1* in yeast was able to complement the hypersensitivity of mutant strains to Zn, and transgenic *Arabidopsis* exhibited enhanced zinc tolerance (Blaudez et al., 2003). The vacuolar membrane-localized TgMTP1 of hyperaccumulator *Thlaspi goesingense* confers tolerance to a broad spectrum of heavy metals including Ni, Cd, Zn, and Co, and complements the metal sensitivity of the yeast COT1/ZRC1 mutant strains (Persans et al., 2001), and could particularly increase zinc tolerance by initiating a systemic Zn deficiency response including up-regulation of Zn transporter genes (*ZIP3*, *ZIP4*, *ZIP5* and *ZIP9*) (Gustin et al., 2009). The *Stylosanthes hamata* ShMTP8 conferred manganese tolerance when heterologously overexpressed in yeast and *Arabidopsis*. Further analysis demonstrated that ShMTP8 is localized to the tonoplast, and the Mn tolerance in yeast was managed by internal sequestration rather than by efflux of Mn<sup>2+</sup> (Delhaize et al., 2003).

It's interesting to note the other side, releasing metal ions from the vacuole into the cytosol if required by metabolism. Which transporter takes the challenge? The NRAMPs might be a possible candidate. The plant NRAMP (natural resistance associated macrophage protein) family transport divalent metal cations into the cytoplasm. *Arabidopsis* AtNRAMP3 and AtNRAMP4 can be induced by iron starvation, complement Fe-uptake yeast mutant, and mediate the remobilization of Fe from vacuolar stores, which is crucial for seed germination during early Fe deficiency period (Lanquar et al., 2005; Thomine et al., 2000). AtNRAMP3 protein, localized to the vacuolar membrane, affects metal accumulation and gene expression of Fe uptake transporter *IRT1* and a root ferric chelate reductase *FRO2* by mobilizing vacuolar metal pools to the cytosol (Thomine et al., 2003). In the metal hyperaccumulator *Thlaspi caerulescens*, TcNRAMP3 and TcNRAMP4, the closest homologues to AtNRAMP3 and AtNRAMP4, have been characterized as highly expressed, vacuolar membrane-localized, and transporting Fe, Mn, Cd and Zn with respective preferences (Oomen et al., 2009; Wei et al., 2009).

Progressive reports implicated that *Arabidopsis* NRAMP proteins have an important role in manganese homeostasis and cadmium toxicity. The *nramp3nramp4* double mutant displayed lower Mn release from mesophyll vacuoles, and it's suggested that AtNRAMP3 and AtNRAMP4 export Mn from vacuoles to maintain mitochondrial MnSOD activity and optimal photosynthesis under Mn deficiency (Lanquar et al., 2010). The transgenic plants with disruption of *AtNRAMP6* exhibits enhanced cadmium tolerance whereas the overexpression causes Cd<sup>2+</sup> hypersensitivity (Cailliatte et al., 2009). *AtNRAMP3* showed the similar result (Thomine et al., 2000), implying these two metal transporters might affect remobilization and distribution of cadmium within the cell.

## 1.2 Chelation of cadmium ions by phytochelatin

Chelation of metals in the cytosol is a very important mechanism of heavy metal detoxification and tolerance (Fig 1). The principal classes of known metal chelators in plant



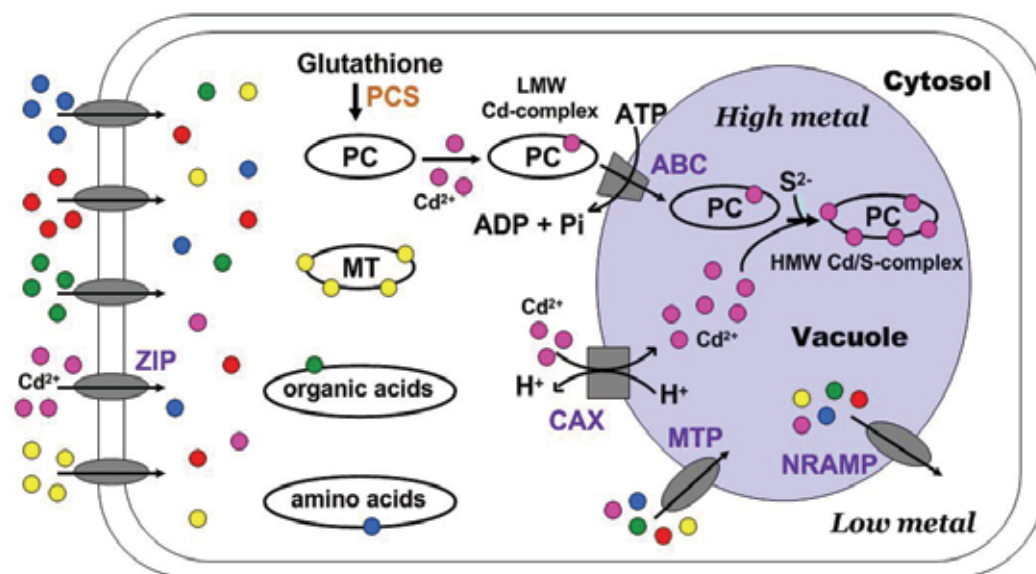


Fig. 1. Vacuolar sequestration of heavy metals in plant cell. Following the uptake through transporters such as ZIP (zinc/iron-regulated transporters) family members, heavy metal ions like  $\text{Cd}^{2+}$  enters the cytosol and it stimulates the glutathione-derived synthesis of phytochelatin (PCs) by PC synthases (PCS). PCs bind cytosolic  $\text{Cd}^{2+}$  to form the low-molecular-weight (LMW) complex first, which is transported into the vacuole via a tonoplast-localized ATP-binding-cassette (ABC) transporter. In the vacuole, LMW Cd-complex then accumulate into high-molecular-weight (HMW) complex with more  $\text{Cd}^{2+}$ , which may enter the vacuole via a direct exchange with protons by tonoplast-localized cation/proton exchanger (CAX) transporters. Transporters MTPs (metal tolerance protein) and NRAMPs (natural resistance associated macrophage protein) residing in the tonoplast mediate passage of metal ions for compartmentation or remobilization. Other chelators including metallothioneins (MTs), organic acids and amino acids help buffering the cytosolic metal concentrations to the safe low metal state.

are metallothioneins (MTs), phytochelatin (PCs), organic acids and amino acids (Clemens, 2001). Metallothionein and phytochelatin are proteins or peptides with low molecular weight, high cysteine content, and unique metal-binding capacity. In early reports lack of detail amino acid sequence data, metal-binding proteins in plants were generally assumed to be MTs, which in fact covered at least part of PCs. In an old classification system of three-class MT proteins, phytochelatin are somewhat confusingly described as enzymatically-synthesized class III whereas other two are gene-encoded class I and II (Cherian & Chan, 1993). Later the classification system has been improved and now it's clear plants express both PCs and MTs, which play relatively independent roles in metal detoxification and/or metabolism (Cobbett & Goldsbrough, 2002).

Phytochelatin have been identified in many plants and photosynthetic organisms, ranging from algae, gymnosperms to monocots and dicots. Phytochelatin (PCs) are synthesized from glutathione (GSH) (in some cases, related compounds) by PC synthases (PCS), and play a role in the distribution and accumulation of Cd and some other highly toxic metals

like Ag, Hg, As (Cobbett, 2000; Rauser, 1999). Modern techniques including X-ray absorption spectroscopy (XAS), high performance liquid chromatography-mass spectrometry (HPLC-MS), inductively coupled plasma optical emission spectrometer (ICP-OES) help to reveal that cadmium ions are generally bound to phytochelatins in plant. The percentage of Cd bound to PCs in Indian mustard seedlings increased from 34% after 6 hours of Cd exposure to 60% after 72 hours (Salt et al., 1997). In a Cd-hyperaccumulator desert plant tumbleweed (*Salsola kali*), cadmium was attached to oxygen and sulfur groups in stems and leaves, implying a great possibility of phytochelatins production in the stems, which later coordinates the absorbed cadmium for transport and storage in cell structures (de la Rosa et al., 2004). The mushroom *Boletus edulis* presented PC-Cd complex under Cd exposure and the more PC complexes were correlated with reduced level of GSH (Collin-Hansen et al., 2007). In wheat, phytochelatins bound 82% of the Cd in roots, 19% in young leaves and 12% in old leaves, suggesting the speciality of PC-based Cd sequestration varies with tissues even in the same plant (Marentes & Rauser, 2007). And it's demonstrated that the chemical structure of thiol and carboxyl groups is essential for the metal-binding ability and formation of a Cd-PCs complex (Satofuka et al., 2001).

There are two types of Cd-PC complexes produced during Cd sequestration: low-molecular-weight (LMW) and high-molecular-weight (HMW). The LMW complex serves as the transient form for transporting Cd<sup>2+</sup> from cytosol to vacuole where more Cd and sulfide are incorporated to produce the HMW complex, which turns the main storage form of Cd<sup>2+</sup> (Rauser, 1995). The first molecular insight into transporting the PC-Cd complex comes from the *S.pombe hmt1* mutant, which is unable to form the HMW complexes. SpHMT1 is a half size ATP-binding cassette (ABC) transporter protein, located in the vacuolar membrane, and mediates the ATP-required transport of LMW PC-Cd complexes into vacuolar membrane vesicles (Ortiz et al., 1992; Ortiz et al., 1995). An ATP-dependent, similar-to-SpHMT1 activity has been identified capable of transporting both PCs and PC-Cd complexes in oat roots (Salt & Rauser, 1995). Using a cDNA-microarray approach, some ATP-binding cassette (ABC) transporters in *Arabidopsis* genome were found to be differentially regulated under cadmium treatments, implying their role in Cd sequestration and redistribution (Bovet et al., 2005). A subfamily of ABC transporters, MRPs (multidrug resistance-associated protein) have been implicated mediating PC-Cd complex transport across the tonoplast in plants (Rea, 2007). Expression of *Chlamydomonas reinhardtii* CrMRP2 not only complements the yeast mutant, but also helps accumulating and sequestering more Cd in the stable HMW PC-Cd complex (Wang & Wu, 2006). Song et al. report the identification of the long-sought and major vacuolar PC transporters recently (Song et al., 2010). Two *Arabidopsis* ABC-type transporters, AtABCC1 and AtABCC2 mediating transport of As(III)-PC though, may as well offer us a good perspective of identifying more specific PC vacuolar transporters for other heavy metals in addition to Cd-PC complex.

Besides the phytochelatin-Cd<sup>2+</sup> complex transported by ABC transporters, cadmium ions can also reach the vacuole via a direct exchange with protons by tonoplast-localized cation/proton exchanger (CAX) transporters. In oat roots, the pH-dependent Cd<sup>2+</sup> accumulation in vesicles was accompanied by efflux of protons, which offers the first clue of Cd<sup>2+</sup>/H<sup>+</sup> antiport in plant (Salt & Wagner, 1993). Then several *Arabidopsis* CAX genes have been cloned and characterized. Expression of AtCAX2 in tobacco increased Cd<sup>2+</sup> and Mn<sup>2+</sup> transport in isolated root tonoplast vesicles (Hirschi et al., 2000). The *cax4* loss-of-function mutant and CAX4 RNAi lines displayed altered root growth in response to Cd<sup>2+</sup>, Mn<sup>2+</sup> and auxin treatment (Mei et al., 2009). The transgenic tobacco overexpressing AtCAX4 and

*AtCAX2* exhibited high  $\text{Cd}^{2+}$  transport and certain selectivity in tonoplast vesicles, indicating some CAX transporters are more selective for particular divalent cations (Korenkov et al., 2007). Comparative analysis of CAX2 transporters between different plant species including *Arabidopsis*, tomato and barley proposed that there are diverse regulatory mechanisms with regard to CAX antiporter diverse functions (Edmond et al., 2009).

### 1.3 Metallothionein: metal-binding protein and more

Metallothioneins (MTs) are ubiquitous low-molecular-weight, cysteine-rich proteins that can bind metals via mercaptide bonds. Since the first MT was characterized from horse kidneys as cadmium-binding proteins in 1957 (Margoshes & Vallee, 1957), plenty of MT genes have been identified in a wide variety of organisms including bacteria, fungi, and all eukaryotic animal and plant species (Robinson et al., 1993).

The spatial structures of MTs have been uncovered as a dumbbell-like shape with two separate domains,  $\alpha$  and  $\beta$ , containing in their core clusters built up of several tetrahedral Metal-Cys units (Fig 2). The different metal reactivity and metal affinity of two domains prompt different functional roles of the two metal clusters, that is, N-terminal  $\beta$  domain is involved in the homeostasis of essential metal ions (Kagi & Schaffer, 1988; Willner et al., 1987), and C-terminal  $\alpha$  domain, the tight binding sequestration of excess and/or toxic metal ions (Cherian et al., 1994; Wright et al., 1987). As for the spacer region linking the  $\alpha$  and  $\beta$  domains, it may contribute to stability or subcellular localization of MT proteins (Domenech et al., 2005), and is necessary for MT metal detoxification function (Domenech et al., 2007; Zhou & Goldsbrough, 1994).

MT proteins are generally classified into mammalian Class I and plant Class II, and plant MTs can be further subdivided into four types based on the number and arrangement of cysteine residues and the length of spacer region (Cobbett & Goldsbrough, 2002). These four-type plant MTs exhibited certain tissue-preferential expression patterns. Type 1 MTs

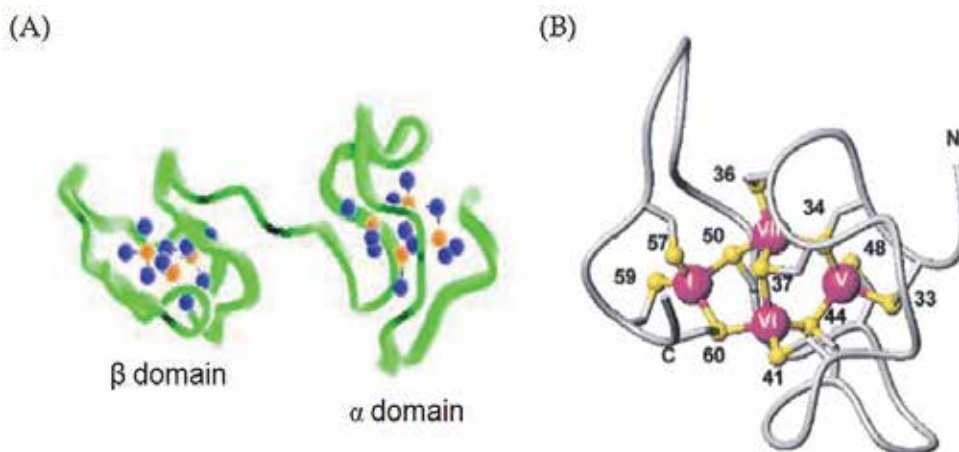


Fig. 2. The spatial structure of metallothionein. (A) general structure of MTs: a dumbbell-like shape with two separate globular domains  $\alpha$  and  $\beta$ ; (B) structure of the  $[\text{Cd}_4]$   $\alpha$ -domain of rat MT-2 showing the example of a tetrahedral Me(II)-Cys units formed by MTs (adapted from Blindauer et al., 2001)

are expressed much higher in roots than in shoots (Hudspeth et al., 1996), whereas Type 2 MTs are found mainly in leaves (Hsieh et al., 1995; Zhou & Goldsbrough, 1994). Type 3 MTs are expressed abundantly in the ripe fruits (Clendennen & May, 1997; Ledger & Gardner, 1994; Reid & Ross, 1997), and expression of Type 4 MTs, also known as the Ec type, was only found in developing seeds so far (Chyan et al., 2005; Lane et al., 1987).

A vast number of stimuli have been demonstrated capable of inducing MT genes expression in plants, including natural senescence (Bhalerao et al., 2003), hormones like ABA (Reynolds & Crawford, 1996), ethylene (Coupe et al., 1995), wounding and virus infection (Choi et al., 1996), heat shock (Hsieh et al., 1995), sucrose starvation (Hsieh et al., 1996), UV-light (Foley & Singh, 1994), cold and salt stress (Reid & Ross, 1997), etc. Apparently, different types of MTs respond to different factors, which is especially true when treated with heavy metal stress under different concentrations. Copper increased *AtMT1a* expression more than 10-fold in 5-to-8-day *Arabidopsis* seedlings, while the expression of *AtMT2a* varied insignificantly during the same stage under the same treatment (Garcia-Hernandez et al., 1998). 1 mM Cu<sup>2+</sup>, 100 μM Cd<sup>2+</sup> and 1 mM Fe<sup>2+</sup> were found to be efficient to decrease the bean MT1 expression, when other concentrations had no pronounced effect (Foley et al., 1997).

Ever since the first identification of MTs, its striking metal-binding property has been brought into sharp focus, which suggests MTs play the principal role in metal homeostasis and detoxification. In animals, MTs are well-known metal-binding proteins protecting against cadmium toxicity (Klaassen et al., 1999), while in plant PCs mainly take the charge of Cd detoxification (Zenk, 1996). MTs seem to have a broader spectrum of metal affinity than PCs, which points to more complicated functions. It's proposed that MTs participate in maintaining the homeostasis of essential copper (Cu) or zinc (Zn) at micronutrient levels, and also in the detoxification of non-essential toxic metals such as cadmium (Cd) and arsenic (As) (Lee et al., 2004; Merrifield et al., 2004; Roosens et al., 2004).

Though modulation of metal concentrations has great impact on cellular redox balance (Bell & Vallee, 2009), MTs may just scavenge reactive oxygen species (ROS) directly. With a large quantity of nucleophilic sulphhydryl groups in the structure, MTs provide a fine nucleophilic "sink" to trap electrophiles and free radicals, that is, the multiple cysteine residues can react with superoxide ( $\cdot\text{O}_2^-$ ) and hydroxyl radicals ( $\cdot\text{OH}$ ) leading to their degradation (Klaassen & Cagen, 1981; Sato & Bremner, 1993). Moreover, MTs can be recycled via thiolate exchange with GST (Vasak et al., 1985). Now accumulating evidences support hypothesis that MTs function as an antioxidant in plants. In wild watermelon, drought-induced CLMT2 showed an extraordinarily high activity for detoxifying hydroxyl radicals *in vitro* (Akashi et al., 2004). Three recombinant metallothionein proteins, the rice OsMT2b (Wong et al., 2004), cotton GhMT3a (Xue et al., 2009) and rubber tree (*Hevea brasiliensis*) HbMT2 (Zhu et al., 2010), possessed hydroxyl radical-scavenging activities, even higher than the positive control GSH in the hydroxyl radical inhibition assays.

Thanks to dynamic instability of metal ions in clusters, MTs can exchange metal ions with other metalloproteins universally necessary for a life cycle. There's zinc transfer between metallothionein and zinc transporter ZnT1 (Palmiter, 2004), chelator EDTA (Leszczyszyn & Blindauer, 2010), SOD (Koh & Kim, 2001; Suzuki & Kuroda, 1995), and other zinc proteins (Jacob et al., 1998). The metal-transfer mechanism should be a cornerstone for MTs' dual role abstracting the toxic metals arsenic (Ngu et al., 2010) or cadmium (Roesijadi, 2000), as well as donating the essential metals like zinc or copper (Liu et al., 2000). In this sense, metal-binding protein MTs are involved not merely in the

coordination of metal concentrations, but contribute more to diverse physiological processes like development or senescence. The wheat type 4 MT Ec gene was specially expressed during pollen embryogenesis, and its accumulation correlates well with increase of the plant hormone abscisic acid (ABA). It's suggested that induced by the ABA signal, this zinc-containing Ec may regulate certain gene expression via zinc trafficking with zinc-dependent DNA/RNA polymerase or zinc-finger proteins (Reynolds & Crawford, 1996). MTs have been implicated during senescence in many plants (Bhalerao et al., 2003; Breeze et al., 2004; Buchanan-Wollaston & Morris, 2000), and hypotheses for MT's role in senescence primarily reckon on either MTs' chelating and detoxifying abilities which alleviate the senescence-induced metal ion disturbance and oxidative burst, or the release of necessary metal ions to required places for nutrient recycling.

The positive correlation between MT expression in diverse organisms and the environmental metal concentration suggests that MTs can be effective biomarkers of heavy metal pollution. Such monitoring programs have already gained great potential comprehensively in aquatic and terraneous invertebrates (Chu et al., 2006; Dallinger et al., 2004; Navarro et al., 2009). In plants, MTs are favorable candidates for phytoremediation of heavy metal contaminants, a low-cost, effective, and sustainable plant-based approach for environment governance (Eapen & D'Souza, 2005; Memon & Schroder, 2009). On the other side, biofortification of mineral micronutrients in food crops for the benefit of human health, is another application and extension for metal research in plants, and MTs could also be contributive. The *rgMT*-overexpressing rice had the cysteine content in seed protein increased about seven-fold, which promises further enhancement of iron bioavailability (Lucca et al., 2002). Overexpression of *OsMT1a* in transgenic rice yielded significant increase of the zinc content in grain by 40-50% compared to wild type, making first step of possibility to fight zinc deficiency with zinc-rich rice (Yang et al., 2009).

#### 1.4 Organic acids, amino acids and chaperones

The reactive interactions between metal ions and S, N, and O made organic acids and amino acids potential ligands for metal chelation. Citrate has been proposed the major ligand for Cd<sup>2+</sup> at low Cd concentration within cell (Wagner, 1993), and can form Nickel-citrate complex in Ni-hyperaccumulating plant *Sebertia acuminata* (Sagner et al., 1998). The efflux of organic acids including citric acid has been elucidated for aluminium (Al) tolerance mechanisms in plant (Delhaize & Ryan, 1995). Malate and oxalate are also implicated in metal tolerance, metal transport through xylem sap and vacuolar metal sequestration (Rausser, 1999).

The coordination of nickel with histidine has been confirmed with analyses of Ni-hyperaccumulating and non-accumulating species. Upon Ni exposure, a large and proportional increase of free histidine was detected in xylem sap in Ni-hyperaccumulating *A. lesbiacum*. When supplying histidine to a non-accumulating species *A. montanum*, transgenic plant exhibited great increase of both nickel tolerance and capacity for nickel transport to the shoot (Krämer et al., 1996). And such histidine-dependent root-to-shoot translocation of Ni was also observed in *Brassica juncea* (Kerkeb & Kramer, 2003). Nicotianamine (NA), synthesized from three molecules of S-adenosyl-L-methionine by nicotianamine synthase (NAS), has been primarily linked with Fe and Cu homeostasis (Hell & Stephan, 2003; Herbik et al., 1996; Pich et al., 2001). Through studies on NAS, NA has also been implicated in Zn homeostasis and tolerance (Weber et al., 2004). Other amino acid chelators including proline, glutathione, polyamines, etc, appear to play roles in metal

binding, metal hyperaccumulation, metal stress defence as well as signalling and antioxidation (Sharma & Dietz, 2006).

Copper chaperones are a novel class of proteins involved in intracellular trafficking and delivery of copper to copper-containing proteins such as copper-ATPases or copper/zinc superoxide dismutase. *Arabidopsis* AtCCS is necessary for activation of all three types of Cu/ZnSOD activity (Chu et al., 2005). AtATX1 interacts *in vivo* with two Cu-transporting P-type ATPases HMA5 (Andres-Colas et al., 2006) and RAN1 (Puig et al., 2007) by yeast two-hybrid. The intracellular metal trafficking pathway model composed of Cu transporter, Cu pump and Cu chaperone has been proposed (O'Halloran & Culotta, 2000), and based on such cooperative work, chaperones make a great contribution to the metal transport, detoxification and remobilization (Himelblau & Amasino, 2000; Robinson & Winge, 2010).

## 2. Heavy metal-induced oxidative stress and stress tolerance: Cross-talk

Seen from a systemic view, different abiotic stresses may bring general effects on plant growth and development. For example, drought, salt, and cold stresses can all interrupt the cellular water balance leading to osmotic stress, and generate a phytohormone abscisic acid (ABA) for osmotic adjustment (Wang et al., 2003). ABA acts as a key endogenous messenger in stress response, and hence the ABA signalling pathway is more or less involved during plant cross-adaptive processes (Tuteja, 2007). In addition, all abiotic stresses can accumulate excess ROS (reactive oxygen species) at certain stage of stress exposure leading to oxidative stress. However, ROS are not only toxic compounds, but sometimes play as important regulators for many biological processes in plants such as cell cycle, programmed cell death, hormone signaling, biotic and abiotic cell responses (Laloi et al., 2004). As common consequences of abiotic stresses, osmotic stress and the ubiquitous oxidative stress have been extensively studied and offer more and more evidences for cross-talk at various steps or levels in the complicated network of abiotic stress signalling pathways.

Reactive oxygen species (ROS) such as  $\cdot\text{O}_2^-$ ,  $\text{H}_2\text{O}_2$  and  $\cdot\text{OH}$  are unavoidable by-products of aerobic metabolism, and also commonly generated under various stress conditions. The unwelcome result of ROS overproduction is the oxidative stress, which can cause extensive cellular damages (Miller et al., 2008). Therefore, a delicate antioxidant system is indispensably required to supervise the cytotoxic effects of ROS tightly. The plant antioxidant system consists of ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), as well as low-molecular-weight antioxidants like glutathione, ascorbate, carotenoids, metallothionein, etc (Table 1). Analysis with transgenic plants overexpressing these antioxidant genes revealed that maintenance of a high antioxidant capacity in cells is linked to increased tolerance against various adverse conditions (Guo et al., 2009; Jayaraj & Punja, 2008; Tseng et al., 2007; Wang et al., 2010).

Heavy metal stresses can shift the cellular balance of free radical homeostasis into terrible accumulation of  $\text{H}_2\text{O}_2$ . For those redox-active transition metals like copper or iron, autoxidation in Fenton reaction and Haber-Weiss reaction will convert  $\text{H}_2\text{O}_2$  to the highly reactive  $\cdot\text{OH}$  molecule in a metal-catalyzed way. Non-redox-active metals like cadmium or mercury can also result in  $\text{H}_2\text{O}_2$  accumulation and an oxidative burst via depletion of the antioxidant glutathione (GSH) pool and inhibition of antioxidative enzymes, especially glutathione reductase (GR) (Mithofer et al., 2004; Schutzendubel & Polle, 2002). To cope with heavy metal stress and associated oxidative stress, metallothionein, a well-known metal chelator and also antioxidant would possibly be a good way out.

Low molecular weight antioxidants	
Compounds	Target
Ascorbate	$O_2 (^1\Delta_g)$ , $\cdot OH$ , $O_2 \cdot$ , $HO_2 \cdot$
$\beta$ -Carotene	$O_2 (^1\Delta_g)$ , $RO_2 \cdot$
$\alpha$ -Tocopherol	$RO_2 \cdot$
Glutathione	Nonspecific
Urate	$O_2 (^1\Delta_g)$ , metal
Metallothionein	$\cdot OH$ , metal
Flavonoid	$\cdot OH$ and $HOCl$
Phytochelatin	Metal
Enzyme antioxidants	
Enzyme	Reaction catalyzed
Superoxide dismutase	$2O_2 \cdot^- + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$
Glutathione peroxidase	$H_2O_2$ or $ROOH + 2GSH \rightarrow 2H_2O$ or $ROH + GSSG$
Ascorbate peroxidase	$H_2O_2 + Ascorbate \rightarrow H_2O + Monodehydroascorbate$
Thioredoxin	$Prot-S_2 + Prot'(SH)_2 \rightarrow Prot(SH)_2 + Prot'-S_2$
Peroxiredoxin	$ROOH + R'(SH)_2 \rightarrow ROH + R'S_2 + H_2O$
Glutathione reductase	$GSSG + NAD(P)H + H^+ \rightarrow 2GSH + NAD(P)^+$

Table 1. Cellular antioxidants including low molecular weight antioxidants and enzymes of the ROS-scavenging system. (Adapted from Pinto et al., 2003)

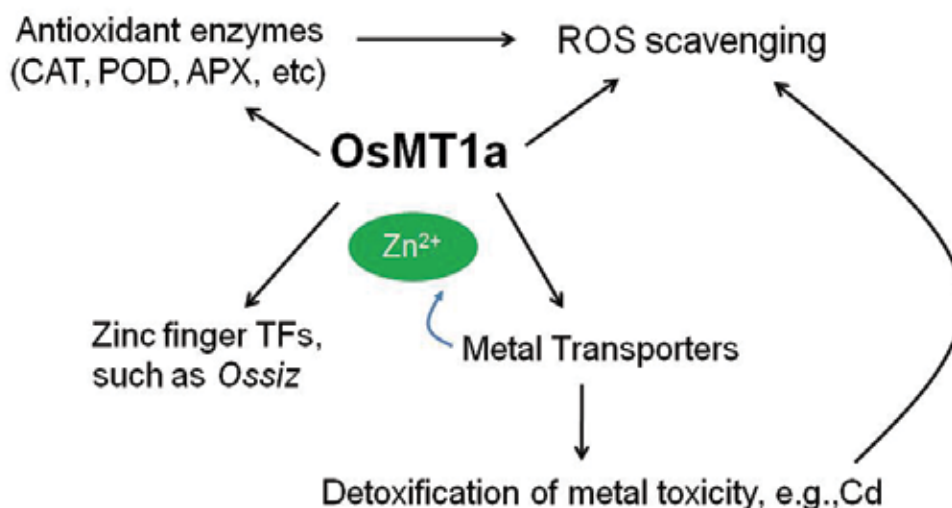


Fig. 3. The proposed model for OsMT1a's role in stress tolerance and metal detoxification.

Take OsMT1a for example. Yang et al. reported functional characterization of a rice type 1 metallothionein, OsMT1a. A model has been proposed to elucidate how OsMT1a plays a role in drought tolerance in plant (Yang et al., 2009). On the one hand, OsMT1a can directly scavenge ROS via increasing activities of antioxidant enzymes CAT, POD and APX. On the

other hand, OsMT1a lies upstream of some zinc finger transcription factors like Ossiz, and may tune up downstream defense genes in virtue of these transcription factors through Zn<sup>2+</sup> trafficking. Additional data reveal that some zinc/cadmium transporter genes including forecasted vacuolar-membrane-localized ABC transporters *ABC1*, *MRP4* were up-regulated in *OsMT1a* transgenic plants, which probably accounts for uptake enhancement of Zn, as well as detoxification of toxic Cd via compartmentation into vacuole (Yang et al., unpublished data). Whether stomatal closure or ABA signalling is involved in OsMT1a-mediated drought tolerance in rice will be further examined. Altogether, researches on this metal-binding protein metallothionein provide a convincing insight into plant cross-talk combined with zinc homeostasis, cadmium detoxification, ROS scavenging and stress tolerance (Fig 3).

Despite their toxicity, ROS have been reevaluated in recent years as key signal molecules for regulating cell function and development (Rhee, 2006). In plants, the elaborate and efficient network of scavenging mechanisms allowed overcoming ROS toxicity and using some of these toxic molecules, mainly the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) produced by cytosolic membrane-bound NADPH oxidases, as a signal in a wide range of abiotic stress responses (Bailey-Serres & Mittler, 2006; Mittler et al., 2004; Neill et al., 2002). For instance, in response to drought stress, ABA-induced H<sub>2</sub>O<sub>2</sub> regulates the stomatal closing of *Arabidopsis* guard cells via activation of calcium-permeable channels in the plasma membrane (Pei et al., 2000), and such ABA-induced ROS production may also be involved in the phosphatidylinositol 3-phosphate (PI3P)-mediated stomatal closure (Park et al., 2003). A vast network of genes have been activated by ROS accumulation, many of which are also central regulators of stress responses, including zinc finger protein Zat family, heat shock and WRKY transcription factors, multiprotein bridging factor 1c, and Rboh genes (Miller et al., 2008). It's implicated that ROS could be an essential intermediate integrating different signals during cross-talk between abiotic stress signalling pathways.

### 3. Outlook and challenges

As the global population and food demand keep increasing fast, and yet the environment has been endangered worse and worse by water deficit and soil salinization, abiotic stress becomes one of the most harmful factors that limit the growth and productivity of crops worldwide. Although we keep moving forward with the understanding of heavy metal stress and detoxification in plant, there are many components of the complex network yet to be identified. Especially much remains unknown about the signalling molecules of the metal-induced signal transduction, including sensing of the cellular metal change and subsequent transcription regulation of metal-responsive genes (DalCorso et al., 2008). In recent years, next-generation sequencing techniques emerge and develop fast, and the microarray-based analyses become available and efficient for transcriptome or proteome high-throughput screenings, which help to identify regulatory factors for the metal homeostasis and still more metal transporters, low-molecular-weight chelators, chaperones as well. In addition, some heavy metal responsive transcription factors can also be induced by other abiotic stresses such as cold, dehydration, Salicylic Acid (SA) and H<sub>2</sub>O<sub>2</sub>, suggesting cross-talk exists between heavy metal response and other abiotic stress defense signalling (Fusco et al., 2005; Singh et al., 2002; Suzuki et al., 2001; Weber et al., 2006). Nevertheless, determining the underlying regulatory and cross-talk mechanisms remain a future challenge.



Heavy metal hyperaccumulators are unique plants capable of accumulating high amounts of various toxic elements (Reeves & Baker, 2000), and the active hyperaccumulation is based on mechanisms of internal hypertolerance to cytotoxic metals and a powerful scavenging system compatible for efficient uptake of the pollutants (Salt, 2006). Therefore, comparative studies on hyperaccumulator and non-hyperaccumulator plants will provide us a good view of naturally selected metal hypertolerance and hyperaccumulation. The first core set of candidate genes with high expression in hyperaccumulators has been identified and will be analyzed at biochemical and genetic level (Krämer et al., 2007). Dissecting these genes opens up a wide avenue for understanding the plant metal homeostasis network, and also agricultural genetic engineering for crop tolerance and biofortification, as well as phytoremediation of environmental metal pollution.

#### 4. Acknowledgment

This work was supported by grants from Chinese Academy of Sciences (grant numbers: KSCX2-YW-N-010 & KSCX2-YW-N-056), and National Science Foundation of China (grant numbers: 30825029 & 30621001).

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# Plant N Fluxes and Modulation by Nitrogen, Heat and Water Stresses: A Review Based on Comparison of Legumes and Non Legume Plants

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## 1. Introduction

**Nitrogen (N) is one of the most limiting mineral elements** for plant growth and yield (Davidson et al., 2007). This element is an essential component of plant cells at the structural, genetic and metabolic levels, getting involved in many processes of plant growth and development which finally lead to yield as well as the quality of harvested organs (seeds or shoot biomass). While soil N availability can be enhanced in agricultural areas through fertilizers supply, under natural conditions mineral N generally limits plant growth because of spatial and temporal fluctuations in its availability in the soil. Moreover, in the context of climate change, plants will experience more abiotic stresses, including those impacting water availability (drought, flooding), extreme temperatures (chilling, heat), presence of heavy metals, nutrients availability, and soil structure, which can have dramatic consequences on yield and quality, as they directly or indirectly modify plant N acquisition and utilization. Among plants species, legumes have the capacity to acquire soil N *via* roots and, in addition atmospheric dinitrogen through symbiosis with bacteria. While in legumes both these N retrieval ways interact (the interaction being mediated by soil nitrate availability), they also complement for an optimum N nutrition. However, even in legumes, because of the high energy cost of N<sub>2</sub> fixation (Voisin et al., 2003b) and the high sensitivity of nodules to environmental factors (Salon et al., 2001; Serraj et al., 1999), N acquisition is also very frequently spatially and temporally limited. Hence, details concerning the differences between legumes and non-legume plants can set the foundation for the understanding of the stress impacts on plants.

One of the **biggest challenge for European agronomy** in the next years will be to cope with the necessary reduction/optimization of fertilizer use in order to minimize agricultural detrimental impacts on the environment (reducing fossil use energy and greenhouse gas emissions, maintaining/enhancing biodiversity through pesticides/herbicide use reduction) while producing more feed/food for the ever growing population. One way to solve this dilemma consists in the identification/selection of crops having **enhanced N use efficiency**

(NUE, yield per unit of soil N) in the context of increased abiotic constraints. Agro-ecological advantages of legume cultures are thus obvious, as they naturally enrich soil with N, leading to a high reduction of N amendments, and consequently avoiding pollutions linked to amendment synthesis, their transport and their spreading.

**In this chapter**, the main features concerning the physiological pathways involved in NUE, that is to say N uptake efficiency (NUpE) and N utilization efficiency (NUtE) including assimilation, allocation and mobilization within/from plant organs will be described highlighting differences between legumes and non legume plants. **A first section** will depict the system when plants encounter no environmental constraints. **In a second section**, the main changes in the plant physiological mechanisms depicted in section 1, mediated by heat stress, water stress and soil N limitation will be illustrated using up to date literature. Using recent results from multidisciplinary integrative approaches we will illustrate how plants cope with these constraints during the different phases of their growth cycle.

## 2. Plant N fluxes without environmental constraints

### 2.1 Nitrogen forms available for plants

In soil, inorganic N forms are produced by soil microorganisms and represent less than 5% of total soil N which is mostly sequestered in soil organic matter. Nitrogen is acquired in a variety of forms by most plant species but inorganic N forms (*ie* nitrate and ammonium) are preferred over organic forms in most agricultural soils (Crawford & Forde, 2002; Harrison et al., 2007) and are the most abundant in temperate climatic conditions. Nitrate, unlike ammonium does not get adsorbed by soil and is very mobile. As such, and despite that reducing nitrate to ammonium requires more energy than the direct uptake of ammonium (Noctor & Foyer, 1998), plants from most of agricultural soils and/or dry environments privilege nitrate for their N nutrition while in forest habitats and humid environments the preference goes for ammonium. Because soil nitrate concentration highly varies according to the soil and its microbial content (Miller et al., 2007) plants have to set up a strategy to adapt to such spatially and temporally fluctuations of resource availability. Inorganic N nutrition mostly concerned nitrate and impressive results arose from molecular research on the model plant *Arabidopsis thaliana* (Gifford et al. 2008; Scheible et al., 2004). Several recent studies otherwise examined ammonium regulation of gene expression in various plant systems (Lopes & Araus, 2008; Ruffel et al., 2008).

Although legumes also assimilate some soil mineral N by their roots, they indirectly acquire this element from atmospheric N<sub>2</sub>, through endosymbiosis with N<sub>2</sub>-fixing bacteria that involves the formation of a specific symbiotic organ (nodules) on roots. Interestingly, non legumes plants such as rice (Chi et al., 2005), wheat (Iniguez et al., 2004) or maize (Perin et al., 2006) are also able to interact with N<sub>2</sub> fixing bacteria for N acquisition (Kraiser et al., 2011; Rosenblueth & Martin-Romero, 2006). For instance, using <sup>15</sup>N dilution methods, it has been demonstrated that wheat plants inoculated with *Klebsiella pneumonia* and cultivated under low mineral N conditions assimilated up to 49% of the plant N from the atmosphere while plants inoculated with mutant of *Klebsiella pneumonia* unable to fix N<sub>2</sub> presented symptom of N deficiency (Iniguez et al., 2004).

### 2.2 Nitrogen acquisition and assimilation

During plant vegetative stage, meristems and young developing organs need ample supply of N for synthesis and storage of their amino compounds, which are further incorporated in

proteins. Water and minerals diffuse osmotically in roots *via* both an apoplastic (extra cellular free spaces) and a symplastic (cytoplasmic channels) pathway up to the endodermis cells surrounding xylem and phloem. Reaching the endodermis, ions have to be actively pumped from the symplasm to tracheids of xylem as apoplastic transport is precluded by the Casparian strip (Enstone & Peterson, 2002). Ammonium and nitrate reduction/assimilation does not take place in the same tissue, ammonium being assimilated in roots, and sometimes translocated to shoots (Schjoerring et al., 2002). Nitrate reduction and further assimilation occurs in roots and/or shoots (most of the times) depending as example upon the plant species, the amount of available soil nitrate or energy.

Adapted to the fluctuating nitrate concentrations, root N uptake relies at the molecular level on nitrate transporters, whose affinity varies. For soil nitrate concentration below 1 mM, high affinity transport systems (HATS comprising the NRT2 gene family) are predominant, while for soil nitrate concentration higher than 1 mM, low affinity transport systems (LATS comprising the NRT1 gene family) are predominant. Both of these nitrate transporters systems are inducible. Part of the LATS is the protein expressed by the gene NRT1.1 belonging to the NRT1 family (Tsay et al., 2007). NRT1.1 is also involved in the activation of nitrate-related genes (Ho et al., 2009), the signalling network for regulation of root development (Ho & Tsay, 2010) and auxin transport (Krouk et al., 2010). It is inducible and located in the epidermis of the root tip and in the cortex and endoderm of upper root parts (Huang et al., 1996). NRT1.2, which expression occurs in root hairs and epidermis of mature root parts (Huang et al., 1999), is a constitutive part of LATS. The main component of HATS (Li et al., 2007) is the plasma membrane protein expressed by the NRT2.1 gene (Filleur et al., 2001; Orsel et al., 2004) which in *Arabidopsis* (AtNRT2.1) occurs in the epidermis, cortex, and endodermis of mature root parts (Nazio et al., 2003). NRT2.1 protein is physically associated with the protein expressed by NAR2 (also named AtNRT3.1) with which it constitutes the nitrate transporter. The gene AtNRT2.2 is also, although for a minor part, involved in the HATS. Both NRT1 and NRT2 transport nitrate together with protons (Miller et al., 2007). Thereafter nitrate is reduced within root tissues to nitrite by nitrate reductase (NR) and then either reduced to ammonium by nitrite reductase (NiR) (Crawford & Forde, 2002) or translocated to upper plant parts for further assimilation or transient storage.

Plants can also uptake ammonium by ammonium transporters (AMT, Crawford & Forde, 2002), further assimilated into glutamate *via* the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle. Some plants such as legumes have the ability to establish symbioses with a bacterium housed within specialized plant organs (nodules) and acquire N through biological N<sub>2</sub> fixation. In nodules of legumes, atmospheric N<sub>2</sub> is converted to NH<sub>3</sub> by nitrogenase and diffuses from the nodule bacteroid alkaline protoplasm into the acidic peribacteroid space where it is converted to ammonium. Ammonium is then transported across the peribacteroid membrane into the cytoplasm of infected cells (Tyerman et al., 1995) and assimilated, as explained above, by the GS/GOGAT system.

**In legume plants, the two N nutrition pathways of display complementarities for N uptake while they are antagonist for C use within the plant.** Indeed, on the one hand, N<sub>2</sub> fixation occurs in situations of low nitrate availability in the soil: as a result, N<sub>2</sub> fixation in nodules decreases as nitrate availability in the soil increases, while nitrate assimilation by the roots increases (Voisin et al., 2002b). On the other hand, bacteria provide the plant with reduced N<sub>2</sub> through nitrogenase activity, while the energy needs for nodule synthesis and functioning are sustained by plant photo assimilate supply to nodules (Kouchi & Yoneyama, 1984). Nodules are strong sinks for assimilates within the plant (Hacin et al., 1997), that

compete for C assimilates both with the shoot and the roots (Schulze et al., 1994, 1999; Voisin et al., 2003a, 2003b). As such, it has been shown that N<sub>2</sub> fixation globally induces higher C requirements for the plant, as compared to nitrate assimilation (Ryle et al., 1979; Schulze et al., 1994, 1999; Silsbury, 1977). Therefore, the C budget at the plant level can be unfavourable when plant N nutrition relies exclusively on symbiotic N<sub>2</sub> fixation (Minchin et al., 1981). Nevertheless, interactions between legume plant, N source (N<sub>2</sub> fixation or nitrate assimilation) and C metabolism can be species- or genotype-dependent (Bethlenfalvay et al., 1978; Radin, 1983; Ryle et al., 1979; Vos et al., 2005). How acquisitions of NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup> and N<sub>2</sub> in legumes are co-ordinately regulated to fulfil the plant N demand is not known. The fact that the assimilatory pathways involved in the acquisition of these N sources share the same end-product suggests that the three pathways might be under a general control exerted by a systemic signalling pathway related to the level of downstream product of N assimilation in the whole plant (Forde, 2002). Split-root experiments were used to induce short term fluctuations of N availability supplied on a localized part of the roots, and to evaluate the systemic responses triggered at the whole plant level that are revealed on the untreated side of the root system. Supplying a high level of NH<sub>4</sub>NO<sub>3</sub> to one part of the root system of *Medicago truncatula* induced a strong repression of the apparent N acquisition capacity of the other side, whatever the N source (NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, N<sub>2</sub>) (Ruffel et al., 2008). This demonstrates that the three pathways are controlled by feed-back repression mechanisms, which involve systemic signalling pathways. This regulation results in the adjustment of the acquisition capacities of the roots to the N demand of the whole plant. At the molecular level, a transcriptomic study has revealed that gene networks involved in these responses are rather specific of the N source (Ruffel et al., 2008).

Plants are also capable of taking up amino acids available in the rhizosphere. Indeed, some amino acid transporters have been identified. They seem rather specific, as for example cationic amino acid transporters (CAT) or lysine/histidine transporter (LHT) (Chen et al., 2001; Liu & Bush, 2006; Rentsch et al., 1996; Scheible et al., 2004; Stacey et al., 2002). LHT1 has a dual role, participating in the uptake of neutral and acidic amino acids in root and providing of leaf mesophyll cells with xylem-derived amino acids (Hirner et al., 1998). An early rice nodulin gene (OsENOD93-1) has been implicated in amino acid accumulation in roots and transport towards the shoot (Bi et al., 2009). Oligopeptide transporters (OPT) seem to have a role for peptide and amino acid transport during the early phase of embryo development (Stacey et al., 2002). Amino acid permeases (AAP) are involved in phloem-loading with various implications in amino acids transport (Sanders et al., 2009; Schmidt et al., 2007).

## **2.3. Distribution of N compounds within the plant**

### **2.3.1 Amino compound transport**

Long distance transport of water and ions from roots to shoots is apoplastic under tension and occurs through mass flow across xylem cells, driven by the shoot evaporative loss of water. In addition, the uptake of water following that of ions creates root pressure which mainly helps re-establishing flow continuity in xylem vessels when transpiration rate is either very high or in some plants reduced due to high air humidity. In *Arabidopsis*, nitrate is transported in roots either passively into the xylem or at the site of pericycle cells by the AtNRT1.5 nitrate transporter while the AtNRT1.8 acts in both root nitrate loading and unloading from root or shoot xylem vessels (Li et al., 2010). The compounds exported by roots in xylem come not only from soil mineral N assimilation by roots or atmospheric N<sub>2</sub> symbiotic fixation within nodules, but also from other catabolic processes, export of

previously stored soluble compounds, or recycling from phloem to xylem in the roots. These latter processes however represent only about 15% of the N exported by the roots.

### 2.3.2 Composition of saps

Xylem sap pH is mildly acidic (between 6 and 6.5) and is characterized by a low osmolarity. Among the few organic and inorganic solutes, xylem sap is mainly composed of N compounds (amino acids, amides, ureides), organic acids and nitrate ions content being much lower. The C skeletons of N compounds originate from C transported by shoots to roots *via* the phloem. Carbon (C) xylem sap originates mainly from amino compounds although sugars may accumulate in xylem sap to concentrations up to 50 mol m<sup>-3</sup>. The main solutes in xylem sap are highly specific to the species, the source of N in soil or the stages of development. In legumes, aminotransferases form the main N compounds exported from the nodules. These compounds are glutamine and asparagine for temperate legumes and ureides (allantoin and allantoic acid) for tropical legumes (Parsons & Sunley, 2001). The N compound spectrum found in xylem also depends upon the N nutrition regime: temperate legumes supplied with large amounts of nitrate in the nutrient solution will shift from asparagine export to glutamine export from roots to xylem following nitrate assimilation. Similarly, tropical legumes will export amides instead of ureides following nitrate supplementation. In non legume plants such as maize supplied with nitrate, the xylem sap has higher glutamine concentration while maize fed with ammonium has higher asparagine concentration in xylem (Murphy & Lewis, 1987). Glutamine and asparagine are also the predominant amino compounds in oilseed rape (Balint & Rengel, 2011) or barley (Lewis et al., 1982).

Phloem sap has an alkaline pH (7 to 8.5) and transports mostly sucrose, in some species fructanes, raffinose-family oligosaccharides (raffinose, stachyose, verbascose), and a tiny amount of hexoses (glucose and fructose) except in wounded tissues, alcohol sugars and amino acids. Nitrogenous compounds in phloem are similar to those in xylem but more concentrated (5 to 40-fold, Pate et al., 1975). Their concentration varies among plant species, from around 0.1 mol m<sup>-3</sup> (Hunt et al., 2010), to over 1.2 mol m<sup>-3</sup> (Faria et al., 2007). Aspartate and glutamate and their corresponding amides, asparagine and glutamine often predominates (Girousse et al., 1996; Hunt et al., 2010; Sanders et al., 2009). The relative concentrations of threonine, histidine, tryptophane, and valine vary between plant species. Sulfur amino acids are less concentrated in phloem sap, cysteine is present as a trace in phloem sap, glutathione is usually also found in the sap (Fisher & Macnicol, 1986; Jongebloed et al., 2004), S-methylmethionine (Bourgis et al., 1999). Phloem never contains either ammonium (Riens et al., 1991) or nitrate (Fan et al., 2009; Hayashi & Chino 1986; Lohaus et al., 2000; Patrick et al., 2001). The precise composition of phloem sap is influenced by abiotic factors, such as temperature, N and water availability (Balint & Rengel, 2011; Mitchell & Madore, 1992; Tilsner et al., 2005). It has been also reported that amino acid composition of phloem sap changed as function of genotype performance in term of NUE. For instance, the phloem sap of oilseed rape genotypes more efficient in remobilising N were proposed to contain greater amounts and/or proportions of asparagine, which is the most efficient N transporter (N : C ratio equal to 0.5, compared with 0.4 for glutamine) (Seiffert et al., 1999).

### 2.3.3 Carbon transport associated with N compounds

Nitrogenous compounds are exported by roots to shoots *via* the xylem but the transfer from xylem to phloem thereafter varies according to the electrical charge of the compounds.

Xylem to phloem transfers occur mostly in the higher part of the plant and allow N from the transpiration stream to be directed to low transpiring organs such as apices, young leaves, fruits. Amino acids such as arginine are in xylem sap under the cationic form and bounded on the negatively-charged cell wall of xylem vessels. Vascular tissues of stems, petioles and major veins of the leaves are then able to attract them (McNeil et al., 1979; Pate et al., 1979a) while neutral amino acids and amides (such asparagine, valine, glutamine, allantoin) can be delivered according to the selectivity of cell uptake in stems or be transferred from xylem to phloem (Pate & Layzell, 1981). The amino acids under anionic forms in xylem sap (allantoic acid, aspartate and glutamate) and nitrate can be transported to transpiring organs such as leaves. The presence in xylem sap of compounds not easily transferred to phloem (arginine, asparagine and glutamine) ensures that N compounds arising from root assimilation are retained by mature parts of aerial organs, in particular cell mesophyll, for synthesis of leaf proteins needed for C assimilation. Compounds easily exchanged between xylem and phloem (asparagine, glutamine and valine) have a major importance for loading upward flow (mostly asparagine) or downward flow (mostly glutamine) of translocated compounds with N, hence providing N to meristematic tissues during their growth, and developing fruits (Pate et al., 1981). Amides (asparagine and glutamine) which are the main compounds concerned with xylem to phloem transfer are therefore beneficial for the N nutrition of such tissues because their low C/N ratio (2 and 2.5, respectively) enriches the phloem in N.

#### **2.3.4 Source – sink relationships for N**

In legumes, there is a preferential N transfer toward apices as compared to roots and 40% of this N is issued from the xylem transpiration stream, the remaining coming from phloem (Layzell et al., 1981). Young developing leaves import most of their C and N needs from phloem up to the point where they transpire actively. Thereafter xylem furnishes up to 80% of the total N necessary for leaf growth while leaves behave as a source for C. Minor veins of source leaves are the main sites of exported sugars and amino compounds in phloem companion cells or xylem transfer cells which then supply sieve elements with carbohydrates, amino compounds, proteins or RNAs which are involved in long distance signalling in response to developmental or stress triggers (Lough & Lucas, 2006; Turgeon & Wolf, 2009). Sucrose synthesised within photosynthetic organs attracts xylem water by osmosis. Amino compounds may additionally be loaded *via* the apoplasm from xylem in phloem. Control of symplasmic loading may occur through either plasmodesmata density or conductance. Apoplastic loading in turn is supposed to be regulated by sucrose pool size or phytohormones (arising from roots *via* the transpiration stream or in phloem imported water from recycled xylem) responding to the sink/source ratios: an increase in this ratio would enhance membrane protein levels of sucrose transporter. Phloem loaded compounds further flow through the phloem by osmotic pressure differences (Minchin & Lacomte, 2005; Thompson, 2006) either following symplastic or apoplastic pathway toward sinks which maintain an osmotic gradient by either 1) respiring sucrose, 2) storing it in vacuoles, or 3) converting it to osmotically inactive forms (starch, cellulose, proteins etc.) (van Bel, 2003). This allows elaborated phloem sap to be delivered to sink organs having low transpiration rates such as growing organs, fruits, apices.

### **2.4 Nitrogen remobilization and storage are crucial for plant N management**

#### **2.4.1 Remobilization**

Optimization of N remobilization efficiency (NRE) in crop plants is probably one of the main lever to improve NUE. Indeed, increasing N remobilisation from senescent organs or



damaged tissues represents an important adaptive trait allowing plants (1) to recycle N resources from the vegetative parts, (2) to limit N loss in the dry remains and (3) to reduce N fertilizer inputs (Masclaux-Daubresse et al., 2010). In legumes or non-legume plants, leaves usually represent the main source organ for N, even during sequential senescence (which occurs leaf rank per leaf rank at vegetative stage) or monocarpic senescence (characterized by a general senescence of leaves and other vegetative tissues) where N content of phloem sap increases because of enhanced mobilisation of N.

Proteolysis is one of the most important processes of N remobilization (Hörtensteiner & Feller, 2002; Zimmerman and Zentgraf, 2005). Moreover, the effective recycling of the N compounds from source leaves to sink growing tissues requires a fine coordination between sink demand and the processes of proteolysis. This is particularly important when plants are confronted by stresses that could lead to strong modifications of the source/sink relationships (see §3). Based on a study of N flux using <sup>15</sup>N tracing methods, the physiological events and proteomic changes involved in the remobilisation of N associated with sequential senescence were recently investigated in leaves of oilseed rape at vegetative stage (Desclos et al., 2009). This work reveals that four proteases were specifically involved in N remobilisation: FtsH, a chloroplastic protease, is induced transiently during early stages of N remobilisation and could be involved in the earlier degradation of chloroplastic proteins such as Rubisco; an Aspartic protease that increases at the beginning of senescence and is maintained at a high level until the abscission, would be implied in proteolysis in vacuolar and chloroplastic compartments during the course of leaf senescence; Proteasome  $\beta$  subunit A1 and SAG12 (cysteine protease), are strongly induced during the later phase of senescence, suggesting that these proteases are more specifically involved in the proteolysis processes occurring at the final stages of leaf senescence.

During the reproductive stage, N compounds provided *via* the recycling and remobilization associated to leaf senescence are largely requisitioned for optimum flowering and grain filling (Brouquisse et al., 2001; Gallais et al., 2006; Guiboileau et al., 2010; Malagoli et al., 2005; Masclaux-Daubresse et al., 2008). Indeed, at this stage, N assimilation usually decreases, mostly due to abiotic stress and as such, leaves are solicited for supplying amino acids to the reproductive organs, between 50 to 80% of their N needs, for legumes (Salon et al., 2001, Schiltz et al., 2005), oilseed rape (Malagoli et al., 2005), wheat (Kichey et al., 2007), maize (Masclaux et al., 2001) or rice (Tabuchi et al., 2007). Mobilised amino acids are issued from a) proteolysis of chloroplastic proteins (70 to 80% of total leaf - Liu et al., 2008 - mostly consisting of Rubisco, Mae et al., 1993), b) *exo/endopeptidases* attack of cytosolic proteins *via* sequestration within double membrane vesicles called autophagosomes (Ishida et al., 2008; Wada et al., 2009) pathway and senescence-associated vesicle (SAV that contains cysteine protease SAG12; Otegui et al., 2005) trafficking, c) removal of short lived proteins through the ubiquitin-26S proteasome for regular cell maintenance. The photosynthetic activity is then progressively reduced by N remobilisation. During N mobilisation from senescent leaves to filling seeds in pea (Schiltz et al., 2005), a proteomic approach has also reported the induction of chloroplastic proteases such as ATP-dependent Clp protease and ATP-dependent Zn-activated protease (FtsH) which were concomitant with the decline of leaf proteins. During leaf senescence, proteolysis of chloroplast proteins would release glutamate that serves as a substrate of the glutamate dehydrogenase (GDH, Purnell & Botella, 1997). GDH would, in turn, provide 2-oxoglutarate to support respiration and ammonium reassimilated by cytosolic GS1 for export and remobilization (Masclaux-Daubresse et al., 2006). Therefore, several evidences have reported the induction of the

activity of cytosolic GS1 during leaf senescence and supports the role of cytosolic GS1 in the efficient remobilization of amino acids for senescing leaves towards grain-filling (for a review see Masclaux-Daubresse et al., 2010). For instance, the role of GS1 in N management, growth rate, yield, and grain-filling has been suggested by the finding of co-localizations between QTLs (quantitative trait loci) for agronomic traits and GS activity (Hirel et al., 2001; Obara et al., 2004).

Following proteolysis, the resulting amino acids are distributed towards sink tissues by phloem loading that is ensured by amino acids transporters and permeases. In mature leaf of oilseed rape, Tilsner et al. (2005) have reported that remobilization of amino acids in mature leaf of oilseed rape was concomitant with the up-regulation of AAP1 gene expression, suggesting that this permease has a pivotal role in amino acid exportation. Phloem sap supplies around 90% of the fruits' N needs, while xylem sap the rest. During fruit development, phloem amino acid content increases (Pate et al., 1977; Peoples et al., 1985).

### 2.4.2 Storage

When N acquisition and assimilation is superior to N demand for growth or in case of asynchronism between remobilization of N in sources organs and N requirement for growth of sink tissues, numerous plants are capable of modifying their N fluxes in order to transiently store N in their organs (Millard, 1988; Staswick, 1994; Volenec et al., 1996). The predominant N reserves are usually amino acids and soluble proteins that are accumulated in specific sub-cellular compartments (vacuole, chloroplast) of various storage organs: tuber, root, taproot, stolon, stem as well as leaf. Deposition of N as soluble protein form in storage organs may have several advantages for plants. First, the storage of N as proteins in cell vacuoles might avoid potential osmotic problems that accompany the accumulation of N as nitrate. Second, this makes it possible to sequester N for extended periods without major consequences for cellular metabolism (Millard, 1988). Additionally, specific proteins called vegetative storage proteins (VSPs) have been characterized for their preferential function as a temporary N reserve in many herbaceous and woody species (Avice et al., 2003; Bewley, 2002; Ourry et al., 2001). These VSPs may represent between 5 and 40% of total soluble proteins and are strongly remobilized to sustain shoot or fruit growth. For instance, in perennial forage legumes such as alfalfa and white clover, studies performed under environmentally controlled or field conditions have shown that N reserve availability, and particularly VSPs concentration, are closely related to shoot regrowth after defoliation or to the spring shoot growth (Avice et al., 1997; Justes et al., 2002; Simon et al., 2004). In addition to their role in N storage, converging evidences suggest that VSPs possess alternatives roles including defence against pathogen attack and tolerance to various abiotic stresses such as cold, elevated temperature or drought (Avice et al., 2003; Dhont et al., 2006; Erice-Soreasu et al., 2007).

## 3. Impact of N limitation, heat stress and drought on plant N fluxes

### 3.1 Impact of N limitation on plant N fluxes

In non legumes, all the components of NUE are affected by low mineral N availability. To cope with this, plants have evolved a suite of adaptive responses to successfully finish their life cycles to produce offspring, rather than die early and barren due to insufficient N nutrient (Kant et al., 2008; Peng et al., 2007). Several physiological and biochemical changes occur in plants as adaptive responses to N limitation, including an increase in N uptake by high-affinity transporters, remobilization of N from older to younger leaves and

reproductive parts, a delay of growth and development, a decrease of photosynthesis capacity, and increased anthocyanin accumulation (Peng et al., 2007). For legumes, soil mineral N availability is the main environmental factor affecting symbiotic N<sub>2</sub> fixation, and consequently the relative contributions of soil mineral N assimilation by the root and symbiotic N<sub>2</sub> fixation to overall plant N uptake (i.e. the N source) (Moreau et al., 2008; Streeter & Wong, 1988; Voisin et al., 2002b).

### 3.1.1 N limitation affects the morphology of root system

Root architecture is a major determinant of the size of the root-soil interface and of the resulting water and nutrients acquisition by the plants. A low N supply generally leads to decreased root growth, suppression of lateral root initiation, increase in the C/N ratio (ie sucrose-to-nitrate ratio) within the plant, reduction in photosynthesis, and early leaf senescence (Kant et al., 2008; Malamy, 2005; Malamy & Ryan, 2001; Martin et al., 2002; Paul & Driscoll, 1997; Wingler et al., 2006; Zhang, 2007). Several genes of *Arabidopsis thaliana* involved in root development through hormonal signals and/or nutrient perception were reviewed by Casson & Lindsey (2003). But much less is known concerning legume root development. The root system of legumes is less extended than that of cereals (Greenwood et al., 1982; Hamblin & Tennant, 1987). Actually, in legumes, root growth competes for C with nodule formation (Voisin et al., 2003b). As such, when symbiotic N<sub>2</sub> fixation occurs, root growth is limited due to C competition within the plant. Both in field and greenhouse conditions, lower root growth was observed for pea plants grown in conditions of low nitrate availability compared to plants fertilised with nitrate (Voisin et al., 2002a; Voisin et al., 2010). It is also known that nitrate locally stimulates root proliferation, which results in increase of soil-root interface. For pea plants, in the field local nitrate-stimulated root proliferation was essentially observed in the ploughed layer (the upper 20 cm) but nitrate did impact neither root repartition within the profile nor maximal rooting depth (Voisin et al., 2002a). Thus, the root system of pea remains shallow (around 80 cm deep) whatever the N source.

### 3.1.2 Adaptive response to N limitation

Plants have developed adaptive responses, allowing them to modulate the efficiency of root N acquisition as a function of both external N availability and their own nutritional status (for review, Forde, 2002; von Wiren et al., 2000).

*N Limitation and soil mineral N uptake:* In non leguminous species, plant response to low nitrate availability triggers increased activity and affinity of nitrate uptake systems of the roots (Crawford & Glass, 1998; Gazzarrini et al., 1999; Lejay et al., 1999; Rawat et al., 1999), together with enhanced lateral root growth promoting root branching, and thus, soil exploration (Forde & Lorenzo, 2001). This regulation involves local NO<sub>3</sub><sup>-</sup> signalling and the systemic action of long distance signals of the plant N status. Nitrate transporters (NRT2.1; NAR2; NRT1.1) and N assimilation genes (nitrate reductase, glutamine synthetase) are induced by either a fall in the plant N status or the availability of another N source (ie ammonium). Among signals putatively involved, translocation of glutamine from shoot to root by the phloem has been shown to regulate N uptake by decreasing the expression of NRT2.1 (Gansel et al., 2001). Limitation of nitrate may also influence the activity of NRT1.1, considered as a nitrate sensor (Ho et al., 2009). Indeed, both high and low affinity nitrate transport of NRT1.1 depend upon phosphorylation / dephosphorylation by the CBL-interacting protein kinase CIPK23 (Liu & Tsay, 2003) and are mediated by low nitrate

conditions (Ho et al., 2009). Ammonium uptake is known to be repressed by high external N and to be induced under N deficiency, by mechanisms that may act at both the transcriptional and post-transcriptional levels (Gazzarrini et al., 1999; Lanquar et al., 2009; Rawat et al., 1999; Yuan et al., 2007). It was reported that some AMT genes (ammonium transporter gene family) showed increased expression during early N limitation, whereas the expression of other transporters increased only after prolonged starvation (Loqué & von Wirén 2004). In legumes, the adaptive response to local N limitation was investigated using split root systems (Jeudy et al., 2010; Ruffel et al., 2008). As for non leguminous plants, it was shown that *Medicago truncatula* plants have the ability to rapidly compensate local nitrate limitation through the increase of nitrate uptake activity of the roots still exposed to nitrate, thus maintaining N uptake by the plant. However, for plants grown with  $\text{NH}_4^+$ , the short term increase of  $\text{NH}_4^+$  uptake in response to local limitation is insufficient to sustain N uptake and growth (Ruffel et al., 2008), presumably due to overall limitation of the  $\text{NH}_4^+$  capacity of plant roots (Salon et al., 2009). The short-term response to local nitrate limitation is associated to a long term developmental response, with root proliferation of the roots in the nitrate exposed roots, at the expense of the N-limited roots that stop growing (Jeudy et al., 2010). Quantitative studies have shown that the sensitivity to soil mineral N or the  $\text{N}_2$  fixation capacity could differ among species or genotypes within a species (Harper & Gibson, 1984; Streeter & Wong, 1988; Ewing & Robson, 1990; Sulieman & Schulze, 2010). In the field, plant N requirement can generally be sustained with or without fertiliser N application, i.e. whatever the N source (in pea: Sagan et al., 1993; Voisin et al., 2002a). Nonetheless, for some legume species, plant N requirements may not be fulfilled when symbiotic  $\text{N}_2$  fixation is the main source. For example, in soybean, N nutrition can be limiting is case where no N fertiliser is supplied at sowing (Crozat et al., 1994; Gan et al., 2002). As another example, the symbiotic association between *Medicago truncatula* cv Jemalong and *Rhizobium meliloti* strain 2011 (Moreau et al., 2008) or Sm 1021 (Terpolilli et al., 2008), which are most frequently used in genomic studies, lead to N stressed plants when  $\text{N}_2$  fixation is the main N source for plant growth.

*Symbiotic  $\text{N}_2$  Fixation is an original adaptive feature of legumes to low mineral N supply.* In situations of low mineral N availability, legume plants have the ability to sustain the plant N requirement through a symbiotic association with soil bacteria enabling atmospheric  $\text{N}_2$  fixation. Quantitative studies have shown a correlative negative relationship between soil nitrate availability and symbiotic  $\text{N}_2$  fixation (Voisin et al., 2002b). In legumes, numerous studies have shown that nitrate ions can affect symbiotic  $\text{N}_2$  fixation (Streeter, 1985a; 1985b). However, the mechanisms by which nitrate limits symbiotic  $\text{N}_2$  fixation remain a matter of debate. This limitation could involve changes in the N composition of the phloem sap (Neo & Layzell, 1997; Sulieman et al., 2010). Moreover, a long term effect may also play a major role in the limitation of symbiotic  $\text{N}_2$  fixation by nitrate, through a limitation of the photosynthate flow from shoots to roots (Francisco & Akao, 1993), resulting first in a limitation of C supply to nodules (Jeudy et al., 2010) and finally to senescence of nodules exposed to high levels of nitrate (Naudin et al., 2011).

*The finely-tuned regulation of nodule number is the main component of the adaptive response of legumes to N limitation.* Actually, an internal regulation of nodule number occurs through a systemic feedback-regulated inhibition of nodule formation on young root segments by nodules pre-existing in the root system (Bhuvaneshvari et al., 1980; Kosslak & Bolhool, 1984; Pierce & Bauer, 1983). This leads to an “autoregulation of nodulation” (called AON) and the

control of nodule number by the host plant (Caetano-Anollès & Greshoff, 1990) through reciprocal shoot-root signalling (see Kinkema et al., 2006; Oka-kira & Kawaguchi, 2006 for review). Although the nature of the signals involved in the AON regulation has not been unambiguously elucidated, potential candidate compounds include either phytohormones, like ethylene (Schmidt et al., 1999), auxin (van Noorden et al., 2006) or brassinosteroids, jasmonic and abscisic acids (Oka-kira & Kawaguchi, 2006), or long distance signals involving the whole plant N status (Ruffel et al., 2008). Hypernodulating mutants, which are defective in AON, display excessive nodule numbers compared to wild type (Bourion et al., 2007) and maintain nodulation even when roots are exposed to nitrate. It was observed in pea that nodule number can vary in a wide range for a given wild type genotype, not only depending on nitrate but also on light conditions (Voisin et al., 2010). In this study, it was shown that nodulation was initiated after the exhaustion of seed reserves, in situations of N limiting conditions, and that nodule numbers were adjusted to plant N requirement for growth, that varied with climatic conditions. Nodule biomass was shown to be co-regulated with nodule number (Voisin et al., 2010), which is in accordance the co-localisation of QTLs of nodule number and nodule biomass in pea (Bourion et al., 2010). Finally, a positive relationship was observed between nodule growth and C availability within the plant (Voisin et al., 2010), in accordance with authors who have shown that nodule biomass is increased together with photosynthesis level when it is modulated by light intensity (Bethenfalway et al., 1978; Kossalak & Bohlool, 1984) or CO<sub>2</sub> concentration (Hardy & Havelka, 1976; Murphy, 1986). Jeudy et al (2010) showed in *Medicago truncatula* that, unlike NO<sub>3</sub><sup>-</sup> fed plants, N<sub>2</sub>-fixing plants lack the ability to compensate rapidly for a localised N limitation (induced by replacement of N<sub>2</sub> by argon in the below-ground atmosphere) by up-regulating the N<sub>2</sub>-fixation activity of roots supplied elsewhere with N. However, they display a long-term response *via* a growth stimulation of pre-existing nodules, and the generation of new nodules. Nonetheless, due to the delay between N stress perception and the effectiveness of the adaptive response through N<sub>2</sub> fixation by new nodules, N<sub>2</sub>-fixing stressed plants experience a temporary N deficiency that reduces growth. Even if the mechanisms may be different when nodule functioning is disturbed by other abiotic or biotic stresses, the adaptive response for SNF recovery consecutive to stress removal may be similar. As such, following short term exposure to nitrate, nitrate removal induced an extra-wave of nodulation (Naudin et al, 2011).

### **3.1.3 The nutrient contents of xylem and phloem saps are modified by abiotic stress**

N limitation caused impaired N assimilation in soybean and a few non-legumes species (tomato, maize, and sunflower), leading to specific changes in the amino acid composition of the xylem sap with an elevated aspartate/asparagine ratio and a decrease of glutamine concentration compared with plants grown at adequate N supply (Amarante et al., 2006). In spring oilseed rape, Balint & Rengel (2011) reported that xylem sap had higher concentrations of aspartate than asparagine under both limited and ample N supplies, indicating that this ratio might be a specific trait and may differ for legumes and non-legumes species. The composition of phloem sap is also influenced by N availability. Proline synthesis and its accumulation are enhanced. The modes of N nutrition influence the N content (amino acids and amides) of the xylem and phloem saps.

### **3.1.4 N partitioning among organs and mobilization of N compounds**

In *Arabidopsis*, N limitation increases both N remobilization and translocation of the N absorbed post-flowering to the seeds (Masclaux-Daubresse & Chardon, 2011). Under low

mineral N availability, leaf senescence can occur prematurely (Gombert et al., 2006). For instance, in oilseed rape at vegetative stage, N deficiency accelerates senescence in older leaves, enabling the foliar N compounds to be recycled and remobilized towards the developing tissues (Gombert et al., 2006, 2010; Tilsner et al., 2005). N starvation resulted in a significant lower residual N in fallen leaves of oilseed rape than in the control that is due to the reduction of period corresponding from maturity to the initiation of senescence and to the extension of the duration of phase of recycling (from initiation of senescence to leaf abscission). This better recycling and remobilization of N-containing compounds during N starvation-induced senescence is also related to the induction of proteolysis and amino acids exportation with a specific up-regulation of amino acid permease AAP1 (Avice et al., unpublished data). Recently, transcriptome analysis has shown that most of the autophagy genes are up-regulated in response to N limitation (Thompson & Vierstra, 2005; Wingler et al., 2009) and suggesting that autophagy play a role in N management during leaf ageing.

### 3.1.5 N limitation effect on seed quality

In planta, the seed N concentration pattern during the seed filling is the result of two processes which are not controlled similarly: seed dry matter accumulation and seed N accumulation. In both legumes and non legumes plants, the rate of seed dry matter accumulation is not significantly responsive to changes in plant N availability because it is determined before the beginning of the seed filling by the seed cell number (in maize: Jones & Simmons, 1983; in wheat: Jenner et al., 1991; in soybean and pea: Munier-Jolain & Ney, 1998). In contrast, the rate of seed N accumulation can vary during the seed filling upon N availability in plant (in wheat: Jenner et al., 1991; in soybean: Hayati et al., 1996; in pea: Lhuillier-Soundélé et al., 1999a; 1999b). N limitation can affect negatively plant N availability during the seed period and consequently decrease the rate of seed N accumulation and seed N concentration (in wheat: Debaeke et al., 1996; in soybean: Streeter, 1978; Hayati et al., 1996; in pea: Lhuillier-Soundélé et al., 1999a).

### 3.2 Impact of heat stress on plant N fluxes

Temperature is one of the main environmental factors explaining the variations of yield and quality in crop plants (Wheeler et al., 2000 in different annual crops; Karjalainen & Kortet, 1987 in pea; Sidlauskas & Bernotas, 2003 in oilseed rape ; Peng et al., 2004 in rice ; Schlenker & Roberts, 2009 in corn, soybean and cotton; Peltonen-Sainio et al., 2010 in barley, wheat and oilseed rape ; Asseng et al., 2011 in wheat). Temperature changes are not necessary deleterious to plants: air or soil temperature regulates the rate of many growth and development processes of plants. A threshold high temperature refers to a value of daily mean temperature at which a detectable reduction in growth begins (Wahid et al., 2007). Such heat stress can have damaging impacts on both vegetative and reproductive tissues by causing proteins to unfold, affecting membrane fluidity, metabolism and cytoskeleton rearrangements (Ruelland & Zachowsky, 2010). Although, it is difficult to define a limiting temperature for an integrative process like growth, it is known that heat stress threshold temperatures vary in plant species with the origin of their habitats. For the temperate legume crop pea (*Pisum sativum* L.), a temperate above 25°C can be considered as limiting (Guilioni et al., 2003). Regarding the differing effects of temperature on the plant physiology in each range, stress-inducing temperature has been defined as severe when temperature exceeds 35°C for a few hours a day for a few days (Guilioni & Jeuffroy, 2010). The threshold

temperature during flowering, which results in seed yield losses, was 29.5 °C for three *Brassica* species (*B. napus*, *B. napu* and *B. juncea* L.) (Morrison & Stewart, 2002). In temperate cereals as wheat, two heat ranges may produce distinct negative reactions: a moderate high temperature range between 15 and 32°C, and a severe heat stress above 32°C (Wardlaw & Wrigley, 1994). Schlenker & Roberts (2009) found that temperature above a threshold is harmful to yields of tropical crops (close to 30°C for corn, soybean and cotton). Although photosynthesis and C metabolism are considered as the physiological processes most sensitive to high temperature (Wahid et al., 2007) and were widely studied, N fluxes in crops plants can be significantly affected by this environmental factor and progress are currently made on this subject.

### 3.2.1 N acquisition and assimilation

#### 3.2.1.1 Root morphology

Soils in the field show temperature gradients and diurnal oscillations that can strongly affect root growth (Walter et al. 2009). However, depending on soil depth, changes in soil temperature are delayed and display lower amplitude than those encountered for the atmospheric temperature. At moderate temperature, the length of main and lateral roots increases almost linearly with temperature in oilseed rape (Nagel et al., 2009) and various plant species, such as cereals, cotton, sunflowers and forage legumes (Al-Ani & Hay, 1983; McMichael & Quisenberry, 1993). The temperature effect seems to be more pronounced on root branching than on tap root lateral growth, thus root branching increases with higher temperature up to the optimal temperature of a plant (McMichael & Quisenberry, 1993; Nagel et al., 2009). Wahid et al. (2007) suggested that heat stress can cause root growth inhibition. Moreover root viability decreases at high soil temperature (Rachmilevitch et al., 2006).

#### 3.2.1.2 Nitrate uptake and assimilation

In non legume plants, N accumulation relies on nitrate and ammonium assimilation. Rates of N assimilation usually decrease at high temperature (Rachmilevitch et al., 2006). Moreover N allocation to root growth increases at high temperature (Rachmilevitch et al., 2006), thus the translocation of N from roots to shoots may be modified by high temperatures (DeLucia et al., 1992). In legumes, nitrate assimilation is also affected by high temperature, with decreased levels and activities of nitrate reductase, glutamine synthetase and glutamate synthase and lowered synthesis of ureides (Sahulka & Lisa, 1979 in pea ; Hungria & Vargas, 2000).

#### 3.2.1.3 Symbiotic N<sub>2</sub> fixation

The responses of legume-Rhizobium symbiosis to a wide range of elevated temperatures were observed in temperate and tropical legumes. The optimum temperatures for N<sub>2</sub> fixation vary widely between legumes species and reflect their environmental adaptation (Chalk et al., 2010). For temperate legumes the optimum temperature for N<sub>2</sub> fixation is between 15 and 25°C (Sprent et al., 1988), while for tropical legumes, upper limits for N<sub>2</sub> fixation range between 27 and 40°C (Hungria & Vargas, 2000). High temperatures decrease significantly the survival of rhizobia (Chalk et al., 2010; Hungria & Vargas, 2000) and affect the competitive ability of Rhizobium strains (Bordeleau & Prévost, 1994; Chalk et al., 2010). Rhizobium is most vulnerable to stress when it is free-living outside the symbiotic relationship (Chalk et al., 2010). So, repeated inoculation and higher rates of inoculation of

grain legumes may be needed to guaranty optimum N<sub>2</sub> fixation in tropical soils (even when the strain is already present in the soil) (Hungria & Vargas, 2000). High temperatures can also inhibit all steps of nodule establishment by affecting the exchange of molecular signals between host plants and rhizobia, the root-infection process being probably the most affected (Hungria & Vargas, 2000). If nodules are formed, high temperatures may either decrease N<sub>2</sub> fixation efficiency by affecting nitrogenase activity or nodule longevity by accelerating nodule senescence (Bordeleau & Prévost, 1994 ; Hungria & Vargas, 2000).

### 3.2.2 N partitioning among organs and mobilization of N compounds

**Assimilate partitioning** can be affected by high temperature (Wahid et al., 2007). Increased remobilisation efficiency of reserves from leaves, stems or other plant parts has been suggested as potential strategy to improve grain filling and yield in wheat under heat stress (Wahid et al., 2007). This suggestion is mostly based on C assimilate movements : heat stress in wheat significantly increased total non-structural carbohydrates (TNC) remobilization efficiency with significant differences observed among genotypes (Blum et al., 1994 ; Tahir & Nakata, 2005). Some results have suggested that the acceleration of senescence by temperature was due to an increase in N assimilate remobilization to seeds (Spiertz, 1977 in wheat). However, for a moderate temperature range, this hypothesis was not verified in pea or soybean as the rate of apparent N remobilization was similar among temperatures (Grangirard et al., 2001; Larmure et al., 2005). Moreover, more recent results in wheat suggest on the contrary that heat stress reduced N remobilization (Ercoli et al., 2010; Tahir & Nakata 2005); in agreement with the results of <sup>15</sup>N assimilate labeling experiments in rice, suggesting that high temperatures induce a decrease in N transport from shoots to the ears *via* the phloem (Ito et al., 2009). Further investigations may improve the understanding of the effect of high temperature on N assimilate partitioning and their physiological basis.

**Seed N accumulation** comes from daily N accumulation by the plant and from N remobilised from vegetative parts in both legumes and non-legumes plants (in wheat: Jeuffroy et al., 2000; in pea : Pate, 1985; Schiltz et al., 2005). The rate of seed N accumulation is mostly determined by plant N availability from these two sources (in pea: Lhuillier-Soundélé et al., 1999b; in soybean: Hayati et al., 1996; in wheat : Jenner et al., 1991), consequently any effect of temperature on plant N available for seeds should also affect the rate of seed N accumulation. Indeed, in wheat, the influence of high temperatures on seed N accumulation was modified when N supply to plants was modulated (Zahedi et al., 2004). In pea, a higher N availability for the seed at a higher temperature (in a moderate range of 13-23°C) resulted in an increase in the rate of seed N accumulation (Larmure et al., 2005). Moreover, high temperatures affect seed N accumulation because elevated temperature during the reproductive phase causes earlier cessation of total dry matter accumulation in seeds (in wheat: Spiertz, 1977; Hunt et al., 1991; Blum et al., 1994; in soybean: Egli & Wardlaw, 1980).

### 3.2.3 High temperatures affect both seed N concentration and composition

Literature generally indicates that seed N concentration progressively increases when temperatures rise during the reproductive period in both legumes and non-legumes plants. This increased seed N concentration seems to result from differing susceptibilities of starch and N accumulation in seed to high temperature. In pea, when high temperature occurs either during or after flowering seed N concentration is increased (Karjalainen & Kortet,



1987; Larmure et al., 2005). In wheat and barley, when temperature is increased in a moderate range of 15-32°C during post anthesis, grain N concentration is increased (Bhullar & Jenner, 1985; Jenner et al., 1991; Passarella et al., 2002; Wardlaw & Wrigley, 1994). In rice after heading, kernel N concentration increases when temperature is raised from day/night values of 27/22°C to 33/28°C but remains thereafter steady when temperature is subsequently increased up to 39/34°C (Tashiro & Wardlaw, 1991). Similarly, in soybean, in the range of temperature encountered by the plants during the reproductive period seed N concentration increases with temperature up to a day/night temperature of 40/30°C (Piper & Boote, 1999; Thomas et al., 2003).

Several authors suggest that the earlier arrest of seed filling due to heat stress interferes with the accumulation and processing of the latest proteins accumulated. In wheat, high temperatures increase the level of globulin protein storage causing a reduction of the albumin/globulin content in mature seeds (Hurkman et al., 2009; Stone & Nicolas, 1996). In pea, the final level of vicilin storage proteins was higher under heat stress (Bourgeois et al., 2009). High temperature during seed filling also affects the accumulation level of stress/defense proteins, like heat shock proteins (Bourgeois et al., 2009; Hurkman et al., 2009; Majoul et al., 2003). The magnitude in which a heat stress during seed filling period affects seed quality depends on the heat tolerance of the genotype, the intensity and the timing of heat stress (Stone & Nicolas, 1996; Passarella et al., 2002; Spiertz et al., 2006). These modifications in final protein composition may have an impact on the digestibility of proteins allergenic potential and seed germination.

### **3.2.4 Impact of heat stress on N rhizodeposition**

**Root exudation increases at elevated temperature**, suggesting that root exudation of organic substances (Uselman et al., 2000 in *Robinia pseudoacacia* L.; Arai-Sanoh et al., 2010 in rice). Moreover, symbiotically N<sub>2</sub>-fixing plants continue to exude organic substances, even under extreme N limitation (Uselman et al., 2000). Roots longevity could also be decreased as soil temperature increases, because maintenance respiration of the roots is probably increased (Pritchard, 2011). Fine root, along with their symbionts, are responsible for a significant input of organic C and N to soil, where much of its is eventually made available to other soils organisms (Pritchard, 2011). These additional organic inputs into the soil at elevated temperature may stimulate the microbial community. Moreover, an interspecific transfer of N between legumes and non legumes crops can take place within a short period (20 days) in legume-based grassland even at relatively low temperature (13°C), suggesting that legumes could lead to a substantial contribution of soil N (Gylfadottir et al., 2007 with white clover *Trifolium repens* and smooth meadow grass *Poa pratensis*).

### **3.3 Impact of water deficit on plant N fluxes**

Water stress is one of the most important factors limiting crop yields world-wide (Kramer & Boyer, 1997), especially in arid and semi-arid areas, although its occurrence fluctuates at different temporal and spatial levels (Bai et al., 2004; Knapp et al., 2001; Xu et al., 2009). In addition, with climate change, plants will be subjected to increased variability of water availability, including increased frequency and intensity of extreme droughts (Gutschick and BassiriRad, 2003; Pereira et al., 2006). It thus appears essential to decipher the impact of water stress on plant N fluxes.

### 3.3.1 Characterization of a water deficit

A water stress can be defined as the situation when the plant cannot cope anymore with a soil water deficit, leading to a decrease of water content in the tissues, and thus importantly modifying its metabolism. Plant responses to water stress can be studied *in situ* but also under controlled conditions, by water withholding or by using osmotica such as polyethylene glycol, mannitol or melibiose. Benefits and drawbacks of each of these experimental methods are presented in Verslues et al. (2006). In order to characterize the plant water status, different variables are used: either variables reflecting *stricto sensu* the plant water status (e.g. leaf water potential ( $\Psi$ ), relative water content (RWC)) (Hsiao, 1973) or a variable reflecting water fluxes within the plant (e.g. fraction of transpirable soil water, FTSW) (Sarr et al., 2004). For example, in pea, a water stress is reached when  $\Psi \sim -1.2$  MPa, and FTSW  $\sim 0.1-0.2$  (Lecoeur & Guilioni, 1998), although these values depend both on the species and on the genotype (Ladrera et al., 2007; Serraj & Sinclair, 1997).

The most obvious plant response to a water stress is a decrease in total plant biomass, which is the consequence of a reduced stomatal conductance, leading to a decrease of leaf expansion, and causing a lesser C assimilation *via* photosynthesis (Bradford & Hsiao, 1982). The impact of a water stress depends on its intensity, on its length but also on the period when it occurs during plant life cycle (Bradford & Hsiao, 1982). However, the most marked effects in term of yield losses are reached when water stress occurs during flowering or grain filling (Meckel et al., 1984; Thomas et al., 2004), which can explain the abundance of studies during these developmental stages. Even if water stress has been the subject of extensive research from the gene to the canopy levels, it still remains difficult to predict the effect of water deficit on plant N status (Gonzalez-Dugo et al., 2010).

### 3.3.2 Impact of water deficit on N supply, acquisition and assimilation

#### 3.3.2.1 Soil N transformation, as affected by water deficit

Water stress conditions lead to decrease soil microbe biomass (Schimel et al., 1999), certainly by the indirect decrease of the soil available  $O_2$  needed by aerobic micro-organisms (Smolander et al., 2005). As a consequence, the mineralization process, converting soil organic matter into  $NH_4^+$ , as well as the nitrification process, converting  $NH_4^+$  into  $NO_3^-$ , which are both achieved by micro-organisms, are reduced (Fierer & Schimel, 2002; Vale et al., 2007). Moreover, after a water stress, the longevity of the root system is reduced, which leads to an increase of the organic matter pool in the soil (Huang & Gao, 2000). When water stress is followed by a rewatering, then mineralization is re-activated and amplified by the high quantity of organic matter (Austin et al., 2004). Finally, soil nutrient status can influence root radial resistance to water movement as, for an example, decreased soil N availability reduces root hydraulic conductivity (Clarkson et al., 2000).

#### 3.3.2.2 Plant N acquisition and assimilation under water deficit

The absorption of N by roots as  $NO_3^-$  or  $NH_4^+$  requires the presence of water in the soil and especially in a close area around the roots, as these minerals are water mobile (Garwood & Williams, 1967). Hence, in a water stressed environment plant N uptake is reduced due (1) to the lower availability of the ions in the soil, (2) to the reduced water flow resulting from the reduced plant transpiration and (3) to an effect on the active transport mechanism, and on the membrane permeability (Hsiao, 1973). Yet studies showed contradictory results (Gonzalez-Dugo et al., 2010). Indeed, numerous authors concluded that N uptake was

independent from plant transpiration in various crops (Bhat, 1982; Gastal & Saugier, 1989; Schulze & Bloom, 1984) while others observed that N uptake decreased under water stress (Buljovic & Engels, 2001). As the latter have found an increase in soluble sugar content of the roots with increasing soil drought, they conclude that low N-uptake ability of roots was not caused by low assimilate supply of roots from shoots (Buljovic & Engels, 2001). In addition, increased abscisic acid (ABA) synthesis during water stress periods regulates aquaporins (either *via* their gene expression modifications or *via* post-transcriptional regulations), leading to a control of root conductance, and thus the absorption of nutrients (Beaudette et al., 2007; Ehler et al., 2009; Parent et al., 2009; Törnroth-Horsefield et al., 2006). In legume plants, the most striking response to water stress is the decrease of N<sub>2</sub> fixation, associated with a decrease in nodule formation, a reduced size of the nodules and a decrease in nodule specific activity (King & Purcell, 2001; Serraj et al., 1999; Streeter, 2003). In any case, the proportional dependence of plant N biomass on symbiotic N<sub>2</sub> fixation decreases with the extended duration of water stress (Kirda et al., 1989). Furthermore, irreversible cessation of symbiotic N<sub>2</sub> fixation has been shown to occur in case of extremely severe water stress conditions (Guerin et al., 1991; Sprent, 1971; Venkateswarlu et al., 1989; Walker & Miller, 1986). And finally, the severity of the N<sub>2</sub> fixation related response is also dependent from the bacteria strains, which can be more or less sensitive to soil drought (Djedidi et al., 2011).

Several mechanisms were proposed to explain the decrease of biological N<sub>2</sub> fixation. A first possibility concerns a decrease in nodule cortical permeability, limiting the O<sub>2</sub> supply to the bacteroids and thus decreasing their respiration. As a consequence, the nitrogenase activity is highly reduced (Durand et al., 1987; Hungria & Vargas, 2000; Walsh, 1995) causing oxidative damages. Moreover, it has been shown that under severe drought conditions, the nodule leghemoglobin content was negatively affected (Manavalan et al., 2009; Naya et al., 2007), impacting once more on the O<sub>2</sub> availability to the bacteroids (Marino et al., 2006). In parallel, a gene coding for a ferritin is largely up-regulated under water stress conditions, suggesting an important iron trafficking in stressed nodules, being the consequence of the leghemoglobin degradation (Clement et al., 2008). Another mechanism related to the inhibition of N<sub>2</sub> fixation involves a N feedback, mediated by various molecules depending on the plant species such as asparagine (Bacanawmo & Harper, 1997), glutamine (Neo & Layzell, 1997), proline, histidine, tryptophane (Larrainzar et al., 2009), ureides (Serraj et al., 2001) or even a combination between high rates of nodule asparagine and ureide and a transport of amino acids from the leaves (King & Purcell, 2005). Synthesis of proline and an induction of amino acid transporter in leaves in response to a water deficit (Rentsch et al., 1996; Bray, 1997.) contribute to explain the dramatic accumulation of proline observed in the growing region of the primary root in response to drought (Ober & Sharp, 1994).

According to Marino et al. (2007), it seems that, contrarily to plant responses to N deficiency, plant response to water stress is localized rather than systemic at least during the early times after applied drought. Some authors have suggested that symbiotic N<sub>2</sub> fixation inhibition and ureide accumulation in shoots following a water stress are concomitant, but are not necessary causally related, because they follow different kinetics (Alamillo et al., 2010). These authors consider that ureide accumulation is part of a general response to stress, in particular because ureides play a key role in cell protection under oxidative stress conditions (Brychkova et al., 2008), such as the nodule senescence induced by drought (Puppo et al., 2005; Yamaguchi et al., 2010). Finally, Planchet and co-authors suggested that the exploration of the putative central role of glutamate in water-deficit tolerance, through ABA

signaling, could lead to more information on changes of N metabolism under adverse environmental conditions (Planchet et al., 2011).

Moreover, C fluxes are considered as playing a major role in the regulation of symbiotic N<sub>2</sub> fixation in pea and soybean. Indeed, in these species, C supply (mainly in the form of malate) to the nodules declines (Arrese-Igor et al., 1999), which can be due to a decrease of the main enzyme responsible for the cleavage of sucrose in nodules : the sucrose synthase (at the gene expression as well as the enzyme activity levels). In the model plant *Medicago truncatula*, an accumulation of sugars, polyols and amino-acids was observed and the authors hypothesized that these compounds could play a role in osmotic regulation processes in the nodules, or could be related to the reduction of the plant transpiration rates, limiting the xylemic flux and thus the transport of the compounds from the nodules to the other plant compartments (Larrainzar et al., 2009).

The effects of water-deficit on N uptake and N assimilation (quantified by <sup>15</sup>N tracing methods) were recently investigated in white clover (*Trifolium repens* L.) by Lee et al. (2009). The water-deficit treatment significantly reduced the maximum NR activity, and also attenuated *de novo* synthesis of amino acids and proteins in the roots. The concentration of proline in the phloem exudates increased rapidly after 3 d of water deficit. Interestingly, the increase in proline concentrations in phloem exudates in response of water stress was closely related to reductions of N uptake, as well as NR activity in the roots and assimilation of newly absorbed N in amino acids. In addition, the accumulation of proline induced in roots by exogenous proline treatment was closely related to the decrease in NR activity. Based on these results, Lee et al. (2009) have suggested that increased proline transport to roots *via* phloem caused by water deficit has a significant influence on the down-regulation of plant N fluxes, particularly N uptake and assimilation of newly absorbed N.

### **3.3.3 Impact of water deficit on N partitioning among organs, mobilization and storage of N compounds**

Water stress doesn't seem to affect xylem nitrate concentration in sunflower (Schurr & Schulze, 1996), probably because of the independence of the transpiration process and the nutrient supply process (Tanner & Beevers, 2001), explained by the fact that the two transpiration independent water flows (flux of water associated with volume expansion and Münch's counterflow in the phloem) were found to ensure plant nutrient transport (Gonzalez-Dugo et al., 2010).

When drought was imposed on one month alfalfa (*Medicago sativa*) plants, N flux determined using <sup>15</sup>N pulse-chase labelling revealed that N compounds in leaves were redistributed mainly towards roots and secondary in stems (Eric & Avice, unpublished data). This was accompanied by increase of proline, and soluble proteins (especially VSP) concentrations in roots (Eric-Soreasu et al., 2007). Interestingly, when water recovery was applied after drought, <sup>15</sup>N analysis demonstrated that N reserves were remobilized from roots and stems to growing leaves. This was concomitant with the decrease of VSP and proline concentrations in roots of alfalfa. Additionally, it was reported that alfalfa plants treated by ABA (a phytohormone highly involved in the mechanisms of drought tolerance ) (1) provoked a massive redistribution of N from leaves to the roots, (2) induced the accumulation of VSP in taproot after 6 d of treatment and (3) stimulated the steady-state 32 kDa VSP transcript within 3d of treatment (Avice et al., 2003). These last data suggest that the drought-induced accumulation of VSP may be regulated by ABA. Thus, the capacity to

remobilize N reserves from leaves and store these recycling N compounds in perennial organs under specific soluble proteins and amino-acids forms during critical phases of alfalfa development, may represent an adaptive trait with regard to plant tolerance against water stress.

In pea, N partitioning between below ground part and above ground part is not modified by water stress (around 15% of N allocated to the roots and 85% to the above ground part), when it has been applied either at the flowering stage or during seed filling stage (Mahieu et al., 2009). In soybean, it has been shown that under a continuous water stress treatment from the beginning of seed filling until physiological maturity, the decreasing rate of leaf N and chlorophyll was more intense (de Souza et al., 1997). In cereals, similar observations were made, considering that these post anthesis water deficits could result in early leaf senescence, and thus more remobilization of pre-anthesis stored assimilates to grains (Yang & Zhang, 2006). Moreover, a water stress induces a shortened seed-filling period, leading to smaller seeds and lower yields, but interestingly, oil and protein content are not modified in soybean (Dornbos & Mullen, 1992) or in pea (Crozat et al., 1992). The origin of the remobilized N for seed filling accounts for 90% from the above ground part in pea, and is significantly reduced under water stress conditions, as well as the amount of N remobilized from roots for seed filling or released into the soil (Mahieu et al., 2009).

### 3.3.4 Impact of water deficit on N rhizodeposition

Root exsudates could play a major role in the maintenance of soil-root contact, which is especially important to the plant under drying conditions, when hydraulic continuity will be lost (McCully & Boyer, 1997; Walker et al., 2003; Wittenmayer & Merbach, 2005). In pea, the rate of N derived from rhizodeposition decreases with water stress, but the proportion of N allocated to rhizodeposition process is not modified. In other words, the amount of rhizodeposited N is directly proportional to the plant total N amount (Mahieu et al., 2009). This results was corroborated by a study carried out on *Lupinus argenteus* (Goergen et al., 2009).

## 4. Conclusion

A decline in the growth trends of cereal and legume yields has been witnessed over the last two decades (Brisson et al., 2010). Abiotic stresses appear to be important factors of climatic change explaining the decrease in yields (Wheeler et al., 2000; Challinor et al., 2005), especially heat stress during seed filling and drought during elongation (Schlenker & Roberts, 2009; Peng et al., 2004, Brisson et al., 2010). Thus, genetic progress made in the second half of the 20<sup>th</sup> century was partly counteracted by climate change. The 21<sup>th</sup> century will be extremely challenging to agriculture because of this global climate change. A global increase in temperature (of about 0.2°C per decade), a change in the distribution of precipitation and an intensification of drought in arid and semiarid areas are projected (Salinger, 2005; IPCC, 2007). Abiotic stresses (heat stress, water stress, and N limitation) will thus occur more frequently and in a simultaneous manner, especially high temperature and water deficit. Finally, changes in the climate variability and increase in temperature will modify the pest and disease distribution and how these need to be managed (Vadez et al., 2011).

The crop productivity is considerably more reduced by combined stresses than by a stress alone (Shah & Paulsen, 2003; Xu & Zhou, 2006). But little is known about their combined impact on plant N fluxes. Xu & Zhou (2006) suggested that high temperature, combined

with severe soil drought weaken N anabolism and strengthen protein catabolism. This emphasizes the need to further understanding of how plants react and adapt to abiotic stresses.

Undoubtedly, the future challenge relies not only on breeding new varieties well adapted to climate change, but also on the development of new cultural practices such as intercropping between non legume plants and legumes.

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# Biotechnological Solutions for Enhancing the Aluminium Resistance of Crop Plants

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## 1. Introduction

Acid soils limit crop yields around the world due to nutrient deficiencies and mineral toxicities. Non-adapted plants grown on acid soils typically have smaller root systems because high concentrations of soluble aluminium ( $Al^{3+}$ ) inhibit root elongation. This restricts their ability to acquire water and nutrients. Plants vary widely in their capacity to tolerate acid soils and even genotypes within species show significant variation. The physiology and genetics controlling this variability have been studied for many years. The analysis of segregating populations and mutants has helped identify the mechanisms and genes controlling aluminium resistance in many species including wheat, rice, *Arabidopsis*, barley and sorghum. The release of organic anions from roots is an important mechanism of resistance in a wide variety of species. This trait is controlled by genes from two distinct gene families that encode transport proteins. Sufficient information is now available for enhancing the aluminium resistance of important crop species using biotechnology. We will review progress in understanding the mechanisms of  $Al^{3+}$  resistance in plants, the genes controlling these mechanisms and the application of genetic engineering to increase the  $Al^{3+}$  resistance of important crop plants. This approach can help maintain and even increase food production on acid soils in the future.

## 2. Acid soils

### 2.1 What is acid soil?

Soil pH is an important consideration for agriculture production (Kochian et al. 2004; von Uexkull and Mutert 1995). Some plants are sensitive to high or low pH, nutrient availability and mineral toxicities are influenced by pH and soil microbial communities are significantly affected by pH (Fierer and Jackson 2006; Osborne et al. 2011). Acid soils present multiple stresses to plants including proton toxicity, nutrient deficiencies (especially calcium, magnesium and phosphorus) and metal-ion toxicities especially aluminium and manganese. The United States Department of Agriculture classifies acid soils into five levels: *ultra acid* soils (below pH 3.5), *extremely acid* (pH 3.5 to 4.4), *very strongly acid* (pH 4.5 to 5.0), *strongly acid* (pH 5.1 to 5.5), *moderately acid* (pH 5.6 to 6.0) and *slightly acid* (pH 6.1 to 6.5). Soils with  $pH \leq 5.5$  can adversely affect the production of many major food crops.

The major limitation to crop growth in acid soils is soluble aluminium. Although aluminium is the third most abundant element in the earth's crust most of it occurs in mineral forms which are harmless to plants (complexes with oxides and silicates). In acid conditions however these minerals dissolve more readily releasing aluminium into the soil solution. Soluble aluminium hydrolyses to form a range of species the prevalence of which depends on soil pH. When the pH is 4.5 or below, the  $\text{Al}^{3+}$  species predominates but as pH increases other mononuclear aluminium species are formed including  $\text{Al}(\text{OH})_2^+$  and  $\text{Al}(\text{OH})_2^+$ . The insoluble  $\text{Al}(\text{OH})_3$  (gibbsite) can also form at higher pH. Trivalent aluminium ( $\text{Al}^{3+}$ ) is highly toxic to many plants but uncertainty continues regarding the relative toxicity of the hydroxyaluminium species (Alva et al. 1986; Kinraide 1997; Noble et al. 1988; Wright et al. 1987).  $\text{Al}^{3+}$  is a reactive metal ion that forms complexes with a variety of organic and inorganic ligands including carboxylates, sulphate, and phosphate and many of these complexes are less toxic to plants than free  $\text{Al}^{3+}$  (Jones 1998; Kinraide 1997; Matsumoto 2000; Takita et al. 1999).

## 2.2 Formation and distribution of acid soils

Acid soils can develop naturally depending on characteristics of the parent rock but human intervention can accelerate the process (Rechcigl and Sparks 1985; Vanbreemen et al. 1983). Ancient and highly-weathered soils are often acid because the basic cations (calcium, magnesium, sodium and potassium) have been leached down the profile, often with nitrate, and replaced by hydrogen ( $\text{H}^+$ ). Other drivers of acidification include acid precipitation (Rechcigl and Sparks 1985; Vanbreemen et al. 1983) and nitrification. Microorganisms can also generate organic acids and nitrate from the decomposition of plant residues which also contribute the soil acidification. Conversely low pH and aluminium mobilization can affect the microbial populations (Fierer and Jackson 2006) which are required for stubble turnover and nutrient recycling.

Approximately 30% of total land area consists of acid soils, and almost 70% of the world's potentially arable lands are acidic (Vonuxkull and Mutert 1995). The two main geographical belts of acid soils include the humid northern temperate zone mainly covered by coniferous forests and the humid tropics which support savanna and tropical rain-forests.

The American continent, Asia, Africa, Europe and Australia and New Zealand account for 40.9%, 26.4%, 16.7%, 9.9% and 6.1% of the world's acid soil respectively. Most acid soils in Asia are distributed throughout Southeast Asia and the Pacific. In Africa large tracts of the acid soil cannot be used for cultivation because they are sandy, nutrient-deprived and water-limited (Vonuxkull and Mutert 1995).

Naturally acidic soils occupy about one third of Australia, but many agricultural soils in the intensive land-use regions have become more acidic as the result of removal of harvestable product, leaching of nitrate and calcium from nitrogen-producing pastures (Australia State of the Environment report, 2001), and high applications of nitrogen fertilizer (Juo et al. 1995; Matsuyama et al. 2005; Sirovy 1979). Rapid acidification associated with the overuse of nitrogen fertilizer is also an emerging problem in China (Guo et al. 2010). Extremely acid soils can mobilise and increase the bioavailability of other toxic metals such as, mercury, zinc, copper, cadmium, chromium, manganese, and vanadium. All these factors may affect plant growth as well as the ecology of soil bacteria, mosses, algae, fungi, and invertebrates.

### 3. Aluminium toxicity

Acid soils are often low in basic cations, prone to crusting, erosion and compaction but physical constraints and nutrient deficiencies are rarely the main reasons crop plants grow poorly on these soils. Instead, soluble  $\text{Al}^{3+}$  is the major factor limiting growth because it inhibits root growth at very low concentrations. Indeed the inhibition of root growth is the primary symptom of plant stress on acid soils (Munns 1965). There are exceptions because many plants endemic to tropical and sub-tropical regions cope well and even thrive on acid soils. The growth of these species can even be stimulated by  $\text{Al}^{3+}$  and some accumulate high concentrations in their leaves. These are discussed in more detail later.

$\text{Al}^{3+}$  can begin to inhibit root growth of wheat (*Triticum aestivum* L.) within minutes or hours in simple hydroponic solutions (Ryan et al. 1992). Longer exposures result in thickened roots, damaged root cap, and lesions in the epidermal and cortical tissues near the apices. The root system becomes small and damaged which limits water and nutrient acquisition. Root apices are the most sensitive part of the root and  $\text{Al}^{3+}$  must contact the apices directly for growth to be affected (**Figure 1**). Exposure of an entire maize root to  $\text{Al}^{3+}$  except the apical 5 mm has no effect on growth in the short term (Ryan et al. 1993). Within this region the zone between the meristematic and elongation zones (distal transition zone) appears to be the most sensitive part of the root (Sivaguru and Horst 1998). The concentration-dependent responses of root growth vary between species and even among genotypes. In some plants root growth remains unaffected at low concentrations of  $\text{Al}^{3+}$  but declines once a threshold is reached. This is called the *threshold for toxicity* response (Barcelo and Poschenrieder 2002). In other species root growth is stimulated by low concentrations of  $\text{Al}^{3+}$  but declines at higher concentrations. This *hormesis-type* response, is interpreted as  $\text{Al}^{3+}$  first alleviating  $\text{H}^+$  toxicity at low concentrations and then becoming toxic itself at higher concentrations. A third response observed shows growth inhibition at low concentrations of  $\text{Al}^{3+}$  (or short exposures) but little or no effect at higher concentrations (or longer exposures). This is called the *threshold for tolerance* model and is indicative of an acclimation response occurring.

For many crops including the cereals, most of the  $\text{Al}^{3+}$  absorbed by roots localises to the apoplast. The fixed negative charges on the membrane surfaces and pectin in the cell walls attract and bind cations, and especially highly-charged cations like  $\text{Al}^{3+}$ . Nevertheless it is still uncertain whether this apoplastic  $\text{Al}^{3+}$  is toxic to plants or if  $\text{Al}^{3+}$  needs to enter the cytosol to cause injury. By binding to pectin in the cell walls  $\text{Al}^{3+}$  can rigidify the walls and restrict solute flow through the apoplast (Horst et al. 2010; Sivaguru et al. 2006). High concentrations of  $\text{Al}^{3+}$  in the apoplast can induce callose production (1,3 beta D-glucan) and affect membrane function by binding with lipids and proteins or by displacing calcium from critical sites on membranes (Foy et al. 1978).  $\text{Al}^{3+}$  can also directly inhibit nutrient uptake by blocking the function of ion channels involved in  $\text{Ca}^{2+}$  and  $\text{K}^+$  influx (Gassmann and Schroeder 1994; Pineros and Tester 1993).

Cytosolic levels of free  $\text{Ca}^{2+}$  ( $[\text{Ca}]_c$ ) are typically below 1.0  $\mu\text{M}$  in most living cells but transient increases act as signals to control cellular functions and responses to hormones and stress.  $\text{Ca}^{2+}$ -sensitive fluorescent compounds have detected transient increases in  $[\text{Ca}]_c$  in root cells treated with  $\text{Al}^{3+}$  (Rincon-Zachary et al. 2010). The rapidity of these responses indicate that  $\text{Al}^{3+}$  is causing damage in the apoplast and that cytosolic  $\text{Ca}^{2+}$  could signal early responses to  $\text{Al}^{3+}$  stress.  $\text{Al}^{3+}$  can interfere with another signal transduction pathway involving inositol 1,4,5-trisphosphate (Jones and Kochian 1995) as well as actin and tubulin stability (Grabski and Schindler 1995; Sivaguru et al. 2003b).

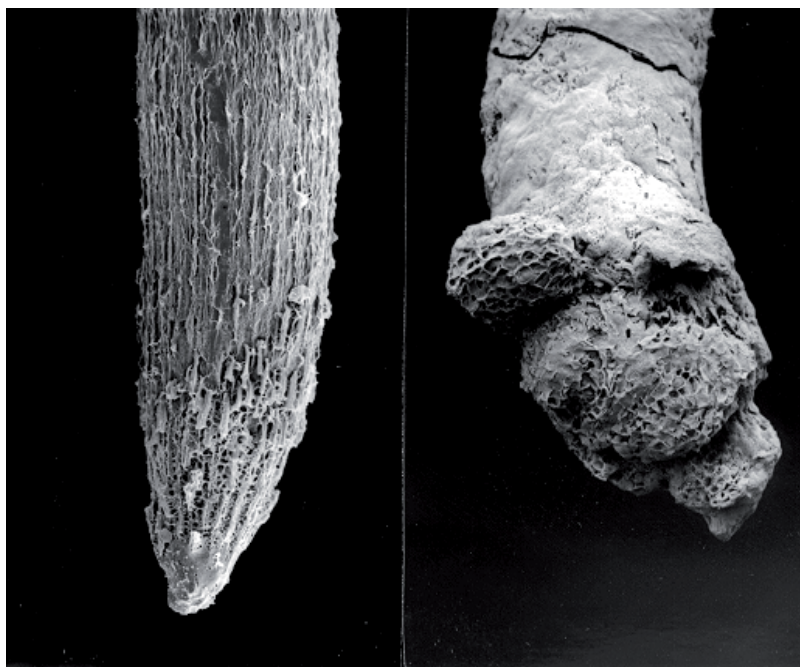


Fig. 1. Effect of  $\text{Al}^{3+}$  toxicity on roots. The scanning electronmicrographs show root apices of near-isogenic wheat plants, ET8 and ES8, that differ in  $\text{Al}^{3+}$  resistance at a single major locus. The plants were grown for 4 days growth in 0.2 mM  $\text{CaCl}_2$  (pH 4.3) with 5  $\mu\text{M}$   $\text{AlCl}_3$ . The resistant line (ET8, on the left) is unaffected by the treatment whereas the sensitive line (ES8, on the right) shows considerable damage to its tissues. The greater  $\text{Al}^{3+}$  resistance of ET8 is controlled by the *TaALMT1* gene on chromosome 4DL. *TaALMT1* encodes an  $\text{Al}^{3+}$ -activated anion channel that facilitates malate efflux from the root apices. (Reprinted from Delhaize and Ryan 1995).

Small but measurable amounts of  $\text{Al}^{3+}$  does enter the cytosol perhaps via non-specific cation channels (Lazof et al. 1994; Rengel and Reid 1997; Taylor et al. 2000). The combination of pH, ionic strength and availability of organic ligands in the cytosol maintain the soluble  $\text{Al}^{3+}$  concentrations to extremely low levels, perhaps less than 1 nM. However even these concentrations may cause damage because  $\text{Al}^{3+}$  can out-compete other cations like  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  for important binding sites and even bind with DNA (Martin 1992).  $\text{Al}^{3+}$  also triggers oxidative stress in root cells by triggering the production of reactive oxygen species (Yamamoto et al. 2001). Whether this response is induced by apoplastic  $\text{Al}^{3+}$  or symplastic  $\text{Al}^{3+}$  is unclear but these highly reactive compounds can rapidly damage membranes, proteins and nucleic acids. Oxidative stress induces callose production which in turn increases cell wall rigidity and decreases the symplastic flow of solutes via the plasmadesmata (Horst et al. 2010; Sivaguru et al. 2000).

In summary,  $\text{Al}^{3+}$  interferes with many cellular functions. The  $\text{Al}^{3+}$ -induced changes to cytosolic  $\text{Ca}^{2+}$  concentration, oxidative stress and callose production are likely to signal the early signs of  $\text{Al}^{3+}$  injury.

#### 4. Natural variations

Plant species vary widely in their ability to grow and yield on acid soils (Foy 1988). Mclean and Gibert (1927) investigated the relative  $Al^{3+}$  resistance of several crop plants. Sensitive crops included *Lactuca sativa* (lettuce), *Beta vulgaris* (beet), *Phleum pratense* (timothy), *Hordeum vulgare* (barley), moderately resistant crops were *Raphanus sativus* (radish), *Sorghum bicolor* (sorghum), *Capitata var. alba* L. (cabbage), *Avena sativa* (oat), *Secale cereale* (rye) and noticeably resistant species included *Zea mays* (maize), *Brassica rapa* (turnip) and *Agrostis gigantea* (redtop) (McLean and Gilbert 1927). Among the cereals rice is significantly more resistant than maize, wheat and sorghum, while barley and durum wheat are among the most sensitive cereal species (Famoso et al. 2010; Garvin and Carver 2003; Khatiwada et al. 1996).

Significant variation in  $Al^{3+}$  resistance occurs within many species as well including maize, wheat, barley, rice, sorghum, snapbean and *Arabidopsis* (Foy 1988; Foy et al. 1993; Furlani et al. 1987; Kochian et al. 2004; Koyama et al. 2003; Magalhaes et al. 2007; Ryan et al. 2011; Toda et al. 1999). This variation provides opportunities for breeders to develop new cultivars better suited to acid soils. Even barley, which is considered one of the most  $Al^{3+}$ -sensitive of the small-grained *Triticeae*, displays significant genotypic variability. A seedling-based screen of barley lines in the South and East Asian Barley Core Collection identified Kearney and Golden Promise as sensitive to  $Al^{3+}$  while Dayton and several Japanese cultivars (Honen, Ohichi and Zairai Tanbo) were among the most resistant (Moroni et al. 2010).

A greater variation occurs in hexaploid or bread wheat where differences in root growth can vary by ten-fold or more in short-term growth assays or in field screens (Bona et al. 1993; Cosic et al. 1994; Dai et al. 2009; Foy 1996; Garvin and Carver 2003; Pinto-Carnide and Guedes-Pinto 1999; Raman et al. 2008; Rengel and Jurkic 1992; Ryan et al. 1995a; Tang et al. 2003). Highly  $Al^{3+}$ -resistant genotypes of bread wheat commonly used in experiments include BH1146 and Carazinho from Brazil and Atlas 66 from the USA. In most cases enhanced  $Al^{3+}$  resistance is associated with reduced  $Al^{3+}$  accumulation in the roots. Therefore the more resistant genotypes of wheat, maize, barley, sorghum and rye are able to exclude  $Al^{3+}$  from their roots cells – especially from the root apices.

#### 5. Genetics

The inheritance and genetics of  $Al^{3+}$  resistance have been widely studied in members of the *Triticeae*. In wheat, barley and sorghum  $Al^{3+}$  resistance is often consistent with a single genetic locus inheritance while in maize and rice it is a more complex multigenic trait.

##### 5.1 Single or few genes: cases of simple inheritance

Crop improvement programs in Brazil and the US led to the development of highly-resistant cultivars of wheat such as BH1146 and Atlas66 which have been used for genetic mapping and quantitative trait loci (QTL) analyses. Many studies indicate a single locus controls most of the variation in  $Al^{3+}$  resistance. For instance, a population of recombinant inbred lines developed with BH 1146 and the sensitive cultivar Anahuac showed a bimodal distribution for Al tolerance, consistent with single gene inheritance. Similar results were obtained with other populations (Raman et al. 2005). The resistance locus in BH 1146, named *Alt<sub>BH</sub>*, was mapped to chromosome 4DL and explained 85% of the phenotypic variation (Riede and Anderson 1996). The location of *Alt<sub>BH</sub>* gene was further confirmed

using 91 recombinant inbred lines and a set of wheat deletion lines (Milla and Gustafson 2001) and Luo et al. (1996) had also linked this chromosome to  $Al^{3+}$  resistance using Chinese Spring deletion lines. More recently a major aluminium resistance gene called *TaALMT1* (Figure 1; see later) was mapped to the same locus on 4DL (Raman et al. 2005) while minor loci were mapped to 4BL and 3BL (Navakode et al. 2009; Ryan et al. 2009).

QTL analyses of 100 F2 barley seedlings derived from the  $Al^{3+}$ -resistant cultivar (Murasakimochi) and the  $Al$ -sensitive cultivar (Morex) identified a single  $Al^{3+}$  resistance locus on chromosome 4H which explained more than 50% of the phenotypic variation (Ma et al. 2004). The *Alp* locus was also mapped to chromosome 4H in a high-resolution map generated from genotypes Dayton and Zhepi 2 (Wang et al. 2007).

Sorghum is closely related to maize and possesses the second smallest genome among cultivated grasses (Mullet et al. 2002). Like wheat and barley the genetics indicate that a single locus, *Alt<sub>SB</sub>*, on chromosome 3 controls most of the variation in resistance (Magalhaes et al. 2004).

## 5.2 Multiple genes: Cases of complex inheritance

Rye is generally regarded as a highly resistant cereal species. Unlike wheat, rye is self-incompatible, so co-segregation experiments in rye generally detect a number of  $Al^{3+}$  resistance loci. Using wheat-rye addition lines, Aniol and Gustafson (1984) identified at least three different  $Al^{3+}$  resistance loci on chromosome 6RS, *Alt2* on 3R, and *Alt3* on 4RL. Two major dominant and independent loci, *Alt1* on chromosome 6RL and *Alt3* were identified (Gallego and Benito 1997) and another on chromosome 7RS (Matos et al. 2005). More recently the 7RS locus was shown to include a cluster of *ALMT*-like genes in the resistant lines (see later) (Collins et al. 2008).

More than 30 QTLs for  $Al^{3+}$  resistance have been reported in rice using populations derived from *indica* and *japonica* cultivars as well as wild relatives like *Oryza rufiogon*. (Ma et al. 2002; Nguyen et al. 2003; Nguyen et al. 2001; Nguyen et al. 2002; Wu et al. 2000; Xue et al. 2007; Xue et al. 2006a; Xue et al. 2006b). Resistance loci on chromosomes 1, 8 and 9 were consistently identified in these studies which confirms resistance is a multigenic trait in this species. Given the conservation of genetic locations among the *Triticeae* (synteny), it will be intriguing whether orthologous loci to the resistance loci from other cereals play a similar role in rice. For instance, a major resistance locus on rice chromosome 3 is homeologous to *Triticeae* 4L where the  $Al^{3+}$  resistance loci on wheat and barley are located (Nguyen et al. 2003). In maize, five QTLs on chromosomes 2, 6 and 8 contribute to  $Al^{3+}$  resistance and these explain 60% of the phenotypic variation. Dominant and additive effects were detected between these loci (Ninamango-Cardenas et al. 2003).

## 6. Mechanisms of $Al^{3+}$ resistance

Some plants have evolved mechanisms that enable them to tolerate  $Al^{3+}$  toxicity and acid soils better than others. The identification and characterization of these mechanisms has been the focus of considerable research. Some very resistant species like tea (*Camelia sinensis*) and *Hydrargenea* sp accumulate high concentrations of  $Al^{3+}$  in their roots and leaves while others, such as resistant members of the *Triticeae*, exclude  $Al^{3+}$  from their root and shoots. For instance the concentration of  $Al^{3+}$  in the root apices of an  $Al^{3+}$ -sensitive wheat cultivar after 24 h in 50  $\mu$ M  $Al^{3+}$  was 10-fold greater than a resistant cultivar (Rincon and Gonzales



1992) and similar results were reported in closely-related wheat lines that differed in  $Al^{3+}$  resistance (Delhaize et al. 1993a). Therefore two main mechanisms have been proposed to account for resistance: exclusion mechanisms and tolerance mechanisms, and evidence is now available for both of these. Exclusion mechanisms prevent  $Al^{3+}$  from entering the cytosol and minimize harmful interactions from occurring in the apoplast. Tolerance mechanisms allow plants to safely take-up and accumulate  $Al^{3+}$  within their cells. Both mechanisms may be operating in the same plant.

### 6.1 Mechanisms of $Al^{3+}$ exclusion

There are several ways  $Al^{3+}$  could be prevented from accumulating in apoplastic and symplastic fractions of root tissues. Cell wall chemistry could affect  $Al^{3+}$  binding, the maintenance of a slightly higher rhizosphere pH could shift the hydrolysis of soluble aluminium from  $Al^{3+}$  to  $Al(OH)^{2+}$  which would reduce accumulation in the cell wall, compounds could be released from the root which bind the harmful  $Al^{3+}$  and limit other more damaging interactions from occurring and  $Al^{3+}$  could be actively exuded from the root cell by some active transport process. Charged residues on cell wall pectin will attract and accumulate cations but pectin content is not consistently correlated with either  $Al^{3+}$  sensitivity or resistance (Horst et al. 2010). Recent studies showing that methylation of the pectin residues is correlated with reduced  $Al^{3+}$  accumulation in the wall support the idea that modifications to cell walls can increase  $Al^{3+}$  resistance.

Currently there are no examples of resistance based on  $Al^{3+}$  exudation and nor are there convincing cases linking higher rhizospheric pH to genotypic variation in resistance despite detailed studies in wheat and maize (Pineros et al. 2005). However there are claims of an  $Al^{3+}$ -resistant *Arabidopsis* mutant (*alr-104*) showing a pH dependent increase in resistance (Degenhardt et al. 1998). Measurements with micro-pH electrodes detected a 0.15 unit higher pH at the root surface of *alr-104* plants compared to wildtype plants and subsequent experiments indicated this relatively small pH change could explain the increased resistance. The molecular biology of the *alr-104* mutation has not been characterised in detail.

The importance of  $Al^{3+}$  exclusion to the very high resistance of rice was confirmed after characterizing two  $Al^{3+}$ -sensitive mutations, *als1* and *c68*, because both of these recessive mutations lead to increased accumulation of  $Al^{3+}$  in the roots (Huang et al. 2009; Ma et al. 2005). *als1* carries a mutation in a gene encoding part of an ATP binding cassette (ABC) transporter (see later) while the *c68* mutation remains uncharacterized at the genetic level.

The exclusion mechanism for which most supporting evidence is available is the release of organic anions from roots (Delhaize et al. 2007; Ma et al. 2001; Ryan et al. 2001). Malate and citrate are the two anions most commonly reported but oxalate efflux occurs from a few species. Once these anions are released from root cells they bind the  $Al^{3+}$  and prevent it from accumulating in the apoplast, damaging the cells and being absorbed by the roots. Efflux is largely restricted to the root apices and in nearly all cases it does not occur continuously but is activated by exposure to  $Al^{3+}$ . The effectiveness of these anions in reducing  $Al^{3+}$  toxicity is demonstrated by adding them to solutions containing toxic concentrations of  $Al^{3+}$ . Root growth improves as the anion concentration increases. This occurs for malate, citrate and oxalate additions but not for anions, such as succinate and acetate, which have lower stability constants for  $Al^{3+}$  (Ryan et al. 2001). This exclusion mechanism has now been reported in species from the Poaceae (e.g. wheat, barley, sorghum, maize and rye), Araceae (e.g. taro), Polygonaceae (e.g. buckwheat), Brassicaceae (e.g. *Arabidopsis*) and the Fabaceae (e.g. soybean, snapbean, common bean, *Cassia tora*).

The first study linking organic anion efflux with  $\text{Al}^{3+}$  resistance was described by Miyasaka et al. (1991). They showed that  $\text{Al}^{3+}$  activated citrate exudation from snapbean roots and that the efflux from a resistant cultivar was 10-fold greater than efflux from a sensitive cultivar. Another example was reported soon after in wheat by Delhaize et al. (1993b) and Ryan et al. (1995a) using near-isogenic wheat lines differing in  $\text{Al}^{3+}$  resistance. These studies showed that addition of  $\text{Al}^{3+}$  to a nutrient solution rapidly stimulated malate release from the root apices of the resistant iso-line but not from the sensitive line. This rapid activation of efflux is termed a Type I response (**Figure 2**). Type I responses are interpreted as  $\text{Al}^{3+}$  activating a transport protein already present in the plasma membrane so little or no delay occurs (Ma et al. 2001). An  $F_2$  population generated from these near-isogenic lines demonstrated that resistance co-segregates with malate efflux. Subsequent analyses revealed a strong positive correlation between malate efflux and  $\text{Al}^{3+}$  resistance in diverse germplasm which supports the importance of this major trait in wheat (Raman et al. 1995a, b; Raman et al. 2005).  $\text{Al}^{3+}$  resistance in barley is correlated with citrate efflux from roots. Organic anion efflux does not appear to be important contributor to the high resistance of rice but it does appear to be a minor contributor in maize. Several maize genotypes display an  $\text{Al}^{3+}$ -activated efflux of citrate but the efflux is delayed by several hours after  $\text{Al}^{3+}$  addition. This is referred to as a Type II response (**Figure 2**). The delay is interpreted as  $\text{Al}^{3+}$  first inducing expression of the transport protein before then activating anion efflux (Ma et al. 2001). Type II responses have also been reported for citrate efflux from *Cassia tora*, rice bean, and rye (Ma et al. 2001; Yang et al. 2006). Some maize genotypes also show a slower  $\text{Al}^{3+}$ -inducible increase of citrate content suggesting that  $\text{Al}^{3+}$  resistance may also rely on internal detoxification (Pineros et al. 2002). Nevertheless no clear correlation has been established between citrate exudation and  $\text{Al}^{3+}$  resistance among a large range of maize genotypes (Pineros et al. 2002) which supports a model where several different mechanisms contribute to  $\text{Al}^{3+}$  resistance in this species.

## 6.2 Mechanisms of $\text{Al}^{3+}$ tolerance

Instead of excluding  $\text{Al}^{3+}$  from their tissues, many highly-tolerant species absorb  $\text{Al}^{3+}$  and store it in their leaves sometimes to concentrations exceeding 3000 mg/kg. This relies on quite different processes involving complexation, detoxification and transport of aluminium within the plant. Aluminium accumulator species are defined as those with 1000 mg/kg aluminium or more in their leaves. Some of these species include tea (*Camelia sinensis*), *Hydrangea sp* and buckwheat (*Fagopyrum esculentum*) as well as a range of tree and shrub species (Haridasan and Dearaujo 1988). Most of the aluminium in tea leaves resides in the apoplast (Tolra et al. 2011) whereas in the leaves of *Hydrangea* and buckwheat the aluminium is bound in vacuoles by citrate and oxalate anions, respectively. *Hydrangea* is an ornamental plant that changes the colour of its flowers from pink to blue when grown in acid soils with high  $\text{Al}^{3+}$  availability (Ma et al. 1997). This colour change is caused by the formation of aluminium delphinidin complexes or aluminium caffeoylquinic acid complexes (Takeda et al. 1985). Buckwheat can accumulate 15,000 mg/kg aluminium in its leaves when grown in acid soils (Ma et al. 1997).

High shoot accumulation of aluminium implies soluble aluminium is transported through the xylem and then stored safely in leaf vacuoles or in the apoplast. To protect the plant cells from damage aluminium is bound by organic ligands as it is transported throughout the plant.  $^{27}\text{Al}$  NMR studies identified aluminium oxalate complexes (1:3) in buckwheat leaves

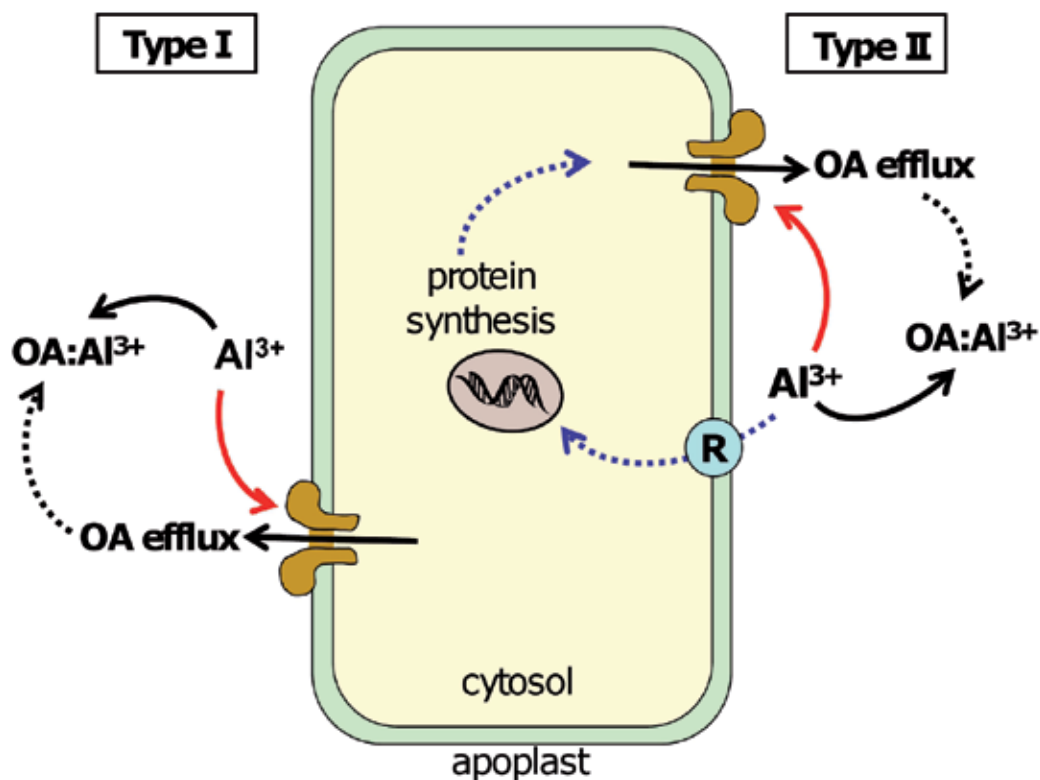


Fig. 2. Al<sup>3+</sup>-activated organic anion efflux. The Type I response illustrates the rapid activation of organic anion efflux in species such as wheat where the anion channel is constitutively expressed. Al<sup>3+</sup> is able to rapidly activate efflux by interacting directly with the pre-existing proteins (red arrows). The Type II response occurs in maize and rye and shows a delay between the addition of Al<sup>3+</sup> and the start of organic anion efflux. This delay is interpreted as Al<sup>3+</sup> first inducing the expression of the transport protein via a signal transduction pathway possibly involving a specific receptor ("R") (blue arrows). Once synthesized and inserted in the plasma membrane, Al<sup>3+</sup> is thought to interact with the protein to activate efflux of organic anion (OA).

(Ma et al. 2001), but aluminium citrate complexes in the xylem (Ma and Hiradate 2000). It appears that aluminium undergoes a ligand exchange with oxalate and citrate depending on whether it is transported into xylem or being sequestered in the leaves.

## 7. Identification of Al<sup>3+</sup>-resistance genes in plants

Several Al<sup>3+</sup> resistance genes have now been mapped and cloned from a range of species (Table 1). Ryan et al. (2011) classifies these resistance genes into three groups: (1) those isolated by analysing segregating populations and therefore explain genotypic variation, (2) those identified from mutant analysis and therefore do not necessarily explain genotypic variation, and (3) likely resistance genes which require additional supporting information.

Species	Genes	Protein Function	Reference
<b>Organic transporters</b>			
Wheat	<i>TaALMT1</i>	Malate transporter	(Sasaki et al. 2004)
<i>Arabidopsis</i>	<i>AtALMT1</i>	Malate transporter	(Hoekenga et al. 2006)
Sorghum	<i>SbMATE</i>	Citrate transporter	(Magalhaes et al. 2007)
Barley	<i>HvAACT1</i>	Citrate transporter	(Furukawa et al. 2007)
Rye	<i>ScALMT1 gene cluster</i>	Malate transporter	(Collins et al. 2008)
<i>Arabidopsis</i>	<i>AtMATE1</i>	Citrate transporter	(Liu et al. 2009)
Maize	<i>ZmMATE1</i>	Citrate transporter	(Maron et al. 2010)
<b>ABC transporters and other proteins</b>			
<i>Arabidopsis</i>	<i>AtSTOP1</i>	C <sub>2</sub> H <sub>2</sub> -type Zn finger transcription factor	(Iuchi et al. 2007)
<i>Arabidopsis</i>	<i>AtSTAR1</i>	ABC transporter-basic detoxification of Al	(Huang et al. 2010)
<i>Arabidopsis</i>	<i>ALS1</i>	Half ABC transporter	(Larsen et al. 2007)
<i>Arabidopsis</i>	<i>ALS3</i>	Half ABC transporter	(Larsen et al. 2005)
Rice	<i>ART1</i>	C <sub>2</sub> H <sub>2</sub> -type Zn finger transcription factor	(Yamaji et al. 2009)
Rice	<i>STAR1,STAR2</i>	ABC transporter-UDP-glucose transport	(Huang et al. 2009)
<b>Likely Al<sup>3+</sup> resistance gene</b>			
Wheat	<i>TaMATE1</i>	Citrate transporter	(Ryan et al. 2009)
Rye	<i>ScMATE2</i>	Citrate transporter	(Yokosho et al. 2010)
<i>Brassica napus</i>	<i>BnALMT1</i>	Malate transporter	(Ligaba et al. 2006)
	<i>BnALMT2</i>	Malate transporter	

Table 1. Al<sup>3+</sup> resistance genes in plants

### 7.1 Organic anion transporters

The genes controlling organic anion efflux from roots were the first Al<sup>3+</sup> resistance genes to be isolated from plants. Those controlling malate efflux belong to the *ALMT* (aluminium activated malate transporter) family of genes and those controlling citrate efflux belong to the *MATE* (multi-drug and toxic compound extrusion) family of genes. The genes controlling oxalate efflux are still unknown. The first aluminium resistance gene cloned from plants was the wheat gene *TaALMT1* which encodes an Al<sup>3+</sup>-activated malate channel (Sasaki et al. 2004). *TaALMT1* was identified by cDNA subtractive hybridization using near-isogenic wheat lines ET8 (resistant) and ES8 (sensitive). *TaALMT1* was identified for being more highly expressed in root tips of ET8 than of ES8 and its expression co-segregated with Al<sup>3+</sup> resistance in a segregating population. Heterologous expression of *TaALMT1* in *Xenopus laevis* oocytes, tobacco suspension cells, barley, wheat and *Arabidopsis* all generate the same phenotype: an Al<sup>3+</sup>-

activated efflux of malate (Sasaki et al. 2004; Delhaize et al. 2004; Pereira et al. 2010; Ryan et al. 2011).  $Al^{3+}$  resistance was not related to the coding alleles of *TaALMT1*, but to the level of expression (Raman et al. 2005). Polymorphisms detected in the promoter of *TaALMT1* are correlated with  $Al^{3+}$  resistance. Tandem repeats in the promoter of resistance genotypes explain the higher expression in resistant plants such that the larger the number of repeats the higher the expression and this is correlated with greater malate efflux (Ryan et al. 2010; Sasaki et al. 2006). Homologues of *TaALMT1* also control  $Al^{3+}$  resistance in *Arabidopsis* (Hoekenga et al. 2006) and rye (Collins et al. 2008). However not all *ALMT* genes confer  $Al^{3+}$  resistance because members of this family in barley and *Arabidopsis* (*HvALMT*, *AtALMT9* and *AtALMT12*) have other functions on the tonoplast of leaves and in guard cells (Gruber et al. 2011; Kovermann et al. 2007; Sasaki et al. 2010).

The first *MATE* gene involved in  $Al^{3+}$  resistance was cloned in sorghum by positional cloning (Magalhaes et al. 2007). *SbMATE* encodes a transport protein located on the plasma membrane that facilitates citrate release from the root cells. *SbMATE* is constitutively expressed in the root apices of resistant sorghum lines but  $Al^{3+}$  treatment increases expression over hours and days and this change parallels the increase in citrate efflux. Interestingly, the coding regions of *SbMATE* in the sensitive and resistant genotypes are identical, with polymorphisms in one of the introns only. It will be interesting to discover how *SbMATE* expression is controlled in sorghum.

*MATE* genes also control the citrate efflux from  $Al^{3+}$ -resistant barley plants and *Arabidopsis*. The *HvAACT1* gene from barley (also known as *HvMATE*) was also isolated by positional cloning (Furukawa et al. 2007). Like *TaALMT1* in wheat, *HvAACT1* is constitutively expressed in roots but  $Al^{3+}$  is still required to activate citrate efflux. Unlike *TaALMT1* and *SbMATE*, *HvAACT1* expression is higher slightly behind the root apices which may influence its effectiveness. Liu et al. (2009) showed that knock-out mutations in *AtMATE* prevent the  $Al^{3+}$ -activated efflux of citrate but the contribution of *AtMATE* to the resistance of this species is relatively small compared to the malate channel *AtALMT1*.

$Al^{3+}$  resistance in maize is likely to involve several mechanisms. Nevertheless citrate efflux does contribute and Maron et al. (2010) isolated a *MATE* gene called *ZmMATE1* which co-localizes with a major QTL for  $Al^{3+}$  resistance. *ZmMATE1* is mainly expressed in roots, is up-regulated by  $Al^{3+}$  and shows higher expression in  $Al^{3+}$ -resistant genotypes. *ZmMATE1* elicits anion efflux when expressed in *Xenopus oocytes* and measurements with labeled [ $^{14}C$ ]-citrate confirmed *ZmMATE1* transports citrate (Maron et al. 2010).

Other candidate genes are likely to control  $Al^{3+}$  resistance but need confirmation. For instance, *TaMATE1* expression in wheat segregates with citrate efflux in some Brazilian genotypes but it needs to be demonstrated directly that *TaMATE* is a citrate transporter (Ryan et al. 2009). The citrate efflux from these few genotypes of wheat differs from other species in one important way: it occurs constitutively and does not require  $Al^{3+}$  to activate it. Two genes from *Brassica napus*, *BnALMT1* and *BnALMT2*, encode functional malate transporters in *Xenopus oocytes* and their expression is induced by  $Al^{3+}$  but no genetic analysis or knockout mutants have confirmed that they contribute to  $Al^{3+}$  resistance.

## 7.2 Other resistance genes

A different set of  $Al^{3+}$  resistance genes was identified using mutant analysis (Table 1). This approach requires no prior knowledge regarding genetics or mechanisms involved. Mutagenized seed is generated by chemical treatments, radiation or the random insertion of a DNA fragment (T-DNA or transposon) into the genome. M2 seedlings are screened and those

that grow similar to wild-type plants under control conditions, but show altered responses to  $Al^{3+}$  stress, are selected for further analysis. Candidate genes can be isolated by mapping or obtaining the sequence flanking the T-DNA region and analysed further. The candidate genes can be characterized by overexpression studies, knockout studies, mutant analysis or association analysis. These genes need not show allelic variation within natural populations.

Using this approach Huang et al. (2009) cloned two genes from rice called *STAR1* and *STAR2* (sensitive to Al rhizotoxicity) which cause plants to be hypersensitive to  $Al^{3+}$  toxicity when knocked out. Both genes are expressed in roots and induced by  $Al^{3+}$  treatment. *STAR1* encodes a nucleotide binding domain of bacterial-type ATP binding cassette (ABC) transporter and *STAR2* encodes the transmembrane domain for an ABC transporter. Huang et al (2009) demonstrated that *STAR1* and *STAR2* interact to form a functional ABC transporter which localizes to vesicles of most root cells except for those in the epidermal layer of the mature zone. *Xenopus laevis* oocytes expressing *STAR1/STAR2* can transport UDP-glucose but a more recent study shows that *STAR1* is also involved in nicotianamine transport, a secondary metabolite used for the long-distance transport of  $Fe^{3+}$  in plants. The role of *STAR1/STAR2* in  $Al^{3+}$  resistance remains unclear but it could be involved with releasing compounds that modify the cell wall during  $Al^{3+}$  stress.

A homologue of *STAR1* in *Arabidopsis*, called *AtSTAR1*, also encodes an ATP-binding domain of a bacterial-type ABC transporter (Huang et al. 2010). A line carrying a knockout mutation showed increased sensitivity to  $Al^{3+}$  and early flowering. Unlike *OsSTAR1*, *AtSTAR1* is expressed in both the roots and shoots, and its expression is not induced by  $Al^{3+}$  stress. *AtSTAR1* may interact with another protein called *ALS3* to form a functional ABC transporter. *ALS3* had been identified previously in similar screens in *Arabidopsis* because plants carrying loss-of-function mutations are more sensitive to  $Al^{3+}$  stress (Larsen et al. 2005; Larsen et al. 2007).

*STOP1* (sensitive to protons) encodes a transcription factor identified by analysing *Arabidopsis* mutants which are hypersensitive to  $H^+$  toxicity. *STOP1* belongs to  $C_2H_2$ -type zinc finger family of proteins. *stop1* mutants are more sensitive to  $Al^{3+}$  but not to a range of other cations including cadmium, copper, lanthanum, manganese and sodium. *STOP1* is required for the induction of a range of genes including *AtALMT1* which encodes the malate transporter. *STOP1* plays a critical role in enabling *Arabidopsis* to resist stress induced by low pH and  $Al^{3+}$  toxicity (Iuchi et al. 2007).

## 8. Transgenic approaches for increasing $Al^{3+}$ resistance

The increasing demands for food from a growing world population highlight the need to overcome the major soil constraints currently limiting crop yields. For acid soils, the application of lime (calcium carbonate) can increase the soil pH but this usually only changes the surface pH in the year of application and it can take decades for acidity to be neutralized at depth. Additionally, in third world countries it can be prohibitively expensive to apply sufficient lime to neutralize soil acidity. Increasing the acid soil tolerance by conventional breeding has been successfully applied to several crop species and this complements liming practices as a way of managing acid soils. However, some species lack sufficient variation in their germplasm and genetic modification provides another avenue for increasing their acid soil tolerance. As described above the mechanisms of  $Al^{3+}$  resistance in species, such as wheat, sorghum and barley have been elucidated and the genes underlying these mechanisms have been isolated. These genes have been used to generate

transgenic plants with enhanced  $Al^{3+}$  resistance. A range of other genes, not necessarily responsible for natural variation in  $Al^{3+}$  resistance, have also been used to enhance the  $Al^{3+}$  resistance of plants. The following discussion summarises these recent attempts to enhance  $Al^{3+}$  resistance using biotechnology (see **Table 2**).

### 8.1 Over-expression of genes involved in organic anion biosynthesis

The important role of organic anion efflux in  $Al^{3+}$  resistance was established 20 years ago, more than a decade before the genes controlling this trait were cloned. Therefore the first attempts to increase organic anion efflux to improve  $Al^{3+}$  resistance focused on increasing organic anion synthesis because the key enzymes and genes involved in those pathways were well known (**Table 2**). This approach was based on the idea that an increased concentration of organic anions in the cytosol would result in increased organic anion transport across the plasma membrane. The underlying assumption was that transport of organic anions across the plasma membrane is not the rate-limiting step for efflux. Citrate synthase was a sensible starting point due to the known role of citrate in the  $Al^{3+}$  resistance of *Cassia tora*, maize, rye and snapbean (Ryan et al., 2001; Ma et al., 2001). Citrate synthase is the first enzyme involved in the tricarboxylic acid and glyoxylate cycles. De la Fuente et al. (1997) transformed tobacco and papaya with the citrate synthase gene (CS) from the bacterium *Pseudomonas aeruginosa* to increase the biosynthesis and efflux of citrate for enhanced  $Al^{3+}$  resistance. When homozygous lines of tobacco expressing the CS gene were analyzed they were found to accumulate up to 10 fold more citrate than the wildtype plants. Citrate efflux of the transgenics was increased four fold over wildtype and this was associated with enhanced  $Al^{3+}$  resistance. Similar results were reported for transgenic papaya expressing the same transgene. However, subsequent work by Delhaize et al. (2001) was not able to repeat these findings on the same tobacco lines or even when the gene was expressed to a much greater level. More recently other groups have reported enhanced  $Al^{3+}$  resistance when CS expression was increased in alfalfa (Barone et al. 2008), *Arabidopsis* (Koyama et al. 2000; Koyama et al. 1999) and tobacco (Han et al. 2009). In most of these cases the increases in  $Al^{3+}$  resistance were marginal except for tobacco transformed with a rice CS gene where transgenic lines showed up to 4.5-fold greater  $Al^{3+}$  resistance than the wildtype.

Malate dehydrogenase (MDH) which oxidises oxaloacetate to form malate is another enzyme involved in organic anion biosynthesis and this gene has now been over-expressed in several species. An MDH gene highly expressed in root nodules of alfalfa (*neMDH*) was over-expressed in alfalfa and this was associated with enhanced malate efflux and greater  $Al^{3+}$  resistance (Tefaye et al. 2001). Similarly, when MDH genes from *Arabidopsis* and *Escherichia coli* were expressed in tobacco, the transgenic plants showed enhanced malate efflux and improved  $Al^{3+}$  resistance (Wang et al. 2010).

### 8.2 Over-expression of genes involved in organic anion transport

Once the  $Al^{3+}$  resistance genes controlling organic anion efflux were identified and cloned they were transformed into plants (**Table 2**). These genes belong to the *MATE* or *ALMT* gene families and they encode transport proteins that mediate organic anion movement across the plasma membrane to the external medium.

*TaALMT1* has now been expressed in several species and in nearly all cases the transgenic plants showed  $Al^{3+}$ -activated malate efflux and enhanced  $Al^{3+}$  resistance. The one exception was rice, where *TaALMT1* expression conferred  $Al^{3+}$ -activated malate efflux but not

Gene function	Source of gene	Species transformed	Phenotype (RRG)	Reference
<i>Organic anion metabolism</i>				
Citrate synthase	<i>Pseudomonas aeruginosa</i>	Tobacco and papaya	2-fold	(De la Fuente et al. 1997)
Citrate synthase ( <i>AtCS</i> )	<i>Arabidopsis</i>	Carrot	1.3-fold	(Koyama et al. 1999)
Citrate synthase ( <i>DcCS</i> )	Carrot	<i>Arabidopsis</i>	1.2-fold	(Koyama et al. 2000)
Citrate synthase ( <i>OsCS1</i> )	Rice	Tobacco	4.5-fold	(Han et al. 2009)
Citrate synthase	<i>Pseudomonas aeruginosa</i>	Alfalfa	2.5-fold	(Barone et al. 2008)
Citrate synthase ( <i>AtmtCS</i> )	<i>Arabidopsis</i>	Canola	2-fold	(Anoop et al. 2003)
Malate dehydrogenase	Alfalfa	Alfalfa	2-fold	(Tesfaye et al. 2001)
Malate dehydrogenase	<i>Arabidopsis E coli.</i>	Tobacco	2.4-fold	(Wang et al. 2010)
Blue-copper-binding protein gene ( <i>AtBCB</i> )	<i>Arabidopsis</i>	<i>Arabidopsis</i>	1.7-fold	(Ezaki et al. 2000)
<i>Stress response</i>				
Glutathione S-transferase gene ( <i>parB</i> )	Tobacco	<i>Arabidopsis</i>	1.7-fold	(Ezaki et al. 2000)
Peroxidase gene ( <i>NtPox</i> )	Tobacco	<i>Arabidopsis</i>	1.7-fold	(Ezaki et al. 2000)
GDP-dissociation inhibitor gene ( <i>NtGDI1</i> )	Tobacco	<i>Arabidopsis</i>	1.7-fold	(Ezaki et al. 2000)
Dehydroascorbate reductase	<i>Arabidopsis</i>	tobacco	1.5-fold	(Yin et al. 2010)
Manganese superoxide dismutase	wheat	<i>Brassica napus</i>	2.5-fold	(Basu et al. 2001)
<i>Organic anion transporter</i>				
<i>TaALMT1</i>	wheat	wheat	8-fold	(Pereira et al. 2010)
<i>TaALMT1</i>	wheat	barley	20-fold	(Delhaize et al. 2004)
<i>TaALMT1</i>	wheat	<i>Arabidopsis</i>	4-fold	(Ryan et al. 2011)



<i>SbMATE</i>	sorghum	<i>Arabidopsis</i>	2.5-fold	(Magalhaes et al. 2007)
<i>Frd3</i>	<i>Arabidopsis</i>	<i>Arabidopsis</i>	2-fold	(Durrett et al. 2007)
<i>ZmMATE1</i>	maize	<i>Arabidopsis</i>	3-fold	(Maron et al. 2010)
<i>HvAACT1</i>	barley	tobacco	2-fold	(Furukawa et al. 2007)

Phenotype (RRG) shows the reported increase in Al<sup>3+</sup> resistance of the transgenic plants based on measurement of relative root growth (RRG).

Table 2. Studies which have used biotechnology to increase Al<sup>3+</sup> resistance in plants.

enhanced Al<sup>3+</sup> resistance (Sasaki et al. 2004). The inability of *TaALMT1* to increase resistance in this case was attributed to the very high endogenous level of Al<sup>3+</sup> resistance of rice.

Barley is among the most Al<sup>3+</sup>-sensitive cereal crops but the small genotypic variation in resistance that does occur is correlated with low rates of citrate release, but not malate efflux (see above). Expression of *TaALMT1* in barley was associated with increased Al<sup>3+</sup>-activated malate efflux and a significant increase in Al<sup>3+</sup> resistance when compared to wildtype plants and null segregant lines (Delhaize et al. 2004). The transgenic barley showed enhanced Al<sup>3+</sup> resistance when grown in both hydroponic culture and in acid soil. In hydroponic culture root growth of transgenics was more than 10-fold greater than wildtype (Delhaize et al. 2004). More recently it was shown that these transgenic barley had enhanced phosphorus-use efficiency and improved grain yield when grown on an acid soil (Delhaize et al. 2009).

Similarly Al<sup>3+</sup>-activated malate efflux and Al<sup>3+</sup> resistance were enhanced when *TaALMT1* was over-expressed in wheat (Pereira et al. 2010) and *Arabidopsis* (Ryan et al. 2011). Some of the transgenic wheat lines displayed greater Al<sup>3+</sup> resistance than ET8 (the source of the *TaALMT1* gene) in both hydroponic and soil experiments (Pereira et al. 2010).

*MATE* genes encoding citrate transporter proteins in sorghum (*SbMATE*), barley (*HvAACT1*), maize (*ZmMATE1*) and *Arabidopsis* (*AtMATE* and *Frd3*) were transformed into *Arabidopsis* or tobacco plants (Durrett et al. 2007; Furukawa et al. 2007; Magalhaes et al. 2007; Maron et al. 2010). *Frd3* is not an Al<sup>3+</sup>-resistance gene but it does encode a transporter which releases citrate into the xylem to assist iron movement to the shoots. In all cases these genes increased citrate efflux and enhanced Al<sup>3+</sup> resistance of the transgenic plants.

These findings indicate that the *MATE* and *ALMT* genes can be effectively used to enhance the Al<sup>3+</sup> resistance of not only model species, but also important crop species. The observation that organic anion efflux can be increased by expression of a transport protein suggests that biosynthesis of organic anions is not a limiting factor for many plant species. To date, the transport proteins, and *TaALMT1* in particular, have provided the most effective means to increase the Al<sup>3+</sup> resistance of plants.

### 8.3 Genes not associated with organic anions

One of the first biotechnological strategies to increase Al<sup>3+</sup> resistance sought to over-express genes induced by Al<sup>3+</sup> stress, and especially those involved in combating oxidative stress.

Ezaki et al. (2000) first identified a range of genes whose expression is induced by Al and then overexpressed these genes in *Arabidopsis*. They found that an *Arabidopsis* blue-copper-binding

protein gene (*AtBCB*), a tobacco glutathione S-transferase gene (*parB*), a tobacco peroxidase gene (*NtPox*) and a tobacco GDP-dissociation inhibitor gene (*NtGDI1*) conferred a degree of tolerance to  $Al^{3+}$  when over-expressed. In particular, overexpression of the *parB* gene simultaneously conferred resistance to both  $Al^{3+}$  and oxidative stresses. Other stress-related genes, such as dehydroascorbate reductase from *Arabidopsis* and manganese superoxide dismutase from wheat, were expressed in tobacco and *Brassica napus*, respectively with the transgenic plants showing enhanced  $Al^{3+}$  tolerance (Basu et al. 2001; Yin et al. 2010). Overexpression of these stress-related genes in transgenic plants exhibited a 1.5-2.5-fold increase in relative root growth compared to wildtype.

Genes encoding proteins involved in various stress responses, endocytosis, lipid biosynthesis or Al-induced programmed cell death have also conferred a degree of  $Al^{3+}$  tolerance when over-expressed in *Arabidopsis* or tobacco. These genes encode WAK1, a auxilin-like protein, a  $\Delta 8$  sphingolipid desaturase and a Ced-2 protein (Ezaki et al. 2000; Ryan et al. 2007; Sivaguru et al. 2003a; Wang et al. 2009). The details of how these genes function to confer  $Al^{3+}$  resistance are not well understood and it is not yet clear that they would be sufficiently effective to enhance the  $Al^{3+}$  resistance of crop species.

## 9. Conclusions

Much information has been gathered on the mechanisms of  $Al^{3+}$  toxicity and tolerance over the last 20 years. Our understanding of the mechanisms involving organic anion release is more complete than other mechanisms operating in species like rice and maize. Genes belonging to the *MATE* and *ALMT* families encode organic anion transport proteins that facilitate anion efflux from the roots. Transgenic plants expressing these genes show increased organic efflux and significantly greater resistance to  $Al^{3+}$  stress. Strategies based on enhanced efflux of organic anions appear to be effective and combining them with  $Al^{3+}$  tolerance mechanisms that act within the plant could provide even greater protection from  $Al^{3+}$  toxicity. These advances pave the way for biotechnological approaches to enhance the acid-soil tolerance of important food crops through genetic engineering and by marker-assisted selection in traditional breeding programs.

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# Soil Bacteria Support and Protect Plants Against Abiotic Stresses

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## 1. Introduction

Numerous stresses caused by complex environmental conditions, e.g. bright light, UV, too high and low temperatures, freezing, drought, salinity, heavy metals and hypoxia, lead to substantial crop losses worldwide (Boyer, 1982; Mahajan & Tuteja, 2005; Mittler, 2006;). These abiotic stresses might increase in the near future even because of global climate change (<http://www.ipcc.ch>). Among the abiotic factors that are shaping plant evolution, water availability is the most important (Kijne, 2006). Water stress in its broadest sense encompasses both drought and salt stress. Soil salinity affects extensive areas of land in both developed and developing countries. The agricultural intensification, together with unfavourable natural conditions, has accelerated soil salinity in many part of the world. According to the FAO Land and Plant Nutrition Management Service, over 6% of the world's land is *salt-affected* (Table 1).

Regions	Total area (Mha)	Saline soils		Sodic soil	
		Mha	%	Mha	%
Africa	1,899	39	2.0	34	1.8
Asia, the Pacific and Australia	3,107	195	6.3	249	8.0
Europe	2,011	7	0.3	73	3.6
Latin America	2,039	61	3.0	51	2.5
Near East	1,802	92	5.1	14	0.8
North America	1,924	5	0.2	15	0.8
<b>Total</b>	<b>12,781</b>	<b>397</b>	<b>3.1</b>	<b>434</b>	<b>3.4</b>

Source: FAO Land and Plant Nutrition Management Service.

Table 1. Regional distribution of salt-affected soils, in million hectares

The term *salt-affected* refers to soil that are saline or sodic (Szabolcs, 1989). Saline soil has an excess of soluble salt in the soil solution, the liquid located between aggregates of soil. A sodic soil has too much sodium associated with the negatively charged clay particles. Salinity occur thought natural or human-induced processes that result in the accumulation of dissolved salts in the soil water to an extent that inhibits plant growth. Natural salinity

results from the accumulation of salts over long period of time and is caused by two natural processes. The first is the weathering process that breaks down rock and release soluble salts of various type, mainly chloride of sodium, calcium and magnesium, and to a lesser extent, sulphates and carbonates. Sodium chloride is the most soluble salt. The second is the deposition of oceanic salt carried in wind and rain. Human-induced salinity results from human activity that change the hydrologic balance of the soil between water applied (irrigation or rainfall) and water used by crops (transpiration). The most common causes are (i) land clearing and the replacement of perennial vegetation with annual crops, and (ii) irrigation schemes using salt-rich irrigation water or having insufficient drainage (Munns & Tester, 2008).

Compared to salt stress, the problem of drought is even more pervasive and economically damaging. Nevertheless, most studies on water stress signalling have focused on salt stress primarily because plant responses to salt and drought are closely related and the mechanisms overlap. Salt and drought stresses affect virtually every aspect of plant physiology and metabolism. Although some of the changes observed under these stresses are adaptive, many may be consequences of stress injury (Mahajan & Tuteja, 2005).

Water deficit and salinity disrupt photosynthesis and increase photorespiration, altering the normal homeostasis of cells and cause an increased production of Reactive Oxygen Species (ROS) such as the super oxide radical, hydrogen peroxide and hydroxyl radical (Miller et al., 2010). Under optimal growth conditions, ROS are mainly produced at low level in organelles such as chloroplasts, mitochondria and peroxisomes (Apel & Hirt, 2004). The enhanced production of ROS during stress can pose a threat to cells but it is though that ROS also act as signals for the activation of stress-response and defence pathways (Pitzschke et al., 2006).

The direct effects of salt on plant growth also involve nutrient imbalance caused by the loss of control on nutrient uptake and/or transport to the shoot leading to ion deficiencies (Munns, 2002).

The main reason for these nutrient deficiencies can be related to the abundant presence of ions, like  $\text{Na}^+$  and  $\text{Cl}^-$ , in the soil solution. Abundance of these soluble ions can decrease the activity of other essential elements in the soil and can lead to reduction in accessibility and uptake of some elements by the plants. Several studies show that plants exposed to environmental stresses require additional supplies of mineral nutrients to minimize the adverse effects of stress (Endris & Mohammed, 2007; Heidari & Jamshid, 2010; Kaya et al., 2002; Khayyat et al., 2007;). In particular, it is known that salt stress causes reduction in P accumulation in plants, which developed P-deficiency symptoms. The addition of soluble P to saline growth medium increased crop growth and yield (Awad et al., 1990; Grattan and Grieve, 1999; Mohammad et al., 1998; Naheed et al., 2008; Satti & Al-Yahyai, 1995).

To deal with saline soil and minimize crop loss, scientists have searched for salt-tolerant cultivars, and have attempted to develop salt-tolerant crops through breeding (Araus et al., 2008; Dwivedi et al., 2010; Sreenivasulu et al., 2007; Witcombe et al., 2008). However, gaps in understanding the complex physiological, biochemical, developmental, and genetic mechanisms that underlie environmental stress tolerance, and the subsequent difficulty in combining favourable alleles to create improved high yielding genotypes, are the major constraint to improve crop yield under abiotic stress. Furthermore, it appears certain that domestication has narrowed the genetic diversity within crops for stress tolerance, and thus limited options in traditional crop breeding.

To overcome salinity effects, scientists are also using transgenic approaches to obtain genetically modified plants (Ashraf & Akram, 2009; Mittler & Blumwald, 2010; Valliyodan & Nguyen, 2006; Vinocur & Altman, 2005; Zhang et al., 2000). These approaches are time consuming and costly due to the impressive charges required to validate the consumption or cultivation of genetically modified plants. Indeed, the development of transgenic plants with increased stress tolerance is primarily based on the performance of transgenic lines produced and tested under controlled conditions as greenhouse, and can be found only few reports where the performance of transgenic cultivars was tested under field conditions. Several factors limit the success of producing salt-tolerant cultivars through genetic engineering. 1) In most cases only a single gene has been transformed, although salt stress resistance is polygenic. If, for example, osmoprotectant-producing, transcription factor-expressing, ion homeostasis-maintaining, and antioxidant enzymatic activities are all incorporated into a single species, there is a strong possibility that all these activities could work in concert to overcome concurrently present abiotic stresses. Transforming recipient plants with many genes or crossing plants containing different stress tolerance genes is very time consuming. 2) Transformation of agronomic important crops and identification of uncovered tolerance determinants or stress inducible promoters that direct the expression at proper time and place must be further explored to maximize salt tolerance.

Plants in their natural environment are colonized both by endocellular and intracellular microorganisms (Gray & Smith, 2005). Rhizosphere microorganisms, particularly beneficial bacteria and fungi, can improve plant performance under stress environments and, consequently, enhance yield both directly and indirectly (Dimkpa et al., 2009a). Some plant growth-promoting rhizobacteria (PGPR) may exert a direct stimulation on plant growth and development by providing plants with fixed nitrogen, phytohormones, iron that has been sequestered by bacterial siderophores, and soluble phosphate (Hayat et al., 2010; Rodriguez & Fraga, 1999). Others do this indirectly by protecting the plant against soil-borne diseases, most of which are caused by pathogenic fungi (Lugtenberg & Kamolova, 2009). Common adaptation mechanisms of plants exposed to environmental stresses, such as temperature extremes, high salinity, drought and nutrient deficiency, or heavy metal toxicity, include changes in root morphology (Potters et al., 2007), a process in which phytohormones are known to play a key role (Spaepen et al., 2007; Spaepen & Vanderleyden, 2010). The majority of root associated bacteria that display beneficial effects on plant growth produce indole-3-acetic acid (IAA) (Hayat et al., 2010). Inoculation of various plant species with such bacteria lead to increased root growth and/or enhanced formation of lateral roots and root hairs that can result in enhanced tolerance to abiotic stress. Bacterial IAA production also stimulates the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase involved in the degradation of the ethylene precursor ACC (Glick, 2005). ACC deaminase activity could be helpful in sustain plant growth and development under stress conditions by reducing stress-induced ethylene production. Modulation of other major plant hormones could improve crop salt tolerance by reducing the toxic effects of salinity (Bianco & Defez, 2009). A number of nitrogen-containing compounds accumulate in plants exposed to saline stress (Mansour, 2000; Parida & Das, 2005). The accumulation of the amino acid proline is one of the most frequently reported modifications induced by water and salt stress as well as other stresses in plants (Hare & Gress, 1997; Kavi Kishor, 2005; Verbruggen & Hermans, 2008). It has been found that *Medicago* plants infected by IAA-overproducing PGPR strains are able to overcome different stressful environmental conditions and accumulate high levels of proline. The increased expression levels of two genes involved in the first two steps of proline biosynthesis from glutamic acid confirmed these results (Bianco & Defez, 2009).

When plants are subjected to environmental stress conditions such as those listed above, the balance between the production of ROSs and the quenching activity of the antioxidants is upset, often resulting in oxidative damage (Jubany-Mari et al., 2010; Miller et al., 2010). Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative damage (Ahmad et al., 2008; Kohler et al., 2008). The activities of the antioxidative enzymes such as catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POX), glutathione reductase (GR), and superoxide dismutase (SOD) increase under salt stress in plants, and a correlation between these enzyme levels and salt tolerance has been described (Apel & Hirt, 2004). It has been found that Medicago plants infected with IAA-overproducing PGPR strains showed high antioxidant enzymes activity which contributed to enhance plant protection against salt stress (Bianco & Defez, 2009).

Considering the positive effects of PGPR strains on different plant cultivars and lines grown under salt stress conditions, we propose that such bacteria might be tested in field trial offering an economical and simple treatment to salt sensitive plants.

A fruitful strategy to alleviate negative effects of salt stress in plants might be the co-inoculation of seeds with different PGPR species, such as *Rhizobium* and *Azospirillum*. Indeed, dual inoculation with *Rhizobium* and *Azospirillum* and other plant growth promoting rhizobacteria was shown to increase the total nodule number of several legumes, acetylene reduction activities, and the total N content of mineral macro- and micronutrients as compared to inoculation with *Rhizobium* alone (Burdman, 1996; Molla et al., 2001a; Remans et al., 2008b). The presence of *Azospirillum* in the rhizosphere was reported to elicit or activate the hydrolysis of conjugated phytohormones and flavonoids in the root tissue, thus bringing about the release of compounds in their active forms (Dardanelli et al., 2008; Saikia et al., 2010; Spaepen et al., 2007).

In addition, even under stress conditions, the use of PGPR inoculants in intercropping systems such as legume-cereal might contribute to the improvement of cereal crop yield that take advantage from the legume release of multiple nutrients and growth promoters (Banik et al., 2006; Dahmardeh et al., 2009; Davies et al., 2010; Dhima et al., 2007; Hauggaard-Nielsen et al., 2001; Javanmard et al., 2009; Tsubo et al., 2005; Li et al., 2006).

The objective of this synthesis paper is to review the pivotal role of plant growth-promoting bacteria in developing sustainable systems for crop production under abiotic stress conditions. In this review, much research information about salt-stress has been gathered because soil salinity, which limits crop yield and restricts use of land, is a major constrain to food production. We start by reviewing the root zone bacteria that have been found to possess plant growth-promoting properties. We then review how plant growth-promoting bacteria act as enhancers of the main biochemical and molecular mechanisms developed by plants to cope with salt stress. We then discuss the potential role that agronomic manipulations can play in ameliorating the impact of salinity stress on plants. The body of studies suggests that, under abiotic stress conditions, the use of improved PGPR inoculants might be advantageous for the development of sustainable agriculture in which yield losses are reduced and plant growth is improved.

## 2. Beneficial rhizobacteria

Populations of microorganisms live in close contact with the plants root zone called rhizosphere. Here the number of microorganisms is usually higher than in other soil area. Thus, the plant root is thought to be a major source of nutrients for microorganisms living in

the rhizosphere. Indeed, plants supply organic carbon to their surroundings in the form of root exudates and rhizobacteria respond to this exudation by means of chemotaxis towards the exudate source modulating their metabolism to optimize nutrient acquisition (Hardoim et al., 2008).

Soil bacteria beneficial to plant growth are usually referred to as plant growth promoting rhizobacteria (PGPR), capable of promoting plant growth by colonizing the plant root (Hayat et al., 2010). Bacteria of diverse genera such as *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Pseudomonas* and *Serratia* (Gray & Smith, 2005), as well as *Streptomyces* spp. (Dimkpa et al. 2008, 2009b; Tokala et al. 2002) were identified as PGPR.

According to their residing sites, PGPR can be divided in iPGPR, which live inside the plant cells and are localized in specialized structures, the so-called nodules, and ePGPR which live outside the plant cells and do not produce organs like nodules, but still prompt plant growth (Gray & Smith, 2005).

Although the exact mechanisms of plant growth stimulation remain largely speculative, possible explanation includes: (1) production of hormones like abscisic acid, gibberellic acid, cytokinins, and auxin, i.e IAA; (2) production of essential enzymes, 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene in the root of developing plants; (3) nitrogen fixation; (4) production of siderophores; (5) solubilization and mineralization of nutrients, particularly mineral phosphate; (6) improvement of abiotic stresses resistance (Hayat et al., 2010).

### **3. Abiotic stresses in plant: Improving mechanisms of stress response by rhizobacteria**

Dehydration, salinity, low as well as high-temperature stresses and other abiotic stresses lead to metabolic toxicity, membrane disorganization, generation of ROS, inhibition of photosynthesis, reduced nutrient acquisition and altered hormones levels. Accumulation of osmoprotectants, production of superoxide radical scavenging mechanisms, exclusion or compartmentation of ions by efficient transporter and symporter systems, production of specific enzymes involved in the regulation of plant hormones are some of the mechanisms that plants have evolved for adaptation to abiotic stresses (Des Marais & Juenger, 2010; Mahajan & Tuteja, 2005; Parida & Das, 2005; Santner et al., 2009; Shao et al., 2009). Many studies have been published on beneficial effects of bacterial inoculation on plant physiology and growth under abiotic stress conditions and some examples are summarized in Table 2.

#### **3.1 Phytohormones synthesis and modulation**

Plants are sessile organisms with a high level of physiological plasticity, enabling survival under a wide variety of environmental insults. This is due to the continuously active shoot and root meristems and their capability to generate new organs after embryogenesis (Wolter & Jurgens, 2009). They have developed an extensive array of defensive responses that includes changes in the root morphology. The root architecture of the plants, which is determined by the pattern of root branching (lateral root formation) and by the rate and direction of growth of individual roots (Malamy, 2005), constitutes an important model to study how developmental plasticity is translated into growth responses under several environmental stresses. Morphogenesis is tightly linked to hormonal homeostasis, with several hormones controlling cell elongation, cell division and re-orientation of growth. The

Stress type	Bacterial inoculate	Plant Species	Reference
Salt	<i>Pseudomonas pseudoalcaligenes</i> , <i>Bacillus pumilus</i>	Rice ( <i>Oryza sativa</i> )	Jha et al. (2010)
Salt	<i>Bacillus megaterium</i>	Maize ( <i>Zea mays</i> L.)	Marulanda et al. (2010)
Salt	<i>Azospirillum brasilense</i>	Barley ( <i>Hordeum vulgare</i> )	Omar et al. (2009)
Salt	<i>Pseudomonas mendocina</i>	Lettuce ( <i>L. sativa</i> L. cv. Tafalla)	Kohler et al. (2009)
Salt	<i>Azospirillum</i> sp.	Pea ( <i>Phaseolus vulgaris</i> )	Dardanelli et al. (2008)
Salt	<i>Bacillus subtilis</i>	<i>Arabidopsis thaliana</i>	Zhang et al. (2008)
Salt	<i>Pseudomonas syringae</i> , <i>Pseudomonas fluorescens</i> , <i>Enterobacter aerogenes</i>	Maize ( <i>Zea mays</i> )	Nadeem et al. (2007)
Salt	<i>P. fluorescens</i>	Groundnut ( <i>Arachis hypogaea</i> )	Saravanakumar & Samiyappan (2007)
Salt	<i>Azospirillum</i>	Lettuce ( <i>Lactuca sativa</i> )	Barassi et al. (2006)
Salt	<i>Achromobacter piechaudii</i>	Tomato ( <i>Lycopersicon esculentum</i> )	Mayak et al. (2004)
Salt	<i>Aeromonas hydrophila/caviae</i> <i>Bacillus insolitus</i> , <i>Bacillus</i> sp.	Wheat ( <i>Triticum aestivum</i> )	Ashraf et al. (2004)
Salt	<i>Azospirillum</i>	Maize ( <i>Z. mays</i> )	Hamdia et al. (2004)
Salt	<i>A. brasilense</i>	Chickpeas ( <i>Cicer arietinum</i> ), faba beans ( <i>Vicia faba</i> L.)	Hamaoui et al. (2001)
Drought	<i>Pseudomonas</i> spp.	Maize ( <i>Zea mays</i> L. cv. Kaveri)	Sandhya et al. (2010)
Drought	<i>Pseudomonas</i> spp.	Asparagus ( <i>Asparagus officinalis</i> L.)	Liddycoat et al. (2009)
Drought	<i>Pseudomonas mendocina</i>	Lettuce ( <i>Lactuca sativa</i> L.)	Kohler et al. (2008)
Drought	<i>Rhizobium tropici</i> , <i>Paenibacillus polymyxa</i>	Common bean ( <i>Phaseolus vulgaris</i> L.)	Figueiredo et al. (2008)
Drought	<i>Bacillus</i>	Lettuce ( <i>Lactuca sativa</i> L.)	Arkipova et al. (2007)
Drought	<i>Ensifer meliloti</i> bv. <i>mediterraneense</i>	Bean ( <i>Phaseolus vulgaris</i> cv. Flamingo)	Mnasri et al. (2007)
Drought	<i>Bradyrhizobium elkanii</i>	Flat crown ( <i>Albizia adianthifolia</i> )	Swaine et al. (2007)
Drought	<i>Achromobacter piechaudii</i>	Tomato ( <i>L. esculentum</i> ), pepper ( <i>Capsicum annuum</i> )	Mayak et al. (2004)
Drought	<i>Azospirillum</i>	Wheat ( <i>T. aestivum</i> )	Creus et al. (2004)
Drought	<i>A. brasilense</i>	Maize ( <i>Z. mays</i> )	Casanovas et al. (2002)
Drought	<i>A. brasilense</i>	Common bean ( <i>P. vulgaris</i> )	German et al. (2000)
Osmotic stress	<i>Bacillus subtilis</i>	<i>Arabidopsis</i>	Zhang et al. (2010)
Osmotic stress	<i>A. brasilense</i>	Rice ( <i>Oryza sativa</i> L.)	Cassan et al. (2009)
Osmotic stress (45% PEG)	<i>Arthrobacter</i> sp., <i>Bacillus</i> sp.	Pepper ( <i>C. annuum</i> )	Sziderics et al. (2007)
Osmotic stress (20% PEG)	<i>Azospirillum</i>	Wheat ( <i>T. aestivum</i> )	Pereyra et al. (2006)
Flooding	<i>Enterobacter cloacae</i> , <i>Pseudomonas putida</i>	Tomato ( <i>L. esculentum</i> )	Grichko and Glick (2001)
Temperature	<i>Burkholderia phytofirmans</i>	Grapevine ( <i>Vitis vinifera</i> )	Barka et al. (2006)
Temperature	<i>Pseudomonas fluorescens</i> , <i>Pantoea agglomerans</i> , <i>Mycobacterium</i> sp.	Wheat ( <i>Triticum aestivum</i> )	Egamberdiyeva & Hoflich (2003)
Temperature	<i>B. phytofirmans</i>	Potato ( <i>Solanum tuberosum</i> )	Bensalim et al. (1998)



Stress type	Bacterial inoculate	Plant Species	Reference
Temperature	<i>Aeromonas hydrophila</i> , <i>Serratia liquefaciens</i> , <i>Serratia proteamaculans</i>	Soy bean ( <i>Glycine max</i> )	Zhang <i>et al.</i> (1997)
Temperature	<i>Burkholderia phytofirmans</i>	Grapevine ( <i>Vitis vinifera</i> )	Barka <i>et al.</i> (2006)
Temperature	<i>B. phytofirmans</i>	Potato ( <i>Solanum tuberosum</i> )	Bensalim <i>et al.</i> (1998)
Temperature	<i>Aeromonas hydrophila</i> , <i>Serratia liquefaciens</i> , <i>Serratia proteamaculans</i>	Soy bean ( <i>Glycine max</i> )	Zhang <i>et al.</i> (1997)
Nutrient deficiency	<i>Azospirillum sp.</i> , <i>Azotobacter chroococcum</i> , <i>Mesorhizobium ciceri</i> , <i>Pseudomonas fluorescens</i>	Chickpea ( <i>Cicer arietinum</i> L.)	Rokhzadi & Toashih (2011)
Nutrient deficiency	<i>Azotobacter chroococcum</i> , <i>Azospirillum brasilens</i> , <i>Pseudomonas putida</i> , <i>Bacillus lentus</i>	<i>Zea maize</i> L. ( <i>Zea maize</i> L.)	Yazdani <i>et al.</i> (2009)
Nutrient deficiency	<i>Bacillus sp.</i> , <i>Burkholderia sp.</i> , <i>Streptomyces platensis</i>	<i>Zea maize</i> L.	Oliveira <i>et al.</i> (2009)
Nutrient deficiency	<i>Bacillus sp.</i> ,	<i>Zea maize</i> L.	Adesemoye <i>et al.</i> (2008)
Nutrient deficiency	<i>Bacillus polymyxa</i> , <i>Mycobacterium phlei</i> , <i>Pseudomonas alcaligenes</i>	<i>Zea maize</i> L. ( <i>Zea maize</i> cv. Felix)	Egamberdiyeva (2007)
Heavy metals toxicity	<i>Sanguibacter sp.</i> , <i>Pseudomonas sp.</i>	<i>Nicotina tabacum</i>	Mastretta <i>et al.</i> (2009)
Heavy metals toxicity	<i>Bacillus subtilis</i> , <i>Pantoea agglomerans</i>	Oat ( <i>Avena sativa</i> )	Pishchik <i>et al.</i> (2009)
Heavy metals toxicity	<i>Pseudomonas fluorescens</i> , <i>Microbacterium sp.</i>	Rape ( <i>Brassica napus</i> )	Sheng <i>et al.</i> (2008)
Heavy metals toxicity	<i>Methylobacterium oryzae</i> , <i>Burkholderia sp.</i>	Tomato ( <i>Lycopersicon esculentum</i> L.)	Madhaiyan <i>et al.</i> (2007)
Heavy metals toxicity	<i>Bacillus subtilis</i> , <i>Bacillus megaterium</i> , <i>Bacillus sp.</i>	Rice ( <i>O. sativa</i> )	Asch & Padham (2005), Terré <i>et al.</i> (2007)

Table 2. Bacterially mediated plant tolerance to abiotic stress. Some of the data reported in this Table were adapted from Dimkpa *et al.* (2009a), whereas recent publications have been included *de novo*.

physiologically most active auxin in plants is indole-3-acetic acid (IAA), and the fact that no fully auxin-deficient mutant plants have been identified so far reflects the importance of auxin in plant development. There is a high capacity for auxin biosynthesis not only in young aerial tissues, but also in roots, particularly in the meristematic primary root tip (Teale *et al.*, 2006). Auxin, and its fine concentration gradients have powerful effects on plant development and in particular on lateral root formation and branching, two key components of the response phenotype induced in plants under stress conditions (Potters *et al.*, 2007, 2009). Alteration in the pattern of lateral root formation and emergence in response to P availability is mediated by changes in auxin sensitivity in *Arabidopsis thaliana* roots. These changes alter the expression of auxin-responsive genes and stimulate pericycle cells to proliferate (Pérez-Torres *et al.*, 2008).

Exogenous auxin application results in formation of branched root and, similarly, mutants that accumulate high levels of auxin, or mutants with an altered auxin distribution, produce excess of lateral roots. A broad range of abiotic stresses induce lateral root formation, therefore auxin may be an intermediate between the action of a stressor and the realization of response phenotype. Several mechanisms have been proposed to explain stress-induced changes in auxin metabolism and/or receptiveness; however, evidences for stress-induced changes in auxin transport and catabolism are predominantly found in literature. For example, water and osmotic stresses impact on auxin transport by altering the expression of PIN genes and/or by inhibition of polar auxin transport (Potters et al., 2009). Moreover, auxin conjugates and the respective hydrolases were shown to be involved in the reaction of plant to stress (Muller, 2011). Interestingly, overexpression of an auxin-amidohydrolase in *Arabidopsis* is associated with a reduced inhibition of root elongation and increased resistance to salt stress. This effect was probably due to the increase in the content of free auxin sufficiently to provide a protective effect against salt stress (Junghans et al., 2006).

As more plant tissues are analyzed for the presence of bacteria, an increased number of IAA-producing PGPR strains are detected inside the plant tissue (Spaepen et al., 2007). Various plant species inoculated with such bacteria showed increased root growth and/or enhanced formation of lateral roots and roots hairs (Dimkpa et al., 2009a). For example, the stimulatory effect of *Azospirillum* strains on the development of roots is well documented. Morphological plant root changes have been observed repeatedly upon *Azospirillum* inoculation and have been attributed to the production of plant-growth promoting substances: auxins, cytokinins and gibberellins, with auxin production being quantitatively the most important (Spaepen et al., 2008). Specific evidences for the involvement of auxins produced by *Azospirillum* in roots proliferation were obtained in many cases. Addition of filter-sterilized culture supernatants of *A. brasiliense* to rice roots grown in hydroponic tanks increased root elongation, root surface area, root dry matter, and development of lateral roots and root hairs, compared with untreated roots (El-Khawas & Adachi, 1999). Similarly, a cell-free supernatant of *A. brasiliense* Cd applied to soybean plants induced many roots and increased root length (Molla et al., 2001a). Exogenous application of IAA to bean roots resembled responses of these plants to inoculation with *Azospirillum* (Remans et al., 2008a). More direct evidence for the importance of IAA was provided when several IAA-attenuate mutants were compared with their parental wild types for their effect on plant growth. A mutant of *A. brasiliense* with low production of phytohormones, but high N<sub>2</sub>-fixing activity, did not enhance root growth over uninoculated controls (Kundu et al., 1997).

Considering the relationship between IAA and ethylene precursor ACC (Dimkpa et al., 2009a), the positive effects of IAA on root growth can be either direct or indirect through the reduction of ethylene levels (Lugtenberg & Kamilova, 2009).

Indeed, under stress conditions, including drought and salinity, the plant hormone ethylene endogenously regulates plant homeostasis and results in reduced root and shoot growth.

It has been shown that plants produce ethylene at two different phases in response to stressful stimuli. In the first phase, the small amount of ethylene produced promotes the activity of stress-related genes. In the second phase (1–3 days after stimulus application) the larger amount of ethylene produced lead to inhibition of growth and harmful effects on plants including senescence, chlorosis, and abscission (Glick et al., 2007).

Degradation of the ethylene precursor ACC into 2-oxobutanoate and ammonia by bacterial ACC-deaminase lowers the ethylene concentration in plant roots, relieves the ethylene repression of auxin response factors synthesis, and indirectly increases plant growth (Glick

et al., 2007; Kang et al., 2010). It has been proposed that ACC might be exuded from plant roots and that soil bacteria containing ACC-deaminase could convert this for their growth. As result, the hydrolyzed ACC products would enhance bacterial growth. Taken together, the ACC-deaminase function seems to be mutually beneficial between plants and PGPR, because ethylene in plants can be reduced by continuous ACC secretion and degradation by bacteria, and bacteria can use metabolized ACC (Glick et al., 1998).

ACC deaminase-containing PGPR strains have found practical application in protecting different plant species against growth inhibition caused by various environmental stresses. Mayak et al. (2004) reported that *Achromobacter piechaudii* having ACC deaminase activity significantly increased the fresh and dry weights of tomato seedlings grown in the presence of NaCl salt (up to 172 mM). *Pseudomonas fluorescens* strain TDK1 containing ACC deaminase activity enhanced the saline resistance in groundnut plants and increased yield as compared to plants inoculated with *Pseudomonas* strains lacking ACC deaminase activity (Saravanakumar & Samiyappan). *Pseudomonas putida* UW4, which produces IAA and ACC deaminase, protected canola seedling from growth inhibition by high levels of salt. Siddikee et al. (2010) have also confirmed that inoculation with 14 halotolerant bacterial strains ameliorate salt stress in canola plants through the reduction of ethylene production *via* ACC deaminase activity. Inoculation of maize plants with *Pseudomonas fluorescens* containing ACC deaminase boosted root elongation and fresh weight significantly under saline conditions (Kausar & Shahzad, 2006). Inoculation with *Pseudomonas* spp. containing ACC-deaminase partially eliminates the effects of drought stress on growth, yield, and ripening of pea (*Pisum sativum* L.) (Arshad et al., 2008). Nadeem et al. (2010) reported that rhizobacteria capable of producing ACC deaminase mitigate salt stress in wheat.

We also analysed the growth of *Medicago truncatula* plants nodulated by *Sinorhizobium meliloti* strains under severe salt stress conditions. Medicago plants nodulated by the IAA-overproducing RD64 strain (*Mt*-RD64) showed a phytohormones re-modulation, with a higher IAA content in nodules and roots and a reduced accumulation of IAA in the shoot strain as compared to plants nodulated by the wild-type strain 1021 (*Mt*-1021). Transcriptional analysis of the main ethylene signalling genes showed that, when compared to *Mt*-1021 plants, *Mt*-RD64 plants did not showed and induction of this pathway when 150 mM NaCl was applied, which means less plants stress damages (Bianco & Defez, 2009).

### 3.2 Accumulation of protective compounds

Several studies correlated accumulation of nitrogen-containing compounds (NCC) with drought and salt tolerance in plants (Parida & Das, 2005). The most frequently accumulating NCC includes amino acids, amides, imino acids, proteins, quaternary ammonium compounds and polyamines.

Very high accumulation of cellular proline (up to 80% of the amino acids pool under stress and 5% under normal conditions) due to increased synthesis and decreased degradation under a variety of stress conditions such as salt and drought has been documented in many plant species (Szabados & Savourè, 2009). Several comprehensive studies using transgenic plants or mutants demonstrate that proline metabolism has a complex effect on development and stress responses. Proline has been proposed to act as a compatible osmolyte and to be a way to store carbon and nitrogen. Saline and drought are known to induce oxidative stress. Several studies showed that proline may have an antioxidant activity acting as a ROS scavenger. Proline may also function as molecular chaperones able to stabilize the structures of proteins and enhance the activity of different enzymes, and its

accumulation play a role in maintenance of cytosolic pH and regulation of intracellular redox potential (Hare & Cress, 1997; Kavi Kishor et al., 2005; Verbruggen & Hermans, 2008). Under abiotic stress conditions, increased proline biosynthesis was observed for various plant species inoculated with different PGPR (Barka et al., 2006; Jha et al., 2010; Kohler et al., 2009; Sandhya et al., 2010; Vardharajula et al., 2011). The synthesis of proline as well as other compatible solutes require an energy cost (41 moles of ATP) and occur at the expense of plant growth, but may allow the plant to survive and recover from the presence of high external salt concentration (Munns & Tester, 2008).

We found a significant correlation between reduced symptoms of senescence, such as chlorosis, necrosis and drying, and 2-fold increased proline content in the shoot of *Mt-RD64* as compared to *Mt-1021* plants, after exposure to 150 mM NaCl (Bianco & Defez, 2009).

### 3.3 Biosynthesis of antioxidative enzymes

In plants ROS such as superoxide ( $\cdot\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hydroxyl radical ( $\cdot\text{OH}$ ), and singlet oxygen ( $^1\text{O}_2$ ) are continuously produced as byproducts of various metabolic pathways localized in different cellular compartments (Apel & Hirt, 2004). A common feature of these species is their capacity to cause oxidative damage to proteins, DNA, and lipids. Since internal  $\text{O}_2$  concentrations are high during photosynthesis, chloroplasts are especially prone to generate activated oxygen species (Gill & Tuteja, 2010). Under physiological steady-state conditions, these molecules are scavenged by different antioxidative defence components that are often confined to particular compartments (Apel & Hirt, 2004). Under normal growth conditions, the production of ROS in cells is low, whereas, during stress their rate of production is enhanced. ROS accumulation during stress results from the imbalance between production and scavenging of ROS. Major ROS-scavenging mechanisms of plants include SOD, APX and CAT enzymes. Antioxidants such as ascorbic acid and glutathione, which are found in high concentration in chloroplasts and other cellular compartments, are also crucial for plant defence against oxidative stress (Miller et al., 2010). For the detoxification of excess ROS in plant, the overall balance between different antioxidants is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide, and has to be tightly controlled (Mittler, 2002).

Induction of antioxidant enzymes (catalase and total peroxidase) is involved in the alleviation of salinity stress in lettuce plants inoculated with PGPR strains (Kohler et al., 2010). Under non-saline conditions, inoculation with *Pseudomonas mendocina* and fertilization led to similar increases in plant growth (about 30% greater than the control plants). Salinity decreased the dry weight of the shoots and roots for all lettuce plants. However, the plants inoculated with *P. mendocina* had significantly greater shoot biomass than the control plants at both medium and high salinity levels. We reported that salt-stressed *Mt-RD64* plants showed much less oxidative damage (reduced chlorosis, necrosis, and drying) compared with salt-stressed *Mt-1021* plants. These effects were connected to the enhanced activity of the antioxidant enzymes SOD, APX, GR and POX (Bianco & Defez, 2009).

Recent study reports the potential of PGPR strains in alleviating drought stress effects in maize. Maize plants inoculated with five drought tolerant plant growth promoting *Pseudomonas* spp. strains namely *P. entomophila*, *P. stutzeri*, *P. putida*, *P. syringae*, and *P. montelli* were subjected to drought stress and the effects of inoculation on growth, osmoregulation and antioxidant status was investigated. Inoculated plants showed significantly lower activity of antioxidant enzymes plants as compared to uninoculated

plants (Sandhya et al. 2010). Reduction in the activity of antioxidant enzymes was also observed in barley plants. Omar et al. (2009) reported that, without inoculation, salinity led to a significant increase of catalase and peroxidase activities in salt-stressed leaves of two barley cultivars differing in salinity tolerance. Inoculation of the two cultivars with *Azospirillum brasilense* lowered the magnitude of increase and significantly ameliorated the deleterious effects of salinity improving crop productivity.

These results, which apparently seem to be in contradiction with the assumption that stress resistance in plants is related to more effective antioxidant systems, are an implication of the same positive effect and indicate that inoculated plants felt less stress as compared to uninoculated plants.

### 3.4 Enhancement of nutrients up-take

Survival and productivity of crop plants exposed to environmental stresses are dependent on their ability to develop adaptive mechanisms to avoid or tolerate stress (Munns & Tester, 2008). Accumulating evidence suggests that mineral nutritional status of plants greatly affects their ability to adapt to adverse environmental conditions and in particular to abiotic stress factors. Impairment of the mineral nutrition status of plants exacerbates the adverse effects of abiotic stresses and the exogenous addition of high levels of macronutrients can alleviate the adverse effects of stress on plant growth (Baligar et al., 2001; Endris & Mohammed, 2007; Grattan & Grieve, 1999; Heidari & Jamshid, 2010; Kaya et al., 2001; Kaya et al., 2002; Khoshgoftarmanesh et al., 2010).

After nitrogen, phosphorous is the second major nutrient for plant growth as it is an integral part of different biochemicals like nucleic acids, nucleotides, phospholipids and phosphoproteins. In most cases salinity decreased P accumulation in plant, which developed P-deficiency symptoms (Martinez & Lauchli, 1994; Navarro et al., 2001; Parida & Das, 2004; Rogers et al., 2003). The reduction in P availability in saline soils was suggested to be a result of ionic strength effects that reduce the activity of phosphate and the tight control of P concentrations by sorption processes and by low solubility of Ca-P minerals. The concentration of soluble P in soil is usually very low (1 ppm or less) (Hinsinger, 2001). The cell might take up several P forms but the major part is adsorbed in the forms of  $\text{HPO}_4^{2-}$  or  $\text{H}_2\text{PO}_4^{-1}$ . Phosphorus exists in two forms in soil, as organic and inorganic phosphate, and like other nutrient elements such as potassium, iron, zinc and copper, possesses limited mobility in the soil (Hayat et al., 2010; Rodriguez & Fraga, 1999). The conversion of insoluble phosphate compounds (both organic and inorganic) in a form accessible to the plant is an important trait of PGPR strains. PGPR strains belonging to various genera have the ability to solubilize insoluble inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Richardson et al., 2009; Khan et al., 2009; Rodriguez & Fraga, 1999).

It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms (Rodriguez & Fraga, 1999). Production of organic acids results in acidification of the microbial cell and its surroundings. Consequently, P may be released from a mineral phosphate by proton substitution for  $\text{Ca}^{2+}$ . The production of organic acids has been well documented for different PGPR genera such as *Pseudomonas*, *Erwinia*, *Rhizobium* and *Bacillus* (Rodriguez & Fraga, 1999).

As discussed previously, soil contains a wide range of organic substrates, which can be a source of P for plant growth. To make this form of P available for plant nutrition, it must be hydrolyzed to inorganic P. Mineralization of most organic phosphorous compounds is

carried out by means of phosphatase enzymes (phosphohydrolases). Considering that the pH of most soils range from acid to neutral values, acid phosphatases should play the major role in this process (Rogriguez & Fraga, 1999).

Under P-limiting conditions, the IAA-overproducing RD64 strain showed high P-mobilizing activity that is connected to the synthesis of high levels of acid phosphatase enzymes and the secretion into the growth medium of malic, succinic, and fumaric acids in large quantities. As compared to *Mt*-1021 plants, *Mt*-RD64 plants released large amount of another P-solubilizing organic acid, 2-hydroxyglutaric acid and showed significant increase in both shoot and root fresh weights, when grown under P-deficient conditions (Bianco & Defez, 2010a).

#### **4. Agronomic approaches for crop improvement under abiotic stress conditions**

The increase in the frequency and severity of abiotic stresses is one of the main consequences of climate change. In particular, extreme weather events will result in more frequent drought and salinity.

To mitigate the effects of these stresses, appropriate crop management techniques will be needed to ensure sufficient production of food from crop plants by increasing the productivity per unit of land area. Several low-technology management systems, such as biofertilization (single or mixed inoculation treatments), crop rotation, intercropping, skip rows (decreasing planting density by omitting rows), mulching (with natural or synthetic mulches), and protected cropping (enclosing the aerial environment of the crop under glass, plastic or netting) can be used to improve crop productivity (Davies et al., 2010). The impact of two of the techniques described above is considered in the current review.

##### **4.1 Co-inoculation of plant growth-promoting bacteria**

Co-inoculation is based on mixed inoculants, combination of microorganisms that interact synergistically, or when microorganisms such as *Azospirillum* are functioning as “helper” bacteria to enhance the performance of other beneficial microorganisms. In the rhizosphere the synergism between various bacterial genera such as *Bacillus*, *Pseudomonas* and *Rhizobium* has been demonstrated to promote plant growth and development. Compared to single inoculation, co-inoculation improved the absorption of nitrogen, phosphorus and mineral nutrients by plants (Figueiredo et al., 2010; Yadegari et al., 2010). A significant increase in root and shoot biomass was observed in chickpea plants when co-inoculated with *Mesorhizobium* and *Pseudomonas* (Sindhu et al., 2002a, b). Increased nodule weight, root and shoot biomass and total nitrogen of chickpea plants was also reported due to co-inoculation of *Rhizobium*, *Pseudomonas* and *Bacillus* (Parmar & Dadarweal, 1999). Co-inoculation with *Bradyrhizobium japonicum* and *Pseudomonas fluorescens* increased colonization *B japonicum* on soybean roots, nodule number and acetylene reduction assay (Tchebotar et al., 1998). Combined inoculation of *Rhizobium* with *Pseudomonas striata* or *Bacillus megaterium* led to increased dry matter, grain yield and phosphorus uptake significantly over the uninoculated control in legumes (Elkoca et al., 2008). Verma et al. (2010) have reported the application of *Rhizobium* spp. and plant growth promoting rhizobacteria on nodulation, plant biomass and yields of chickpea plants. In field studies, the grain and straw yield were significantly increased in co-inoculation of *Rhizobium* with *P. fluorescens* followed by *B. megaterium* and *Azotobacter chroococcum* over uninoculated control. Co-inoculation of

*Pseudomonas* spp. with *Rhizobium* improves growth and symbiotic performance of fodder galega (Egamberdieva et al., 2010). The greenhouse experiment showed that co-inoculation of fodder galega with *R. galegae* and *P. trivialis* or with *R. galegae* and *P. extremorientalis* improved plant growth, nodulation and N content compared to plants inoculated with *R. galegae* alone in potting soil containing low levels of nitrogen. Co-inoculation of plants with *P. trivialis* and *R. galegae* showed the highest stimulatory effect.

The mechanisms behind these effects are only partially understood. One of the mechanisms used by these PGPR strains is the production of phytohormones such as auxins, gibberellins and cytokinins, which steadily contribute to the plant auxin "pool" in a way that the effect of PGPR inoculation can be mimicked by exogenous auxin application.

The endogenous IAA level in plant regulates growth of the shoots and roots, and in the case of legumes, nodules formation (Teale et al., 2006). It has been observed that low concentrations of exogenously given pure IAA stimulated shoot and root growth of wheat in non-saline and saline conditions, and similar effects were induced by IAA-producing PGPR strains (Egamberdieva, 2009).

Bacteria of the genus *Azospirillum* are free-living, surface colonizing and, sometimes, endophytic diazotroph and plant growth promoting rhizobacteria. *Azospirillum* strains had no preference for crop plants or weeds, or for annual or perennial plants, and can be successfully applied to plants that have no previous history of *Azospirillum* in their roots. Although reports about isolating *Azospirillum* from graminaceous plants are common, other reports showed that the bacterium is a natural inhabitant of many non-graminaceous plants. It appears that *Azospirillum* is a general root colonizer and is not a plants-specific bacterium (Bashan & Holguin, 1997). *Azospirillum* strains are capable of increasing yield of important crops growing in various soils and climatic regions. It has been reported that root elongation rate, mineral N, P and K and microelements uptake are consequently improved after *Azospirillum* inoculation (Bashan et al., 2004), even under stressful environmental conditions (Askary et al., 2009). Dual inoculation of legumes with *Rhizobium* and *Azospirillum* significantly increase several plant-growth variables when compared with single inoculations (Hamaoui et al., 2001; Itzigsohn et al., 2000; Remans et al., 2007; Remans et al., 2008b; Tchebotar et al., 1998). *Azospirillum* is considered a *Rhizobium* helper by stimulating nodulation, nodule function, and possibly plant metabolism (Molla et al., 2001; Verma et al., 2010). Phytohormones produced by *Azospirillum* promote epidermal-cell differentiation in root hairs that increased the number of potential sites for rhizobial infection leading to the formation of more nodules. Morphological and physiological changes in root system are also stimulated (Bashan & Levanony, 1990; Pacovsky, 1990; Sarig et al., 1992; Volpin & Kapulnik, 1994). An increase in the number of lateral roots and root hairs cause addition of root surface available for nutrients and water uptake. Higher water and nutrient uptake by inoculated roots cause an improved water status of plant, which in turn could be the main factor enhancing plant growth (Boddey et al., 1986; Dalla Santa et al., 2004; Fallik & Okon, 1996; Mostajeran et al., 2002).

Positive effects of co-inoculation were also observed on symbiotic performance of common bean, which is usually considered a poor nitrogen-fixing legume. Poor nodulation and variable response to inoculation is mainly attributed to intrinsic characteristics of the host plant, particularly the great sensitivity to nodulation-limiting factors, such as high rate of N fertilizer used in intensive agriculture, high temperature and soil dryness (Bais et al., 2006; Egamberdiyeva, 2007). Indeed, Yadegari & Rahmani (2010) showed that co-inoculation of

three *Phaseolus vulgaris* cultivars with two *Rhizobium* strains, *Pseudomonas fluorescens* and *A. lipoferum* resulted in increased seed yield, number of pods per plant, weight of seeds, seeds protein yield and number of seeds per pod.

Inoculation of common bean or alfalfa (*Medicago sativa*) with *Azospirillum brasilense* in the absence of *Rhizobium* resulted in a more persistent exudation of flavonoids by legumes roots. *Azospirillum*-*Rhizobium* co-inoculation positively affected the expression of *nod*-genes and production of nodulation factor patterns in *Rhizobium tropici* and *Rhizobium etli* in the presence or absence of NaCl at 50 mM. A significant increase of total and upper nodule numbers was observed at different concentrations of *Rhizobium* inoculum (Dardanelli et al., 2008).

Several greenhouse and field experiments demonstrated the potential of co-inoculation to increase grain yield of various legumes (Bashan and Holguin, 1997; Galal et al., 2002; Itzigsohn et al., 1993; Sarig et al., 1986; Yahalom et al., 1989).

#### 4.2 Intercropping systems

Due to climate changed, suitable land area for agricultural production remains fixed or is diminishing and farmers are faced with the task of increasing production demands (Zhang & Cai, 2011). Raising productivity is possible through the introduction of improved genotypes with enhanced resilience to abiotic stresses. In addition to this, agronomic manipulation may impact significantly on crop productivity. Because of restricted availability of water and fertilizer in many part of the world, productivity increase must be accompanied by increase in use efficiency. The cultivation of two or more species in the same field at the same time (intercropping) can boost productivity per unit land area (Davies et al., 2010). Crop mixtures may consist of legume/legume or legume/non-legume systems. Some of the intercropping advantages include: higher yield than sole crop yields, greater yield stability, more efficient use of environmental resources, better weed control and improved quality by variety (Malèzieux et al., 2009). Intercropping is a common practice where land is scarce: beans are mostly intercropped in sub-Saharan Africa, with the major exception of southern Africa where nearly half are monocropped (Kimani et al., 2005); groundnut is often grown as an intercrop in West Africa (Ndjeunga et al., 2008); pigeonpea has been traditionally grown as an intercrop in India; more than half of lentil grown in Bangladesh is planted under mixed cropping (Sarker et al., 2004).

However, the increased awareness of environmental degradation arising from the use of non-renewable artificial fertilizers and pesticides is encouraging the use of mixed cropping even in developed countries (Fujita et al., 1992).

The N<sub>2</sub> fixed by *Rhizobia* in legumes can also benefit associated non-legume via direct transfer of biologically fixed N to cereals growing in intercrops when fertilizer N is limited, which has both economic and environmental benefits. Among the various combinations of cereals and legumes used by small-scale farmers maize-cowpea is one of the most widely used because cowpea fixes atmospheric nitrogen and produces proteins, while maize depletes the soil nitrogen and produce carbohydrates. Maize and cowpea mixtures improve the diets as well as the soil fertility and productivity (Dahmardeh et al., 2009, 2010). PGPR strains may contribute to the benefits of legumes in cropping systems in more way than just fixing atmospheric nitrogen. Indeed, as previously discussed, these bacteria have the ability to promote modifications of root architecture, enabling those plants to accumulate more mineral nutrients than control plants, increase disease resistance and improve plant response to environmental stresses. Therefore, the use of PGPR-inoculated legumes and



non-legumes in mixed cropping systems would be a promising agricultural practice for rehabilitation of extreme wasted lands, after a careful selection of appropriate tolerant bacterial strains and legume genotypes to the prevailing stressful conditions.

## 5. Discussion and conclusions

A large number of PGPR representing diverse genera have been described over the past 50 years. Despite their appeal as a “natural” means of plant protection few strains have been developed commercially. This is partly because uneconomically large doses often must be applied and performance can be inconsistent in the field. There are several advantages of developing genetically-modified PGPR over transgenic plants for improving plant performance under a variety of stresses: (1) it is far easier to modify a bacterium than complex higher organisms; (2) several plant growth-promoting traits can be combined in a single organism, and (3) instead of engineering crop by crop, a single, engineered inoculant can be used for several crops, especially when using a non-specific genus like *Azospirillum*.

PGPR strains development is hampered mainly by the fact that these organisms not always survive harsh environmental conditions including high concentrations of environmental contaminants, salts, extremes of pH and temperature, and compete with other organisms. Genetically engineered strains offer a means to develop PGPR that are effective at low inoculum doses and under a variety of environmental conditions. Many rhizobacteria produce phytohormones that undoubtedly affect root growth leading to the formation of roots systems with increased exploratory capacity. This morphological modification plays an important role in the mechanisms of stress response (Potterset al., 2007, 2009). Efforts to engineer the rhizosphere through hormone manipulation have focused mainly on degradation of so-called “stress” ethylene, which is synthesized by plants upon exposure to stresses such as flooding, drought, salt, and the presence of metals, organic contaminants and pathogens. The production of ACC deaminase enzyme, which catalyzes the cleavage of ACC, the immediate precursor of ethylene, may be used to modulate ethylene levels. Available data are consistent with the proposed model of plant growth facilitation by ACC deaminase-producing PGPR strains (Glick et al. 2007). We have described an engineered PGPR strain, RD64 (Pii et al., 2007), a derivative of *Sinorhizobium meliloti* 1021, able to release into liquid growth medium up to 78-fold more IAA than wild-type 1021 (Camerini et al., 2008). For this strain, as well as for IAA-treated *Escherichia coli* cells (Bianco et al., 2006a, 2006b), a more resistance to salinity and other abiotic stresses and the induction of tricarboxylic acid cycle (TCA) enzymes was observed as compared to 1021 strain (Bianco & Defez, 2009; Imperlini et al., 2009). In addition, RD64 strain showed enhanced long-term cell survival (Defez, 2006), has improved nitrogen fixation ability (Bianco & Defez, 2010b; Imperlini et al., 2009) and is highly effective in mobilizing P from insoluble source such as Phosphate Rock (Bianco & Defez, 2009). *Mt*-RD64 plants showed an higher degree of protection against oxidative damage induced by salt stress (Bianco & Defez, 2009) and significant increases in both the shoot and root fresh weight under P-starved condition when compared to salt-stressed and P-starved *Mt*-1021 plants (Bianco & Defez, 2010a).

For *Mt*-RD64 plants we also observed a re-modulation of phytohormones, with a higher IAA content in nodules and roots and a decreased IAA levels in shoots, as compared with *Mt*-1021 plants. The modulation of IAA levels in these plants lead to alterations of other important hormones that control plant growth. Indeed, the expression levels of Medicago genes encoding members of cytokinin signalling pathway were induced in the root of *Mt*-

RD64 plants (Bianco et al., 2009). In addition, the analysis of the expression levels of the main ethylene signalling genes showed that severe salt stress triggered a high induction of ethylene signalling in *Mt-1021* plants whereas this pathway was not significantly altered in *Mt-RD64* plants (Bianco & Defez, 2009).

We speculate that the growth promoting effects observed under stressful environmental conditions for the model legume Medicago might be extended to other plant species. Indeed, for legumes such as pea, alfalfa and bean plants, we previously reported an increase in the shoot or seed production for the plants nodulated by IAA-overproducing strains. In addition, for tropical legumes such as soybean and peanut plants, we also have preliminary data indicating the positive effects triggered by the specific IAA-overproducing rhizobia strains (Bianco et al., 2010c).

A PGPR strain with the characteristics described above is a good candidate to promote plant yield under stressful environmental conditions either in mono-cropping or mixed cropping systems.

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# Soil Salinisation and Salt Stress in Crop Production

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## 1. Introduction

For optimal grow and development, cultivated plants require balanced presence of water and dissolved minerals (salts) in their rhizosphere. In that respect, quality and availability of two natural resources, water and soils, are crucial in cultivation. Although Earth abounds in water, an almost negligible portion (~2.5% or 35 million km<sup>3</sup>) is fresh or with low salt concentration (<1 dS/m) (Shiklomanov & Rodda, 2003; Ondrasek et al., 2010), i.e. water that may be conditionally used for irrigation in crop production, whereas the rest is salty and therefore unsuitable for irrigation. However, irrigated agriculture consumes ~70% (and >90% in many developing countries) of total water withdrawal to produce ~36% of global food (Howell, 2001). According to recent estimates (ICID, 2009), almost 300 million ha in the world are irrigated, with ~2/3 of that in most populated and the fast growing Asian countries. In many irrigated agricultural areas, especially in developing countries, water scarcity is pronounced because of environmental conditions (e.g. arid and semiarid climate zones) and the rising population (i.e. food demand). As a consequence, there is an increasing trend of inappropriate use of restricted water (e.g. over/pumping of salinised aquifers) and continuous degradation of land resources (e.g. salt-affected soils), representing a large burden to human food supply and natural ecosystems.

Some of the most produced and widely used crops in human/animal nutrition such as cereals (rice, maize), forages (clover) or horticultural crops (potatoes, tomatoes) usually require irrigation practices, but are relatively susceptible to excessive concentration of salts either dissolved in irrigation water or present in soil (rhizosphere) solution. In a majority of cultivated plants, yields start declining even at relatively low salinity in irrigation water ( $EC_w > 0.8$  dS/m) (e.g. Ayers & Westcot, 1994) or soil ( $EC_{sc} > 1$  dS/m in saturated soil extracts) (see Table 1 in Chinnusamy et al., 2005). Increased soil salinity may induce various primary and secondary salt stress effects in cultivated plants (section 4.3.1). Salt stress as one of the most widespread abiotic constraints in food production may also result in the negative ecological, social and/or economic outcomes. For instance, recent deposition of toxic salt sediments and sea intrusion in tsunami-affected areas of Maldives damaged >70% of agriculture land, destroyed >370,000 fruit trees and affected around 15,000 farmers, with

costs estimated at around AU\$6.5 million (FAO, 2005). Successful remediation of salt-degraded areas for crop production, besides using relatively salt-tolerant species/genotypes, is highly dependent on sustainable management practices that are usually costly, time consuming and may be difficult or impossible to implement fully in certain ecological situations (e.g. seepage soil salinity; section 3.1). Accordingly, in response to the salinity issue, Australia's National Action Plan for Salinity and Water Quality from 2000, resulted in investments of about AU\$1.4 billion over 7 years to support actions by communities and land managers in salt-affected regions (Williams, 2010). However, recent advances in plant breeding and molecular biology technologies suggest that increasing salt tolerance in cultivated plants could be one of the most promising and effective strategies for food production in salt-affected environments.

## **2. Salinisation - A global land degradation issue**

Different types of physical, chemical and/or biological land degradation processes (e.g. compaction, inorganic/organic contamination, diminished microbial activity/diversity), mostly under excessive anthropogenic pressures during the last century, have resulted in serious consequences to global natural resources. Among them, soil salinisation, arising from either natural or human-induced causes, led to an increase in concentration of dissolved salts in the soil profile to a level that impairs food production, environmental health and socio-economic wellbeing (Ondrasek et al., 2009a; Rengasamy, 2006). Despite numerous adverse environmental consequences (e.g. crop yield/quality decline, disruption of soil aggregates/structure, desertification), areas already affected by salinity are among the most intensively exploited ones in global agriculture. Salinisation is among the three soil degradation processes [in addition to organic matter (OM) decline and contamination] recognized as the main threats to environmental resources and human health in EU (Ondrasek et al., submitted a) and many other developed (Helal et al., 1999) or developing countries (Rengasamy, 2006), affecting almost 1 billion ha worldwide.

## **3. Soil salinisation processes**

Depending on soils, the extracted solutions differ in the content of dissolved salts (cations/anions); if total salt concentration, i.e. electrical conductivity ( $EC_{se}$ ), exceeds 20 mM (~2 dS/m), they can be categorised as salt-affected (e.g. Abrol et al., 1988). The underlying salinisation processes may be primary (natural) and secondary (anthropogenic) (Ghassemi et al., 1995). Main driving forces of naturally-induced (inherent) soil salinity are: i) intrusion of highly salinised water in coastal (oceans, sea) or continental (e.g. fossil salt aquifers) regions, ii) aeolian i.e. wind borne salt from salt surface waters (oceans, lakes) deposited inland, and iii) dissolution of soil parent minerals. In contrast, some agricultural practices (fertigation, application of inorganic/organic soil amendments) may contribute to secondary salinisation processes. In contrast to that categorised classification, soils salinisation principally inherent to Australian environmental conditions is different (Biggs et al., 2010; Rengasamy, 2006).

### **3.1 An Australian example**

Australia is the most salt-affected continent with ~1/3 (~260 million ha) of total global salinised area, and ~1/2 of total world's alkaline area (e.g. Szabolcs, 1989). The most



widespread Australian soil salinity (>250 million ha), called subsoil or transient, is present in alkaline (pH >8.5) and Na-enriched soils (e.g. ESP $\geq$ 6) (Rengasamy, 2002) and specific climatic surrounding, where evapotranspiration demand frequently exceeds rainfall by multi-fold. Transient salinity occurs preferentially in higher ecobiotopes (e.g. slight uplifts and terraces), in illuvial subsoil (B) horizons (20-100 cm from the soil surface) enriched with topsoil-leached Na salts (EC<sub>se</sub> 4-16 dS/m) and dispersed clay particles (e.g. Rengasamy, 2002, 2006). In such subsoil circumstances, whereby excessive Na<sup>+</sup> presence disturbs soil structure and hydraulic properties (e.g. Ondrasek et al., 2010), after watering i.e. during wet season, dissolved salt ions fluctuate over the solum and cause its salinisation. Therefore, subsoil salinity is triggered by water/solute flux and hydraulic conductivity of (sub)pedosphere (e.g. Rengasamy, 2002), rather than by the fluctuation of salinised groundwater that is the main cause of second most abundant type of salinisation in Australian soils.

Groundwater-induced (seepage, dryland) salinity, in contrast to transient salinity, occurs in the lowest positions in the landscape (e.g. base of slopes/valleys). In this type, also called catena form - type 1, water flows relatively easily through the lighter textured soils in upslope locations, but cannot move as quickly through the heavy soils in the footslope, causing waterlogging and seepage (Biggs et al., 2010). It is induced by upwards intrusion and/or capillary rising of highly saline (EC<sub>w</sub> up to 150 dS/m) and relatively superficial (<2 m from land surface) watertable, even up to topsoils strongly salinising them (EC<sub>se</sub> up to 80 dS/m) by secondary processes (Rengasamy, 2002). From economical/ecological perspective, soils affected by dryland salinity are more adversely affected than transiently saline subsoils, are highly restricted for cultivation and could be high costly to remediate.

Total annual cost to Australian economy caused by dryland/transient salinity and associated subsoil constraints (e.g. excessive pH, Na, Cl, B, Al, carbonates; see review by Dang et al., 2006) are estimated at more than A\$1.6 billion (e.g. Rengasamy, 2002). One of relatively cost-effective and widely used strategies in coping with groundwater salinity in Australia is lowering/controlling of watertables (e.g. around 1 m from soil surface) by withdrawing from underlying salt-affected aquifers and using the water in irrigated agriculture (directly or after mixing with fresh water), thus enables salt leaching and improved permeability in the rhizosphere (e.g. Bethune & Batey, 2002). Also, a short-term use of slightly to moderately salinised water for irrigation of perennial pastures (EC<sub>w</sub> 0.8-2.4 dS/m) or legume lucerne crops (EC<sub>w</sub> 2.5 dS/m) would have little effect on their production (e.g. Burrow et al., 2002; Rogers, 2001).

However, over the long-term period, such irrigation practices will most likely induce (among other constrains discussed later) irrigation salinity as a widespread soil degradation problem. In many similar arable, (semi)arid areas around the world, secondary salinisation induced with inappropriate irrigation/drainage practices (e.g. re/using of saline ground/surface waters, using of industrial/municipal waste waters/effluents, over-pumping of coastal aquifers, lacking/improper subsoil drainage, etc) affects ~50% of global irrigated areas, with an annually increment of up to 500,000 ha (e.g. Martinez-Beltran & Manzur, 2005; Ondrasek et al., 2009a), representing a serious threat to sustainable food production and deterioration of natural terrestrial resources.

Recently, ICID (2009) estimated that >20% of water used in Australia is derived from groundwater sources, and out of that the most is used for irrigation of totally ~2.55 million ha irrigated land area. A substantial part of that land is probably exposed to secondary irrigation salinisation because (re)using of saline groundwater for crop irrigation is one of common

strategies in Australian soil salinity management (Bethune & Batey, 2002). According to the same authors, irrigated pastures, mostly developed on lands with highly salinised watertables, are spread on around 30% or 0.7 million ha of irrigated land in Australia.

There are many other types of primary/secondary land (water) salinisation processes (see report by Biggs et al., 2010) such as i) closed depression salinity i.e. primary salinity that occurs in natural deep depressions (e.g. lakes, swamps, etc: Figure 1a) with restricted leaching and pronounced surface (10-30 mm) salt encrustation (Figure 1c), and ii) so-called dam-form salinity, occurring in many water storage areas (Figure 1b).

### 3.2 An European (Croatian) example

Soil degradation processes are widespread in European countries (e.g. wind/water erosion on ~160 million ha or ~15% of total Europe's area; compaction on ~36% of European subsoils, etc) and have been either driven or exacerbated by human activities (European



Fig. 1. Closed depression (a, c) and dam (b) type salinity with the white surface salt encrustation (a, b, c) in the Lake Grace area, Western Australia (Photos taken by G. Ondrasek, 2011).

Commission, 2006). According to the same source, total annual costs of some of the most important land degradations (erosion, OM decline, salinisation, landslides and contamination) that could be estimated amount to AU\$52 billion, and do not include the damage to the soil ecological functions as these are exceedingly difficult to quantify. Among continents, Europe has the lowest portion of degraded soils by excessive salinity (~31 million ha), ~8-fold lower compared to the most salt-affected Australia (e.g. Szabolcs, 1989). According to General Soil Map of Croatia (1:50,000) permanent halomorphic soil classes (Solonchak and Solonetz) are minor i.e. distributed on <600 ha or 0.01% of total land area (Bogunovic et al., 1998). However, recent multidisciplinary studies, related to environmental salinisation processes (land/water) and their influence on irrigated agriculture and natural ecosystems of intensively agro-exploited coastal estuaries (Ondrasek et al., 2010; Romić et al., submitted) suggest potentially large influence of salinisation in Mediterranean areas of Croatia. Also, seems that substantial portion of salinised land areas is still fortunately periodically characterised because of specific geo(pedo)logical and climate interactions. From geological perspective, along coastline of Adriatic Sea there are many fertile and arable karstified fields and alluvial estuaries with similar properties and exposure to salinisation processes as can be explained in case of Neretva River delta (Figure 2).

The geological parent material in down stream of Neretva river consists of: i) Mesozoic and Paleogene carbonates with certain Paleogene flysch materials (creating the basement of the fractured and deeply karstified valley that is very susceptible to sea water intrusion; Figure 2a), and ii) Quaternary sediment and poorly lithified deposits (with heterogeneous water permeability and other hydraulic properties) representing surface of lito(pedo)sphere underlain by clay, sand and/or gravel alluvial (Holocene) fractions as well as Pleistocene conglomerates (Romić et al., submitted). Also, direct sea water intrusion, in the shape of salty wedge, through Neretva river and its natural/artificial tributaries is possible even up to several tens of kilometres upstream, depending on river watertable and seasonal (dry/wet) conditions (Figure 2b). Irrespective of which way seepage of salty water is manifested (directly through porous karstified materials or via river flows, laterally or upwardly through alluvial deposits), salty water is mixing with groundwater, restricting its usage for agriculture or as drinking water (Figure 2c).

As confirmed by chemical analyses of surface and groundwater sources and topsoil saturation extracts, primary and/or potential secondary salinisation processes are present on >2,500 ha of irrigated land in Neretva River estuary, given that  $EC_w/EC_{se}$  and/or SAR (Sodium Adsorption Ratio) values exceed those recommended for agriculture by FAO (e.g. Abrol et al., 1988; Ayers & Westcot, 1994; Ondrasek et al., 2010). Detected processes of primary salinization caused by the capillary rising of highly salinised groundwater (e.g.  $EC_w > 23$  dS/m at <4 m depth) may initiate salt accumulation in (sub)soil horizons (Ondrasek et al., 2010). Also, multi-annual monitoring of quality of surface water sources (ameliorative drainage channel network, Romić & Vranjes, 2010) confirmed their periodically strong salinisation and serious restrictions of their irrigation usage (i.e.  $EC_w > 3$  dS/m) (Ayers & Westcot, 1994). Meanwhile, at most analysed locations, surface water is used in intensive horticultural production employing drip/micro sprinkler fertigation practices (Romić et al., 2008), inducing secondary soil salinisation. However, favourable climate and soil conditions such as i) intensive rainfall (>650 mm) in between two vegetation seasons, ii) relatively homogenous/stable solum structure, and iii) light soil texture and therefore good water permeability of majority of alluvial sediments, enable leaching of accumulated topsoil salts

into deeper horizons (below the root zone) and underpin resilience of these agricultural ecosystems (Ondrasek et al., 2010). Finally, recently initiated monitoring of salinisation downstream of Neretva River estuary (Romic & Vranjes, 2010 and references therein) will provide new insights into the subsoil constrains in this area (e.g. salinity/sodicity).

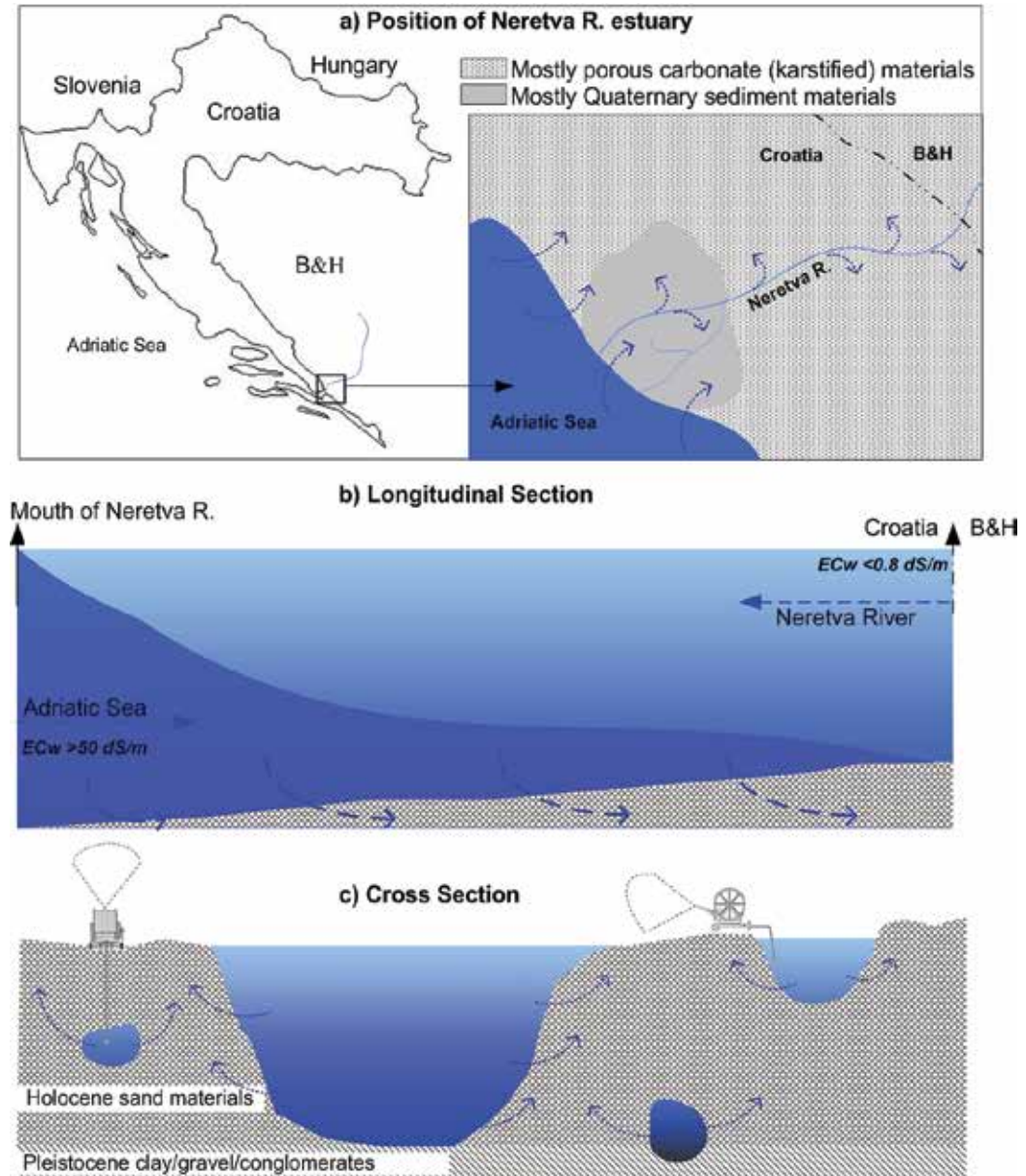


Fig. 2. Salinisation processes in Neretva river estuary, Croatia, adopted from Ondrasek, 2008; Romic et al., submitted and references therein (dashed arrows represent intrusion of sea/salted water, whereas the straight arrows are main flow directions of river/sea)

## 4. Environmental consequences of soil salinity

### 4.1 Impact on pedosphere

Soil salinity is often accompanied by a wide range of pedosferic (low soil fertility, high exposure to erosion processes), atmospheric (high air temperature, low precipitation and air humidity) and/or hydrospheric (water scarcity) constraints, which negatively influence agricultural production. However, secondary consequences of salinity, such as those due to long-term usage of salinised water for irrigation, may cause permanent soil degradation because of dispersion of soil aggregates. Sodium ( $\text{Na}^+$ ), as the most frequent causative agents of salinity, is the most pronounced destructor (by dispersion) of secondary clay minerals. Dispersion occurs because of  $\text{Na}^+$  replacement of calcium ( $\text{Ca}^{2+}$ ) and other coagulants ( $\text{Mg}^{2+}$ , OM) adsorbed on the surface and/or inter-layers of soil aggregates (e.g. Ondrasek et al., 2010 and references therein). The replacement is mainly caused by specific physical characteristics, such as ionic radii, electrical charge and hydration ability of particular elements, whereas some chemical properties (e.g. increased pH) may facilitate the clay dispersion process.

For instance, 6-co-ordinated  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions have radii of 0.100 and 0.072 nm, respectively, whereas that of  $\text{Na}^+$  is 0.102 nm (Shannon, 1976). Also, single-charged Na has lower clay-binding ability than double-charged Ca (or Mg), which in turn is not so strongly hydrated as Na (Rashad & Dultz, 2007). Thus, after  $\text{Ca}^{2+}$  ( $\text{Mg}^{2+}$ ) replacement by  $\text{Na}^+$  and its intrusion into secondary clay minerals (e.g. 1:1 or 2:1 phyllosilicate sheets) and watering (i.e. hydration), interlamellar space increases and may cause decoupling of lamellae (sheets), i.e. clay dispersion. Finally, under alkaline conditions ( $\text{pH}>8$ ) rate of dissolution (dispersion) of silicate minerals increases because excessive  $\text{OH}^-$  interacts with the clay interface and generates strong negative charge (e.g. Rashad & Dultz, 2007).

Dispersed clay particles undergo leaching through the soil and may accumulate and block pores, especially in fine-textured soil horizons (Burrow et al., 2002) i.e. cause pedospheric waterlogging. Furthermore, dispersion of clay usually induces topsoil crusting, thus reducing infiltration, enhancing surface runoff and other related degradation processes (soil erosion/desertification, surface waterlogging, nutrient leaching, etc). Over time, in saline (sodic) soils crusted surface layer (Figure 3b) constrains hydraulic properties (water permeability, infiltration rate, etc) as well as aeration of topsoil horizons and the root zone.

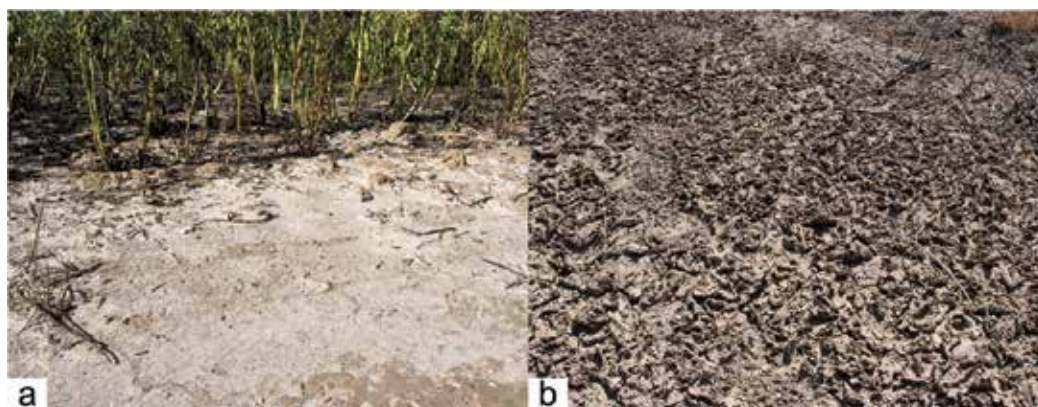


Fig. 3. Surface salt crystallisation (a) and topsoil crusting (b) in a faba bean paddock (Esperance area, W. Australia) (Photos taken by G. Ondrasek, 2010)

During the dry periods, salts accumulated in the soil profile or on the soil surface (Figure 3a) may further affect soil properties. For instance, ground observations and radiometric measurements confirmed that quantity and quality (i.e. mineralogy) of salts, together with soil moisture, colour, and roughness, affect the soil surface reflectance (Metternicht & Zinck, 2003), and consequently influence the topsoil physical properties (e.g. warming). It was shown that abundance of  $\text{Na}_2\text{SO}_4$  (puffy crusts) or  $\text{NaCl}$  (smooth crusts) increases reflectance in the visible and near-infrared wavelengths compared with the nonsaline soil surface (e.g. Metternicht & Zinck, 2003). According to the same authors, the surface of saline soils is brighter compared to sodic (alkaline) soils due to OM dispersion in the latter.

#### **4.2 Impact on hydrosphere**

Increased salinity in soil solution, especially increased concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  ions, significantly influence solubility i.e. mobility of potentially toxic trace elements (Helal et al., 1999; Khoshgoftar et al. 2004; Khoshgoftarmanesh et al., 2006; Ondrasek et al., 2009a, 2009b; Weggler et al., 2004). Excessive salinity may cause desorption of particular metal and other cations from the soil adsorption matrix, increasing concentration of bioavailable forms in the soil solution. In particular, an exposure to increasing  $\text{NaCl}$  salinity increased concentration of trace elements (e.g.  $\text{Cu}$ ,  $\text{Zn}$ ,  $\text{Cr}$  and/or  $\text{Cd}$ ) in the rhizosphere soil solution (Helal et al., 1999; Khoshgoftar et al. 2004; Ondrasek et al., submitted b) or in unplanted humic solution (Lores & Pennock, 1998). Namely, organic (humics, black carbon) and inorganic (clay, hydroxides) surfaces, mostly negatively charged, compete with salt anions ( $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ) for metal cations ( $\text{Cu}^{2+}$ ,  $\text{Al}^{3+}$ ) via adsorption and complexation reactions (e.g. Adriano et al., 2004). Therefore, under excessive concentrations of  $\text{Na}^+$ , it is really expected displacement of weakly bound metal forms ( $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ ) and their accelerate release from soil solids to solution and enhance their mobility i.e. expose surrounding aquatic ecosystems to increased metals contamination (Ondrasek et al., submitted a).

#### **4.3 Impact on biosphere, particularly plants**

In general, bulk and rhizosphere soil, as well surface and subsurface soil profiles, differ in their chemical, physical and biological properties (pH, salinity, redox potential, porosity, water retention, abundance and diversity of microbial species, etc). Increased salinity/sodicity (EC/Na) in the rhizosphere affects root exudation (e.g. of low-molecular-weight organic substances) and biochemical transformations i.e. decomposition of organic matter by microorganisms (Ondrasek et al., submitted b). Contradictory effects of increased EC and Na on soil biological processes (e.g. OM decomposition) have been reported (Li et al., 2006 and references therein). Recently, Ondrasek et al. (submitted a) observed a decrease in dissolved OM (dependent on soil microbe-root interactions) of 5% (at  $\sim 4$  dS/m and 20 mM Na) and  $>20\%$  (at  $\sim 8$  dS/m and 60 mM Na) compared to control ( $\sim 2$  dS/m and 1.5 mM Na). Similar observation were reported by Li et al. (2006), who noticed significant negative correlations between soil EC and total  $\text{CO}_2$  emission or microbial biomass C, suggesting that salinity had an adverse effect on microbial biomass and activity. Therefore, naturally-occurring soil OM decomposers may be sensitive to salt-induced stress.

##### **4.3.1 Salt stress in crop production**

###### **4.3.1.1 Background**

A majority of cultivated plant species, especially widely grown horticultural and cereal crops (e.g. Table 1 from Chinnusamy et al., 2005), are glycophytes i.e. susceptible to

excessive concentration of dissolved ions (e.g. >30 mM or >3.0 dS/m) in the rhizosphere solution. Depending on salt concentration and the length of exposure, the stage of growth/development and the environmental conditions (e.g. humidity, temperature, insolation, soil moisture, etc), in glycophytes increased salinity may induce different physiological malfunctions, such as osmotic, ionic and different secondary (e.g. oxidative) disorders (Zhu, 2001), generally known as salt stress. Osmotic stress, as primary reaction triggered by relatively low/moderate salinity levels, decreases soil water potential i.e. reduces water uptake and causes possible cell dehydration (Ondrasek et al., 2009a and references therein). For instance, increased concentration of dissolved salts may reduce soil water potential from -1 to -2.5 (-5 in extreme cases) MPa (Flowers & Flowers, 2005), whereas at water field capacity its potential is 0.033 MPa. In saline conditions, osmotic pressure in the rhizosphere solution exceeds that in root cells, influencing water and nutrient uptake. Further plant responses to osmotic stress are stomata closure (partially or fully) i.e. transpiration/C assimilation reduction, decrease in cell growth and development, reduced leaf area and chlorophyll content, accelerated defoliation and senescence i.e. mortality of plant organism (Shannon & Grieve, 1999).

An increase in concentration of certain dissolved ions, such as those in relatively shallow Australian Na-subsoils solution, will enhance their uptake i.e. ionic stress, and ultimately cause phytotoxic effects (e.g. Cl<sup>-</sup>, B, Al toxicity). Specific ionic stresses disrupt integrity/selectivity of root plasma membrane, homeostasis of essential ions and numerous metabolic activities (e.g. Zhu, 2001). For instance, in rice, as one of the most widely grown cereals, salinity is the main limiting variable of mineral nutrition (Marschner, 1995). Moreover, approximately half of global saline (i.e. alkaline) soils used for cereal production are overlain on soils with low levels of plant-available Zn i.e. Zn-deficient soils, due to Zn complexation/competition with dissolved salts (CO<sub>3</sub>S, SO<sub>4</sub>S, Na<sup>+</sup>) at alkaline pHs (Ondrasek et al., submitted b). Therefore, given that the food crop consumption is the principal route of most essential minerals into the human organism, salinity may indirectly contribute to mineral deficiency in billions of people.

The primary salinity effects give rise to numerous secondary ones such as oxidative stress, characterised by accumulation of reactive oxygen species (ROS, e.g. H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>, ·OH), potentially harmful to biomembranes, proteins, nucleic acids and enzymes (Gomez et al., 2004) (see 5.2. section). Antioxidative enzymes such as superoxide dismutase (SOD), catalases, peroxidises, etc enhance detoxication of ROS. The relatively salt-tolerant species (e.g. pea genotypes) have increased activities of certain antioxidative enzymes (e.g. SOD; Hernandez et al., 2001), whereas in salt-sensitive species (e.g. cowpea) Na<sup>+</sup> causes a stronger inhibition effect on particular SOD forms than Cl<sup>-</sup> ions (Hernandez et al., 1994).

#### **4.3.1.2 Nutrient uptake under salt stress - Impact on food production**

Two most important ions that induce salt stress in plants are Na<sup>+</sup> and Cl<sup>-</sup>. Sodium is nonessential but beneficial element, whereas Cl is essential phytomicronutrient (Marschner, 1995); however, both are potentially toxic in excessive concentrations, triggering specific disorders and causing substantial damages to crops. Under excessive Na<sup>+</sup> and Cl<sup>-</sup> rhizosphere concentration (activity), there are competitive interactions with other nutrient ions (e.g. K<sup>+</sup>, NO<sub>3</sub><sup>-</sup>, H<sub>2</sub>PO<sub>4</sub><sup>-</sup>) for binding sites and transport proteins in root cells, and thereafter for (re)translocation, deposition and partitioning within the plant (see reviews by Grattan & Grieve, 1999; Tester & Davenport, 2003; White & Broadley, 2001). Significantly enhanced uptake and accumulation of Na and Cl (from 11-fold in radish up to >100-fold in

strawberry and muskmelon leaves), accompanied with a decrease in K concentration in the same tissues (from ~30% in strawberry and ~40% in radish to 4-fold in muskmelon) was obtained under moderate (60 mM) NaCl salinity (Ondrasek et al., 2009a, 2009b; unpublished). In the same studies, salinity stress reduced all vegetative parameters (e.g. number of strawberry runners by up to 7-fold and length of the longest runner by 3-fold), decreased total fruit yield (in radish by 35%, muskmelon by 50% and strawberry by 60%), accelerated leaf senescence and reduced the strawberry growing period by up to 22 days i.e. induced plant mortality after 65-day treatment with salinised (60 mM NaCl) nutrient solution.

The high K/Na and Ca/Na ratios are essential for normal plant functioning (e.g. Chinnusamy et al., 2005). The Ca/Na declined under salinity by 13-fold in radish, 113-fold in strawberry and 150-fold in muskmelon leaf tissue (Ondrasek et al., 2009a, 2009b; unpublished). It appears that disturbance of cytosolic Ca<sup>2+</sup> homeostases in root cells is one of the fastest signals in glycophytes to increasing salinity; hence, a decrease in Ca/Na could be one of the most reliable salt-stress indicators (reviewed by Rengel, 1992). An addition of Ca may significantly mitigate certain salt stress-induced dysfunctions in crops that may be used as one of management strategies in salt-affected agro-ecosystems (section 5.1).

Crops with perturbed nutrients relations (e.g. Ca/Na, K/Na etc) are more susceptible to invasion of different pathogenic (micro)organisms and/or physiological dysfunctions (e.g. blossom-end rot, blackheart), whereas their edible parts have markedly less economic and nutritional value due to reduced fruit size and shelf life, non-uniform fruit shape, decreased vitamin content, etc. (Ondrasek, 2008 and references therein). Consumption of edible crop parts grown in salt-affected and metal contaminated surrounding could pose a high risk of metals intake into the human/animal organisms (Ondrasek et al., submitted b). It was well documented that in rhizosphere/soil solution salinised by Na<sup>+</sup>, Cl<sup>-</sup> and/or SO<sub>4</sub><sup>2-</sup> ions, potentially toxic metals such as Cd, Zn and Cu are taken up and accumulated to a large extent in different horticultural (McLaughlin et al., 1998; Ondrasek et al., 2009a, 2009b; Smolders et al., 1997), cereal (Khoshgoftar et al. 2004; Khoshgoftarmanesh et al., 2006; Weggler et al., 2004) and forage (green manure) crops (Helal et al., 1999), although complete mechanisms are unexplained. Matrix of saline (sodic) soil is naturally poor and/or depleted in metal-binding interfaces (e.g. aluminosilicate minerals, organic C), thus predisposes crops to potentially strong metal influence.

## 5. Options for mitigating salt stress in crop production

A majority of arable salt-affected areas are distributed in the intensively cropped food producing regions such as those in Asia and Australia. The excessive concentration (EC) and ratios (e.g. ESP, SAR) among particular salts (Na, Cl, Ca, Mg) as well pH conditions need to be taken into account when considering appropriate strategies for reclamation of salt-affected soils in crop production. Such salt-affected soils can be categorised into: i) saline (ECe > 4 dS/m, ESP < 15 and pH < 8.5), ii) saline-sodic (ECe > 4 dS/m, ESP > 15 and pH < 8.5) and iii) sodic (ECe > 4 dS/m, ESP > 15 and pH > 8.5), each requiring specific approaches for reclamation (Horney et al., 2005) and sustainable land management practices, that are usually costly and difficult to implement, and may even result in further degradation.

### 5.1 Sustainable agricultural management in salinised conditions

Sustainable agricultural management in salt-affected conditions is principally based on two main approaches; prevention (with aim to elude further salinisation processes) and



remediation management (i.e. reclamation of existing salinised land/water), but they usually overlap (e.g. Biggs et al., 2010). In selecting the most appropriate managing approaches analysis of several critical variables should be taken into account: i) the nature of soil geochemistry (i.e. salt type and concentrations, acidity, alkalinity), ii) groundwater hydrology (e.g. watertable fluctuation), iii) climate conditions (precipitation, evapotranspiration), and iv) plant species selection.

In natural environment, indigenous flora can counteract negative influences of excessive salinity by maintaining salinised groundwater below a harmful level and/or by its genetic predisposition i.e. pronounced salinity resistance. With introduction of cultivated species, many interrelations in natural terrestrial ecosystems were disturbed. Many primary salinised areas, such as those in alluvial floodplains (e.g. section 3.2), after land amelioration by (sub)surface drainage (open channel and pipeline network) had groundwater salinity decreased to a level suitable for agricultural production (Romic et al., submitted). These authors confirmed that with proper selection of modern irrigation systems (e.g. drippers vs. micro sprinklers) and mulch cropping technology, negative ecological consequences (e.g. yield/vegetative growth declining, mortality of cultured crop, increased  $EC_{se}$ ) arising from usage of salinised water for irrigation ( $EC_w$  up to 7 dS/m) may be markedly reduced (Romic et al., 2008). Thus, establishing adequate drainage and/or ensuring sufficient volume of good quality (low salinity) water for irrigation and salt leaching from the root zone may be an appropriate strategy for at least partially reclaiming saline soils. Burrow et al. (2002) used channel/rain water ( $EC_w=0.1$  dS/m) to leach salts from the topsoil, but not in subsoil where another constraints were detected, i.e. increased clay dispersion and sodicity. Therefore, saline-sodic and/or sodic (sub)soils require an application of appropriate soil amendments for Na replacement to aid in remediation (e.g. Horney et al., 2005).

Application of certain amendments (inorganic or organic fertilisers, lime, gypsum, etc) to pedosphere with excessive concentration of salts/Na has multiple beneficial roles. One is cation exchange of Ca for Na (e.g. with Ca-based amendments) given that the addition of electrolyte such is Ca (also Mg) helps to maintain micro-aggregate integrity in the soil profile (discussed in 4.1). With the application of 12 t/ha of gypsum, Wheaton et al. (2002) markedly decreased the ratio of  $Na^+$  to  $Ca^{2+} + Mg^{2+}$  (i.e. SAR) and reclaimed sodic subsoil (ESP 6-10) to non-sodic. In the same study, gypsum improved physical soil properties i.e. decreased clay dispersion (i.e. increased flocculation), reduced spontaneous dispersion (air-dry aggregates) and dispersion of remoulded aggregates, increased hydraulic conductivity and probably ensured better aeration. Under acidic pHs,  $CaCO_3$  application (liming) provides an electrolyte source for ensuring sufficient Ca-for-Na replacement, whereas in naturally  $CaCO_3$ -sufficient soils,  $H_2SO_4$  application (or its precursor, elemental S) in reaction with carbonates ultimately produces gypsum i.e. exchangeable Ca (e.g. Horney et al., 2005). For achieving positive effects of different soil amendments in salt-affected conditions, several critical factors need to be considered, such as: i) uniform soil incorporation (mixing), ii) adequate soil water content for their dissolution, iii) presence of certain microbial population (e.g. sulphuric bacteria for S oxidation), etc. For example, Wheaton et al. (2002) reclaimed moderately sodic subsoils by applying 12 t/ha gypsum and 2,850 mm of water (as irrigation plus rain). To be dissolved, an average gypsum dosage (~10 t/ha) would need ~1,200-1,500 mm water (Greene & Ford, 1985; Wheaton et al., 2002), an amount hardly achievable by natural precipitation in short term in (semi)arid conditions (in contrast, irrigation may be too costly to implement).

Given that excessive  $\text{Na}^+/\text{Cl}^-$  salinity mostly impairs macro/micronutrient balance (discussed before), the direct practice to recover nutrient uptake and homeostasis is by specific (e.g. Ca, K, P) fertilisation. For instance, it was shown that supplementary application of Ca may result in many benefits (e.g. reduced accumulation of Na and improved K and Ca uptake, dry matter production, yield, etc) for crops grown under saline conditions (Cuartero & Fernandez-Munoz, 1999). Also, the same authors suggested that increasing K (to 10-15 mM) and P (to 10-100  $\mu\text{M}$ ) in the rhizosphere solution could be recommendable for saline conditions, although the upper levels of these nutrient concentrations should be further investigated.

Application of Zn fertiliser may be beneficial in saline/sodic environment. It was confirmed that  $\text{ZnSO}_4$  may improve salt tolerance in cereals and results in several other important benefits such as crop micronutrient enrichment (e.g. by 90% for Zn) and reduced uptake/phytoaccumulation of toxic elements (e.g. by >100% for Cd) (Khoshgoftar et al., 2004). However, Zn fertilisation is not always acceptable because of ecological/economic consequences, whereas cropping of Zn-efficient species/genotypes on such Zn-deficient soils is one possible approach for reducing land degradation and minimizing the use of fertilizers (Khoshgoftar et al., 2006; Rengel & Graham, 1995).

Cropping system adaptation i.e. opportunity cropping, is another possible and widely used strategy in combating salinity and some other accompanied (sub)soil constrains (e.g. low soil fertility and water retention capacity, water deficit, high evaporation, increased pH) or land degradations (e.g. OM depletion, desertification). It assumes implementation of conservation tillage systems to restrict loses of soil moisture, soil erosion, reduce soil compaction, disturbance and energy consumption, i.e. conserve plant-available water and OM (Ondrasek et al., submitted b). Under such land management, at least 30% of the crop residues may remain on the soil surface (e.g. straw residues and stubble between rows, Figure 4). Furthermore, opportunity cropping assumes a wide range of other measures, such as double cropping (consociation of cereals and forages), selection of crop species (e.g. perennial deep rooted, more salt-tolerant, etc.), presence of pasture and tree species (for windbreaks, controlling groundwater level), etc. (see report by Biggs et al., 2010). Lucerne, as relatively salt-tolerant and deep-rooted legume, is shown to be the most effective perennial pasture for controlling groundwater recharge, with the strongest dewatering in the soil profile within the first 18 months of establishment (Powell, 2004).



Fig. 4. Conservation tillage systems in barley (a; Esperance area, W. Australia), and canola and wheat (b, c; Dalwallinu area, W. Australia) (Photos taken by G. Ondrasek, 2010)

Grafting is a widely used technique in horticulture for asexual propagation and may also be helpful in withstand deleterious salinity effects (e.g. Cl toxicity) in crops. It was well documented that certain rootstock-scion combinations can reduce uptake and root-shoot translocation/accumulation of dissolved salts (Na<sup>+</sup> and/or Cl<sup>-</sup>) in mango (Schmutz & Lüdders, 1999), citrus (reviewed by Storey & Walker, 1999) and grapevines (Stevens & Walker, 2002).

Several attempts have been made to improve salt tolerance by hydropriming (Basma et al., 2005a), pre-sowing chilling treatment (Basma et al., 2005b), halopriming (Kamboh et al., 2000) and ascorbate priming (Borsani et al., 2001; Afzal et al., 2006). Priming can increase the activity of free radicals scavenging enzymes, thus can reduce the damages caused by salt stress. Seed priming (osmoconditioning) with salinised (NaCl) solution prior to sowing is one of possible methods for improving salt tolerance in a wide range of relatively salt-sensitive crops (e.g. tomato, muskmelon, cucumber, etc) (e.g. Sivritepe et al., 2003, 2005). Based on literature review, Cuartero & Fernandez-Munoz (1999) recommended priming in 1 M NaCl for 36 hours in the case of direct sowing, and for seedlings conditioning in moderately saline solution or by withholding water for 20-24 hours, although specific duration and concentrations should be adapted to a particular crop.

Notwithstanding that elucidation of salt tolerance is complex with respect to its quantitative multigenic character (e.g. Flowers & Flowers, 2005), transgenic plants have been produced with high tolerance to salinity (up to 200 mM NaCl or 20 dS/m) (reviewed by Chinnusamy et al., 2005). Therefore, exploitation of genetic approaches (discussed next) in the long-term may achieve some of the most promising and effective outcomes in plant salt tolerance.

## 5.2 Genetic mechanisms of increasing of salt tolerance in plants

Enhanced salt tolerance in crop plants may be achieved via traditional and molecular breeding and transgenic approaches. However, genetic and physiological complexity of salinity tolerance does not lend itself easily to traditional breeding that may for example use a pedigree approach, which consists of screening germplasm for donors of salt tolerance, crossing a donor with an elite line and advancing the F1 hybrid to about the F8 generation while selecting for an elite trait. While the efficiency of this type of breeding is not sufficient (Ashraf & Akram, 2009), the use of wild relatives for breeding gives useful physiological information about the salt tolerance traits (Colmer et al., 2006). In addition, cell and tissue culture techniques are used to identify somaclonal variants (Zhu et al., 2000) and screen germplasm for salt tolerance *in vitro* (Arzani, 2008).

Molecular and transgenic breeding is more expensive than conventional, but represents an efficient way to produce salt-tolerant lines. Advances in genomics are underpinning an alternative approach in which a pre-breeding phase is used to pyramid several known genes and finely-mapped major quantitative trait loci (QTLs) for complementary aspects of salt tolerance. DNA-based selection protocols that are used to pyramid these genes are again employed during the breeding phase to transfer the entire set of genes for salt tolerance into any elite line by backcrossing (Benneth & Khush, 2003; Flowers, 2004).

Given the complexity of salt tolerance, only a few QTLs are identified within any given genome (Yeo et al., 2000). The fact that a QTL represents many genes complicates finding key loci within the QTL. Quesada et al. (2002) found five QTLs associated with salinity in *Arabidopsis* and identified the location of two of them. The other genetic approach currently used to enhance salt tolerance includes generation of transgenic plants to introduce new

genes or to alter expression levels of existing genes to affect the degree of tolerance (avoid or reduce deleterious effects, re-establish the homeostatic conditions and maintain active growth in saline environments; Zhu, 2001).

Important deleterious effects under saline conditions might be due to reactive oxygen species (ROS), which cause oxidative stress as one of the most general stress types (Lee et al., 2001). Plants under salt stress produce stress protein and specific osmolytes for scavenging ROS (Xiong & Zhu, 2002; Zhu et al., 1997). Oxidative stress tolerance is genetically controlled; improvements can be provided by conventional breeding and transgenic techniques (Asraf & Akram, 2009) or by adopting physiological approaches (Afzal et al., 2006). Most transgenic improvements in plant salt tolerance are focused on overexpressing enzymes involved in oxidative protection (Allen et al., 1997). In addition, engineering with the regulatory protein NPK1, a nitrogen-activated protein kinase that mediates oxidative stress responses, is also an efficient way for supporting antioxidant defence (Kovtun et al., 2000). Improvements provided via other proteins, like barley late embryogenesis abundant proteins and C-repeat-binding/dehydration-responsive element-binding proteins (CBF/DREBs) in transgenic plants may have ROS detoxifying effects, but are not specific for salt tolerance (Liu et al., 1998; Stockinger et al., 1997).

There are many proteins that appear related to salt stress response in plants. These proteins can directly regulate the levels of osmolytes and control ion homeostasis. Osmolytes, such as mannitol, fructans, proline and glycinebetaine, are also active in scavenging ROS. Genetic engineering of these osmolytes resulted in increasing salt tolerance (Harinasuth et al., 1996; Sahi et al., 2006; Zhu, 2001).

For improved tolerance to salinity, it is important to re-establish homeostasis by controlling  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$  transporters mediating influx and efflux to fine-tune ion concentrations in the cytoplasm (Zhu, 2001). Nonselective cation channels mediate  $\text{Na}^+$  entry into the cell, and their molecular identity is becoming clear (Munns & Tester, 2008). Anion and cation transporters are a frequent target of genetic engineering to improve crop salt tolerance (Yamaguchi & Blumwald, 2005). Transcriptome analysis can pinpoint genes associated with regulation of RNA and protein metabolism that have a significant role in regulating salt-stress tolerance (Sahi et al., 2006). Mian et al. (2011) identified a large number of important genes using forward and reverse genetics, yeast complementation and transcriptomics. Microarray analysis has clearly shown that transcripts encoding RNA-binding proteins, helicases, cyclophilins, F-box proteins, dynamin-like proteins, and ribosomal proteins are linked to the salt-stress response in *Arabidopsis* (Sottosanto et al., 2004).

Earth is a salty planet, the problem of salinity is increasing together with the demand for food; hence, salt-tolerant crops are required. Great efforts have been made to improve salt tolerance of crops by means of conventional and more recently genetic breeding program. However, the genetic complexity of salt tolerance makes the task extremely difficult.

## 6. Conclusion

Soil salinisation is a widespread soil degradation process, exacerbated by a mismatch between water demands for irrigation in food production and the amount of quality (non-saline) water. Different land, crop and/or water management approaches (e.g. conservation tillage, crop selection/rotation, groundwater level control) have been used to combat salinisation processes in agro-ecosystems and mitigate salt-induced stresses in food/feed production. Although salt resistance in plants is multigenic and thus complex, breeding and

transgenic approaches to improve salinity resistance could contribute to enhancing crop production over millions of ha of salt-affected areas worldwide.

## 7. Acknowledgment

This work was supported by the Croatian Science Foundation (Con. O-3510-2010).

## 8. References

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## **Part 2**

### **Mechanisms and Tolerance**



# Current Knowledge in Physiological and Genetic Mechanisms Underpinning Tolerances to Alkaline and Saline Subsoil Constraints of Broad Acre Cropping in Dryland Regions

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## 1. Introduction

Abiotic stresses are a serious problem to crop production under dryland conditions in arid and semi-arid regions of the world. These abiotic stresses include high and low temperature, water deficit, salinity, sodicity, alkalinity, acidity and ion deficiencies and toxicities. Many Australian agricultural soils accumulate salts under seasonal fluctuations and have multiple subsoil constraints such as alkalinity, acidity, sodicity, and toxic ions (Rengasamy, 2006). Of these, salinity and alkalinity are simultaneously found in soils of southern Australia (Nuttall *et al.*, 2003a; Nuttall *et al.*, 2003b). The simultaneous occurrence of multiple abiotic stresses may result in far greater productivity loss than any single abiotic or biotic factor.

Nearly 800 million ha of land throughout the world is salt affected either by salinity or associated with sodicity (FAO, 2009). The total area under salinity in Australia is estimated to be 32 million ha in arable and permanent cropping land (FAO, 2000). Transient or dryland salinity is probably the biggest factor causing salinity in Australia (Figure 1; Rengasamy 2002). Saline soils are generally defined as those having high concentrations of soluble salt with an electrical conductivity ( $EC_e$ ) of more than  $4 \text{ dSm}^{-1}$ . Among the soluble salts, NaCl is the major component contributing to salinity (USSL, 2005).

Yields of important cereal, oilseed and forage crops are limited by soil salinity in broad acre dryland regions. Therefore, genetic crop improvement by conventional and non-conventional methods for salt tolerance is vital to maintain food production. The ability to grow and reproduce in saline soil differs widely between species, due to differences in the ability to control salt uptake from the soil and to compartmentalise it effectively at the cellular level (Munns & Tester, 2008).

Crops grown under dryland conditions on alkaline soils in south-eastern Australia are potentially limited by many factors, especially water supply and nutrition (Incerti & O'Leary, 1990). Alkaline soils are usually categorized by low availability of plant nutrients, high concentrations of  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$ , and high pH (Marschner, 1995; Misra & Tyler, 1999). By definition, alkalinity is the concentration of soluble alkalis with the ability to neutralize acids (Bailey, 1996). Bicarbonate ( $\text{HCO}_3^-$ ) and carbonate ( $\text{CO}_3^{2-}$ ) are the principal

contributors to alkalinity, whereas hydroxide, borate, ammonia, organic bases, phosphates, and silicates are considered minor contributors (Petersen, 1996). This review will discuss how salinity and alkalinity affect plant growth and the different methods used to identify and improve tolerance in various crop species.

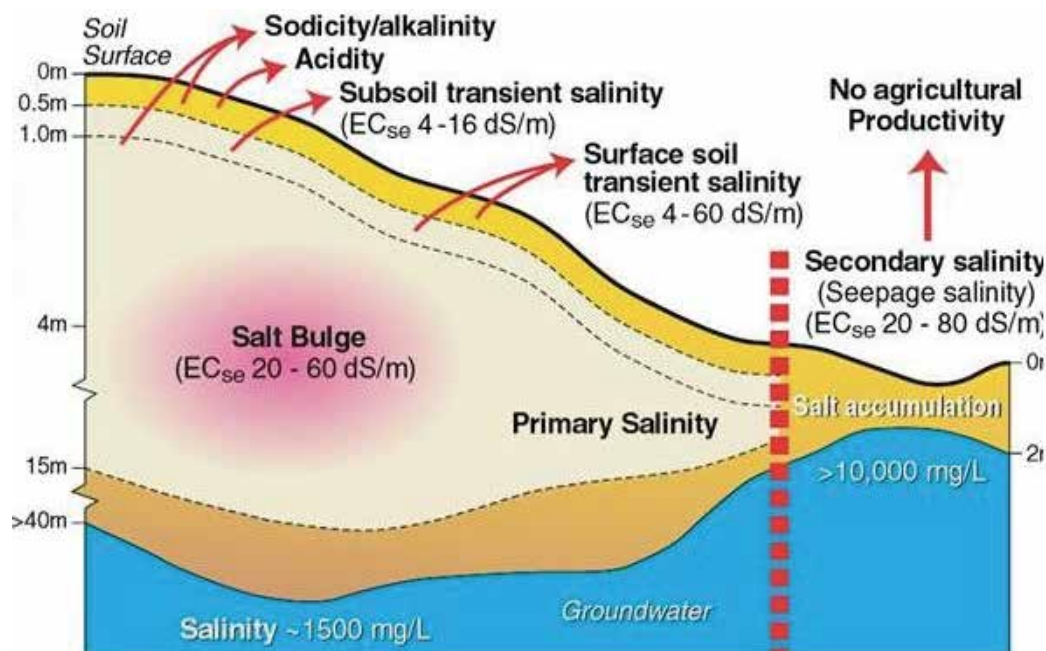


Fig. 1. Different types of salinity in Australian landscapes (after Rengasamy, 2002).

## 2. Salinity effects in plants

The most common effects of salinity on plant growth are smaller and fewer leaves, reduced plant height and poor yield (Kumar, 1995). At the physiological level, salinity imposes an osmotic stress that limits water uptake and ion toxicity can cause nutrition (N, Ca, K, P, Fe, Zn) deficiency and oxidative stress (Munns, 2002). Salinity can differentially affect the mineral nutrition of plants. Nutrient imbalances due to salinity diminish plant growth by affecting the availability, transport, and partitioning of nutrients. Nutrient deficiencies or imbalances result due to competition of Na and Cl with other nutrients such as K, Ca, Mg and NO<sub>3</sub> (Hasegawa & Bressan, 2000; Hu & Schmidhalter, 1998; Hu & Schmidhalter, 2005; Munns, 2002; Netondo *et al.*, 2004). These nutrient imbalances due to salinity also cause reduction in plant growth. Plant responses to salinity can vary with the degree and duration of the stress imposed as well as the plant developmental stage (seedling, flowering, maturity) when the stress is applied (Munns, 1993). To identify truly tolerant germplasm, it is important to gain full information regarding the degree of salt tolerance at all growth stages of a crop species. Otherwise selection at one particular growth stage may result in plants that lose their tolerance at other stages.

### 2.1 Mechanisms of salt tolerance

Plants are generally categorized as either halophytes or glycophytes. Halophytes grow and survive best where salt concentration is 200 mM or more (Flowers & Colmer, 2008). Conversely, glycophytes cannot survive under high saline conditions (Figure 2). A major difference between halophytes and glycophytes is the ability of halophytes to survive salt shock (Braun *et al.*, 1986; Casas *et al.*, 1991; Hassidim *et al.*, 1990).

Several mechanisms for tolerance operate in both halophytes and glycophytes and the differences are presented in Figure 3. However, the main adaptive strategies of salt-tolerant glycophytic plants exposed to salinity are: 1) avoidance through ion exclusion, potentially as a result of low membrane ion permeability; 2) tolerance, through ion inclusion and possible compartmentalisation; and 3) osmotic stress tolerance, which enables the plant to remain functional despite internal ionic stress (Blumwald *et al.*, 2004; Munns, 2005; Munns & Tester, 2008).

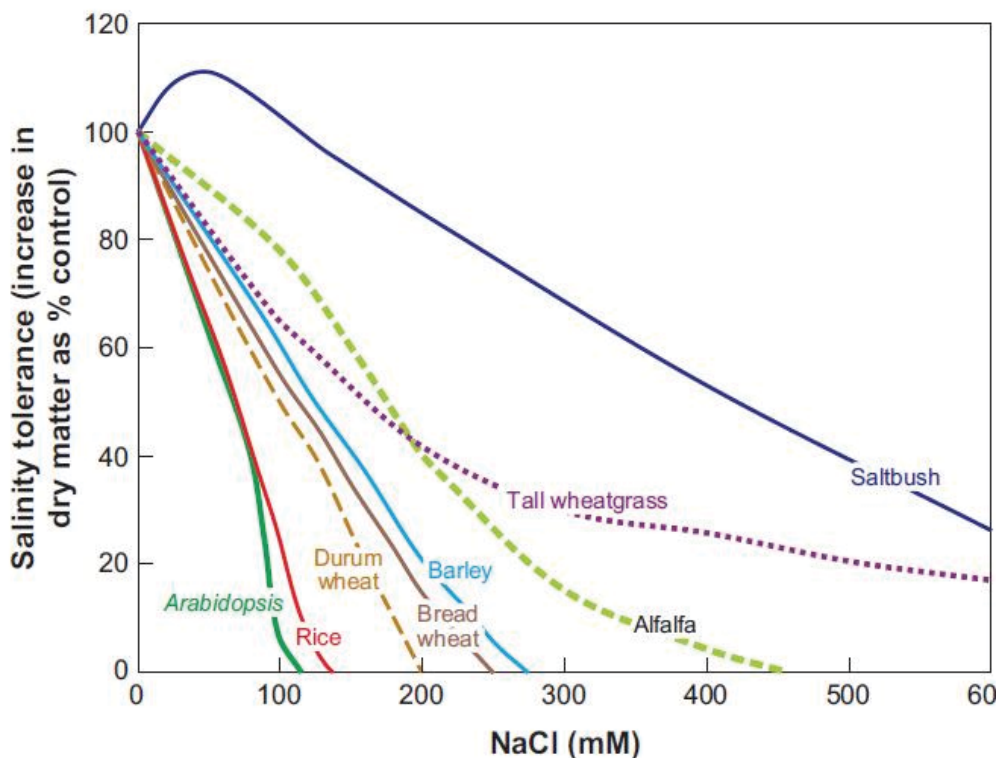


Fig. 2. Response of glycophytes and halophytes to varying concentrations of NaCl after 3 weeks of treatment (after Munns & Tester, 2008).

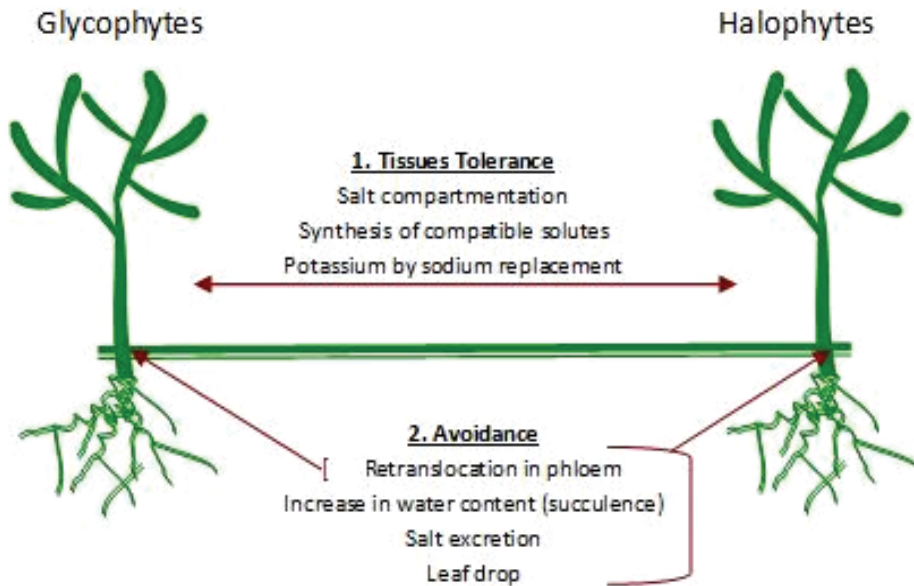


Fig. 3. Adaptive strategies for salt tolerance in plants.

## 2.2 Ion exclusion

Na avoidance is an essential salt tolerance mechanism that operates in several glycophytes such as; wheat (Munns, 2005; Munns & Tester, 2008), *Arabidopsis* (Møller *et al.*, 2009; Møller & Tester, 2007), *B. napus* and *B. juncea* (Ashraf & McNeilly, 2004; Ashraf *et al.* 2001). To achieve this type of tolerance, plant roots must exclude most of the Na and Cl dissolved in the soil solution, and escape from gradual build-up of salt in shoots to toxic levels (Munns, 2005). Salinity tolerance in cereals is largely contributed by Na exclusion. As plants transpire about 50 times more water than they retain in their leaves, exclusion of 98% (1/50) of the salt in the soil solution results in stable leaf Na concentration (Munns, 2005). For instance, bread wheat excluded > 98% of the Na in the soil solution, and consequently Na concentration build-up in leaves remained less than 50 mM (Husain *et al.*, 2004). Barley, on the other hand, excluded < 98% of the Na in the soil solution, and the concentrations reached up to 500 mM (Rawson *et al.*, 1988).

Salinity tolerance in *B. juncea* however, is achieved through partial exclusion (Ashraf & McNeilly, 2004; Ashraf *et al.*, 2001). In some members of the Brassicaceae including *Arabidopsis*, salinity tolerance appears to be mainly achieved by tissue tolerance to accumulated Na rather than by exclusion of Na from the shoot (Møller & Tester, 2007). In two *B. napus* genotypes, the genotype with the higher Na accumulation in the shoot was more Na tolerant and also accumulated increased amounts of K and proline (Huang & Redman, 1995). These results imply that tissue tolerance to Na and tolerance to osmotic stress are more important than Na exclusion from the shoot in members of the Brassicaceae.

## 2.3 Ion compartmentation

Salt tolerance by compartmentation is very important mechanism that operates in many glycophytes such as *Arabidopsis* (Møller *et al.*, 2009; Møller & Tester, 2007), wheat and barley (Munns, 2005; Munns *et al.*, 1995; Munns & Tester, 2008), and *B. juncea* (Ashraf & McNeilly,



2004; Kumar *et al.*, 2009). Ion specific effects of salinity primarily result in accumulation of higher levels of sodium (Na), usually in the older leaves. Entry of Na into the cell is due to the similarity in hydrated ionic radii between Na and K that makes it difficult for the transporters to distinguish between these two ions (Blumwald *et al.*, 2000). To avoid deleterious Na toxicity in the cytoplasm, it must be compartmentalised into cell vacuoles (Munns & Tester, 2008). This allows maintenance of optimum cellular levels of K and calcium (Ca) as well as Na exclusion by the plant. These two activities are known to operate at the plasma membrane and tonoplast levels, as integral components of the ion transport network. This is one of the key physiological criteria of plant salt tolerance, to maintain optimal K/Na ratio in the cytosol (Singla-Pareek *et al.*, 2003; Singla-Pareek *et al.*, 2008; Tester & Davenport, 2003). A higher K/Na ratio essentially indicates that a plant has not only excluded Na to some extent but has also maintained a healthy level of K for normal metabolic activities and injury avoidance under salinity. Hence, manipulation of the salt overly sensitive (SOS) pathway, Na/H antiporters and/or K transmembrane transporters that are involved in ion homeostasis may be the target of future strategies for salt tolerance improvement in a range of crops including canola quality *B. juncea* (Benke *et al.*, 2010; Blumwald *et al.*, 2004).

The role of transport proteins such as antiporters, ion channels, ABC-type transporters, Na and K transporters, plasma membrane and vacuolar ATPases is fundamental for salt tolerance in Na<sup>+</sup> exclusion, ion homeostasis, and compartmentalization of solutes and amino acids under stress (Apse *et al.*, 2003; Takahashi *et al.*, 2009). The over-expression of vacuolar Na/H antiporter in *B. napus* greatly diminished the salt-induced oxidative stress in the vacuoles, highlighting the importance of Na homeostasis during salt stress tolerance (Ruiz & Blumwald, 2002; Zhang *et al.*, 2001b). The *Arabidopsis thaliana* vacuolar alkali cation transporter AtNHX1 has been shown to increase salt tolerance in transgenic plants through the intracellular compartmentation of Na (Apse *et al.*, 1999; Hernández *et al.*, 2009; Venema *et al.*, 2002).

## 2.4 Osmotic adjustment

Salinity is a common feature of arid and semiarid lands, and plants have evolved mechanisms to tolerate the low soil water potential caused by salinity, as well as by drought, and so some level of tolerance to osmotic stress is a feature of most glycophytes and halophytes (Munns & Tester, 2008). Osmotic adjustment in plants exposed to salt stress helps to maintain turgor pressure, which consequently helps plant to achieve tolerance under saline conditions (Ashraf & McNeilly, 2004). A significant genetic variation within species may exist in the osmotic response under saline stress; however this has not yet been documented (Munns & Tester, 2008). In salt sensitive plants, low water potential caused by salinity stress leads to cell membrane damage causing ion toxicity and cell injury (Chen & Murata, 2002; Sreenivasulu *et al.*, 2000). This primarily results in smaller leaves and reduction in leaf area in many crops. Reduction in leaf area development and relative root growth might decrease the water use by the plant, which allows it to preserve soil moisture and avoid an escalation in the salt concentration in the soil (Munns & Tester, 2008). Osmotic adjustment occurs in plants subjected to saline stress, but particularly to a large extent in salt-tolerant Brassica species (Ashraf & McNeilly, 2004).

This is primarily due to accumulation of different types of organic osmotica such as soluble sugars, free amino acids and free proline in most of the salt-tolerant Brassica species (Ashraf & Akram, 2009; Ashraf & McNeilly, 2004). The relative importance of variation in osmotic tolerance remains unknown for most crop species, due to inherent difficulties in quantifying this parameter. However, a close association is likely exists between osmotic tolerance and

tissue tolerance of  $\text{Na}^+$ , because genotypes that tolerate high internal  $\text{Na}^+$  concentrations in leaves by compartmentalizing it in the vacuole may also be more tolerant of the osmotic stress owing to their elevated osmotic adjustment (Munns & Tester, 2008). However, this theory needs further investigation.

## 2.5 Molecular control of salt tolerance

The adaptive physiological and biochemical responses of a plant to salinity are controlled by genes that encode salt tolerance mechanisms (Casas *et al.*, 1992). Since salinity tolerance is a complex trait, it is most likely controlled by interactions of hundreds of salt responsive genes (Sahi *et al.*, 2006; Winicov, 1998). Plants recognise a salinity stress and condition adaptive response mechanisms (Hasegawa & Bressan, 2000). Reported responses involve many molecular processes such as ion homeostasis (membrane proteins involved in ionic transport), osmotic adjustment and water regime regulation (osmolytes), as well as scavenging of toxic compounds (enzymes; Benke *et al.*, 2010; Blumwald *et al.*, 2004). The regulatory molecules conditioning these responses have been found to be cellular signal pathway components and transducers of long distance response co-ordination such as hormones, mediators, transcription factors and regulatory genes (Mishra *et al.*, 2006). The expression of such genetic regulators during plant stress has been studied at the transcriptional level (Fernandez *et al.*, 2008; Hasegawa & Bressan, 2000). Consequently, abiotic stress-inducible genes have been classified into two categories; 1) those that directly protect against environmental stress; and 2) those that regulate gene expression and signal transduction against stress response (Hasegawa & Bressan, 2000; Kawaura *et al.*, 2008; Mishra *et al.*, 2006; Popova *et al.*, 2008; Ueda *et al.*, 2002). Some of the major genes/proteins that are activated under salinity might be involved in tolerance (Table 1). Hence, it is imperative to analyse the functions of stress-inducible genes for amplification of the molecular mechanisms of stress tolerance in plants.

Salt tolerance is attained through three interrelated characteristics; the foremost, salt injury must be avoided or alleviated. Second, homeostatic conditions must be re-established in the new stressful environment. Third, growth must resume, even if at a reduced rate (Fig. 4; Zhu, 2001).

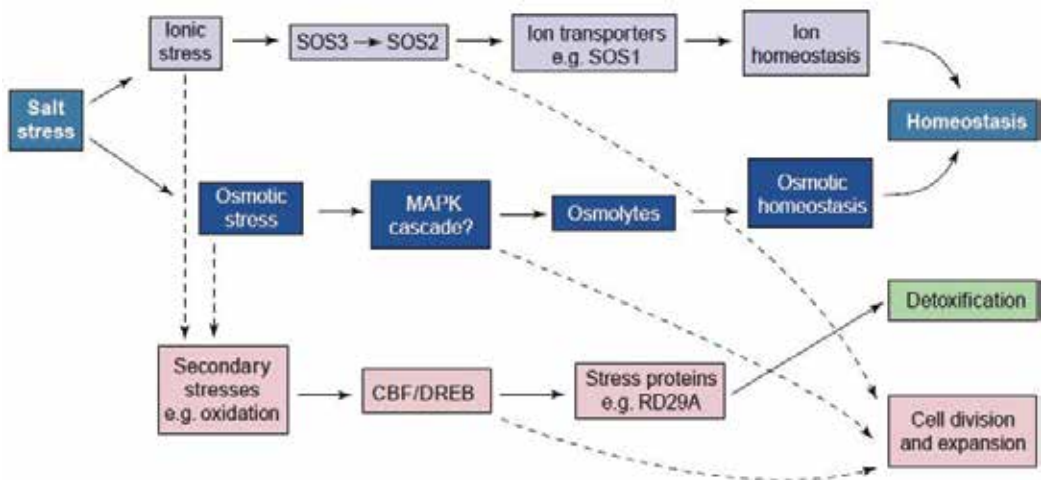


Fig. 4. Three avenues of salt tolerance in plants (after Zhu, 2001).

Functionality class	Possible role in stress	References
Signalling molecules	Stress signal transduction and gene expression	Cardinale et al. 2002; Pardo et al. 1998; Saijo et al. 2000; Ulm et al. 2002
Transcriptional and post-transcriptional machinery	Transcriptional regulation of stress gene expression, transcript stability, turnover, processing	Cooper et al. 2003; Lee et al. 2001; Park et al. 2001; Sanan-Mishra et al. 2005
Translational machinery	Stress-regulated protein translation, selective translation, transport, localization	Wood et al. 2000; Wood and Oliver 1999
Protein folding	Maintenance of protein structures, protein folding, preventing protein denaturation, Protein sorting, targeting	Sun et al. 2001
Protein turnover	Regulation of protein metabolism, targeted protein degradation in response to stress	Khedr et al. 2003; Moon et al. 2004
Osmoprotectants	Osmotic adjustment, protection of cellular structures and macromolecules	Nomura et al. 1998; Tarczynski et al. 1993
Transport protein	Ion homeostasis during stress, compartmentalization of solutes and amino acids	Apse et al. 1999; Gisbert et al. 2000; Shi et al. 2000; Zhang and Blumwald 2001; Zhang et al. 2001a
ROS scavengers, cell death, senescence and ageing	Detoxification of free oxygen radicals, cell death, hypersensitive response	Reddy and Sopory 1999; Roxas et al. 1997
Metal-binding proteins	Affecting cellular metabolism, metal ion homeostasis, acting as cofactors for critical reactions, signaling, metal toxicity, secondary stress responses, oxidative stress	Kawasaki et al. 2001; Sahi et al. 2003
Photosynthesis	Regulation of photosynthesis	Kawasaki et al. 2001; Sahi et al. 2003
Defense-related proteins	Protection against biotic stress including viral, bacterial and fungal infestation	Cheong et al. 2002; Dombrowski 2003; Reymond et al. 2000
Hormone-related proteins	Hormonal homeostasis and gene expression	Kalifa et al. 2004
General metabolism	Overall cellular function, housekeeping metabolic pathways carbohydrate, fatty acid and protein synthesis and modifications membrane fluidity, nitrogen metabolism, carbon and nitrogen fixation	Hoshida et al. 2000; Jeong et al. 2002

Table 1. Major categories of genes/proteins related to salt-stress responses/tolerances in plants (After Sahi *et al.* 2006).

The mitogen-activated protein kinases (MAP kinases), a specific class of serine/threonine protein kinases, play a central role in the transduction of various extra- and intracellular signals for cell division and stress responses in plants. Most of the abiotic stresses; salinity, cold, drought and oxidative stress can induce defence responses in plants through MAP kinase pathways such as osmoregulation, cell growth and differentiation (Mishra *et al.*, 2006; Pitzschke *et al.*, 2009). AtMKK3, AtMPK1, and AtMPK2 genes induced by ABA signalling amplified through MAP kinase-1 or MAP kinase-2 might increase salt tolerance in Arabidopsis (Hwa & Yang, 2008). Similarly, expression of active MKK9 protein enhanced salt tolerance and its loss increased sensitivity of transgenic Arabidopsis seedlings, emphasizing the significant role of MKK9 in salt stress response (Xu *et al.*, 2008b). Reactive oxygen species (ROS) scavengers such as peroxidases and glutathione are known to play a significant role in plant salt tolerance by reducing oxidative damage. For instance, in wild *B. napus*, glutathione synthesis was induced during salt stress, suggesting a possible protective mechanism against salt-induced oxidative damage (Ruiz & Blumwald, 2002).

In another study, genetic manipulation of carotenoid biosynthesis through over-expression of phytoene synthase gene *SePSY* in transgenic Arabidopsis increased the photosynthesis rate from 92% to 132% under 100 mM NaCl stress. The transgenic plants also displayed higher activities of superoxide dismutase (SOD) and peroxidase (POD) and lower concentrations of H<sub>2</sub>O<sub>2</sub> and malondialdehyde (MDA) than the wild plants (Han *et al.* 2008). Therefore, it is important to understand the genetics of detoxification of free oxygen radicals in order to enhance crop salt tolerance.

## **2.6 Methods for improving crop salinity tolerance**

Several methods such as germplasm selection, marker assisted selection, transcriptional profiling, metabolomics, proteomics and transgenics have been successfully used for crop salinity improvement. This chapter will only discuss gene expression analysis for salinity tolerance with a main focus on microarrays.

### **2.6.1 Gene expression profiling for crop improvement**

Messenger RNAs that are differentially transcribed between tolerant and sensitive genotypes under a particular stress may be targets for selection for future crop improvement. However, the expression of genes involved in stress responses is highly affected by the environment in which they are located, and thus care must be taken to accurately represent the stress environment conditions when seeking differentially transcribed gene responses. The responses may also differ among plant growth stages and among genotypes (Ashraf & McNeilly, 2004; Munns, 2005; Munns & Tester, 2008).

In order to determine key genes that are differentially transcribed for metabolic regulation under stress environments and among genotypes, several techniques have been developed. These rapidly provide gene-specific or genome wide expression patterns with high accuracy through biological and technical replication (Kuhn, 2001). Moreover, the information generated can be integrated within functional genomic processes to aid in understanding relationships between gene expressions and observed phenotypes.

### **2.6.2 Types of gene expression techniques**

The last decade has produced several dynamic transcriptional technologies for measuring and interpreting single and multiple gene expressions. These have facilitated the analysis of mRNA

from selected cells/tissues to generate multi-dimensional measurements of differentially expressed genes. Techniques that assess gene expression are grouped into two categories; open and closed systems, based on their architecture. The open system techniques such as AFLP (Amplified Fragment Length Polymorphism), SAGE (Serial Analysis of Gene Expression), MPSS (Massively Parallel Signature Sequencing), and Real-time RT-PCR can permit the discovery of novel genes; however they might not cover the whole genome (Cheng *et al.*, 2008; Drea *et al.*, 2009; Nakano *et al.*, 2006; Sreenivasulu *et al.*, 2010). On the other hand, closed system techniques such as microarrays rely on already annotated information; therefore they can be used to study several thousand genes from a single experiment (Lee *et al.*, 2005; Seki *et al.*, 2002). Due to the flexibility of microarrays to permit the study of multiple stress situations such as salinity, drought and cold in a single experiment, this technique has become a method of choice for many for assessing differential genes expression studies in molecular biology (Dai *et al.*, 2007; Nakashima *et al.*, 2009; Seki *et al.*, 2002).

### 2.6.3 Microarray analysis for salt tolerance

Microarrays utilize the preferential binding of complementary single-stranded nucleic acid sequences. Instead of working on individual genes, the aim of a microarray experiments is to examine the profiles of expression of thousands of genes in a single experiment. Microarrays have been extensively used to study global gene expression profiling of plant responses to abiotic and biotic stresses. Studies on gene expression for abiotic stress include; salinity, drought and cold tolerance in *Arabidopsis* (Do-Young *et al.*, 2010; Lee *et al.*, 2005; Seki *et al.*, 2002; Seki *et al.*, 2010; Zhenxian *et al.*, 2010), rice (Huang *et al.*, 2008; Walia *et al.*, 2009), wheat (Huang *et al.*, 2008; Kawaura *et al.*, 2008), *B. napus* (Dalal *et al.*, 2009).

Several types of microarray platforms are available for gene expression studies: those that are spotted with known sequences comprised of cDNA or oligonucleotides, and those manufactured by Agilent and Affymetrix using GeneChip® technologies, which involve synthesis of oligonucleotides directly onto the microarray support. The cDNA microarray is a fabrication of spotted PCR products resulting from direct amplification of genomic DNA by using ESTs or gene specific primers (Alba *et al.*, 2004; Scott *et al.*, 2009). A number of cDNA microarrays have been developed for a variety of plant species such as *Arabidopsis*, rice, maize, petunia and lima bean (Vij & Tyagi, 2007). These have been used to study gene regulation at different developmental stages and in response to both abiotic and biotic stresses. Seki *et al.*, (2002) developed a full length cDNA microarray in *Arabidopsis* to identify genes transcribed in response to cold, drought and salinity, to examine differences in cross-talk between signalling cascades. Currently there are no specific EST-enriched or cDNA gene libraries from *B. juncea* in response to the abiotic stresses of high salinity, alkalinity and/or boron. However, limited information is reported for genes involved in stress tolerance in the Brassicaceae. For example, the cDNAs of the BjDHN2 and BjDHN3 genes from *B. juncea*, a novel subclass of dehydrin genes conferred salt and freezing tolerance in transgenic yeast (Xu *et al.*, 2008a). Similarly, Wang *et al.* (2003) cloned a new Na<sup>+</sup>/H<sup>+</sup> vacuolar antiporter gene from *B. napus* using a full-length cDNA. The designated vacuolar antiporter gene BnNHX1 was found to be salt-inducible and its transcript level was abundant after 24 hours treatment with 200 mM sodium chloride shock treatment.

## 3. Alkalinity

Alkaline soils are usually categorized by low availability of plant nutrients, high concentrations of HCO<sub>3</sub><sup>-</sup>, CO<sub>3</sub><sup>2-</sup> and high pH (Marschner, 1995; Misra & Tyler, 1999). By

definition alkalinity is the concentration of soluble alkalis with the ability to neutralize acids (Bailey 1996). Bicarbonate ( $\text{HCO}_3^-$ ) and carbonate ( $\text{CO}_3^{2-}$ ) are the principal contributors to alkalinity, whereas hydroxide, borate, ammonia, organic bases, phosphates, and silicates are considered minor contributors (Petersen, 1996). Although alkaline soils have high pH (more  $\text{OH}^-$  than  $\text{H}^+$ ), the  $\text{OH}^-$  ions contribute to alkalinity only at  $> \text{pH } 11$ . Below this pH, alkalinity is mainly caused by  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  ions. Hence, the predominant form of carbonates is determined by the soil pH (Whipker *et al.*, 1996). The carbonate system consists mostly of  $\text{HCO}_3^-$  at pH 8.34. As pH increases due to the availability of atmospheric  $\text{CO}_2$  in the system, the proportion of  $\text{CO}_3^{2-}$  increases and  $\text{HCO}_3^-$  declines (Figure 5; Lindsay, 1979). Hence crop growth is mainly inhibited by  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  ions rather than  $\text{OH}^-$  ions in alkaline soils. This has been demonstrated by growing maize plants in solution at pH 8.0 with the buffer HEPES, without  $\text{HCO}_3^-$ . The high pH due to HEPES buffer did not cause any reduction of root and shoot elongation (Lee & Woolhouse, 1969; Romera *et al.*, 1992).

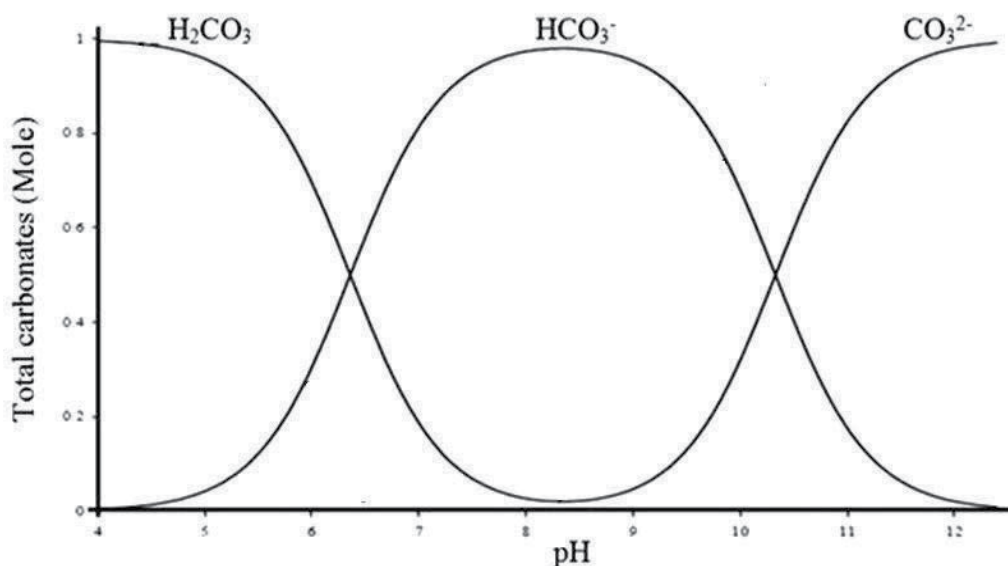


Fig. 5. Three carbonate species at different pH (after Lindsay, 1979)

### 3.1 Nutrient availability in alkaline soils

Several essential micro nutrients such as Fe, Zn and Mn become less available to plants under alkaline stress (Guardia & Alcántara, 2002; Valdez-Aguilar & Reed, 2008; 2010). Nitrogen and Phosphorus deficiencies are also caused by alkalinity. Bicarbonate can significantly decrease Fe uptake, accumulation and/or raise internal Fe precipitation (Fernández-Falcón *et al.*, 1986; Bertoni *et al.*, 1992; Alhendawi *et al.*, 1997; Norvell & Adams, 2006). The alkalization of root tissues due to  $\text{HCO}_3^-$  can either inhibit Fe acquisition or cause Fe to precipitate in the root apoplast (Fernández Falcón *et al.*, 1986; Bertoni *et al.*, 1992 and Römheld, 2000). Alhendawi *et al.* (1997) found that Fe concentrations in roots of barley, maize and sorghum were significantly reduced when grown in solutions containing 5 to 20 mM  $\text{HCO}_3^-$ .

Iron (Fe) chlorosis is a major problem for crops grown in calcareous soils. Soluble bicarbonate has been documented as a contributor to iron (Fe) deficiency and lime-induced chlorosis of crops growing on calcareous soils (Wadleigh & Brown, 1952; Brown, 1978;

Coulombe *et al.*, 1984; Marschner, 1995). In bicarbonate buffer, the pH is in the alkaline range and reduces the uptake or utilization of Fe, leading to deficiency that results in leaf chlorosis (Chaney *et al.*, 1992; Parker & Norvell, 1999; Brand *et al.*, 2002; Lucena, 2000 Norvell & Adams, 2006; Valdez-Aguilar & Reed, 2008; 2010).

Besides Fe, other nutrients that become deficient at high pH include calcium (Ca), copper (Cu), phosphorus (P), and zinc (Zn) (Figure 6; Al-Karaki & Al-Omouh, 2002; Chaves *et al.*, 2006; Naidu & Rengasamy, 1993; Valdez-Aguilar & Reed, 2010). Plant response to alkalinity for nutrient uptake varies from crop to crop. For example, Solaiman *et al.* (2007) found that canola genotypes maintained higher uptake of P and accumulated greater biomass on alkaline soils compared to wheat genotypes. The better growth and P content of the canola genotypes compared to the wheat genotypes was due to the greater root length, leading to exploitation of greater soil volume. However on alkaline soils, P may be rapidly fixed into non plant-labile pools, by precipitation of Ca-P compounds not accessible to plant roots (Bertrand *et al.*, 2006). In a recent study, Valdez-Aguilar & Reed (2008) found that N, K, Ca, Mg and Fe concentrations were higher in roots than shoots of  $\text{HCO}_3^-$  treated tomato plants.

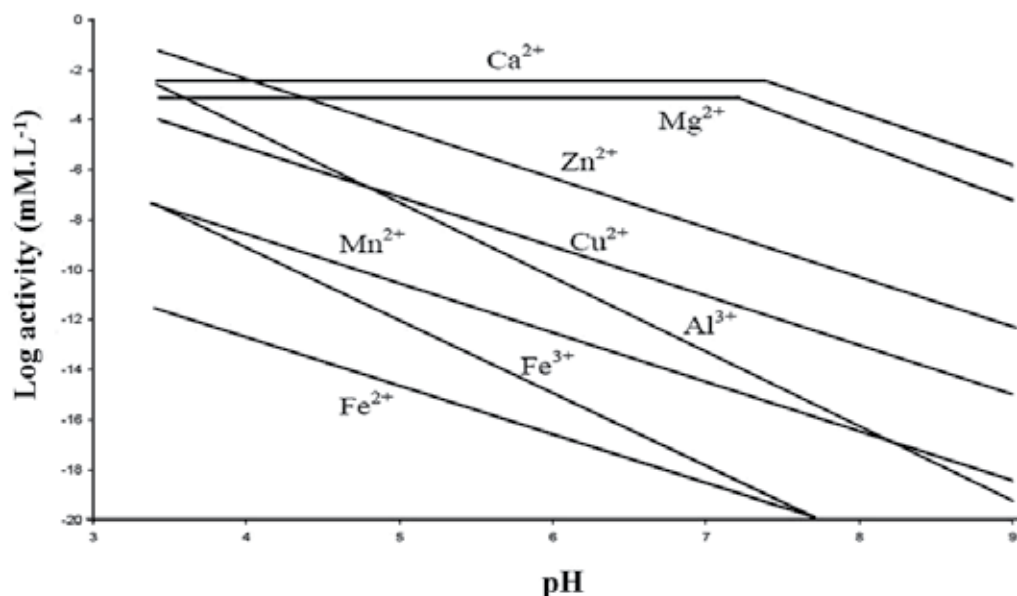


Fig. 6. Effect of increasing pH on availability of various nutrients (after Lindsay 1979).

### 3.2 Effect of alkalinity on plant growth

Plant growth is significantly reduced by alkaline stress mainly contributed by decreased shoot growth with smaller leaves and reduced leaf area as well as reduced root growth and elongation (Pearce *et al.*, 1999). Plants show minor to severe stunting of growth depending on  $\text{HCO}_3^-$  concentration in the soil solution. Growth of many commercial crops such as bean (Valdez-Aguilar & Reed, 2008; 2010), cucumber (Rouphael *et al.*, 2010), wheat (Yang *et al.*, 2008c), sorghum, maize barley (Alhendawi *et al.*, 1997; Yang *et al.*, 2009), soybean (Rogovska *et al.*, 2009), sunflower (Alcántara *et al.*, 1988; Shi & Sheng, 2005), tomato (Bailey & Hammer, 1986; Bialczyk & Lechowsk, 1995; Bialczyk *et al.*, 2004; Navarro *et al.*, 2000), pea (Zribi & Gharsalli, 2002), and rice (Hajiboland *et al.*, 2005; Yang *et al.*, 1994) are reported to be

considerably affected by  $\text{HCO}_3^-$ . Toxic concentrations of bicarbonate can diminish leaf area, leaf length and leaf width, consequently shoot biomass is decreased. This may be due to decreased photosynthetic rate and stomatal conductance in bicarbonate-induced leaf chlorosis (Bie *et al.*, 2004). The reduction in photosynthetic rate is due to impaired chlorophyll synthesis as a result of low translocation of Fe (Bavaresco *et al.*, 1999).

Increasing concentrations of bicarbonate inhibit root growth, which varies with crop species and bicarbonate concentration. Higher  $\text{HCO}_3^-$  concentrations can inhibit root respiration and may result in reduced root growth (Bingham & Stevenson, 1993; Alhendawi *et al.*, 1997). This inhibitory effect may also be related to high accumulation and compartmentation of organic acids such as malate and citrate in root cells (Lee & Woolhouse, 1969; Yang *et al.*, 1994). A bicarbonate-induced build-up of surplus organic acids, particularly malate, in the elongation zone appeared to be related to inhibition of root elongation by bicarbonate in calcifuge plant species (Lee & Woolhouse, 1969). Abscisic acid (ABA), an important stress-induced hormone, is produced in the roots and leaves, transferred from the roots to the leaves in the xylem and from the leaves back to the roots in the phloem (Wolf *et al.*, 1990). Excessive ABA inhibits shoot growth, but increases root growth especially under stress conditions, thus sustaining root growth in drying soils (Saab *et al.*, 1990). In alkaline soils, ABA may leak from the roots to the soil despite being released into the xylem, thereby causing root inhibition as water becomes less available (Daeter *et al.*, 1993; Slovik *et al.*, 1995). Crop species such as corn that can retain root ABA in the face of its tendency to leach into the more alkaline compartment are better able to tolerate these harmful stresses (Degenhardt, 2000).

Crops such as maize, sorghum, and barley have also shown depressed root growth at elevated levels of bicarbonate stress (Alhendawi *et al.*, 1997). Whereas some other crops such as sugar beet, sunflower, pea, and rice are considered better able to tolerate bicarbonate stress (Alcántara *et al.*, 1988; Campbell & Nishio, 2000; Yang *et al.*, 1994; Zribi & Gharsalli, 2002). For instance, root thickness, and lateral root production of sugar beet were increased after three days of Fe deficiency and  $\text{HCO}_3^-$  treatments (Campbell & Nishio, 2000).

### 3.3 Molecular responses to alkalinity stress

Most of the studies to date have focussed on the physiological impacts of alkalinity stress. Recently, Yang *et al.*, (2008) constitutively expressed the high affinity bicarbonate transporter gene “IctB” from *Cyanobacterium* in rice. Under low  $\text{CO}_2$  or alkaline water conditions, cyanobacteria use bicarbonate transporters to pump in bicarbonate as a major carbon source to survive under unfavourable growth conditions. All transgenic rice lines expressing the transporter exhibited enhanced photosynthetic capacity, growth and grain yield (Yang *et al.*, 2008).

As previously mentioned, Fe deficiency is one of the predicted outcomes of alkalinity, therefore an understanding of how plants acquire this ion under stress is needed. Plants have developed two discrete iron uptake strategies by the roots (Marschner *et al.*, 1987). For most plants, including dicots and non-graminaceous monocots, ferrous ion Fe (II) transport from soil into root cells takes place via a transporter after reduction from ferric ion Fe (III) on the plasma membrane (Eide *et al.*, 1996; Robinson *et al.*, 1999; strategy I). However some graminaceous plants synthesise and release iron-chelating phytosiderophores, hence have a specific iron uptake system, the Fe (III)–phytosiderophore complex (Romheld & Marschner, 1986; strategy II).

Barley is the most tolerant species to iron deficiency among the graminaceous plants and Murata *et al.* (2006) identified an iron–phytosiderophore transporter “HvYS1” gene which has



72.7% similarity with ZmYSL1, the first protein identified as an iron(III)-phytosiderophore transporter in maize. The expression of this gene is linked to iron deficient conditions and is expressed in the epidermal root cells. The localization and substrate specificity of HvYSL1 is different from those of ZmYSL1, indicating that HvYSL1 is a specific transporter involved in primary iron acquisition from soil in barley roots (Namba & Murata, 2010).

#### 4. Conclusions

Further research is required to determine the key genes and molecular pathways that underpin the best tolerance responses of our elite crop genotypes to the common abiotic soil constraints including salinity and alkalinity. Once uncovered and assessed to be stably expressed under varying background environments and genomes, these genetic mechanisms may become central to future tolerance breeding programs through advanced selection methods. Also, full characterisation of shared molecular mechanisms to multiple stresses may uncover strategic selection tools for breeding cultivars that are tolerant to stresses that occur simultaneously.

For example, when alkalinity is combined with salinity in the same soil environment, the negative impact on plant growth is significantly increased (Li *et al.*, 2010; Shi & Sheng, 2005). Saline soils containing  $\text{CO}_3^{2-}$  and/or  $\text{HCO}_3^-$  can cause injury to plants through high salts as well as through carbonates and bicarbonate (Shi & Sheng, 2005). The combined stress (alkaline salinity) leads to Na toxicity due to high concentrations of salt and deficiencies of Fe and Zn. Therefore, future genetic studies and screening for selective breeding should incorporate the interactive nature and impacts of multiple concurrent stresses.

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# Trehalose and Abiotic Stress in Biological Systems

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## 1. Introduction

Any non-living factors that affect negatively living organisms are reunited under the general term “abiotic stress” and its effects can be and are mitigated by a variety of defense mechanisms developed by the different biological systems in existence. Examples of abiotic stress are desiccation, salinity, high and low temperature. There are two general mechanisms used to counteract abiotic stress: avoidance and adaptation. In the case of avoidance, organisms migrate to deeper soil layers where temperatures are within tolerable range (Roelofs et al., 2008). Adaptation to stress is based on activation of stress defense gene pathways, which results in the production of heat shock proteins, LEA proteins, redox regulating proteins, different compatible solutes, cytochrome P450s (Roelofs et al., 2008).

Trehalose, also known as tremalose or mycose, a non-reducing disaccharide, is widely spread in biological systems: bacteria, yeast, fungi, lower and higher plants, as well as insects and invertebrates (Elbein, 2003), and its function is associated with tolerance against multiple abiotic stresses. It was discovered in 1832 by H.A. Wiggers in the ergot of rye and later isolated from mooshrooms by Mitscherlich in 1858, who called it mycose (Richards et al., 2002). In the same year Berthelot isolated a novel sugar from trehala-manna, a secretion left by different insects on leaves on Middle East. He named this new sugar “trehalique glucose” or trehalose (Richards et al., 2002). Initially trehalose was considered to be a rare sugar because it could only be extracted from trehala manna or the resurrection plant. Later Koch and Koch in 1925 discovered it in yeast and established basic protocols for trehalose isolation from yeast (Richards et al., 2002). Still, the cost of trehalose production was high enough so that it limited its use for commercial exploitation. In 1990's Hayashibara company in Japan discovered a method to mass-produce trehalose inexpensively from starch. The enzymes used in the process, maltooligosyl-trehalose synthase (MTSase) and maltooligosyl-trehalose trehalohydrolase (MTHase), are derived from a non-pathogenic soil bacteria, *Arthrobacter* sp. (Maruta et al., 1995).

Trehalose has multiple functions, and some of them are species specific. In microorganisms trehalose appears to act as an energy source, during certain stages of development such as spore germination (Elbein, 2003). In anhydrobiotic organisms, trehalose is known to accumulate to high concentrations to survive complete dehydration (Drennan et al., 1993), by preserving the membranes during drought period (Crowe et al., 1984). Trehalose acts as a structural component in mycobacteria, being incorporated into glycolipids (Elbein, 1974). In *Escherichia coli*, trehalose protects against cold stress, presumably by stabilizing cell

membranes and preventing protein denaturation, whereas in yeast it plays a role in osmotic (Hounsa et al., 1998), heat, and desiccation tolerance (Hottiger et al., 1987), and it may act as a free radical scavenger (Benaroudj, 2001). Insects use trehalose from blood as an energy source during flight (Elbein, 1974). Even though in most plants trehalose does not participate directly in the alleviation of abiotic stress, it may act as a signaling molecule. Microarray analyses revealed that both trehalose and trehalose-6-phosphate are affecting the levels of genes involved in abiotic stress (Schluepmann et al., 2004; Bae et al., 2005). Trehalose accumulated by brine shrimp embryos when entering dormancy may act as a stabilizer during dormancy and as an energy source for the embryos when the dormancy period ends (Clegg, 1965). Nematodes, when dehydrated slowly, convert as much as 20% of their dry weight to trehalose, helping them survive dehydration (Crowe et al., 1992).

## 2. Trehalose biosynthesis

There are five known pathways reported in living organisms for trehalose biosynthesis (Fig.1). Some organisms possess only one pathway, whereas others use multiple pathways, which are used depending on the stress affecting the organism (Paul et al., 2008).

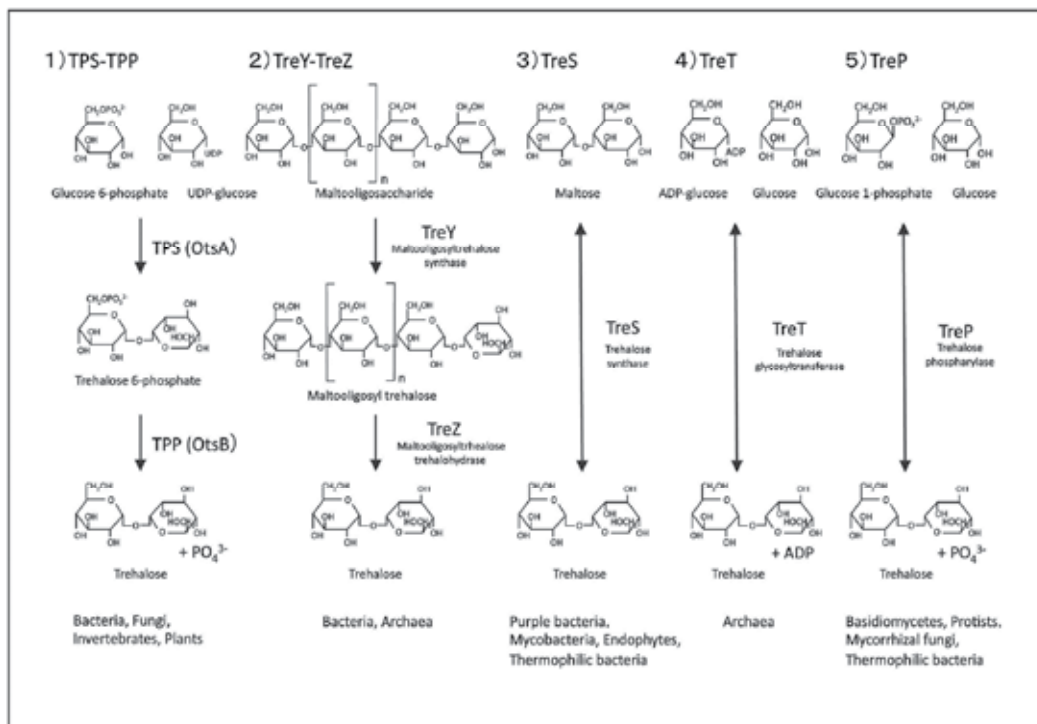


Fig. 1. Trehalose biosynthesis pathways in living organisms

### 2.1 TPS-TPP (OtsA-OtsB) pathway

TPS-TPP (OtsA-OtsB) pathway is a two steps process and it is the most common pathway for trehalose biosynthesis. It is present in both prokaryotes and eukaryotes (archaea, bacteria, fungi, plants and arthropods) (Paul et al, 2008). In plants, trehalose 6-phosphate

synthase (TPS) catalyzes the synthesis of the intermediate trehalose-6-phosphate from glucose-6-phosphate and Uridine Diphosphate (UDP)-glucose, and then trehalose-6-phosphate phosphatase (TPP) catalyzes the dephosphorylation of trehalose-6-phosphate to trehalose. In bacteria OtsA and OtsB enzymes catalyze the conversion of glucose-6-phosphate and UDP-glucose to trehalose, whereas in yeast TPS1 and TPS2 (homologues of TPS and TPP, respectively) are catalyzing the process. If in plants and bacteria the two trehalose biosynthesis enzymes are separate entities, in yeast the TPS1 (TPS homologue) and TPS2 (TPP homologue) are part of a complex that contains two other regulatory subunits, TPS3 and TSL1 (Bell et al., 1998).

## 2.2 TreY-TreZ pathway

Initially this pathway was discovered in *Arthrobacter* sp. (Maruta et al., 1995) and it is also a two-step pathway, in which maltodextrines are converted to trehalose. In the first step, maltooligosyltrehalose synthase (TreY) catalyzes the conversion of maltopentaose into maltooligosyl trehalose by intramolecular transglycosylation, and in the second step, maltooligosyltrehalose trehalohydrolase (TreZ) hydrolyzes the maltooligosyl trehalose, releasing free trehalose (Maruta et al., 1995). This pathway is also found in other bacterial species such as *Rhizobium* (Maruta et al., 1996a), *Bradyrhizobium japonicum* (Sugawara et al., 2010) and *Corynebacterium* (Tzvetkov et al., 2003), but is missing in other major bacterial groups including *E. coli* and *Bacillus subtilis*. Archaea *Sulfolobus* also uses this pathway for trehalose synthesis (Maruta et al., 1996b).

## 2.3 TreS pathway

The TreS pathway is a reversible transglycosylation reaction in which trehalose synthase (TreS) converts maltose, a disaccharide with  $\alpha,\alpha$ -1,4 linkage between the two glucose molecules, to trehalose. Trehalose synthase was first cloned from *Pimelobacter* sp. R48 (Nishimoto et al., 1995) and so far it has been detected only in bacteria (Paul et al., 2008). Due to the reversible nature of the enzyme, TreS contributes trehalose synthesis during osmotic stress in *Pseudomonas syringae* (Freeman et al., 2010), while TreS functions in trehalose catabolism in *B. japonicum* (Sugawara et al., 2010).

## 2.4 TreT pathway

TreT pathway involves the reversible formation of trehalose from ADP-glucose and glucose and it is catalyzed by trehalose glycosyltransferase (TreT) (Qu et al., 2004). TreT appears both in archaea and bacteria (Paul et al., 2008), and it was first reported in the hyperthermophilic archaea *Thermococcus litoralis* (Qu et al., 2004).

## 2.5 TreP pathway

TreP is the second trehalose synthesis pathway found in both prokaryotes and eukaryotes. It is a potential reversible reaction that converts glucose-1-phosphate (G1P) and glucose into trehalose, catalyzed by trehalose phosphorylase. The pathway was first discovered in *Euglena gracilis* (Belocopitow & Marechal, 1970) and later found in mushrooms and bacteria (Paul et al., 2008).

## 3. Trehalose roles in abiotic stresses

Trehalose involvement in tolerance to abiotic stress has been documented in numerous organisms, both prokaryotes and eukaryotes. The effects of desiccation, salt, high and low

temperature stresses have been shown to be averted by trehalose. The information is by no means complete and future studies may reveal trehalose implication in the fight against abiotic stresses in additional species.

### 3.1 Desiccation stress

Water is essential to the existence of life. Not only it is a basic component of living organisms, but also it is critical for their survival. However, there are organisms that can forgo water for extended periods of times, even for decades or centuries. Anhydrobiotic organisms can survive almost complete dehydration, the term anhydrobiosis literally meaning in Greek “life without water”. Such organisms are the invertebrates rotifers, tardigrades, brine shrimp and nematodes, but also certain resurrection plants, and microorganisms like baker's yeast (*Saccharomyces cerevisiae*) (Crowe et al., 1992).

Many of the anhydrobiotic organisms accumulate high concentrations of trehalose during drought stress. In the case of desiccation, water loss can be extremely high, as much as 99% (Strom et al., 1993). Among the protecting disaccharides that accumulate during drought, trehalose is the most effective in stabilizing dry membranes (Crowe et al., 1992). During dehydration, membranes are destabilized because of lipid phase transitions and vesicle fusion (Crowe & Crowe, 1990). Trehalose, even in small quantities, inhibits vesicles fusion completely and depresses the phase transition temperature of dry lipids, maintaining them in liquid crystalline phase in the absence of water (Crowe et al., 1992). It appears that during dehydration or freezing trehalose molecules replace bound water normally associated with biological structures (Donnamaria et al., 1994). Because of its high hydration potential, trehalose may stabilize dry biological membranes and proteins by hydrogen bonding of its hydroxyl groups to the polar groups of proteins and phosphate groups of membranes (Kawai et al., 1992).

Another mechanism by which trehalose protects against desiccation stress is vitrification. Trehalose has the tendency to form a protective glass-like structure that has a low reactivity, making it more stable than other disaccharides due to its non-reducing character. In this hygroscopic glass-like structure, trehalose is extremely stable both at high temperature and when completely desiccated and may hold biomolecules in a form that allows them to return to their native structure and function following rehydration (Crowe & Crowe, 2000). Trehalose glass is suggested to have such a great stability because a small addition of water may form trehalose dihydrate on the outer surface of the glass, which may result in a structure that encloses the inner glass, isolating it (Richards et al., 2002).

#### 3.1.1 Bacteria

Trehalose involvement in bacteria resistance to desiccation stress has been studied extensively. *Nostoc commune* is a terrestrial cyanobacterium that can survive more than a hundred years in desiccated state (Lipman, 1941; Cameron, 1962). When exposed to drought, in addition to the production of large amounts of extracellular polysaccharides, which have an important role in desiccation tolerance, *N. commune* also accumulate trehalose (Sakamoto et al., 2009; Klahn & Hagemann, 2011). Other cyanobacteria that accumulate trehalose during drought stress are *Phormidium autumnale* and *Chroococcidiopsis* sp. (Hershkovitz et al., 1991). Trehalose synthesis is activated in response to water loss due to desiccation process, whereas when the water becomes available and the cells rehydrate trehalose content in the cells decreases (Sakamoto et al., 2009). Other cyanobacteria, like

*Anabena* and *Nostoc flagelliforme*, accumulate trehalose but not in high enough quantities to be able to offer protection as a molecular chaperone. In these cases, trehalose may act indirectly in alleviating drought stress, by regulating the expressions of molecular chaperone-related genes (Higo et al., 2006; Wu et al., 2010).

Rhizobia, soil bacteria that live in symbiosis with legumes are important to agriculture because of their biological nitrogen fixation capacity. *Bradyrhizobium japonicum*, the root nodule symbiont of soybeans, accumulates trehalose using three independent trehalose biosynthesis pathways (Streeter, 2006). Interestingly, mutants of the cells lacking the TreS degradation pathway showed a low survival under desiccation stress (Sugawara et al., 2010). This happened presumably because the high concentrations of trehalose affected the refolding and reactivation of denatured proteins by molecular chaperones and explains the reason why trehalose is quickly degraded after stress has ceased (Singer & Lindquist, 1998). Trehalose accumulation in bacteria influences as well the symbiont plants drought resistance. Rhizobacteria engineered to overexpress trehalose biosynthetic genes improved the drought tolerance of the plants inoculated with the modified rhizobia strains. Common bean plants (*Phaseolus vulgaris*) inoculated with *Rhizobium etli* overexpressing trehalose-6-phosphate synthase recovered completely when exposed to drought stress as opposed to plants inoculated with the wild type strain, which wilted and died (Suarez et al., 2008). Maize plants inoculated with *Azospirillum brasilense* overexpressing trehalose biosynthetic genes had an 85% of survival following drought stress compared to 55% survival rate in the case of the plants inoculated with the untransformed strain (Rodriguez-Salazar et al., 2009).

### 3.1.2 Fungi

Trehalose is widely distributed in fungi and it accumulates in both vegetative and reproductive stages, and at particularly high concentrations in periods with reduced growth rates and during starvation (Thevelein, 1984). Trehalose is also present in the extra-radical mycelium as well as in spores of arbuscular mycorrhizal fungi (Becard et al., 1991).

Log-phase cultures of yeast have low concentrations of trehalose and are quite susceptible to dehydration, but as they enter the stationary phase of growth the levels of trehalose increase (Elbein, 2003). A study of desiccation tolerance of yeast cells subjected to temperature shifts revealed a clear correlation between the cells trehalose content and the changes in desiccation tolerance, demonstrating the trehalose function as a protectant against desiccation (Hottiger et al., 1987).

### 3.1.3 Plants

The presence of trehalose in higher plants was discovered relative recently, and most of the reports were referring to a few desiccation tolerant plants (Bianchi et al., 1993; Drennan et al., 1993; Albin et al., 1994). Following whole genome sequencing, *Arabidopsis* genome has been found to contain eleven putative TPS and ten putative TPP genes, whereas in the rice genome nine TPSs and nine TPPs have been identified. Transgenic *Arabidopsis* plants overexpressing *AtTPS1* (Avonce et al., 2004) exhibited drought stress tolerance as well as glucose and ABA insensitive phenotypes. The altered regulation of genes involved in ABA and glucose signaling during seedling vegetative growth may account for the insensitivity, pointing out *AtTPS1* and/or trehalose-6-phosphate as a major player in gene regulation and signaling during seedling development. *Arabidopsis csp-1* mutant, with a point mutation in the synthase domain of another *Arabidopsis* TPS, *AtTPS6*, is also drought-tolerant (Chary et al., 2008).

Typically, trehalose does not accumulate in plants in quantities high enough to directly protect against abiotic stress as a compatible solute, the way it accumulates in other organisms. Transgenic plants engineered to overexpress microbial trehalose biosynthesis genes accumulated low levels of trehalose and still became tolerant to abiotic stresses, specifically to drought stress. Tobacco and tomato plants transformed with yeast *TPS1* gene under the control of 35S promoter proved to be drought tolerant (Romero et al., 1997; Cortina & Culianez-Macia, 2005). The drawbacks of the studies were the growth aberrations of the transgenic plants – they presented stunted growth (tobacco) and abnormal root development (tomato). The growth abnormalities present in the engineered plants are most probably due to the accumulation of trehalose-6-phosphate, who proved to be an essential player in plant development, since homozygous *tps1* mutants are embryo-lethal (Eastmond et al., 2002). In addition, trehalose-6-phosphate has been found to act as an inhibitor of SnRK1, a hexokinase that is an important transcriptional regulator of metabolism, growth and development in plants (Zhang et al., 2009; Paul et al., 2010). Drought tolerant tobacco plants which did not present any growth abnormalities were obtained by targeting the *TPS1* gene expression to chloroplast or by using bifunctional fusion yeast trehalose synthesis genes (Karim et al., 2007).

### 3.1.4 Tardigrades

Tardigrades are microscopic animals, also known as water bears, which show an extraordinary tolerance to variety of extreme environmental conditions, particularly anhydrobiosis (Welnicz et al, 2011). In some species, the trehalose levels are increased during the induction of anhydrobiosis, but in others there was no difference in trehalose levels between the desiccated and hydrated specimens. In addition, the absolute levels of trehalose detected in tardigrades are much lower than those detected in other anhydrobiotic organisms, indicating that trehalose may have a specific function connected to desiccation, but the nature of that function is not currently known (Welnicz et al., 2011).

### 3.1.5 Insects

*Polypedilum vanderplanki* lives in temporary rock pools in tropical Africa. When the pool dries up, the larvae become dehydrated and remain desiccated until the next rain (Fig. 2). *P. vanderplanki* is the largest multicellular organism known to be able to withstand almost complete dehydration for up to 17 years. Cryptobiotic larvae can withstand extreme temperatures from -170°C to + 106°C and recover completely within an hour when supplied with water (Watanabe et al., 2003). Desiccation stress induces trehalose synthesis in larvae as well as the *TreT1* gene expression. *TreT1* is a trehalose specific transporter, which during the desiccation stress transports the trehalose synthesized in the fat body to the hemolymph (Kikawada et al., 2007).

## 3.2 Salt stress

Salt stress affects organisms in two major ways. Low water potential causes loss of water and turgor pressure, whereas the high ionic strength of the surrounding environment creates a continuous flux of inorganic ions into the living cells. Living organisms maintain their turgor pressure and cell volumes within acceptable limits by accumulating organic osmolytes, called compatible solutes (sugars, polyols, free aminoacids and their derivatives, quaternary amines and their sulfonium analogues, sulfate esters and small peptides (Kempf



et al., 1998). Compatible solutes participate in alleviating salt stress in two major ways. First, they lower the osmotic potential of the cytoplasm, maintaining the normal turgor pressure of the cells (Kempf et al., 1998). Second, they serve as stabilizers of proteins and cell components against the denaturing effects of high ionic strength (Hinch & Hagemann, 2004). Among the compatible solutes, trehalose occupies an important place, acting as a stress protectant in both prokaryotes and eukaryotes.

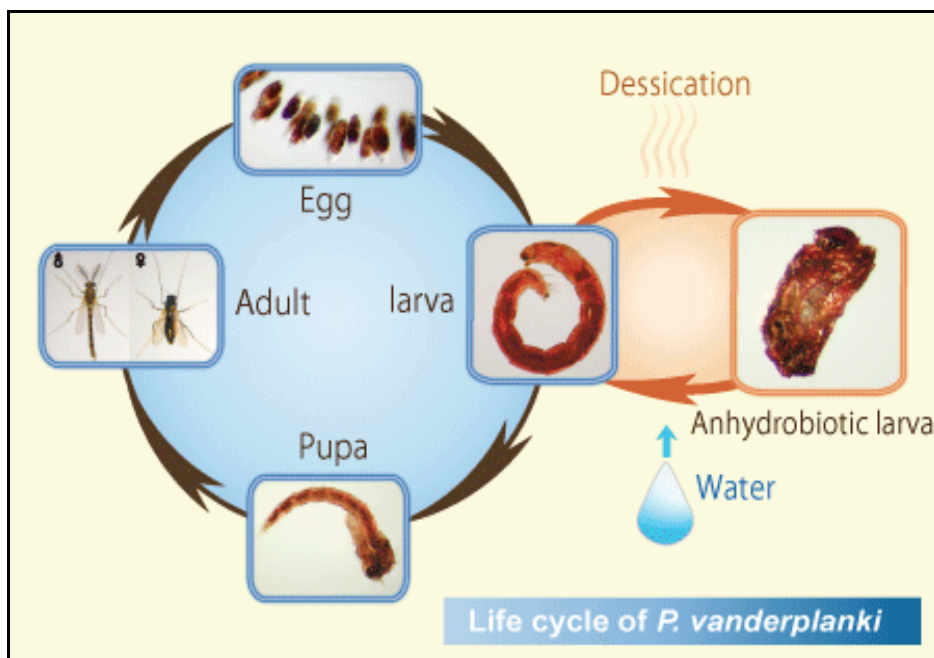


Fig. 2. Life cycle of *Polypedilum vanderplanki*. The larva stage animal shows complete desiccation tolerance (Reproduced with permission from Anhydrobiosis Research Group, National Institute of Agrobiological Sciences, 2009).

### 3.2.1 Bacteria

*E. coli* cells adapt to osmotic stress by accumulating in the cytoplasm trehalose produced endogenously. Excess trehalose is excreted and then degraded by periplasmic trehalase (treA) to glucose, which is subsequently reutilized by the cells (Strom et al., 1993). Furthermore, *E. coli*, *S. meliloti*, *B. japonicum* mutants lacking trehalose biosynthesis genes are sensitive to osmotic stress. (Strom et al., 1993; Dominguez-Ferreras et al., 2009; Sugawara et al., 2010).

Most of cyanobacteria are living in waters of different or changing salinities. Those who dwell in fresh water habitats are adapted to a low osmotic strength environment. Nevertheless, most freshwater cyanobacteria are able to withstand at least partially increasing salt concentrations. Among the compatible solutes that are mostly induced to accumulate in response to low osmotic stress are trehalose and sucrose (Klahn et al., 2011). Trehalose accumulation in cyanobacteria was first demonstrated in *Rivularia atra*, which live in the tidal zone of the coastal waters (Reed & Stewart, 1983). Since then it was revealed that more than forty strains accumulate trehalose when grown on media enriched with NaCl, and in twenty of them trehalose is the major compatible solute accumulated (Hagemann,

2011). The mechanism by which trehalose helps alleviate the stress condition is not salt-specific, but is rather related to the ability of trehalose to stabilize membranes and protein structure. Compatible solutes transport is wide spread in bacteria and many of them (in particular heterotrophic bacteria) prefer to import the stress protectants instead of synthesizing them. However, cyanobacteria use compatible solutes synthesized de novo and use the transporters to uptake their compatible solutes that have diffused into the periplasm (Hagemann, 2011). A transporter with uptake specificity for sucrose, trehalose and glucosylglycerol has been discovered for the first time in *Synechocystis* (Mikkat et al., 1996).

The soil bacterium *Corynebacterium glutamicum* utilizes trehalose as a compatible solute depending on the environmental conditions. When nitrogen is present in sufficient quantities, trehalose plays only a marginal role in osmoprotection and proline is the dominant compatible solute, whereas when nitrogen is scarce, trehalose becomes dominant. In addition, trehalose accumulation increases if maltose is used instead of sucrose as carbon source (Wolf et al., 2003).

Salinity stress affects negatively the symbiotic interactions between rhizobia and legume plants, limiting nitrogen fixation and reducing crop yields. Trehalose accumulates in rhizobium *Bradyrhizobium japonicum* in response to salt treatment. Mutant strains lacking the trehalose biosynthetic genes failed to grow on salt containing medium, indicating that trehalose plays a role as an osmoprotectant for growth under salt-induced osmotic stress (Sugawara et al., 2010). In a different study investigating four rhizobial strains isolated from nodules of *Phaseolus vulgaris* under salt-stress conditions revealed that all strains under study accumulated trehalose (Fernandez-Aunión et al., 2010).

### 3.2.2 Fungi

Arbuscular mycorrhizal fungi colonize plant root systems of over 80% of terrestrial plant species and have been shown to promote plant growth and salinity tolerance by numerous studies (Evelin et al., 2009). *Glomus intraradices* exposed to osmotic stress did not present major changes in trehalose metabolism. Only moderate transient activations of trehalose-6-phosphate phosphatase and neutral trehalase were observed (Ocon et al., 2007).

### 3.2.3 Plants

Rice *OsTPP1* and *OsTPP2* are transiently induced by cold, salt and drought stress as well as external ABA applications (Pramanik & Imai, 2005; Shima et al., 2007). Garcia et al. (1997) reported as well trehalose accumulation in small amounts in rice roots 3 days following salt stress. External applications of low concentrations (up to 5 mM) of trehalose reduced Na<sup>+</sup> accumulation and growth inhibition and higher concentrations (10 mM) prevented chlorophyll loss in leaf blades and preserved root integrity (Garcia et al., 1997). Alfalfa (*Medicago sativa* L.) plants exposed to salt stress accumulated trehalose in roots and bacteroids, but the quantities detected were too low to account for an osmoprotectant role for trehalose (Fougere et al., 1991). In *Medicago truncatula* trehalase activity in nodules was downregulated under salt stress, permitting trehalose accumulation, but again in low quantities to efficiently contribute to osmoprotection (Lopez et al., 2008).

Tomatoes engineered to overexpress yeast trehalose synthesis genes are resistant not only to drought, but also to salt and oxidative stresses (Cortina & Cullanez-Macia, 2005). Rice plants overexpressing bacterial fused trehalose synthesis genes under the control of tissue-specific or stress-dependent promoters accumulate trehalose and are salt, drought, and low temperature stress tolerant, without showing growth abnormalities (Garg et al., 2002).

### 3.3 Low and high temperature stress

Biological membranes are affected during low temperature stress, as their fluidity decreases. Protein denaturation and aggregation happen both under low and high temperature stresses. As a compatible solute trehalose, can prevent protein degradation and aggregation. It can also stabilize biological membranes, by hydrogen bonding with the phosphate groups.

#### 3.3.1 Bacteria

Kandror et al. (2002) reported a protective role of trehalose in cold adaptation of *E. coli*. Strains deficient in trehalose biosynthesis are much more sensitive to cold stress than wild type. However, the deficient strains complemented with trehalose biosynthesis genes have their cold resistance restored. The authors suggest that the mechanisms of trehalose protection against cold include protection of protein integrity, free radical scavenger activity *in vivo* defending against oxidative damage, and cell membranes stabilization. Some of the bacteria that live in high temperature saline environments, such as *Thermus thermophilus*, and hyperthermophilic archaea, like *Pyrobaculum aerophilum*, accumulate primarily trehalose as a compatible solute (Santos & da Costa, 2002).

#### 3.3.2 Fungi

The most studied abiotic stress that induces trehalose in fungi is heat stress. The high level of trehalose in fungal spores increases their resistance to heat stress. Trehalose biosynthesis genes as well as trehalase, responsible for trehalose breakdown are up regulated, resulting in trehalose accumulation. In the yeast *S. cerevisiae* trehalose induced by heat shock not only stabilizes protein structure, but also suppresses aggregation of the proteins that have already been denatured. However, following heat stress, trehalose is degraded rapidly allowing the molecular chaperones to renature the proteins by refolding (Hottiger et al., 1987). Damage done to protein structure and cell membranes during heat stress is due at least partly to reactive oxygen species (ROS) (Davidson et al., 1996). Exposing yeast cells to increased temperatures, trehalose also increased markedly their resistance to oxidative stress (Benaroudj et al., 2001).

Trehalose appears to also have a role in low temperature stress. Hino et al. (1990) reported a correlation between trehalose intracellular accumulation and freeze tolerance of *S. cerevisiae*. When trehalose was accumulating constitutively in the yeast *S. cerevisiae* overexpressing *TPS1* and *TPS2* genes, and *Schizosaccharomyces pombe* overexpressing *TPS1* gene, the strains became resistant to multiple abiotic stresses, including freezing stress (Soto et al., 1999; Mahmud et al., 2010). Following cold stress, when the yeast is returned to non-stress temperature, trehalose and trehalose synthesizing enzymes levels are dropping rapidly (Kandror et al., 2004). *Hebeloma* spp., an ectomycorrhizal basidiomycetes, who can survive sub-zero temperatures, synthesize trehalose, arabitol and mannitol when exposed to freezing stress (Tibbett et al., 2002).

#### 3.3.3 Plants

Rice *OsTPP1* and *OsTPP2* are transiently induced by cold, salt and drought stress as well as external ABA applications (Pramanik & Imai, 2005; Shima et al., 2007). Trehalose was also transiently induced following chilling stress, and its accumulation coincided with the phase change of glucose and fructose levels (Pramanik and Imai 2005). In *Arabidopsis*, *AtTPS5* has a role in thermotolerance. *AtTPS5* interacts with MBF1c, a transcriptional activator that is a key regulator of thermotolerance (Suzuki et al., 2008).

*Arabidopsis* plants engineered with yeast *TPS1* gene under the control of either 35S promoter or a yeast *TPS1-TPS2* fused-genes construct under the control of a stress regulated promoter, accumulated trehalose at low levels and are resistant to abiotic stresses, including freezing and heat stress. If the plants transformed with the first construct displayed aberrant growth, color and shapes, plants transformed with the second construct did not show any morphological or growth abnormalities (Miranda et al., 2007).

### 3.3.4 Nematodes

In nematodes, trehalose induces thermotolerance by preventing damage under heat stress. In addition, trehalose extends the nematodes life-span, possibly by protecting against heat-stress associated damage (Honda et al., 2010). Arctic nematode *Panagrolaimus davidi* accumulates an increasing amount of trehalose following cold temperature acclimation, which may help in membrane stabilization and protect against freeze-induced dehydration (Wharton et al., 2000).

### 3.3.5 Insects

Exposure to a mild cold stress over a period of few minutes to a few hours can increase the cold tolerance of some insects. *Drosophila melanogaster* and *Sarcophaga crassipalpis* flies show an increase of their trehalose levels following cold treatment, increase correlated with an improved chill tolerance (Clark & Worland, 2008). Also, the prepupal larvae of the sawfly *Trichiocampus populi*, who can survive at -30°C for several hours, contain high concentrations of trehalose (Ohtake & Wang, 2011).

## 4. Trehalose uses in relation to abiotic stress

Due to the fact that trehalose proved to be a protectant compound under abiotic stress conditions, it has been and may be used in countless applications in pharmaceutical industry, agriculture, food industry, cosmetics industry, medicine, and research.

### 4.1 Agriculture and food industry

Since trehalose has been found to participate in increasing tolerance to abiotic stresses in other organisms, many attempts have been made to engineer plants with microbial trehalose biosynthetic genes from OtsA-OtsB pathway in order to create stress tolerant plants: tobacco (Holmstrom et al., 1996; Goddijn et al., 1997; Romero et al., 1997; Pilon-Smits et al., 1998; Lee et al., 2003; Han et al., 2005; Karim et al., 2007), rice (Garg et al., 2002; Jang et al., 2003), tomato (Cortina & Culianez-Macia, 2005), potato (Goddijn et al., 1997) and *Arabidopsis* (Karim et al., 2007; Miranda et al., 2007). The first trials were partially successful, trehalose accumulated, albeit at a low level, the plants were stress tolerant, however they displayed abnormal phenotype characteristics (Goddijn et al., 1997; Romero et al., 1997; Pilon-Smits et al., 1998; Cortina & Culianez-Macia, 2005). Nonetheless, subsequent studies solved the phenotype problem by using fused bacterial trehalose biosynthesis genes, directing the gene constructs to chloroplasts (Garg et al., 2002; Jang et al., 2003; Karim et al., 2007; Miranda et al., 2007), or engineering the plants with alternate trehalose biosynthesis genes, as trehalose phosphorylase (TreP), which circumvented the production of trehalose-6-phosphate (Han et al., 2005).

The symbiotic relationship between rhizobia and legumes has a considerable impact not only on the legumes yields but also on the significant amount of the fixed nitrogen that

remains in the soil for future crops use. A way to encourage the formation of the legume-rhizobia symbiosis could be the application of rhizobia to legume seeds prior to planting in the field. However, the percent of survival of rhizobia applied in this manner is very low, less than 5%, because of rapid desiccation (Roughley et al., 1993). By supplying external trehalose (3 mmol l<sup>-1</sup>) to *Bradyrhizobium japonicum* strain USDA 110, the concentration of trehalose in the cells increased threefold and bacteria survival in response to desiccation increased by two to fourfold (Streeter, 2003).

Trehalose role in protecting agricultural products may not be resumed only in helping the direct preservation. For instance, the yeast *Pichia anomala* has antifungal activities and could be used in biocontrol activities against fungal contaminants on fruits and grains. Large quantities of yeast have to be produced and processed for commercial use. The end product should have a long-shelf life, preferably should be able to withstand high temperatures, in the same time keeping to a minimum the production cost in order to be viable from an economical point of view. Trehalose is used in liquid formulations as well as in freeze-drying and vacuum-drying techniques (Melin et al., 2011).

The ability of trehalose to protect protein structure suggested its use in food packaging and preservation. Fresh fruits, herbs, and vegetables are preserving their color, taste and flavors when dried after a brief immersion in trehalose solution (Iturriaga et al., 2009). Superoxide dismutase (SOD) in plants acts as an antioxidant and protects cell components against oxidative damage by reactive oxygen species (Alscher et al., 2002). SOD-like activity of vegetables (carrots, cucumber, spinach onion) was preserved upon drying fresh vegetables with trehalose (Ohtake & Wang, 2011). Trehalose in its glass form encases and protects biomolecules, permitting them to return to their native structure and function upon rehydration (Crowe & Crowe, 2000). Fresh banana, strawberry, mango, avocado, apple, and raspberry, pureed in the presence of trehalose and dried at 25–50°C, kept their color and aroma during prolonged storage. Volatile aromatics are trapped within the trehalose nonpermeable glass and released only after reconstitution of the product (Ohtake & Wang, 2011).

Because of its shielding properties, trehalose has been included as a food additive in Japanese rice cake for protection against low temperature and freezing stresses (Ohtake & Wang, 2011). Freeze-tolerant yeast strains, accumulate trehalose when exposed to freezing (Hino et al., 1990). These strains retain their fermentative ability and bread leavening activity up to six weeks of frozen storage, maintaining their ability to produce good quality bread following freezing (Hino et al., 1987). As trehalose had been found to be able to suppress fatty acid degradation from heat and also from free radical oxidation (Higashiyama, 2002), it is effective in suppressing the formation of unpleasant odors associated with cooking fish (Ohtake & Wang, 2011).

#### 4.2 Cosmetics industry

Trehalose has been found to suppress the unpleasant odors emitted by human skin by up to 70%, which makes it a very good candidate for use in facial and body creams and body deodorants (Higashiyama, 2002). In addition, trehalose incorporated in cosmetic products may enhance their shelf life and mask the odor of active ingredients and their degradation products, due to its antioxidation properties (Ohtake & Wang, 2011).

The high water retention capabilities of trehalose make it useful as a moisturizer in cosmetics (Hyde et al., 2010)

### 4.3 Medicine and pharmacy

As trehalose proved to be able to suppress peptide aggregation (Singer & Lindquist, 1998), it has been used to reduce the symptoms of Huntington's disease. In the cells of patients suffering of this disease, the mutant protein huntingtin forms insoluble aggregates that are thought to produce the disease. Oral administration of trehalose inhibited the formation of huntingtin aggregates and improved the associated motor dysfunction in a transgenic mouse model of Huntington disease (Tanaka et al., 2004).

Researchers at Tokyo University developed an organ preservation solution, extracellular-type trehalose-containing Kyoto (ET-Kyoto) solution, that was successfully used in clinical lung transplantation (Ohtake & Wang, 2011).

Another application for trehalose use is the formulations of heat stable vaccines. An optimized *Salmonella enterica* serovar Typhi oral typhoid vaccine formulated with trehalose, methionine and gelatin proved to be stable for more than four weeks at 37°C. This represents a clear advantage for developing countries as it allows for longer shelf life and the vaccine distribution without the need of refrigeration (Ohtake et al., 2011).

### 4.4 Research

Unstable molecules such as antibodies can be dehydrated at room temperature or 37°C in the presence of trehalose, maintaining their activity after months in storage (Iturriaga et al., 2009). Dried proteins adopt the same configuration as they do in hydrated state when they are stabilized with trehalose, because trehalose prevents degradation by deamidation, oxidation, and aggregation (Ohtake & Wang, 2011).

In plant research, Arabidopsis AtTPS1 gene can be employed as a selectable marker gene during the process of plant transformation, using glucose as a selective agent Iturriaga et al., 2009). The AtTPS1 gene of *A. thaliana* encodes the TPS1 enzyme, which confers glucose insensitivity to seeds and tissues of plants overexpressing this gene when cultivated under tissue-culture conditions (Avonce et al., 2004).

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# Glyoxalase System and Reactive Oxygen Species Detoxification System in Plant Abiotic Stress Response and Tolerance: An Intimate Relationship

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## 1. Introduction

Plants are sessile and sensitive organisms that inevitably encounter a variety of abiotic stresses in nature. Abiotic stresses such as salinity, drought, heavy metal toxicity and extreme temperatures are critical factors that reduce crop yields by more than 50% worldwide (Wang et al., 2003). The scenario is even more aggravated by the predicted forthcoming global changes in climate, foreseen extremization of environmental conditions, continuous increase of world population, ever-increasing deterioration of arable land, and scarcity of fresh water, all underscoring the importance of developing stress-resistant crops that are able to sustain growth and productivity in stressful environments. Plants tolerate abiotic stresses by modulating multiple genes and by coordinating the action of various genes from different pathways or systems (Sasaki-Sekimoto et al., 2005; Ahuja et al., 2010). During the past few years, the complex interrelationship of biochemical pathways that changes during stress has become appreciated, although we are far from understanding this complexity. A thorough understanding of biochemical and molecular responses of plants to various abiotic stresses and the interaction of different molecular pathways is, therefore, essential for a holistic perception of plant resistance mechanisms under stressful conditions. The regulatory roles of the glyoxalase system and reactive oxygen species (ROS) detoxification systems in plant abiotic stress tolerance have increasingly attracted much interest because excessive production of ROS and methylglyoxal (MG) is a common consequence of both abiotic and biotic stresses in plants (Veena et al., 1999; Chen et al., 2004; Yadav et al., 2005a, 2005b; Singla-Pareek et al., 2006; Hossain & Fujita, 2009, 2010; Banu et al., 2010; El-Shabrawi et al., 2010; Hossain et al., 2009, 2010, 2011). ROS and MG are highly toxic to plant cells, and in the absence of adequate protective mechanisms, they can react with proteins, lipids and nucleic acids and inactivate the vital defense system leading to irreparable metabolic dysfunction and death. Plants have a complex network of enzymatic

and non-enzymatic scavenging pathways or detoxification systems which function as an extremely efficient cooperative system to counter the deleterious effects of ROS and MG as well as to perform their signaling function. In plants, MG is detoxified mainly via the glyoxalase system. Besides detoxification of MG, the glyoxalase system could also play a role in oxidative stress tolerance by recycling reduced glutathione (GSH) that would be trapped nonenzymatically by MG to form hemithioacetal, thereby maintaining glutathione homeostasis. In addition, ROS levels are controlled via a versatile antioxidant network in plants. The specific interplay between ROS and components of the antioxidant and glyoxalase pathways could generate compartment-specific changes in both the absolute concentrations of ROS, MG and antioxidant compounds as well as in the ascorbate and glutathione redox ratios. Under stress conditions, these redox signals could interfere with the signaling networks complementary to the antioxidant system and regulate defense gene expression, thus coordinating the necessary readjustments in the redox-regulated plant defense to overcome oxidative stress (Foyer & Noctor, 2005a, 2011; Kuźniak, 2010; Mhamdi et al., 2010).

The results of numerous recent studies have shown that the alleviation of oxidative damage and increased resistance to abiotic stresses are often correlated with the more efficient antioxidative and glyoxalase systems. In this chapter, we will try to provide an overview of MG and ROS metabolism in plants and address a new metabolic relationship of AsA- and GSH-dependent antioxidative and glyoxalase systems in inducing abiotic stress tolerance. Further, we will discuss the progress made over the last few years in our understanding of the interaction between these two important pathways in improving abiotic oxidative stress tolerance either by exogenous chemical treatment (proline, betaine, selenium and nitric oxide) or by genetic engineering of different components of the glyoxalase system and ROS detoxification system in plants.

## 2. Methylglyoxal (MG) and its formation in biological system

MG is a highly reactive  $\alpha\beta$ -dicarbonyl aldehyde compound. MG has a ketone group and an aldehyde moiety and the aldehyde group is more reactive than the ketone. Chemically MG is a yellow liquid with a characteristic pungent odor. Extensive studies have been carried out in mammalian and animal systems and different pathways have been proposed for endogenous MG formation from metabolic intermediates of carbohydrates, protein and lipid metabolism (Fig. 1). However, very little work has been done in plant systems regarding the endogenous production of MG. MG is formed spontaneously in plants by non-enzymatic mechanisms under physiological conditions from glycolysis and from photosynthesis intermediates, glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Espartero et al., 1995; Yadav et al., 2005a). Under stress conditions, the rate of glycolysis increases, leading to an imbalance (in the initial and latter five reactions) in the pathway. Triosephosphates are very unstable metabolites, and removal of the phosphoryl group by  $\beta$ -elimination from 1, 2-enediolate of these trioses leads to the formation of MG (Richard, 1993; Yadav et al., 2005c). Therefore, spontaneous production of MG is an unavoidable consequence of the glycolysis pathway during stress. MG can also be formed enzymatically from G3P and DHAP. Triosephosphate isomerase hydrolyzes G3P and DHAP and removes phosphate to yield MG (Pompliano et al., 1990). Under physiological conditions, minor sources of MG formation include the metabolism of acetone (a metabolite of fatty acids). Acetone monooxygenase catalyzes acetone to acetol, and acetol



monooxygenase (AMO) converts acetol to MG (Casazza et al., 1984). Additionally, MG is also formed during the metabolism of aminoacetone (a metabolite of protein). Semicarbazide-sensitive amine oxidase (SSAO) is able to convert aminoacetone into MG (Lyles, 1996). In a bacterial system, MG is primarily produced from DHAP via MG synthase (Cooper, 1984). Degradation of lipid peroxidation products, in animal systems, generates products like 4-hydroxynon-2-enal and MG. Whether these mechanisms of MG formation are contributing to total MG in plants still needs to be established.

### 3. Detoxification of MG

MG is both a mutagen and a genotoxic agent. At high cellular concentration, it inhibits cell proliferation (Ray et al., 1994) and results in a number of adverse effects such as increasing the degradation of proteins through the formation of advanced glycation end products (AGEs) and inactivating the antioxidant defense system (Wu & Juurlink, 2002; Hoque et al., 2010). Additionally, MG causes increased sister chromatic exchange and endoreduplication (Chaplen, 1998). It also induces DNA strand breaks and increases point mutations (Chaplen, 1998). Therefore, efficient detoxification of MG overproduced during various abiotic or biotic stresses is one of the most important adaptive strategies of plant stress tolerance.

#### 3.1 Glyoxalase system of MG detoxification

The glyoxalase system is an integral component and major pathway of cellular metabolism of MG in living systems present in the cytosol of cells and cellular organelles, particularly mitochondria. The function of the glyoxalase pathway has been studied extensively in animals, primarily because of its putative association with clinical disorders, such as cancer, diabetes and hypertension (reviewed in Chang & Wu, 2006; Desai et al., 2010). It consists of two enzymes: glyoxalase I (Gly I; lactoylglutathione lyase; EC 4.4.1.5) and glyoxalase II (Gly II; hydroxyacylglutathione hydrolase; EC 3.1.2.6). These enzymes act coordinately to convert MG and other 2-oxoaldehydes to their 2-hydroxyacids using GSH as a cofactor in a two-step reaction (Thornalley, 1990). The spontaneous reaction between GSH and MG forms hemithioacetal, which is then converted to S-D-lactoylglutathione (SLG) by Gly I. The second reaction is the hydrolysis of SLG to D-lactate catalyzed by Gly II and GSH is recycled back into the system (Fig. 1). MG detoxification is therefore strongly dependent on the availability of cellular GSH. Deficiency of GSH limits the production of hemithioacetal, leading to the accumulation of MG. The reactions catalyzed by the glyoxalase system are irreversible. The existence and widespread distribution of this shunt pathway documents its fundamental importance in biological systems. Recent investigations in plants have brought new developments in the involvement of the glyoxalase system in stress tolerance and its involvement with oxidative defense systems. Further insights into the biological function of the glyoxalase system came from the molecular cloning of their respective genes. The pioneering work of Dr. Sudhir Kumar Sopory and his associated co-workers (Veena et al., 1999; Singla-Pareek et al., 2003; Saxena et al., 2005; Yadav et al., 2005a, 2005b) provides a potential framework for interpreting the physiological roles of the glyoxalase system in higher plants against various abiotic stresses.

#### 3.2 Non-glyoxalase metabolism of MG

There are other enzymatic systems through which MG could be detoxified in living systems, including plants. MG contains two functional groups that may be either oxidized or

reduced. The enzymes involved in oxido-reductions are capable of catalyzing the conversion of MG to either acetol or lactaldehyde (Kalapos, 1999; Yadav et al., 2008). Among the reductase family of enzymes, aldose/aldehyde reductase (ALR) or aldo-keto reductase (AKR) is currently attracting much interest since it converts MG to acetol and lactaldehyde using NADPH. Overexpression of the *ALR* gene in tobacco increases tolerance against oxidative agents and low temperature, Cd and drought stress (Oberschall et al., 2000; Hegedüs, 2004). Turóczy et al. (2011) reported that transgenic plants overexpressing the *OsAKR1* gene showed oxidative stress tolerance induced by methyl viologen (MV) and high temperature. The transgenic plants also exhibited higher AKR activity and accumulated less MG under heat stress conditions. In addition, pyruvate dehydrogenase has also been shown to catalyze MG detoxification. This enzyme is found in abundance in plants, but its role in MG degradation is not well studied. Other MG metabolizing enzymes include MG reductase and MG dehydrogenase (Ray & Ray, 1982, 1984), which were found in the animal system but are yet to be reported in plants.

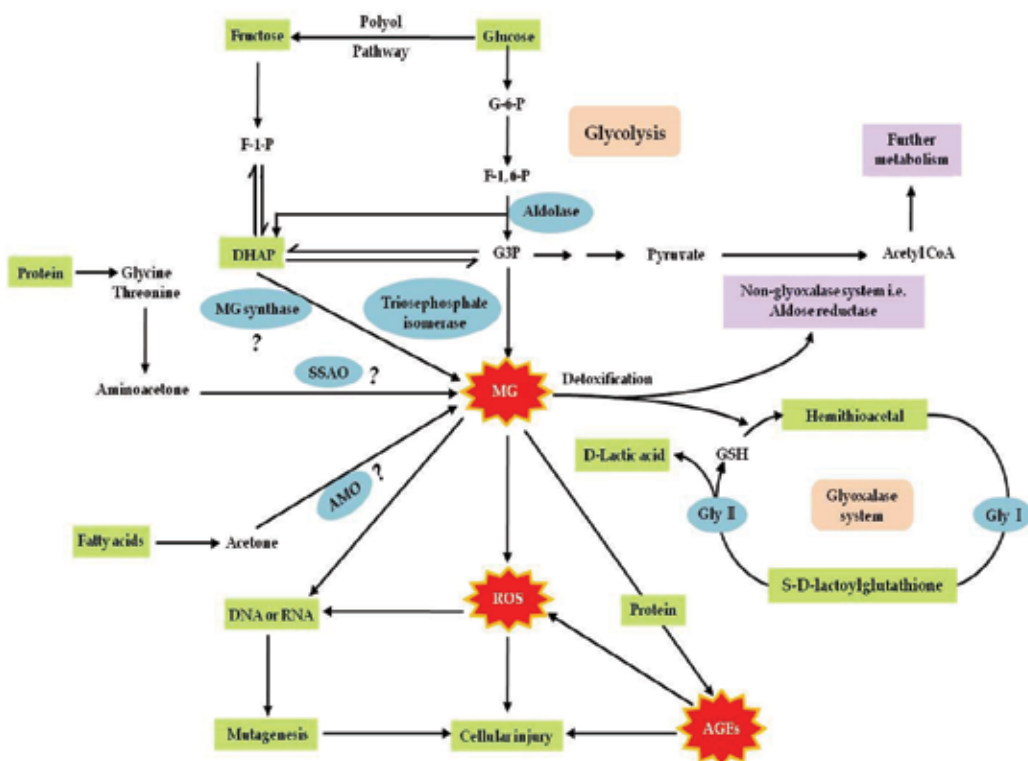


Fig. 1. Formation and detoxification of MG in biological systems (modified from Chang & Wu, 2006; Desai et al., 2010). For further discussion, see text.

#### 4. Induction of MG levels in plants in response to abiotic and biotic stresses

Endogenous production of MG has been reported in all biological systems, including higher plants. In response to stress and diseases, a rapid increase in MG level has been found in animals, mammals, yeast, and bacterial systems (Thornally, 1990; Kalapos et al., 1992; Wu &

Juurlink, 2002) and more recently in plant systems (Yadav et al., 2005a; Singla-Pareek et al., 2006; Hossain et al., 2009; Banu et al., 2010). Yadav et al. (2005a) first showed that induction of the level of MG in response to various abiotic stresses is a common phenomenon in different crop species in which rice, *Pennisetum*, tobacco and *Brassica* seedlings showed a 2- to 6-fold increase in MG levels in response to salt, drought and cold stresses. To further check whether induction of MG in plants in response to various abiotic, hormonal and chemical stresses is a common phenomenon, we measured MG levels in pumpkin (*Cucurbita maxima* Duch.) seedlings subjected to drought, salinity, cold, high temperature, heavy metal, MG, 2,4-D, abscisic acid (ABA) and white light. MG levels were rapidly induced in response to different stresses and white light treatments within a short period of time (24 h) compared to the untreated control, and the levels ranged from 39.96 to 88.40  $\mu\text{mol g}^{-1}$  FW. Importantly, white light (60  $\mu\text{mol photon m}^{-2}\text{s}^{-1}$ ) caused the highest induction (2.21-fold) in the level of MG followed by salinity (1.77-fold), MG (1.69-fold), drought (1.63-fold), heavy metal (1.55-fold) and ABA (1.37-fold) stresses (Hossain et al., 2009). MG was also reported to increase (2.5-fold) in a maize genotype (G4666) susceptible to fungus infected with *Aspergillus flavus* (Chen et al., 2004). Banu et al. (2010) showed that MG level increased significantly ( $\approx$  2-fold) in tobacco BY-2 cells in response to 200 mM NaCl stress. The rapid increase in the level of MG in plants due to different stresses clearly suggests that it is a general stress response. There is a possibility that MG could therefore act as a signal for plants to respond to stress.

## 5. Molecular characterization of Gly I protein and gene, induction of glyoxalase pathway enzymes (Gly I & Gly II) and Gly I protein expression in model plants in response to abiotic stresses

Since proteins are directly involved in plant stress tolerance, proteomics studies can significantly contribute to unravel the possible relationship between protein abundance and plant stress acclimation. Stress-induced Gly I protein and Gly I mRNA expression was first demonstrated by Espartero et al. (1995) in tomato (*Lycopersicon esculentum* cv. Rutgers) seedlings subjected to NaCl, mannitol and ABA treatment. In our study with pumpkin (*Cucurbita maxima* Duch.) seedlings, we found differential induction of Gly I activity and Gly I mRNA expression in response to various stresses and white light. A sharp increase in Gly I activity (1.82-fold) was observed in response to white light followed by salinity (1.42-fold), MG (1.34-fold), drought (1.27-fold) and heavy metal (1.19-fold) stresses. A sharp increase in Gly I activity due to white light suggested that Gly I expression might be under stringent regulation of photoreceptors in plants (Yadav et al., 2008). To know the genetic structure of the pumpkin Gly I gene, the pumpkin Gly I cDNA was also cloned and sequenced. The pumpkin Gly I cDNA (AB 303333) consists of a 975-bp nucleotide encoding a polypeptide of 185 amino acids and belongs to the short-type Gly I sequence of plants (Hossain et al., 2009). However, to clarify the mechanism by which stress-induced alteration of glyoxalase pathway enzymes and Gly I protein expression occurs, and with the hope of obtaining a new genetic sequence of the Gly I gene, we selected the bulbs of a model plant, onion (*Allium cepa* L.), which had very high Gly I activity compared to various other plant species and plant organs (Hossain et al., 2007). Gly I protein was purified from onion bulbs and the Gly I gene was characterized from onion cDNA libraries prepared from onion callus. The specific activity (356  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein) of the purified Gly I was the highest reported to date in plants (Hossain & Fujita, 2009). The relative molecular weight of purified onion

Gly I is approximately 25 kDa. We produced rabbit antiserum using purified protein and immunoscreened onion cDNA libraries with anti-Gly I antiserum. We isolated the onion Gly I cDNA, and purified, cloned and sequenced it. Onion Gly I cDNA (AB 630177) consisted of a 877-bp nucleotide encoding a polypeptide of 187 amino acids. Based on the number of amino acids, the onion Gly I gene was classified as a short-type Gly I, which showed significant homology with other known Gly I sequences of plants present in the database. Further, we studied the regulation of glyoxalase pathway enzymes (Gly I and Gly II) and Gly I protein expression in onion callus subjected to a range of abiotic stresses. Significant increases in Gly I activity and Gly I protein induction were observed following various stress treatments, except for heavy metal stress ( $\text{CdCl}_2$ ). Low temperature stress showed the highest induction (2.57-fold) followed by salinity (2.52-fold) and drought stress (1.43-fold). A similar pattern of induction of Gly II activity was also observed in response to the various abiotic stresses: drought showed a 1.81-fold and chilling showed a 2.5-fold increase in activity. Moreover, the activity followed the same pattern as that of Gly I, except for salinity stress. In the case of heavy metal stress, the activity of both Gly I and Gly II decreased. The protein expression profile of Gly I also showed a true reflection of possible changes in activity levels due to various abiotic stresses. It is conceivable that an elevated level of Gly I activity is required to remove excessive MG produced in ample amounts under normal and various stressful conditions (Yadav et al., 2005a, 2005b; Hossain et al., 2009; Hossain & Fujita, 2009). The concomitant increase in Gly II activity convincingly indicated that both enzymes are critically important in MG detoxification and stress tolerance because, apart from MG, pathway intermediate SLG (substrate for Gly II) has also been found to be cytotoxic at high concentrations by inhibiting DNA synthesis (Thornalley, 1996). The presence and characterization of both Gly I and II has been reported in many plant species and the genes encoding these enzymes have been cloned and found to be regulated under various abiotic stress conditions (Espartero et al., 1995; Veena et al., 1999; Jain et al., 2002; Yadav et al., 2005a, 2005b; Saxena et al., 2005; Singla-Pareek et al., 2006; Hossain et al., 2009; Hossain & Fujita, 2009; Lin et al., 2010). Therefore, the multistress inducibility of Gly I and Gly II genes, and their overexpression, can lead to tolerance of multiple forms of stress in plants.

## **6. Similarity of Gly I activity, gene and protein expression between model plant (onion) and other plant species in response to environmental stresses**

In agreement with our research results of stress-induced alteration of glyoxalase pathway enzymes and protein expression in a model plants species, onion (Hossain & Fujita, 2009), recent proteomic and transcriptomic studies also showed a similar pattern of Gly I activity, Gly I mRNA and protein expression in response to various stresses (biotic and abiotic). Salt-tolerant barley (*Hordeum vulgare* L.) genotype cv. Morex showed up-regulation of Gly I protein expression in response to salt stress but these proteins were down-regulated or remained unchanged in the susceptible line cv. Steptoe (Witzel et al., 2009). Durum wheat (*Triticum durum* Desf. cv. Svevo) subjected to heat stress showed up-regulation of Gly I protein expression (Laino et al., 2010) and rice (*Oryza sativa* L. cv. Dongjin) seedlings subjected to chilling stress (10°C) showed gradual up-regulation of Gly I protein expression (Lee et al., 2009). Consequently, rice seedlings treated with ABA (5  $\mu\text{M}$ ) showed up-regulation of Gly I activity (Li et al., 2010). Recent transcriptomic analysis of a salt-tolerant wild tomato species (*Solanum pimpinellifolium*) showed 2- to 3-fold

increases in Gly I gene transcript whereas the cultivated sensitive variety showed less than a 2-fold increase (Sun et al., 2010). Du et al. (2011) detected the up-regulation Gly I protein expression in rice leaves subjected to UV radiation. However, Tuomainen et al. (2011) did not observe an increase of the Gly I transcript in a metal hyperaccumulator plant *Thlaspi caerulescens*, similar to our finding in onion, in which we did not find an increase in protein expression in response to heavy metal (0.5 mM CdCl<sub>2</sub>) stress (Hossain & Fujita, 2009). However, an increase in Gly I mRNA in response to ZnCl<sub>2</sub> (5 to 10 mM) was reported in *Brassica* and wheat (Singla-Pareek et al., 2006; Lin et al., 2010). This apparent discrepancy may be due to differences in genetic background or differential regulation of different Gly I isoforms (Tuomainen et al., 2011).

The potential for direct involvement of Gly I activity and protein expression in host resistance against aflatoxigenic fungi (*A. flavus*) in maize (*Zea mays* L.) was also investigated. Higher Gly I activity was observed in the kernels of resistant lines with or without *A. flavus* infection. After fungal infection, the level of MG did not increase in resistant genotypes. This lack of increase could be due to the relatively higher levels of Gly I activity and Gly I protein expression observed in resistant kernels (Chen et al., 2004). Later on, the involvement of Gly I protein in the *B. napus*/*Sclerotinia sclerotiorum* pathosystem was investigated by qRT-PCR analysis. Gly I exhibited an increasing trend during a 36-48 h period, the timing of which paralleled the increase in abundance of a protein spot identified as Gly I, suggesting the potentially important role for this enzyme in biotic stress tolerance (Liang et al., 2008).

## 7. Influence of MG on oxidative stress and antioxidant defense system

There is a substantial evidence of MG-induced oxidative stress in various living cells, including those of plants. MG causes mitochondrial oxidative stress by increasing the generation of mitochondrial O<sub>2</sub><sup>•-</sup>, NO and peroxynitrate. Additionally, MG significantly decreased the activities of MnSOD and complex III. MnSOD is the first-line enzyme in mitochondria to dismutate O<sub>2</sub><sup>•-</sup> and complex III transfers electrons from ubiquinone to cytochrome *c*. The inhibition of complex III by MG may disrupt the electron transport chain, leading to electrons leaking out to form O<sub>2</sub><sup>•-</sup> (Wang et al., 2009; Desai et al., 2010). MG was found to modify arginine, lysine and cysteine residues and inhibit a large number of enzymes (Thornalley, 1996). MG can also cause oxidative stress indirectly through the formation of advanced glycation end products (AGEs), the irreversible chemical modifications and cross-links in proteins (Desai et al., 2010). The activities of SOD, GST, CAT, Gly I and II, and GSH content decreased in a time- and dose-dependent manner following the administration of exogenous MG to rat liver cells whereas lipid peroxidation increased (Choudhary et al., 1997). Wu and Juurlink (2002) also found that MG (100 to 500 μM) induced oxidative stress by inactivating antioxidant enzymes such as GR and GPX and by a profound increase in oxidized glutathione (GSSG) content. They proposed that MG enhanced AGEs that in turn could activate receptors of AGEs (RAGE), thereby promoting O<sub>2</sub><sup>•-</sup> production. MG-induced impairment of GR and GPX would also result in oxidative stress because GR plays an important role by reducing GSSG to GSH, whereas GPX scavenges peroxides by utilizing GSH, which can be converted to very reactive free radicals. The MG-induced impairment of GR and GPX activity was inactivated glycation because these proteins are known to be susceptible to glycation inactivation. However, very limited work has been done on the influence of exogenous MG in antioxidative defense systems in higher plants. Hoque et al. (2010) showed that exogenous application of MG (0.5 to 10 mM)

in tobacco (*Nicotiana tabacum* L. cv. BY-2) cells inhibits GST activity, whereas exogenous application of GSH can reverse the phenomenon. Thus, MG weakens the antioxidant enzymes and their modulation may contribute to oxidative stress.

## 8. Engineering glyoxalase pathway enzymes and abiotic stress tolerance of plants

Undoubtedly, the role of MG-scavenging systems in plant stress tolerance has increased through the use of gene transfer technology. A number of experiments clearly demonstrated that the enhancement of the MG-detoxification systems in plants provides partial protection from oxidative damage (Venna et al., 1999; Yadav et al., 2005a, 2005b; Singla-Pareek et al., 2003, 2006, 2008; Bhomkar et al., 2008). Overexpression of glyoxalase pathway genes in transgenic plants has been found to keep a check on the MG and ROS levels under stress conditions, regulate glutathione homeostasis, allowing the transgenic plants to survive and grow under various abiotic oxidative stresses.

Veena et al. (1999) first produced transgenic tobacco plants overexpressing the *B. juncea* short-type *Gly I* gene. The over-expressing transgenic plants showed tolerance to salt (400 and 800 mM NaCl) and toxic concentrations of MG (5 and 25 mM). The degree of tolerance was correlated with the degree of *Gly I* expression indicating the importance of the gene in plant stress tolerance. Similarly, Singla-Pareek et al. (2003) produced transgenic tobacco plants overexpressing glyoxalase pathway genes (*Gly I* and *Gly II*). Although *Gly II* transgenic lines showed improved tolerance to salinity stress and could set normal viable seeds, double transgenic lines showed a better response than either the single gene-transformed lines or non-transgenic line. Additionally, the double transgenic line showed a negligible (5%) yield reduction under salt stress (200 mM NaCl). The transgenic plants also showed lower chlorophyll destruction than wild-type (WT) plants. Later on, the suitability of the glyoxalase transgenic plants against heavy metal ( $ZnCl_2$ ) stress was also tested by Singla-Pareek et al. (2006). Transgenic plants showed restricted MG accumulation and less lipid peroxidation at a high Zn concentration (5 mM  $ZnCl_2$ ) and were able to survive, maintain growth, flower and set normal viable seeds without any reduction in yield. The most striking observation was that double transgenic plants performed better than either of the single-gene transformants as initially observed under salt stress. Maintenance of glutathione homeostasis in transgenic plants was the possible mechanism behind the tolerance against heavy metal toxicity. Subsequently, overexpression of the *Gly II* gene in transgenic rice plants showed higher tolerance to a toxic concentration of MG and salinity compared to non-transformed (NT) plants. The overproduction of *Osgly II* results in detoxification of MG and SLG and recycling of GSH that could be a possible mechanism behind stress tolerance and the *Osgly II*-overexpressing transgenic line. Importantly, the transgenic plants were able to maintain a high selectivity for  $K^+$  over  $Na^+$  uptake in the roots and reduced  $Na^+$  sequestration in the young leaves compared with the WT plants leading to the maintenance of a high  $Na^+/K^+$  ratio in both shoots and roots of transgenic plants. The maintenance of ideal  $Na^+/K^+$  ratio in both the shoots and roots of transgenic plants was correlated with the normal growth of *Osgly II*-overexpressing transgenic tobacco plants that may be the basis of minimizing  $Na^+$  toxicity under salt stress (Singla-Pareek et al., 2008). Additionally, overexpression of the *Gly I* gene using a novel *Cestrum yellow leaf curling virus* (CmYLCV) promoter in blackgram (*Vigna mungo* L.) showed tolerance to salinity stress. Exposure of transgenic plants to salinity stress (100 mM NaCl) revealed that the transgenic

plants survived under salt stress and set seed. In contrast, the untransformed control plants failed to survive. The higher level of Gly I activity in transgenic lines was directly correlated with their ability to withstand salt stress (Bhomker et al., 2008). The above findings clearly indicate that the glyoxalase pathway is one of the most important metabolic pathways that could have both a direct and indirect effect on plant stress tolerance by modulating multiple physiological responses and metabolic pathways.

## 9. Methylglyoxal as an initiator of activation of signal transduction pathways

Information regarding the signaling roles of MG in higher plants is scarce although MG was found to activate several signal transduction pathways in yeast. MG activates transcription factors such as Yap1 and Msn2, and triggers a Hog1 mitogen-activated protein (MAP) kinase cascade in *Saccharomyces cerevisiae*. Regarding the activation of Hog1 by MG, Sln1, an osmosensor possessing histidine kinase activity, functions as a sensor of MG (Maeta et al., 2005; Takatsume et al., 2006). Our understanding of the signaling role of MG in higher plants is at a rudimentary stage. However, critical analysis of glyoxalase transgenic plants reveals that they maintain MG at a certain level, which we predict to be an appropriate level that these transgenic plants maintain under stress, playing a secondary role in stress perception and signaling. In view of the finding in yeast, it would be worthwhile to investigate whether MG might be involved in signal transduction pathways in higher plants. Therefore, the signaling functions of MG in higher plants remain an open question.

## 10. Sites and sources of ROS production in plant cells

Abiotic stresses disrupt cellular homeostasis in plants leading to the onset of oxidative stress or the generation of ROS such as singlet oxygen ( $^1\text{O}_2$ ), superoxide radical ( $\text{O}_2^{\bullet-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radical ( $^{\bullet}\text{OH}$ ). ROS are continuously produced during various metabolic processes. However, certain environmental stresses or genetic defects cause the production of ROS to exceed the management capacity. Organelles with a highly oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria and peroxisomes (Fig. 2), are major sources of ROS production in plant cells (Mittler et al., 2004). In the chloroplast, during photosynthesis, energy from sunlight is captured and transferred to two light harvesting complexes, photoystem I (PS I) and photoystem II (PS II).  $\text{O}_2^{\bullet-}$ , which is produced mainly by electron leakage from Fe-S centers of PS I or reduced ferredoxin (Fd) to  $\text{O}_2$  (Mehler reaction), is then converted to  $\text{H}_2\text{O}_2$  by SOD (Gechev et al., 2006).  $\text{O}_2^{\bullet-}$  can also be produced by the leaking of electrons to molecular oxygen from electron transport chains in PS I and II (Sgherri et al., 1996). Under excess light conditions PS II is able to generate  $^1\text{O}_2$  by energy transfer from the triplet state chlorophyll (Asada, 2006). In peroxisomes, ROS is produced mainly during photorespiration and also during  $\beta$ -oxidation of fatty acids. The ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme, which catalyses the carboxylation of ribulose-1,5-bisphosphate (RuBP) during carbon assimilation, can also use  $\text{O}_2$  to oxygenate ribulose-1,5-bisphosphate. Under abiotic stress conditions, which impair  $\text{CO}_2$  fixation in the chloroplast, the oxygenase activity of RuBisCO increases and the glycolate that is produced moves from the chloroplast to peroxisomes, where it is oxidized by glycolate oxidase (GO) forming  $\text{H}_2\text{O}_2$  (Takahashi & Murata, 2008). In peroxisomes,  $\text{H}_2\text{O}_2$  can also be formed directly from  $\text{O}_2$  by enzyme systems such as xanthine oxidase (XO) coupled to SOD (Mhamdi et al., 2010). The

mitochondrial electron transport chain consists of several dehydrogenase complexes which reduce a common pool of ubiquinone (Q). ROS production is likely to occur mainly in complex I and the Q zone (Blokina & Fagerstedt, 2006). Additional sources of ROS in plant cells include the detoxifying reactions catalyzed by cytochromes in both the endoplasmic reticulum and the cytoplasm (Urban et al., 1989). In glyoxysomes, acyl-CoA oxidase is the primary enzyme responsible for  $H_2O_2$  generation. Plasma membrane-bound NADPH oxidases as well as cell-wall associated peroxidases are the main sources of  $O_2^{\cdot-}$  and  $H_2O_2$  producing apoplastic enzymes (Mhamdhi et al., 2010). In the presence of redox-active metals, the extremely reactive  $\cdot OH$  can be formed from  $H_2O_2$  through the Fenton reaction or from  $H_2O_2$  and  $O_2^{\cdot-}$  through the Haber-Weiss reaction causing extensive oxidative damage of membranes and other macromolecules, including photosynthetic pigments, protein, DNA and lipids (Foyer & Noctor, 2005a; Gechev et al., 2006).

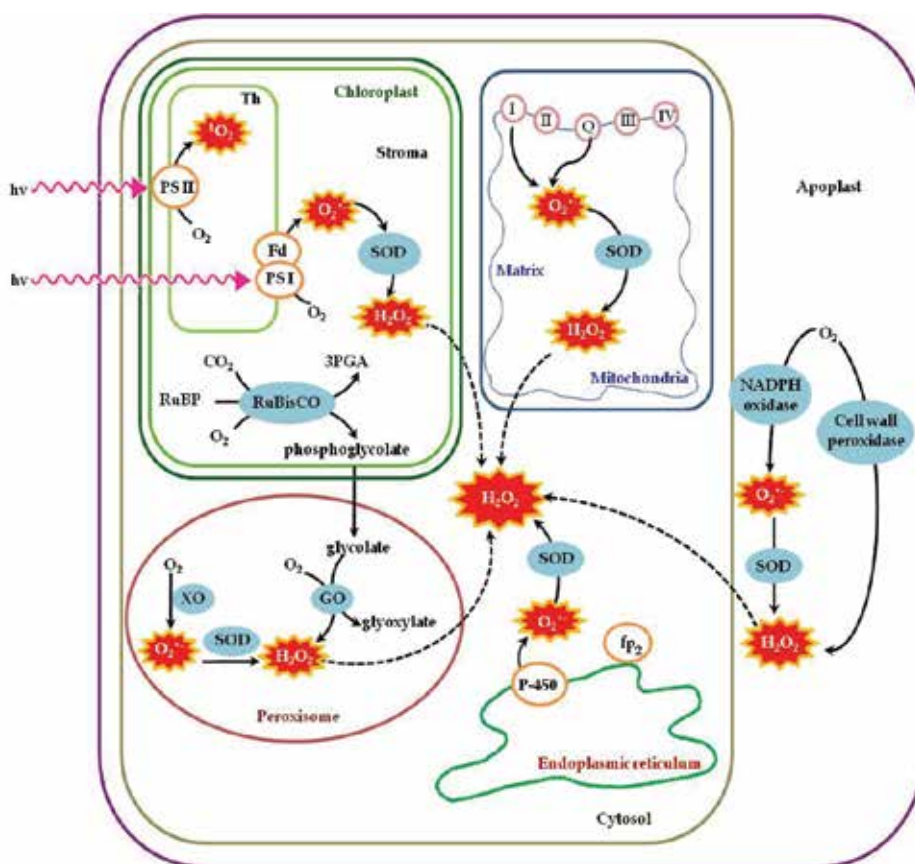


Fig. 2. Major sites and sources of ROS production of plant cells (modified from Blokina & Fagerstedt, 2006; Mhamdhi et al., 2010; Hossain & Fujita, 2011).

## 11. ROS scavenging and detoxification system in plants

Plants use an intrinsic mechanism known as the plant antioxidant system as a defense mechanism to regulate the ROS levels according to the cellular needs at a particular time.



These antioxidants includes the enzymes superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), glutathione reductase (GR; EC 1.6.4.2), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.9), glutathione S-transferase (GST; EC 2.5.1.18) and water-soluble compounds such as ascorbate (AsA) and GSH (Apel & Hirt, 2004; Hossain & Fujita, 2011). Although AsA and GSH function as cofactors of enzymes of the antioxidant and glyoxalase pathways, both can also directly quench ROS and regulate gene expression associated with biotic and abiotic stress responses to optimize defense and survival. Importantly, sustaining the ROS concentration (specially  $H_2O_2$ ) at an appropriate level can promote plant development and reinforce resistant to environmental stressors by modulating the expression of genes and redox signaling pathways (Neill et al., 2002). Abiotic stress induced differential induction of different components of antioxidant defense systems, in particular those related to AsA and GSH briefly described below:

### 11.1 Ascorbate peroxidase (APX)

APX is one of the most important enzymes of the AsA-GSH cycle and plays a vital role in plant defense against oxidative stress by catalyzing the conversion of  $H_2O_2$  to  $H_2O$ . APX activity and APX isoenzyme gene expression are variable even under the same stress conditions and some of them are constitutively expressed for the immediate and efficient detoxification of  $H_2O_2$  under normal and oxidative stress conditions (Ishikawa & Shigeoka, 2008; Hossain & Fujita, 2011). Salt-tolerant plant species showed a significant increase in APX activity in response to salt stress but the activities decreased or remain unchanged in salt-sensitive genotypes (Mittova et al., 2003; Sekmen et al., 2007). A profound increase ( $\approx 5$ -fold) in APX activity was observed in a drought-tolerant tomato variety (Zarina) in response to mild drought stress (Sánchez-Rodríguez et al., 2010). Sato et al. (2011) showed that transgenic plants overexpressing the OsAPX gene in rice showed cold tolerance at the booting stage as indicated by 2-fold lower  $H_2O_2$  levels and MDA content. Spikelet fertility was significantly higher in transgenic lines than in WT plants. These results indicate that higher APX activity enhances  $H_2O_2$ -scavenging capacity and protects spikelets from lipid peroxidation, thereby increasing spikelet fertility under cold stress.

### 11.2 Monodehydroascorbate reductase (MDHAR)

MDHAR, one of the major components of the AsA-GSH cycle, is a monomeric enzyme using NADPH as an electron donor to reduce monodehydroascorbate (MDHA) to AsA (Hossain et al., 1984). MDHAR activity increased in response to salt stress in wild salt-tolerant tomato species while the activity decreased in a salt-sensitive cultivar. Additionally, due to higher antioxidant capacity, salt-tolerant wild relatives maintained a lower level of  $H_2O_2$  and MDA, whereas cultivated species showed greater oxidative damage (Shalata et al., 2001; Mittova et al., 2003). Mung bean and rapeseed seedlings subjected to salinity and heavy metal stresses showed a decrease in MDHAR activity (Hossain et al., 2010, 2011; Hasanuzzaman et al., 2011a). In contrast, an increase in MDHAR activity due to long-term drought stress was observed in diploid hybrid which was accompanied by higher AsA and lower DHA content compared to their parents (Gao et al., 2009). Transgenic tobacco plants overexpressing the *AtMDHAR1* gene showed enhanced stress tolerance in terms of higher net photosynthetic rates under  $O_3$ , salt and osmotic stress and greater PSII effective

quantum yield and a lower level of H<sub>2</sub>O<sub>2</sub> under O<sub>3</sub> and salt stresses (Eltayeb et al., 2007). Additionally, Kavitha et al. (2010) showed that tobacco plants overexpressing the *AmMDHAR* gene showed higher MDHAR and APX activity. The transgenic lines showed an enhanced redox state of AsA and reduced level of lipid peroxidation denoting the vital role of MDHAR in AsA regeneration and oxidative stress tolerance.

### 11.3 Dehydroascorbate reductase (DHAR)

Oxidation of AsA by APX leads to the formation of a short-lived MDHA radical, which is converted to AsA by MDHAR or disproportionates nonenzymatically to AsA and dehydroascorbate (DHA). DHA is recycled to AsA by DHAR, which requires GSH as the reductant (Chen et al., 2003). Mottova et al. (2003) reported that salt stress significantly induced DHAR activity and a higher AsA and AsA/DHA ratio in salt-tolerant cultivar whereas the activity remained unchanged in a salt-sensitive tomato cultivar accompanied by higher oxidative damage. Mung bean and rapeseed seedlings subjected to salt and heavy metal stress showed a significant decrease in DHAR activity (Hossain et al., 2010; Hossain et al., 2011; Hasanuzzaman et al., 2011a). However, a sharp increase in DHAR activity was observed in rice shoot tissues under mild drought stress but the activity decreased under severe drought stress (Sharma & Dubey, 2005). Heat (55°C) altered DHAR activity in tobacco BY-2 cells, showing an important phenomenon in maintaining redox balance when MDHAR activity decreased significantly. The increase in DHAR activity could be a sort of feedback regulation mechanism occurring to improve AsA regeneration from DHA when AsA depletion occurs at its production sites due to enhanced ROS (Locato et al., 2009). Therefore, DHAR may optimize the utilization of the still available AsA in a cellular organelle that is strongly subjected to oxidation by the overproduced ROS. Transgenic tobacco plants overexpressing the *cytDHAR* gene exhibited higher DHAR activity, a higher level of AsA and ascorbate redox state and exhibited enhanced tolerance to O<sub>3</sub>-, drought-, salt-, and PEG-induced oxidative stresses (Eltayeb et al., 2006). Wang et al. (2010) showed that *DHAR* overexpression in *Arabidopsis thaliana* increased AsA and GSH levels and their redox state relative to WT showed oxidative stress tolerance induced by high light and high temperature (40°C). Therefore, increasing AsA content through enhanced ascorbate recycling could limit the deleterious effects of environmental oxidative stress.

### 11.4 Glutathione reductase (GR)

GR is a flavoprotein oxidoreductase that catalyzes the reduction of GSSG to GSH by utilizing NADPH. The adaptive behaviors of salt-tolerant and -sensitive genotypes suggest that GR plays a significant role in maintaining the glutathione redox state under oxidative stress. Salt-tolerant plant species showed an increase in GR activity in response to salt stress whereas in the sensitive genotypes the activity decreased or remain unchanged (Shalata et al., 2001; Sekmen et al., 2007). In our recent study, mung bean and rapeseed seedlings showed a decrease in GR activity under a high level of salt and Cd stress (Hossain et al., 2010; Hasanuzzaman et al., 2011a, 2011b). A drought-tolerant rice genotype significantly increased GR activity and antioxidant metabolites such as AsA and GSH when the level of H<sub>2</sub>O<sub>2</sub> decreased. However, in the susceptible genotype the activity of GR decreased as the level of GSH and AsA decreased. Selote & Khanna-Chopra (2004) proposed that during water stress an integrated antioxidant defense system, including the AsA-GSH cycle, in

developing panicles, is an important factor related to enhanced spikelet fertility in the drought-resistant rice genotype under upland conditions. Similarly, a drought-tolerant cultivar (Zarina) showed a sharp increase ( $\approx 3$ -fold) in GR activity under mild drought stress (Sánchez-Rodríguez et al., 2010). Recently, Martret et al. (2011) reported that co-expression of DHAR+GR or GR+GST in tobacco chloroplasts exhibit altered metabolism and improved abiotic stress tolerance. The level of AsA and GSH was significantly increased in both the double transformants (DHAR+GR and GR+GST). In response to chilling stress, the H<sub>2</sub>O<sub>2</sub> content increased nearly 3-fold in WT plants whereas the double transgenic plants reduced H<sub>2</sub>O<sub>2</sub> levels more efficiently than WT or single gene transformants.

### 11.5 Catalase (CAT)

CATs, the first antioxidant enzymes to be discovered and characterized, are predominantly localized in the peroxisomes and glyoxysomes for scavenging H<sub>2</sub>O<sub>2</sub>. Purev et al. (2010) reported a significant induction of the *PgCat1* gene in response to various stimuli such as heavy metals, plant hormones, osmotic agents, UV and chilling stress. Salt-tolerant *P. maritima* and *L. pennellii* showed an increase in CAT activity in response to salt stress (Mittova et al., 2003; Sekmen et al., 2007) indicating that an increase in CAT activity may be an adaptive response to overcome oxidative stress. However, a decrease in CAT activity in response to various stresses was also reported (Sharma & Dubey, 2005; Hossain et al., 2010; Hossain et al., 2011; Hasanuzzaman et al., 2011a). The *Cat1*-deficient mutant under a high light regime showed leaf necrosis and was unable to maintain glutathione in the reduced state when exposed to elevated light indicating that the recycling of GSH is defective because of a general shortage of reducing power for the AsA-GSH cycle to allow continuous recycling of the substrates (Mhamdi et al., 2010). A 'gain-of-function' study also revealed the importance of the CAT gene in plant stress tolerance. Overexpression of *E. coli* catalase gene (*katE*) in Indica rice (*Oryza sativa* cv. BR5), conferred tolerance to salt stress; transformed plants formed flowers and produced normal seeds (Moriwaki et al., 2008).

### 11.6 Glutathione peroxidase (GPX)

GPXs are key enzymes of the antioxidant network in plants present in different subcellular organelles. Their principal activity is thought to catalyze the reduction of H<sub>2</sub>O<sub>2</sub>, organic hydroperoxides (ROOH) and lipid hydroperoxides to H<sub>2</sub>O and alcohol using GSH and/or other reducing equivalents (Foyer & Noctor, 2011). Most identified plant GPX genes were shown to have high homology to the mammalian phospholipid hydroperoxide glutathione peroxidases (PHGPX), which have a higher affinity to lipid hydroperoxides than to H<sub>2</sub>O<sub>2</sub>. However, at least two plant PHGPXs probably represent novel isoforms of TRX peroxidase, which are generally more active against H<sub>2</sub>O<sub>2</sub> than lipid peroxides (Foyer & Noctor, 2005b). The specific expression pattern of PHGPX in salt-induced tolerant foxtail millet seedlings suggests that its product plays a crucial role in the defense reaction against salt-induced oxidative damage (Sreenivasulu, 2004). In addition, gene expression and the activity of GPX were found to increase in response to salt, drought and heavy metal stresses (Mittova et al., 2003; Hossain & Fujita, 2010; Hossain et al., 2011). Despite this, transgenic plants overexpressing GPX were more tolerant to oxidative stress caused by treatments with H<sub>2</sub>O<sub>2</sub>, MV, and environmental stress conditions, such as chilling, salinity and drought (Gaber et al., 2006) indicating the potential physiological role of GPX in higher plants against oxidative stress.

### 11.7 Glutathione S-transferases (GSTs)

GSTs are a superfamily of multifunctional enzymes best known for their role in enzymatic detoxification of xenobiotics. GST acts by catalyzing the conjugation of GSH with electrophilic, often hydrophobic toxic compounds to form derivatives that can be secreted from the cell, sequestered in the vacuole, or catabolized (Dixon & Edwards, 2010). In addition, plant GSTs also provide protection against oxidative stress induced by abiotic stresses and oxidants (Fujita & Hossain, 2003a, 2003b; Hossain et al., 2006a, 2006b; Dixon & Edwards, 2010). Functioning as GPX and DHAR, plants GSTs can catalyze the reduction of hydroperoxides to less harmful alcohols and safeguard protein function from oxidative damage and maintain redox homeostasis by regenerating AsA from DHA (Dixon & Edwards, 2010). Due to its high GSH content, we used pumpkin as a model plant and studied the induction pattern and role of pumpkin GSTs in oxidative stress tolerance and detoxification, by exposing pumpkin seedlings and callus to different types of stresses viz. environmental stresses (dehydration, high and low temperatures), hormones such as 2,4-D and methyl jasmonate (MJ), aldehydes and alcohols including  $\alpha,\beta$ -unsaturated carbonyl compounds, heavy metals including Cd, Mn, Cr and As, and antioxidants and oxidants. High temperature (42°C) and dehydration induced pumpkin GSTs to different degrees (Hossain & Fujita, 2002; Fujita & Hossain, 2003a). Pumpkin GSTs are significantly induced by  $\alpha,\beta$ -unsaturated carbonyl compounds, saturated chain aldehydes and alcohols (Fujita & Hossain, 2003b). However,  $\alpha,\beta$ -unsaturated aldehydes were the most effective inducers and their potency is related to the Michael acceptors reaction. Pumpkin GSTs were also induced by heavy metals, different antioxidants and oxidants (Hossain et al., 2006a). *CmGSTF1* was not responsive to all the applied stresses except for MJ (Fujita & Hossain, 2003b; Hossain et al., 2006a). Induction of *CmGSTF1* by MJ alone is therefore indicative of the possibility of involvement of pumpkin GSTs in developmental processes. Transgenic tobacco plants overexpressing the *PjGSTU1* gene showed drought tolerance and Green Fluorescent Protein (GFP) fusion studies revealed the presence of PjGSTU1 in the chloroplast of transgenic plants which was correlated with its role in ROS removal (George et al., 2010).

### 11.8 Ascorbate (AsA)

AsA is one of the most abundant and powerful antioxidants in plant cells. It is an integral weapon in the defense against ROS generated by various abiotic and biotic stresses in different subcellular organelles and the apoplast. AsA has the ability to donate electrons in a number of cellular redox reactions and serves as a major cellular redox regulatory antioxidant (Smirnoff, 2000). AsA can directly quench  $^1O_2$ ,  $O_2^{\bullet-}$  and  $\bullet OH$  and regenerate  $\alpha$ -tocopherol from  $\alpha$ -chromanoxyl radical thereby providing protection to membranes (Thomas et al., 1992). It is the substrate of APX, which is a critical component of the AsA-GSH cycle for  $H_2O_2$  detoxification. A wealth of evidence suggests that stress-resistant plants are up-regulated or maintained higher AsA levels than stress-sensitive plants (Shalata et al., 2001; Mittova et al., 2003). In our recent study with mung bean, rapeseed and wheat seedlings, the level of AsA decreased in response to salt stress (Hossain et al., 2011; Hasanuzzaman et al., 2011a, 2011b). AsA level increased in a drought-tolerant rice genotype (N22) while in the susceptible genotype (N118) its levels decreased (Selote & Khanna-Chopra, 2004). However, decrease in AsA content in response to drought and heavy metal stresses was reported (Sharma & Dubey, 2005; Sečenji et al., 2010; Hossain et al., 2010). Zhang et al. (2011) showed that transgenic tomato plants overexpressing the GDP-Mannose

3',5'-epimerase (an important enzyme of the ascorbate biosynthesis pathway) gene (*SIGME*) exhibited a significant increase in total AsA content in different plant organs and showed enhanced oxidative stress tolerance induced by MV. The transgenic plants showed a higher survival and growth rate under chilling and salt stress. They proposed that improved stress tolerance was closely related to higher endogenous AsA content that increased the ability to scavenge ROS.

### 11.9 Glutathione (GSH)

The thiol tri-peptide GSH is one of the major antioxidant and redox buffers in plants found abundantly in all cell compartments. GSH takes part in the control of H<sub>2</sub>O<sub>2</sub> levels through the AsA-GSH cycle (Foyer & Noctor, 2005a). It can also function directly as a free radical scavenger by reacting with <sup>1</sup>O<sub>2</sub>, O<sub>2</sub><sup>•-</sup>, and •OH (Larson, 1988). It is also involved in the transfer and storage of sulfur and in the detoxification of heavy metals where phytochelation (PC) derived from GSH forms heavy metal complexes. Additionally, GSH has been associated with several growth- and development-related events in plants, including cell differentiation, cell death and senescence, pathogen resistance and enzymatic regulation (Ogawa, 2005). Up-regulation of the GSH level is of pivotal importance, because it induces the signal transduction and defense against ROS and MG which is achieved through different pathways with various control points (Fig. 3) which include orchestrated activation of genes encoding enzymes related with GSH and AsA (Hossain & Fujita, 2011). A sharp increase in GSH content was found in mung bean, rapeseed and wheat seedlings subjected to salinity and Cd stress (Hossain & Fujita, 2010; Hossain et al., 2010, 2011; Hasanuzzaman et al., 2011a). A desiccation-tolerant plant (*Boea hygroskopica*) showed an almost 50-fold increase in GSH content under dehydration stress (Sgherri et al., 1994). Additionally, a drought-tolerant rice genotype showed an increase in GSH content while in the susceptible genotype the GSH content decreased (Selote & Khanna-Chopra, 2004). Ivanova et al. (2010) found that overexpression of  $\gamma$ -glutamylcysteine synthetase gene (*gshI*) in the cytosol led to a 2-fold increase of foliar GSH content. Biomass accumulation of WT poplar hybrid decreased in heavy metal-contaminated soil by more than 30-fold, whereas transformants showed a 2-fold decrease. Thus, poplars overexpressing  $\gamma$ -ECS in the cytosol were more tolerant to heavy metal stress.

## 12. Coordinated role of ascorbate and glutathione during oxidative stress tolerance

Glutathione and ascorbate co-operation is the key for the cellular redox homeostasis in the antioxidative AsA-GSH cycle as well as redox regulation of signaling pathways, gene expression and plant metabolism. Positive correlations between high contents of AsA and GSH and higher activities of AsA and GSH utilizing and regenerating enzymes in inducing stress tolerance have frequently been found. Correlative response of AsA and GSH and other antioxidant enzymes were observed in wheat seedlings subjected to salt stress. The fresh and dry weights of salt-stressed seedlings were significantly reduced, but least reduced in the tolerant cultivar (H 168). The salt stress treatment caused a temporary increase in the activities of SOD, APX, GR and CAT. The increases were consistent in the tolerant genotypes, but mostly stopped or even inverted in the susceptible cultivar. Importantly, the AsA and GSH contents increased significantly in the tolerant cultivar but decreased in the susceptible cultivars. It can, therefore, be concluded that higher AsA and

GSH content and antioxidative enzymes conferred salinity tolerance of the resistant cultivars (El-Bastawisy, 2010). Liu et al. (2009) showed that mild oxidative shock induced by exogenous PQ pretreatment in cucumber leaves modifies the functioning of AsA and GSH utilizing and regenerating enzymes and showed drought-induced oxidative stress tolerance. Drought stress and PQ pretreatment increased the activities of SOD, CAT, GPX, APX, DHAR, MDHAR, GR, and non-enzymatic antioxidants such as AsA and GSH in leaf tissues. However, PQ-pretreated drought-stressed seedlings resulted in higher activities of those enzymes and non-enzymatic antioxidants such as AsA and GSH and AsA/DHA and GSG/GSSG ratios compared to seedlings subjected to drought stress without a pretreatment. Subsequently, Lin et al. (2011) further showed that simultaneous induction of both AsA and GSH content and their metabolizing enzymes by PQ pretreatment increased the tolerance to salt-induced oxidative stress in cucumber leaves. Salt stress significantly increased the activities of SOD, APX and GR but decreased the activities of CAT, GPX, MDHAR, DHAR and AsA accompanied by higher  $O_2^{\bullet-}$ ,  $H_2O_2$  and MDA levels. However, PQ pretreated salt-stressed seedlings maintained higher activities of SOD, GPX, MDHAR, DHAR, GR as well as AsA, GSH, AsA/oxidized ascorbate, GSH/GSSG ratios, accompanied by lower levels of  $O_2^{\bullet-}$ ,  $H_2O_2$  and MDA.

Xu et al. (2010) showed the  $H_2O_2$ -induced upregulation of AsA and GSH metabolism in inducing Al-induced oxidative stress tolerance in wheat seedlings. Al stress increased the  $O_2^{\bullet-}$  and  $H_2O_2$  level leading to more predominant lipid peroxidation, programmed cell death, and inhibited root elongation in both Al-tolerant and -sensitive genotypes. Al-stress increased the activities of SOD, POD, CAT, MDHAR, DHAR, GR, GPX and AsA and GSH content and their redox state. However, Al-stress seedlings pretreated with  $H_2O_2$  showed higher SOD, POD, CAT, MDHAR, DHAR, GR, GPX activities and AsA and GSH content and their redox state than non-treated Al-stressed seedlings. Importantly, antioxidant capacity was more enhanced in the Al-sensitive genotype than in the tolerant one. Therefore,  $H_2O_2$  pretreatment makes the plant more tolerant to Al-induced oxidative stress by inducing AsA and GSH levels and their metabolizing enzymes.

### **13. Simultaneous expression of two or more transgenes related to AsA- and GSH-metabolism in plants and abiotic stress tolerance**

Only few recent studies in plants explored how transgenic plants overexpressing multiple genes related to AsA and GSH metabolism showed better tolerance against abiotic oxidative stress by co-regulation of their antioxidant machinery. Zhao et al. (2009) studied the co-expression of GST and CAT gene in transgenic plants under Cd and heat stress and in combination with heat and Cd stress conditions to understand the influence on other function-linked components of the antioxidant defense system, including AsA-GSH cycle enzymes and metabolites such as AsA, GSH and their redox state. Transgenic plants under Cd stress and combined stress (Cd and heat) conditions showed a sharp increase in CAT, GST, APX, MDHAR, DHAR, GR activities and maintained a higher ascorbate and glutathione redox state, a higher photosynthetic rate and a lower level of  $H_2O_2$  and chloroplast destruction under stress. Their results denote that co-expression of GST and CAT ultimately affects the AsA-GSH cycle and coordinates up-regulation of AsA-GSH pathway enzymes rendering the plants more tolerant to Cd and heat-induced oxidative stress. Additionally, tobacco plants overexpressing three antioxidant enzymes (CuZnSOD, APX and DHAR) showed greater tolerance to oxidative stress than double transgenic

(CuZnSOD and APX) or WT plants (Lee et al., 2007). Transgenic plants overexpressing three antioxidant genes had higher DHAR activity, and higher ratios of AsA to DHA, and GSSG to GSH compared to double transgenic (CuZnSOD and APX) plants.

Consequently, Ahmad et al. (2010) found that transgenic plants overexpressing SOD, APX and choline oxidase (*codA* gene, gene for betaine synthesis) in naturally betaine non-accumulator plants (potato) showed enhanced protection against oxidative stress as indicated by lower levels of H<sub>2</sub>O<sub>2</sub> compared with double transgenic (SOD+APX) and non-transgenic plants after MV-mediated oxidative stress. Additionally, transgenic plants overexpressing three genes synergistically enhanced tolerance to salt and drought stresses by maintaining higher activities of SOD, APX and CAT and betaine level. These results are strongly coherent with the findings of our studies on exogenous proline- and betaine-induced oxidative stress tolerance in mung bean seedlings (Hossain & Fujita, 2010; Hossain et al., 2010, 2011). Additionally, transgenic tobacco plants overexpressing *cytSOD* or *cytAPX* alone, or in combination, enhanced tolerance to drought-induced oxidative stress. The most striking observation was that the transgenic plants overexpressing both enzymes showed higher MDHAR, DHAR, GST, POX and CAT activity in addition to SOD and APX activity in the soluble fractions. Moreover, an increase in the activity of some antioxidant enzymes (APX, SOD & POD) was also observed in the chloroplastic fraction of the transgenic plants. These results indicate that co-regulation among the antioxidant enzymes in different subcellular organelles is also vital to obtain substantial tolerance against oxidative stress (Faize et al., 2011).

The tolerance mechanism of oxidative stress in response to various abiotic stresses was investigated in transgenic potato tubers overexpressing D-galacturonic acid reductase gene (*GalUR*) with enhanced accumulation of AsA (Hemavathi et al., 2010). Enhanced activity of SOD, CAT, APX, DHAR and GR were observed in transgenic potato tubers subjected to various abiotic stresses induced by MV, NaCl and ZnCl<sub>2</sub>. The ascorbate redox state (AsA:DHA) and ratio of reduced to oxidized glutathione (GSH:GSSG) were significantly higher in transgenic tubers than in WT tubers. Therefore, transformation of one gene related to ROS metabolism can have a substantial influence on other enzymes; moreover, AsA-producing transgenic plants modulate glutathione metabolism because they are interdependent in keeping the AsA-GSH cycle fully functional and thereby reducing oxidative stress (Foyer & Noctor, 2011). Dixit et al. (2011) noted that overexpression of even one gene can have a profound influence on other antioxidant enzymes in plants. Tobacco plants overexpressing the GST gene (*TvGST*) showed better Cd tolerance, as indicated by lower Cd accumulation and lipid peroxidation, than WT plants. Most importantly, the transgenic plants showed significantly higher SOD, GST, GPX, APX and CAT enzyme activities under Cd stress than WT plants. Their results further proved that co-regulation among antioxidant enzymes is essential to maintain the correct balance between overproduction of ROS and their scavenging to keep them at the required levels to execute their signaling function and to improve oxidative stress tolerance.

#### **14. Involvement of AsA/DHA, GSH/GSSG ratios in abiotic stress response, redox regulation and signaling**

During abiotic stress-driven oxidative stress, higher plants have the ability to sense and translate ROS signals into specific cellular responses. Additionally, ROS have the ability to oxidize redox-sensitive proteins directly or indirectly through the use of molecules like

AsA and GSH. AsA and GSH are united together through redox flux and coordinate their action during the metabolism of ROS (Foyer & Noctor, 2005a, 2005b, 2011). Compartment-specific variations in AsA/DHA and GSH/GSSG ratios may have a substantial significance in redox signaling. The ascorbate redox state in the apoplast is critically important in a number of stress responses such as in the control of guard cell signaling, stomatal movement and plant growth (Chen et al., 2003). Under stressful conditions AsA oxidation to DHA takes place and, in turn, this molecule can modulate plant responses to stress (Lopez-Carbonell et al., 2006). DHA is believed to signal the redox state of the apoplastic environment, and hence to allow the cell to perceive stress in the environment. DHA accumulation in the apoplast may trigger the arrest of cell growth (Latowski et al., 2010). Moreover, DHA may act as a potential factor in signaling pathways. Reversible modification of specific proteins by DHA could be important in cell signaling. Unlike ascorbate, the redox potential of glutathione is a function of both the GSH/GSSG ratio and the concentration of GSH. According to the Nernst equation the glutathione redox state is a second order of function of GSH concentration (Kuźniak, 2010). The redox state of the GSH/GSSG couple is altered under abiotic stress conditions because two molecules of GSH are converted to GSSG through oxidation. Conditions that trigger the accumulation of GSSG often also lead to a subsequent increase in total glutathione content and are related to stress-induced changes in H<sub>2</sub>O<sub>2</sub> content. Stress-induced changes in the H<sub>2</sub>O<sub>2</sub> content and GSH/GSSG ratio have a central role in signaling due to their effects on transcription, translation and post translational modification of proteins and metabolic processes (Neill et al., 2002; Szalai et al., 2009). Unfortunately, the role of the glyoxalase pathway in regulating GSH concentration and the glutathione redox state is often neglected. However, our recent studies showed that simultaneous induction of glyoxalase pathway enzymes (Gly I and Gly II) and GR by exogenous chemical treatment (proline, betaine, Se and NO) increased the GSH content and the GSH/GSSG ratio (Hossain & Fujita, 2010; Hossain et al., 2010, 2011; Hasanuzzaman et al., 2011a, 2011b) and several studies conducted in a number of plant species under abiotic stress conditions have elucidated the fact that a high GSH/GSSG and/or AsA/DHA ratio sustained by increased GSH and AsA or decrease of GSSG and DHA may be key element for efficient protection against abiotic oxidative stress.

### **15. Intimate relationship between GSH-dependent MG detoxification system and AsA- and GSH-based ROS detoxification system: clues from stress-tolerant and transgenic plants**

Some of the most exciting advances in understanding, sensing and response networks of abiotic stress tolerance by using stress-tolerant, stress-sensitive and transgenic plants lead to a cross talk between the AsA- and-GSH dependent ROS detoxification system and the GSH-dependent MG detoxification system in counteracting abiotic stress-induced oxidative damage (Fig. 3). In deciphering the molecular insights of salinity-induced oxidative stress tolerance in transgenic tobacco overexpressing glyoxalase pathway enzymes, Yadav et al. (2005b) first revealed and discussed the interconnection of GSH-based ROS and MG metabolism in plants. Transgenic plants overexpressing both Gly I and II genes showed better antioxidative protection under salt stress (200 mM NaCl) while WT plants suffered from serious oxidative stress. Furthermore, transgenic plants reflect higher basal antioxidant enzyme activities such as APX, GR, GST and GPX, although the activities of these enzymes



increased sharply when salt stress was imposed. Based on their findings it can be concluded that glyoxalase transgenic plants showed enhance salinity tolerance by regulating multiple biochemical pathways and also by having multiple functions, including: (i) prevention of excessive accumulation of MG, which could deplete GSH; (ii) maintenance of higher antioxidative activities of AsA-GSH cycle enzymes that regulate the level of H<sub>2</sub>O<sub>2</sub> and ascorbate and glutathione redox ratios; (iii) maintenance of higher activities of GST and GPX, which utilize GSH in degrading lipid peroxide, organic hydroperoxide and H<sub>2</sub>O<sub>2</sub>; (iv) protection of non-enzymatic antioxidants from oxidative and antioxidative enzymes from inactivation through advanced glycation. These four mechanisms hold true for various abiotic stresses and recent studies further demonstrated that co-ordinated induction of both detoxification pathways showed enhance abiotic stress tolerance in different plant species and cultured cells (see later in the next section).

El-Shabrawi et al. (2010) further pointed out the interaction among glyoxalase and ROS detoxification systems while identifying biochemical markers for enhanced salt tolerance in two rice cultivars differing in salt tolerance. Analysis of non-enzymatic antioxidants and their redox state (AsA, DHA, AsA/DHA, GSH, GSSG and GSH/GSSG) and the antioxidant and glyoxalase pathway enzyme activities (SOD, APX, CAT, GPX, GR, POX, Gly I and Gly II) and isozyme expression of antioxidant enzymes depicts that the salt-tolerant cultivar Pokkali maintained higher enzymatic activities - reflected by isozyme analysis - and showed oxidative stress tolerance - as indicated by a lower level of H<sub>2</sub>O<sub>2</sub> and oxidative DNA damage - than the salt-sensitive cultivar (IR64) suggesting that Pokkali possesses a more efficient antioxidant defense system to cope with salt-induced oxidative stress. Furthermore, Pokkali exhibited a higher GSH/GSSG ratio and a higher AsA/DHA ratio than IR64. Their results showed that fine modulation of AsA and GSH metabolism and regulation of ROS via the antioxidant and glyoxalase systems and higher proline content in the tolerant rice cultivar allowed it to show better tolerance against salinity-induced oxidative stress. Based on the above findings we therefore infer that ROS and MG metabolism are tightly correlated and that a plant induces both pathways in response to abiotic stress tolerance.

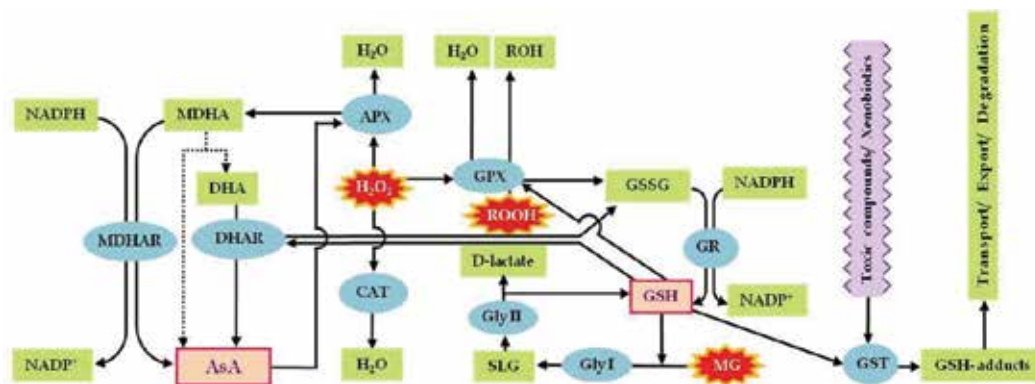


Fig. 3. Schematic illustration of possible metabolic interaction of AsA- and GSH-based antioxidative system and GSH-based glyoxalase system in plant cells (modified from Hossain et al., 2011). Dotted lines indicate non enzymatic reactions. For further discussion, see text.

## 16. Induction of abiotic stress tolerance in plants by simultaneous induction glyoxalase system and AsA- and GSH-based ROS detoxification system through exogenous chemical treatments

Abiotic stress tolerance is a multigenic trait and acquired tolerance must be a cumulative result of different multiple metabolic pathways and genes. However, up-regulation of at least two detoxification pathways (ROS and MG) provides substantial tolerance against abiotic oxidative stress (Hoque et al., 2008; Kumar & Yadav, 2009; Hossain & Fujita, 2010; Hossain et al., 2010, 2011; Hasanuzzaman et al., 2011a, 2011b). Although a close relationship between MG and ROS metabolism was first described by Yadav et al. (2005b) in a transgenic system, later on, Hoque et al. (2008) further showed experimental evidence of a close relationship among ROS and MG detoxification systems in tobacco (*N. tabacum* L. cv. BY-2) cells by applying exogenous proline and betaine under salt stress (200 mM NaCl) conditions. Salt stress increased protein oxidation, contents of thiol, disulfide, GSH and GSSG, and the activity of GST and Gly II enzymes, but decreased the redox state of both thiol-disulfide and glutathione, and the activity of GPX and Gly II enzymes involved in ROS and MG detoxification. Exogenous application of proline or betaine (20 mM) resulted in reduced protein oxidation, and in an increase in the glutathione redox state and activity of GPX, GST and Gly I under salt stress. In previous studies, Hoque et al. (2007) showed that exogenous proline and betaine increased the activity of APX, DHAR and GR under salt stress (200 mM NaCl). These results suggest that exogenous application of proline or betaine rendered the plants more tolerant to salinity-induced oxidative damage by modulating antioxidant and glyoxalase systems. Consequently, Kumar and Yadav (2009) reported that simultaneous induction of both MG and ROS detoxification by exogenous proline and betaine induces cold tolerance in *Camellia sinensis* (L.) O. Kuntze. MG and lipid peroxidation levels increased in tea bud (youngest topmost leaf) in response to cold stress (4°C) whereas this increase did not occur when tea bud was exposed to proline and betaine (25 mM). Exposure of tea bud to proline and betaine helped to maintain the thiol/disulfide ratio and enhanced the activities of GST and GR during cold stress. Furthermore, both proline and betaine showed a protective effect on Gly I and activated Gly II. Finally, they concluded that proline and betaine might provide protection against cold stress by regulating the formation of MG and lipid peroxidation and by activating or protecting some antioxidant and glyoxalase pathway enzymes.

A similar correlated regulation of glyoxalase and antioxidant pathways in inducing heavy metal tolerance was also observed in mung bean (*Vigna radiata* cv. Binamoog-1) seedlings (Hossain et al., 2010). Seven-day old seedlings were subjected to 1 mM CdCl<sub>2</sub> for 48 h with or without proline and betaine. Cadmium stress caused a profound increase in GSH and GSSG content, while the AsA content decreased with a sharp increase in H<sub>2</sub>O<sub>2</sub> and lipid peroxidation (MDA). APX, GST, GPX, and Gly I activities increased in response to Cd stress while the activity of CAT, MDHAR, DHAR, GR and Gly II decreased. Exogenous application of proline and betaine (5 mM) showed an increase in GSH and AsA content, maintained a high GSH/GSSG ratio and increased the activity of APX, DHAR, MDHAR, GR, GST, GPX, CAT, Gly I and Gly II involved in ROS and MG detoxification systems more than the control and most Cd-stressed plants, with a concomitant decrease in GSSG content, H<sub>2</sub>O<sub>2</sub> and MDA levels. These findings suggest that both betaine and proline provide protection against Cd-induced oxidative stress by reducing H<sub>2</sub>O<sub>2</sub> and MDA levels and by

increasing the antioxidant defense and MG detoxification systems. Recently, we further demonstrated that coordinated induction of antioxidative and glyoxalase defense systems by using exogenous proline and betaine protects mung bean seedlings from salinity-induced oxidative damage (Hossain et al., 2011). Salt stress (200 mM NaCl, 48 h) caused a sharp increase in GSH and GSSG content while the GSH/GSSG ratio and AsA content decreased. The GR, GPX, GST and Gly II activities increased in response to salt stress while the MDHAR, DHAR, CAT and Gly I activities decreased sharply with an associated increase in H<sub>2</sub>O<sub>2</sub> and MDA. Contrarily, salt-stressed seedlings pre-treated with proline or betaine (5 mM proline or betaine, 24 h) showed an increase in AsA, GSH content, GSH/GSSG ratio and maintained higher activities of APX, DHAR, GR, GST, GPX, CAT, Gly I and Gly II involved in ROS and MG detoxification systems than untreated control seedlings and most salt-stressed plants with a simultaneous decrease in GSSG content, H<sub>2</sub>O<sub>2</sub> and MDA levels. These results further coincide with our previous results denoting that simultaneous induction of both detoxification pathways make the plant more tolerant to salinity-induced oxidative stress. A similar concomitant induction of ROS and MG detoxification systems by exogenous selenium (Se) pretreatment were reported in rapeseed (*Brassica napus* cv. BINA sharisha 3) seedlings under salt stress (Hasanuzzaman et al., 2011a). Twelve-day-old seedlings were supplemented with Se (25 µM Na<sub>2</sub>SeO<sub>4</sub>) and salt (100 and 200 mM NaCl) separately and in combination. The AsA content in leaves decreased significantly with increasing salt stress. The amount of GSH and GSSG increased as the level of salt stress increased, while the GSH/GSSG ratio decreased. The activity of APX and GST increased with an increase in salt concentration while GPX activity increased only at moderate salt stress (100 mM NaCl). GR activity remained unchanged at 100 mM NaCl but it was decreased under severe (200 mM NaCl) salt stress. The activities of MDHAR, DHAR, CAT, Gly I, and Gly II decreased following the imposition of salt stress, whereas a sharp decrease in these activities was observed under severe salt stress (200 mM NaCl) with higher H<sub>2</sub>O<sub>2</sub> and MDA levels. Importantly, Se-supplemented salt-stressed seedlings showed an increase in AsA and GSH contents, GSH/GSSG ratio, and the activities of APX, MDHAR, DHAR, GR, GST, GPX, CAT, Gly I, and Gly II more than the control and in most cases than seedlings subjected to salt stress without Se supplementation. Additionally, Se-supplemented salt-stressed seedlings showed lower oxidative damage as indicated by lower H<sub>2</sub>O<sub>2</sub> and MDA levels. These results suggest that the exogenous supplementation of Se induces salt stress-induced oxidative stress tolerance in rapeseed seedlings by enhancing their antioxidant defense and MG detoxification systems.

The latest findings by Hasanuzzaman et al. (2011b) further prove that modulation of the glyoxalase and ROS detoxification systems by exogenously applied SNP (an NO donor) improved oxidative stress tolerance of wheat (*Triticum aestivum* L. cv. Pradip) seedlings subjected to salt stress (150 and 300 mM NaCl, 4 d). The AsA content decreased in leaf tissues in response to salt stress while the GSH and GSSG contents and the GSH/GSSG ratio increased as the level of salt stress increased. GST activity increased in response to salt stress while APX, MDHAR, DHAR, CAT and GPX activities remained unchanged. GR, Gly I and Gly II activities decreased following the imposition of salt stress with a concomitant increase in the levels of H<sub>2</sub>O<sub>2</sub> and MDA. Furthermore, salt-stressed seedlings pretreated with NO (1 mM SNP, 24 h) showed an increase in the AsA and GSH contents and the GSH/GSSG ratio as well as the activities of MDHAR, DHAR, GR, GST, GPX, Gly I, and Gly II compared to seedlings subjected to salt stress without pretreatment. The authors concluded that NO-

induced coordinated induction of AsA- and GSH-based ROS and MG detoxification systems make the plant more tolerant to salinity-induced oxidative stress. The evidence described above clearly illustrates that the appropriate induction of a detoxification system (both ROS and MG) renders the plant more tolerant to various abiotic stresses in a synergistic manner by efficient regulation of both AsA and GSH levels and their utilizing and regenerating enzymes.

## 17. Conclusion and future perspective

MG and ROS are clearly emerging as leitmotifs in plant life, being involved in most physiological responses to stress as well as developmental processes (Paulus et al., 1993; El-Shabrawi et al. 2010; Hossain & Fujita, 2011). Imbalances in metabolic processes due to abiotic or biotic stresses or certain genetic defects may lead to increased accumulation of ROS and MG, forming a potential threat for plant growth and survival. Usually a dynamic balance has to be maintained between ROS and MG generation and scavenging in order to guarantee normal plant growth. The glyoxalase system and AsA- and GSH-based antioxidant systems play a central role in regulating ROS and MG levels in plants. It is now clearly evident that ROS regulate a complex signal transduction network within plant development and its response and adaptation to both biotic and abiotic stressors although signaling roles of MG in higher plants is scarce. Considerable progress has been made over the last few years in understanding how plants protect themselves against MG and ROS while several genes encoding the components of both MG and ROS detoxification systems have been cloned, characterized and used in the construction of transgenic lines. Although gene manipulation seems to be a sound approach to counteract oxidative or MG stress, attempts to improve stress tolerance, particularly by manipulation of a single antioxidant gene or either Gly I or Gly II genes, have seen limited success because of the need for a balanced interaction of protective enzymes and other metabolites of MG and ROS detoxification systems (Yadav et al., 2005b; Lee et al., 2007; Martret et al., 2011). Several recent studies using enzyme protectants (proline and betaine) or a signaling molecule (NO) also proved that simultaneous induction of different components of both MG and ROS detoxification pathways showed substantial tolerance to abiotic oxidative stress. Inhibition of one component of the glyoxalase system or ROS detoxification system strongly influence the activity of other enzymes or metabolites and thereby lead to deterioration of the system because both systems unite together through a multifunctional redox molecule (GSH). Therefore, it is important to clarify of the bottlenecks affecting the performance of both glyoxalase and ROS detoxification systems under various abiotic stresses in the future. Complete elucidation of MG metabolism by integration of proteomics and metabolomics, and dissecting its signaling roles by using model plant species would be worthwhile research to improve multiple abiotic stress tolerance. Pyramiding both H<sub>2</sub>O<sub>2</sub> and MG detoxifying genes in one genetic background and to study their consequence in stress response and tolerance will also be a fascinating future area of study. However, a major gap exists in our understanding about how plants sense MG and oxidative stress in different subcellular compartments and how this stress signal is transduced, thus activating large-scale and coordinated expression of different enzymes and metabolites of their detoxification pathways. A complete understanding of the interaction between ROS, MG and plant hormones and transcription factors (Sasaki-Sekimoto et al., 2005; Takatsume et al., 2006) and components of ROS and MG detoxification pathways in different subcellular

compartments will reveal more subtle regulatory roles of both detoxification systems in abiotic stress tolerance. In addition, identification of master regulators that control stress response activation will accelerate the process to improve and strengthen plant fitness to changing climates.

## 18. Acknowledgement

We thank Miss. Pukclai Piyatida, PhD student, Laboratory of Plant Biochemistry, Kagawa University, for artwork. We apologize to our colleagues whose primary work could not be cited due to space constraints.

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# Stomatal Responses to Drought Stress and Air Humidity

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## 1. Introduction

Water is one of the most important substances for both plant and animal survival. Plants require water for photosynthesis, nutrient uptake and transportation as well as cooling (Farooq et al., 2009). Plants are sessile organisms and in contrast to most animals they are unable to move when the environment becomes unfavorable. Accordingly, plants have to be able to respond and adapt to the local environmental changes. Since water is essential for plant survival, the ability to tolerate water stress is crucial.

To be able to grow plants need to take up water from the soil and CO<sub>2</sub> from the atmosphere and use it in photosynthesis. This is done by CO<sub>2</sub> uptake through the stomatal pore, where water is simultaneously transpired. Water transpiration drives the water uptake by the roots and transport through the xylem. When the stomata are open CO<sub>2</sub> is taken up while water is transpired. When the stomata are closed little CO<sub>2</sub> is taken up and the transpiration is lowered. By opening and closing the stomata plants can regulate the amount of water lost, by sacrificing CO<sub>2</sub> uptake, when the environmental conditions are unfavorable.

Water stress can be defined as reduced water availability; either by water scarcity (drought) or osmotic stress (high salt concentrations) or water logging; too much water. Water stress may reduce photosynthesis, respiration and ion uptake, change the metabolic and growth patterns in the plant and in severe cases result in plant death (Jaleel et al., 2009a). In nature water stress is common either for long or short periods of time, depending on the local climate. Most plants therefore have some adaptation or response to enhance the growth and survival rate during water stress and subsequent recovery.

In agriculture and horticulture drought stress is one of the major problems, causing major crop losses every year as well as loss of aesthetic value in ornamentals. In agriculture crop loss is due to reduced numbers of tillers, spikes and grains per plant and reduced grain weight (Farooq et al., 2009). With the global human population rapidly increasing, simultaneously as water scarcity increases, the loss of crop will be even more serious than before. The discovery and development of stress tolerant crops to avoid yield loss during water stress is therefore very important. In the greenhouse industry, energy saving for economic profit is important to be able, but it also affects the plants. To reduce the amount of energy needed for CO<sub>2</sub> and heating in the greenhouses, energy-efficient semi-closed

greenhouses can be used. In these greenhouses the ventilation is reduced to a minimum, which consequently results in increased relative air humidity inside. This increase in air humidity affects the plants in different ways and might result in plants that are less tolerant to water stress (Torre and Fjeld, 2001).

In this review different plant responses to water stress will be discussed, with most attention to drought and the role for abscisic acid (ABA) as a plant stress hormone. In addition, consequences of plant development under high relative air humidity, which reduces the plants ability to respond to water stress, will be discussed.

## 2. Plant responses to water stress

Plants growing in deserts or high salinity habitats are all exposed to more or less constant water stress. To survive such conditions plants have developed growth strategies such as increased water use efficiency with C<sub>4</sub>- or CAM metabolism (Keeley and Rundel, 2003), succulent growth and extensive root systems (Henry et al., 2011). These strategies are good in a dry environment, but in more “favourable” conditions at least some of these plants may, due to lower growth rates, more easily be outcompeted by other less drought tolerant plants. Other adaptations to plant life in dry environments are thick cuticula and wax layers, depressed stomata and high density of trichomes. Thick cuticula and wax layers reduce extra-stomatal transpiration, and depressed stomata and trichomes create a thicker boundary layer outside the stomata, where the humidity gradient is more gradual, thereby reducing the stomatal transpiration.

Plants living in saline environments (e.g. beaches, salt marches) commonly keep a low osmotic potential in their cells, which facilitates water uptake. They usually also have the ability to exclude or excrete salt from their cells to avoid too high salt concentrations. A variety of perennials commonly avoid water stress during the winter by entering dormancy and often shedding leaves (deciduous woody species) before the onset of the harsh conditions when water is unavailable due to frost. However, plants keeping the leaves on through the winter commonly face water stress in the spring when air temperatures are high while the soil is still frozen.

Even if they do not live in particularly dry places, most plants will occasionally encounter water stress for shorter or longer periods of time. Most of these plants do not have many of the adaptations of desert plants and must respond to the water stress in other ways. When these plants are exposed to water stress, such as drought or saline conditions, to survive they must be able to retain as much water as possible. If the plants are not able to cope with the water stress, they will not be able to survive. The sensitivity and response time to drought differs between different species and slow growing species have been found to be more sensitive (Aasamaa and Sober, 2011). Repeated drought encounters increases the sensitivity to environmental changes that induce stomatal closure, while the sensitivity to changes that induce stomatal opening is decreased (Aasamaa and Sober, 2011). In response to water stress plants have developed several different mechanisms that increase the desiccation tolerance and water retention. These responses can be divided into short term and long term responses (Figure 1).

### 2.1 Long term responses

During prolonged water stress plants must be able to survive with low water content and maintain a minimum amount of water, through water uptake and retention. To cope with



prolonged drought stress plants respond with energy demanding processes that alter the growth pattern, chemical content of the plants and the up or down regulation of genes.

### 2.1.1 Biochemical changes

When the water availability is reduced, plants change the biochemistry to be able to retain as much water as possible and take up whatever water they can. During water stress plants produce and accumulate compatible solutes such as sugars, polyols and amino acid to lower the osmotic potential in the cells to facilitate water absorption and retention (Xiong and Zhu, 2002). Some of the compatible solutes also contribute to maintaining the conformation of macromolecules by preventing misfolding or denaturation (Xiong and Zhu, 2002). Plants also produce higher levels of the plant stress hormone ABA during water stress and this affects their growth pattern and stress tolerance (details under growth changes and stomatal functioning).

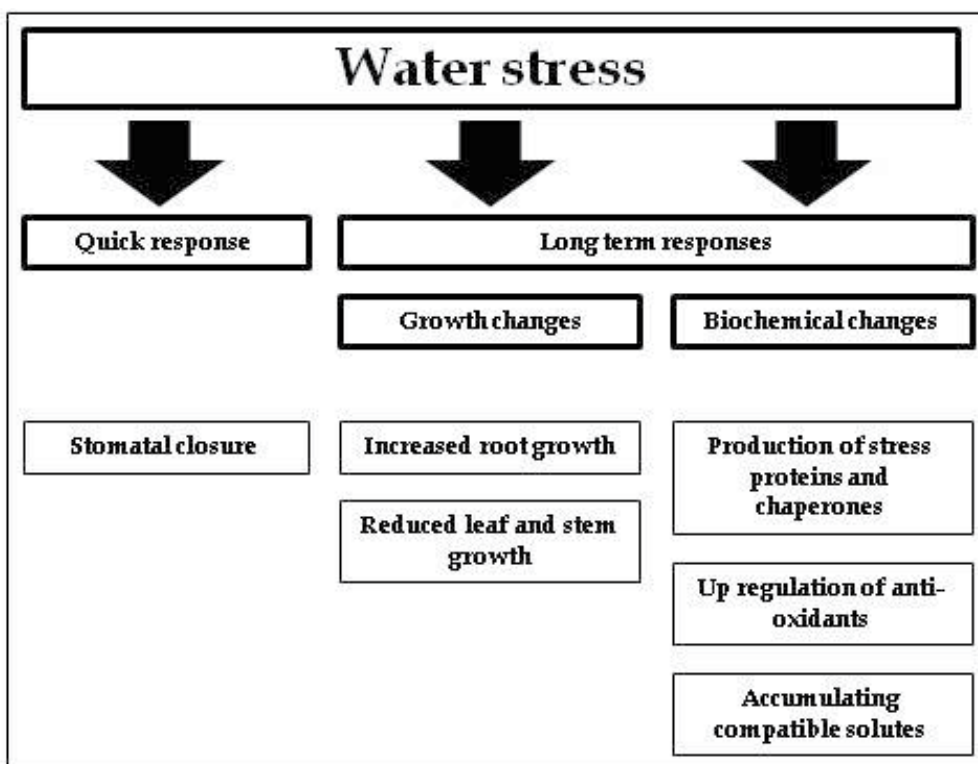


Fig. 1. Plant responses to water stress.

A group of proteins called late embryogenesis abundant like (LEA) proteins are also produced during water stress. These LEA-like proteins are highly hydrophilic, glycine-rich and highly soluble and have been found to be regulated by ABA (Xiong and Zhu, 2002). The LEA-like proteins are thought to act as chaperones, protecting enzymatic activities (Reyes et al., 2005) and preventing misfolding and denaturation of important proteins (Xiong and Zhu, 2002). Some of the LEA-like proteins have similar features as ribosomal proteins and are thought to interact with RNA (Garay-Arroyo et al., 2000).

Decreased transpiration and decreased CO<sub>2</sub> and nutrient uptake during water stress result in changes in metabolic pathways such as photosynthesis and respiration, as well as changes in ion uptake, transport and extrusion (Xiong and Zhu, 2002). Some of these changes can lead to oxidative damage. Reactive oxygen species, such as H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>, OH and OH<sub>2</sub>, are by-products in electron transport chains and have unpaired electrons that can attract electrons from other components. Reactive oxygen species can therefore cause damage to a variety of compounds such as DNA, RNA, proteins, lipids and chlorophyll and thus damage membranes and change cell metabolism and eventually lead to senescence. Many antioxidant systems, both enzymatic and non-enzymatic, are up-regulated in response to the increased reactive oxygen species levels during water stress. These antioxidants scavenge the reactive oxygen species and reduce the oxidative damage. The enzymatic antioxidants, such as superoxide dismutase, peroxidase, ascorbate peroxidase, catalase, polyphenol oxidase and glutathione reductase can detoxify reactive oxygen species (Prochazkova et al., 2001; Jaleel et al., 2009b). The non-enzymatic anti oxidants, including vitamins (A, C and E), glutathione, carotenoids and phenolic compounds, can scavenge reactive oxygen species by donating an electron or a hydrogen atom (Prochazkova et al., 2001; Jaleel et al., 2009b).

### 2.1.2 Growth changes

During water stress the water content of the plant decreases, which causes the cells to lose turgor pressure and shrink. The loss of turgor pressure in the cells inhibits turgor dependent activities such as cell expansion, which affects the growth of the whole plant. Some studies show that ABA can function as a signal to reduce leaf growth rate, both when ABA is applied exogenously or generated by water stress (Wilkinson and Davies, 2010). Reduced cell growth during water stress has e.g. been found to decrease the stem length in *Arabidopsis thaliana* soybean (*Glycine max*), potato (*Solanum tuberosum*), oca (*Abelmoschus esculentus*) and parsley (*Petroselinum crispum*) (Heuer and Nadler, 1995; Specht et al., 2001; Park et al., 2007; Petropoulos et al., 2008; Sankar et al., 2008). Similarly reduced cell enlargement reduces the leaf expansion in *Populus* (Ren et al., 2007). By reducing the leaf expansion the leaves become smaller and therefore transpire less. In some cases water stress can even lead to leaf abscission. This has e.g. been seen in *Populus* and paper birch (*Betula papyrifera*) (Giovannelli et al., 2007; Gu et al., 2007). The reduction of cell volume also concentrates the solutes in the cells and compresses the plasma membranes causing them to increase in thickness.

To increase water uptake and maintain a minimum osmotic pressure during drought many plants increase their root growth, either deeper or laterally. By increasing the root growth the area for water uptake becomes larger and water further away and deeper in the soil may be reached. This growth response has been found in e.g. maize, madagaskar periwinkle (*Catharanthus roseus*) and date palm (*Phoenix dactylifera*) (Djibril et al., 2005; Jaleel et al., 2008; Trachsel et al., 2010).

### 2.2 Short term response

When plants suddenly encounter drought it is important to respond as quickly as possible. A faster drought response means that less water is lost and the survival rate of the plants is increased. The most important quick response is stomatal closure. Stomata consist of two guard cells surrounding the stomatal pore. When the stomata are open water is transpired and CO<sub>2</sub> enter the leaf through the stomatal pore. During water stress the stomatal pore can be closed to reduce water loss. By closing the stomatal pore the

water use efficiency is increased (Farooq et al., 2009), reducing the amount of water lost per CO<sub>2</sub> molecule assimilated. Several mechanisms work together to close the stomata, such as hydro passive closure and chemical signals from the plant stress hormone ABA. Increased levels of ABA also causes increased hydraulic conductivity in the roots and xylem, enabling the plants to transport more water and thereby recover more rapidly after water stress (Kudoyarova et al., 2011).

### 3. Stomatal functioning

Development of stomata is often considered one of the most important developments in plant evolution (Brodribb and McAdam, 2011). By being environmentally controlled gateways into the plants controlling CO<sub>2</sub> uptake and transpiration they are central determinants of photosynthesis, cooling and nutrient uptake (Farooq et al., 2009). To be able to balance CO<sub>2</sub> uptake and water transpiration through stomatal movement is therefore an important response to changes in the environmental conditions. Low transpiration due to stomata closure means less cooling of the leaves and less uptake and transportation of nutrients.

#### 3.1 Stomatal signaling and movement

Stomatal closure occurs when the two guard cells surrounding the stomatal opening lose turgor pressure and close the opening (Outlaw, 2003). There are many signals that induce stomatal closure, among these the best known signal is probably ABA. In the signaling pathway towards stomatal closure there are several secondary messengers, such as Ca<sup>2+</sup>, H<sub>2</sub>O<sub>2</sub> and NO (Atkinson et al., 1990; Zhang et al., 2001; Neill et al., 2002; Garcia-Mata and Lamattina, 2009) that contribute to the stomatal closure. Passive loss of turgor pressure also results in stomatal closure.

Since stomatal closure has negative effects on CO<sub>2</sub> uptake, photosynthesis, transpirational cooling as well as water and nutrient uptake it is important to close the stomata only when the benefit of water retention outweighs the negative effects. To be able to close the stomata during unfavourable conditions there are several mechanisms and signalling pathways leading to stomatal closure. These pathways can be divided into hydro passive and active stomatal closure (Figure 2).

##### 3.1.1 Hydro passive stomatal closure

Hydro passive stomatal closure occurs when the water evaporation from the guard cells is too low to be balanced by water movement into these cells. The water content in the cells is then rapidly reduced to the extent where the osmotic pressure is reduced and the cells lose turgor pressure and shrink (Luan, 2002). When this happens the guard cells are unable to maintain the shape and the stomatal pore is covered.

Some studies have shown that passive stomatal closure is important in ferns and Lycopods, but not in Angiosperms and Gymnosperms (Franks and Farquhar, 2007; Brodribb and McAdam, 2011). This is because in Angiosperms and Gymnosperms the guard cells closely interact with their subsidiary cells. When the guard cells lose turgor pressure the subsidiary cells also lose turgor pressure and the force from the subsidiary cells pulls the guard cells apart, opening the stomata. This hydro passive opening is called the "wrong-way" response (Franks and Farquhar, 2007). In contrast the guard cells of ferns and Lycopods do not interact closely with their subsidiary cells.

The loss of turgor pressure in the subsidiary cells in these plants does therefore not result in the guard cells being pulled apart. The simultaneous loss of turgor in the guard cells will in these plants be enough to close the stomata.

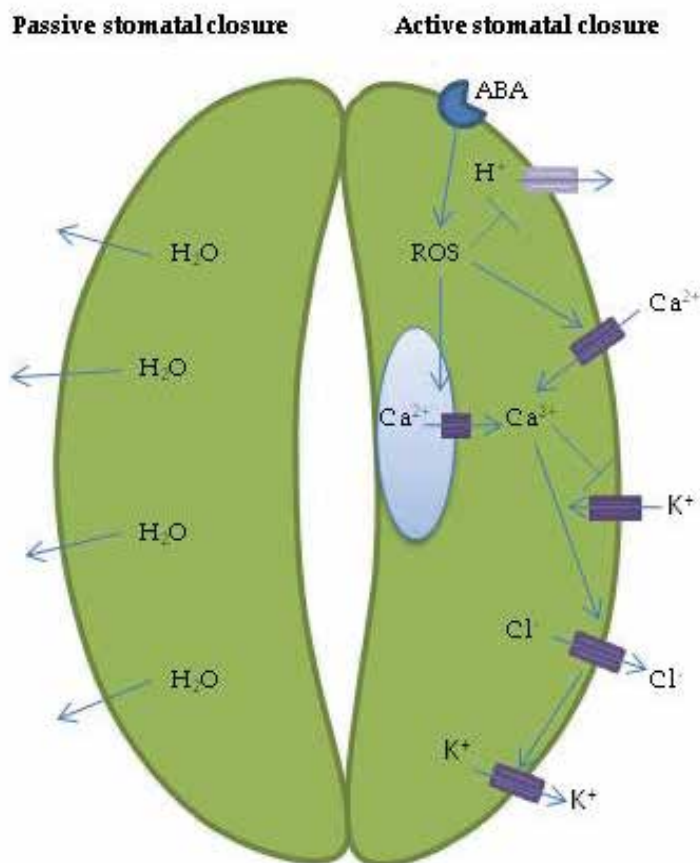


Fig. 2. Hydro passive and active stomatal closure pathways.

### 3.1.2 Active stomatal closure

ABA as well as elevated levels of CO<sub>2</sub> activates signalling pathways leading to stomatal closure (Kim et al., 2010). ABA is produced in the roots and leaves during water stress and is transported to the guard cells. ABA is transported into the guard cells by ATP-binding cassette (ABC) transporters that are located in the plasma membrane (Kang et al., 2010). When the ABC transporters are knocked out the ABA uptake is lower, stomata remain more open during drought and the stress tolerance is decreased (Kang et al., 2010). The ABA signals are first recognised by several receptors. PYR/PYL/RCAR (PYRABACTIN RESISTANCE/ PYRABACTIN RESISTANCE -LIKE/REGULATORY COMPONENT OF ABA RESPONSE) proteins have been shown to function as ABA receptors (Klingler et al., 2010). Another protein GCR2 (G protein coupled receptor) has also been shown to be a ABA receptor (Liu et al., 2007).

The size of the stomatal opening is regulated by the turgor pressure and cell volume of the guard cells (Schroeder et al., 2001; Kim et al., 2010). Regulation of stomatal opening is linked

to transport of ions and water through channel proteins across the plasma and vacuole membrane (Kim et al., 2010). ABA induces the production of reactive oxygen species (e.g.  $\text{H}_2\text{O}_2$ ), which in turn acts as a trigger for NO production, inhibition of membrane proton pumps and  $\text{Ca}^{2+}$  influx across both the plasma and vacuole membranes.  $\text{H}^+$ -ATPases that are hyperpolarizing the plasma membranes must be inhibited to induce ABA mediated stomatal closure (Merlot et al., 2007). The increased  $\text{Ca}^{2+}$  levels activate slow and rapid type anion channels, generating an anion efflux from the cells. The anion efflux depolarizes the membrane, which in turn causes  $\text{K}^+$  efflux through  $\text{K}^+$ <sub>out</sub> channels across both the vacuole and the plasma membrane. Simultaneously  $\text{Ca}^{2+}$  also inhibits  $\text{K}^+$ <sub>in</sub> channels (Wasilewska et al., 2008). Malate is also converted to starch reducing the osmotic potential and turgor pressure further (Kim et al., 2010). The plasma membrane is thus depolarised, the turgor pressure and cell volume reduced and the stomata close (Kim et al., 2010).

#### 4. ABA biosynthesis and metabolism

Increased content of ABA during water stress has been found in all photosynthetic organisms. The biosynthesis of ABA have previously been thought to occur only in the roots, but more recent studies show that ABA is also synthesized in mesophyll cells, vascular tissue and stomata. As stated above increased levels of ABA in leaves induces and regulates stomatal closure, while the increased levels of ABA in roots increase the hydraulic conductivity increasing the water uptake and transportation (Parent et al., 2009). The amount of ABA in the tissue is regulated in several metabolic steps, both in the biosynthesis and inactivation steps.

ABA is synthesized from phytoene (Figure 3), a carotenoid produced from pyruvate and glyceraldehydes-3-phosphate (Cutler and Krochko, 1999; Liotenberg et al., 1999). In the plastids phytoene is converted to  $\zeta$ -carotene by phytoene desaturase and then to  $\beta$ -carotene, lycopene and zeaxanthin. Zeaxanthin is converted first to antheraxanthin and then to violaxanthin by zeaxanthin epoxidase (ZEP). Violaxanthin is then converted to xanthoxin by 9-cis-epoxycarotenoid dioxygenase (NCED). Xanthoxin is then converted further in the cytosol. The main pathway from xanthoxin to ABA is through abscisic aldehyde. Xanthoxin is then converted to abscisic aldehyde by an enzyme related to a short-chain dehydrogenase/reductase (SDR). Abscisic aldehyde is further oxidized to ABA by abscisic aldehyde oxidase (AAO) (Seo and Koshiba, 2002). It has been found that genes regulating at least the last steps in the ABA biosynthesis (NCED and AAO) are the most important and are strongly up regulated during water stress, showing the important role of ABA as a rapid stress response (Qin and Zeevaart, 1999; Seo et al., 2000).

ABA is further regulated by several inactivation pathways (figure 3) (Cutler and Krochko, 1999). There are two main such pathways. The first is inactivation by oxidation. ABA is then oxidized to 8'-hydroxy ABA and subsequently to phaseic acid (PA) and 4'-dihydrophaseic acid (DPA). The conversion of ABA to 8'-hydroxy ABA is catalysed by the enzyme (+)-ABA 8'-hydroxylase (Kushiro et al., 2004) and the enzyme phaseic reductase catalyzes the conversion of PA to DPA (Cutler and Krochko, 1999). (+)-ABA 8'-hydroxylase is highly regulated by environmental factors, such as air humidity (Okamoto et al., 2009). The other inactivation pathway is by conjugation to ABA glucose ester, which is hypothesised to be a storage form of ABA (Cutler and Krochko, 1999). This conjugation is catalyzed by ABA glucosyltransferase (Lee et al., 2006). Several experiments provide evidence that ABA

glucose ester can be cleaved enzymatically by  $\beta$ -D-glucosidase (Dietz et al., 2000; Lee et al., 2006). The liberated ABA can then induce metabolic and changes and stomatal closure.

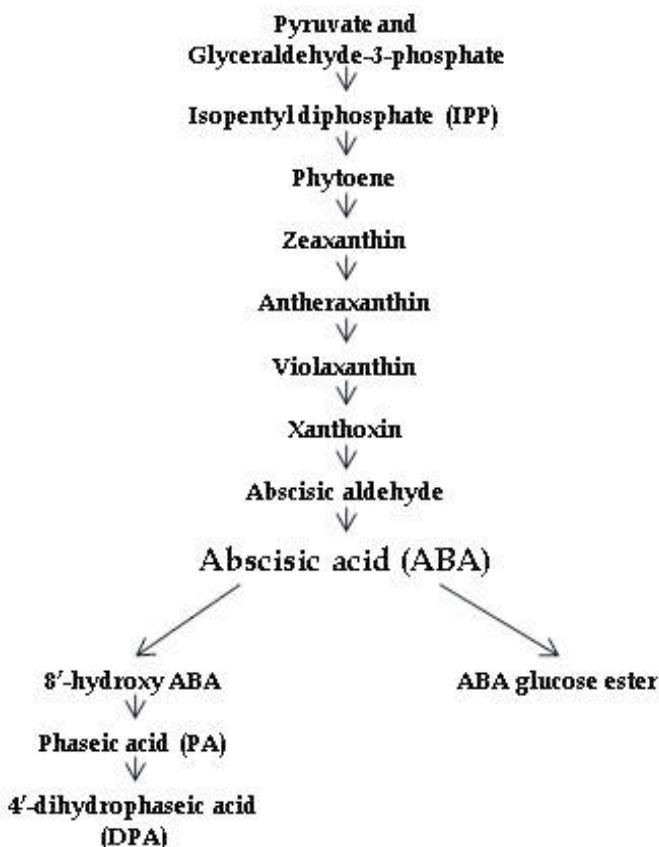


Fig. 3. Biosynthesis of ABA from pyruvate and glyceraldehydes-3-phosphate and ABA metabolism by oxidation to PA and DPA and conjugation to ABA glucose ester.

## 5. Stomatal development under high relative air humidity

Plants grown under high relative air humidity have malfunctioning stomata that are unable to close in response to darkness, ABA and desiccation (Fordham et al., 2001). This results in high stomatal conductance and frequent leaf drying. Also, plants grown *in vitro* under high relative air humidity have low ABA levels, but when moved to an *ex vitro* environment with lower relative air humidity the ABA levels increase (Hronkova et al., 2003). Furthermore, *Wrightia tomentosa* plants grown under high relative air humidity *in vitro*, had 29.4 % malformed stomata (Joshi et al., 2006). These stomata were described as large, spherical and wide open, lacking the ability to close. In comparison stomata of *in vivo* developed plants were smaller, elliptical and depressed. Other studies have shown similar results, where *in vitro* propagation has resulted in stomata that are unable to close in response to environmental and biochemical stimuli (Brainerd and Fuchigami, 1982; Santamaria et al., 1993; Sciutti and Morini, 1995).

The efficiency of stomatal openings for CO<sub>2</sub> uptake and water transpiration is not only determined by the size of the opening, but also by the number of stomata (Metwally et al., 1971). More stomata can take up more CO<sub>2</sub> and transpire more. In research done in different humidities it has also been found that the number of stomata per leaf increased with development in higher soil humidities, but when calculated as number of stomata per area the number decreased in higher humidities (Metwally et al., 1970; Metwally et al., 1971). The stomatal index, the number of stomata relative to the number of epidermal cells, was also found to increase with soil moisture (Schürmann, 1959). Similar experiments have been performed with air humidity, increased air humidity results in increased stomatal density (Sciutti and Morini, 1995). The stomatal density has been found to increase in plants with decreased ABA concentrations, which also have increased transpiration (Lake and Woodward, 2008). In *Vicia faba* drought and salinity stress has been found to increase the stomatal density and stomatal index, facilitating water uptake under water stressed conditions (Gan et al., 2010).

In the greenhouse industry the stomatal functioning and transpiration influences the post harvest quality of the plants. The value of ornamental plants is dependent on the aesthetic condition. Loss of aesthetic value can be due to water stress, where high transpiration rates shorten the shelf life. When ornamental plants are grown in large scale industries it is important to produce stress tolerant plants that have long shelf lives. In greenhouses there is an artificial environment, where the day length, temperature, relative air humidity (RH) and watering regimes are controlled to be able to produce as many plants as possible with as little cost as possible, without reducing the quality of the plants. This has resulted in energy-efficient greenhouses, which conserve energy (CO<sub>2</sub> and temperature) by rarely opening the ventilation. This consequently increases the relative humidity inside the greenhouses. Furthermore, much of the plant breeding is done in greenhouses, particularly when it comes to ornamentals.

Roses developed under high relative humidity (>85%) have 6-8 days shorter shelf life and greater water loss than plants grown under lower humidities (Mortensen and Fjeld, 1998; Torre and Fjeld, 2001). When roses are cultivated in high relative humidity environments in greenhouses they develop large, malfunctioning stomata, similar as the malfunctioning stomata produced under *in vitro* conditions (Torre and Fjeld, 2001; Torre et al., 2003). When these plants are moved to a dryer environment the stomata are unable to close, which results in high water loss and less stress tolerant plants that quickly lose their ornamental value (Torre and Fjeld, 2001).

The shorter shelf life of plants developed under high humidity is a major problem in the greenhouse industry. One of the important challenges is therefore to find new environmental regimes that save energy, but still produce high quality and stress tolerant plants. When plants grown in high relative humidity are treated with a 6 hour low humidity period in the middle of every day, the stomata remain functional (Mortensen et al., 2007; Pettersen et al., 2007). Similarly using 18 hour light period instead of 24 hours also result in more water retention and longer shelf life in roses (Mortensen et al., 2007).

Plants grown under constant high relative humidity contain less ABA than plants grown under lower relative humidities and some of the stomata of these plants are larger and malfunctioning (Nejad and van Meeteren, 2005, 2007). One of the main hypotheses explaining the malfunctioning stomata in high humidity is development with low ABA concentrations (Nejad and van Meeteren, 2007; Okamoto et al., 2009). If the plants developed under high relative humidities are treated with ABA during development, the

stomata respond as if they were developed under lower relative humidities (Nejad and van Meeteren, 2007). In plants moved from high humidity to lower humidities regained stomatal functioning in leaves that were still actively expanding, but not in fully developed leaves (Nejad and van Meeteren, 2008). Similarly if leaves developed under high relative humidity were given ABA application, the stomatal functioning was restored in young expanding leaves, but not in fully developed leaves (Nejad and van Meeteren, 2008). These experiments implicate that ABA is involved in the development of functioning and malfunctioning stomata, although there is also contradicting results. In *Arabidopsis thaliana* it has been shown that ABA-deficient and ABA-insensitive mutants responded similarly as wild type plants to changes in humidity (Assmann et al., 2000). Plants developed under low ABA conditions also have higher stomatal density (Lake and Woodward, 2008), indicating that ABA is important in both the development of stomata size and density.

ABA application in lower concentrations, applied to plants can reduce transpiration rate and increase the shelf life of *Salvia splendens* and a number of other ornamentals, by inducing stomatal closure (Pompodakis et al., 2004; Waterland et al., 2010a; Waterland et al., 2010b; Kim and van Iersel, 2011). On the other hand, application of high ABA concentration caused early leaf abscission in *Salvia* (Kim and van Iersel, 2011). Also, ABA application decreased the shelf life of miniature potted roses (Muller et al., 1999), possibly due to high concentrations.

## 6. Conclusion

The ability of plants to be able to regulate the size of the stomatal opening is a very important mechanism to control water loss and survive. This ability is especially important during water stress, when loss of water can have serious consequences for the plants. Water stress can cause reduced growth and in severe cases plant death. To minimize the negative effects of water stress the plants respond by changing their growth pattern, producing stress proteins and chaperones, up-regulation of anti-oxidants, accumulation of compatible solutes, increasing the amount of transporters involved in water and ion uptake and transport and by closing the stomata. If the plants are unable to quickly respond to water stress, by closing the stomata and thereby conserve as much water as possible, the consequences are more severe and plants wilt and die more quickly. This is a major problem in plant propagation of ornamentals. Plants developed under high relative air humidity develop malfunctioning stomata, which are unable to close in response to water stress. When these plants are later placed in dryer conditions they quickly lose their ornamental value and wilt. Treatments with ABA or periods of high temperature or low relative air humidity during development can offset this malfunctioning and produce functioning stomata, even in high humidity.

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## **Part 3**

### **Genetics and Adaptation**



## Plant Genes for Abiotic Stress

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### 1. Introduction

Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50%. Plants as sessile organisms are constantly exposed to changes in environmental conditions. When these changes are rapid and extreme, plants generally perceive them as stresses. However stresses are not necessarily a problem for plants because they have evolved effective mechanisms to avoid or reduce the possible damages.

The response to changes in environment can be rapid, depending on the type of stress and can involve either adaptation mechanisms, which allow them to survive the adverse conditions, or specific growth habitus to avoid stress conditions. In fact, plants can perceive abiotic stresses and elicit appropriate responses with altered metabolism, growth and development. The regulatory circuits include stress sensors, signalling pathways comprising a network of protein-protein interactions, transcription factors and promoters, and finally the output proteins or metabolites (table 1).

A number of abiotic stresses such as extreme temperatures, high light intensity, osmotic stresses, heavy metals and a number of herbicides and toxins lead to over production of reactive oxygen species (ROS) including H<sub>2</sub>O<sub>2</sub> causing extensive cellular damage and inhibition of photosynthesis.

Normally, ROS are rapidly removed by antioxidative mechanisms, but this removal can be impaired by stresses themselves (Allan & Fluhr, 2007), causing a rise in their intracellular concentration and an increase of the damage. To prevent or repair these damages, plant cells use a complex defence system, involving a number of antioxidative stress-related defence genes that, in turn, induce changes in the biochemical plant machinery. Studies have shown that ROS probably require additional molecules to transduce and amplify defence signals. ROS production and anti-oxidant processes, all act in a synergistic, additive or antagonistic way, related to the control of oxidative stress.

Responses to stress are not linear pathways, but are complex integrated circuits involving multiple pathways and in specific cellular compartments, tissues, and the interaction of additional cofactors and/or signalling molecules to coordinate a specified response to a given stimulus (Dombrowski, 2009). Onset of a stress triggers some (mostly unknown) initial sensors, which then activate cytoplasmic Ca<sup>2+</sup> and protein signalling pathways, leading to stress-responsive gene expression and physiological changes (Bressan et al., 1998;

Stress	Consequences	Plant Responses
Heat stress	High temperature lead to high evaporation and water deficit. The consequent increased turnover of enzymes leads to plant death.	Efficient protein repair systems and general protein stability support survival, temperature can lead to acclimation.
Chilling and cold stress	Biochemical reactions proceed at slower rate, photosynthesis proceeds, carbon dioxide fixation lags, leading to oxygen radical damage. Indeed, freezing lead to ice crystal formation that can disrupt cells membranes.	Cessation of growth in adaptable species may be overcome by changes in metabolism. Ice crystal formation can be prevented by osmolyte accumulation and synthesis of hydrophilic proteins.
Drought	Inability to water transport to leaves leads to photosynthesis declines.	Leaf rolling and other morphological adaptations. Stoma closure reduces evaporative transpiration induced by ABA. Accumulation of metabolites, consequently lower internal water potential and water attracting.
Flooding and submergence	Generates anoxic or microaerobic conditions interfering with mitochondrial respiration.	Development of cavities mostly in the roots that facilitate the exchange of oxygen and ethylene between shoot and root (aerenchyma).
Heavy metal accumulation and metal stress	In excess, detoxification reactions may be insufficient or storage capacity may exceeded.	Excess of metal ions may be countered by export or vacuolar deposition but metal ions may also generate oxygen radicals.
High light stress	Excess light can lead to increased production of highly reactive intermediates and by-products that can potentially cause photo-oxidative damage and inhibit photosynthesis.	Exposure of a plant to light exceeding what is utilized in photochemistry leads to inactivation of photosynthetic functions and the production of reactive oxygen species (ROS). The effects of these ROS can be the oxidation of lipids, proteins, and enzymes necessary for the proper functioning of the chloroplast and the cell as a whole.

Table 1. Consequences of abiotic stress and plant responses

Xiong et al., 2002). Also, accumulation of abscisic acid (ABA) plays an important role in abiotic stress signalling and transduction pathways, mediating many responses (Wasilewska et al., 2008).

It is well known that abiotic stresses in general, through regulation of both gene expression and protein turnover, alter the abundance of many transcripts and proteins (Wong et al., 2006; Yan et al., 2006; Jiang et al., 2007), indicating that transcriptional and post-transcriptional regulation play an essential role in the adaptation of cellular functions to the environmental changes.



Recent advances in molecular biology, genomics, proteomics and metabolomics have provided insight into plant gene regulatory network system, which is mainly composed of inducible-genes (environmental factors and developmental cues), expression programming and regulatory elements (*cis*-element and *trans*-element), corresponding biochemical pathways and diverse signal factors (Tang et al., 2003; Wang et al., 2003; Zhu, 2003; Munns, 2005). Genetic studies revealed that stress tolerance traits are mainly quantitative trait loci (QTLs), which make genetic selection of traits difficult.

Responses to abiotic stress require the production of important metabolic proteins such as those involved in synthesis of osmoprotectants and of regulatory proteins operating in the signal transduction pathways, such as kinases or transcriptional factors (TFs). In addition, new transcripts are made and within a few hours a steady level of stress adaptation has been reached. In general, the transcriptional regulation of genes is directly controlled by a network of TFs and transcription factor binding sites (TFBS) (Chaves & Oliveira, 2004). TFs are proteins with a DNA domain that binds to the *cis*-acting elements present in the promoter of a target gene. They induce (activators) or repress (repressors) the activity of the RNA polymerase, thus regulating gene expression. TFs can be grouped into families according to their DNA-binding domain (Riechmann et al., 2000). The presence or absence of transcription factors, activators and suppressors regulating transcription of target genes often involves a whole cascade of signalling events determined by tissue type, developmental stage or environmental condition (Wyrick & Young, 2002).

Environmental stress-inducible genes can be mainly divided into two groups in terms of their protein products: one type of genes, whose coding products directly confer to plant cells the resistance to environmental stress such as late embryogenesis abundant (LEA) protein, anti-freezing protein, osmotic regulatory protein, enzymes for synthesizing betaine, proline and other osmoregulators; the other groups of genes, whose coding products play an important role in regulating gene expression and signal transduction such as the transcriptional elements. At least four different regulons can be identified, two ABA independent (1 and 2) and two ABA dependent (3 and 4): (1) the CBF/DREB regulon; (2) the NAC (NAM, ATAF and CUC) and ZF-HD (zinc-finger homeodomain) regulon; (3) the AREB/ABF (ABA-responsive element-binding protein/ ABA-binding factor) regulon; and (4) the MYC (myelocytomatosis oncogene)/MYB (myeloblastosis oncogene) regulon.

Our knowledge of the molecular mechanisms underlying the responses of plants to such environmental stresses is still rather limited, but an increasing number of genes have been identified in recent years that mediate these responses. Some of these genes are induced by stress stimuli and encode products that confer tolerance to adverse conditions, whereas others encode upstream regulators that function within signalling pathways controlling the stress response.

The aim of this book chapter is to describe the regulation of gene expression under abiotic stresses and report recent advances in the stress-response mechanisms.

## 2. Abiotic stress-inducible genes

The complex plant response to abiotic stress involves many genes and biochemical-molecular mechanisms. The analyze of the functions of stress-inducible genes is an important tool not only to understand the molecular mechanisms of stress tolerance and the responses of higher plants, but also to improve the stress tolerance of crops by gene

manipulation. Hundreds of genes are thought to be involved in abiotic stress responses (Seki, 2003; Avni Öktem et al., 2008).

Many drought-inducible genes are also induced by salt stress and cold, which suggests the existence of similar mechanisms of stress responses.

These genes are classified into three major groups: (1) those that encode products that directly protect plant cells against stresses such as heat stress proteins (HSPs) or chaperones, LEA proteins, osmoprotectants, antifreeze proteins, detoxification enzymes and free-radical scavengers (Bray et al., 2000; Wang et al., 2000); (2) those that are involved in signalling cascades and in transcriptional control, such as Mitogen-activated protein kinase (MAPK), Calcium-dependent protein kinase (CDPK) (Ludwig et al., 2004) and SOS kinase (Zhu et al., 2001), phospholipases (Frank et al., 2000) and transcriptional factors (Cho et al., 2000; Shinozaki et al., 2000); (3) those that are involved in water and ion uptake and transport such as aquaporins and ion transporters (Blumwald et al., 2000).

### 3. Transcriptional factor genes involved in abiotic stress

Plant growth and productivity are under constant threat from environmental changes in the form of various stress factors. The most common abiotic stresses are drought, flooding or submergence, salinity, extreme temperatures (heat and freezing) and high light. Furthermore, the continued modification of the atmosphere by human activities lead to increase in the concentration of ozone in the troposphere and this can generate oxidative stress, which leads to the destruction of proteins and cells, premature ageing and reduced crop yields.

Tolerance or susceptibility to these abiotic stresses is a very complex phenomenon, both because stress may occur at multiple stages of plant development and more than one stress simultaneously affects the plant. Therefore, the perception of abiotic stresses and signal transduction to switch on adaptive responses are critical steps in determining the survival and reproduction of plants exposed to adverse environments (Chinnusamy et al., 2004).

During the past few years, transcriptome analysis has indicated that distinct environmental stresses induce similar responses. Overlap between stress responses can explain the phenomenon known as cross-tolerance, a capability to limit collateral damage inflicted by other stresses accompanying the primary stress.

Responses to abiotic stresses require the production of important metabolic proteins such as those involved in synthesis of osmoprotectants and regulatory proteins operating in signal transduction pathways, that are kinases or transcription factors (TFs). The regulation of these responses requires proteins operating in the signal transduction pathways, such as transcriptional factors, which regulate gene expression by binding to specific DNA sequences in the promoters of respective target genes. This type of transcriptional regulatory system is called regulon. At least four different regulons that are active in response to abiotic stresses have been identified. Dehydration-responsive element binding protein 1 (DREB1)/C-repeat binding factor (CBF) and DREB2 regulons function in abscisic acid (ABA)-independent gene expression, whereas the ABA-responsive element (ABRE) binding protein (AREB)/ABRE binding factor (ABF) regulon functions in ABA-dependent gene expression (Saibo et al., 2009). In addition to these major pathways, other regulons, including the NAC (or NAM, No Apical Meristem) and Myeloblastosis-Myelocytomatosis (MYB/MYC) regulons, are involved in abiotic stress-responsive gene expression (Fig. 1). Particularly, NAC- type TF OsNAC6 is induced by abiotic stresses, including cold, drought

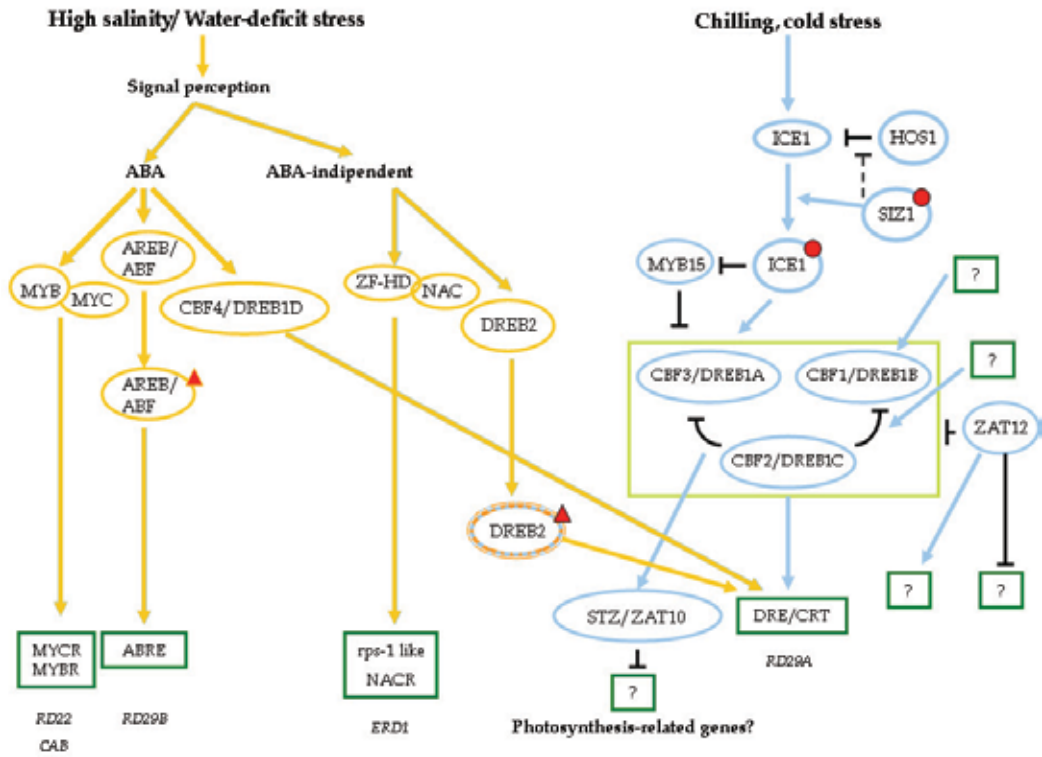


Fig. 1. Transcriptional network of abiotic stress responses.

and high salinity. Microarray analysis showed that many abiotic inducible genes were up regulated in rice plants over-expressing OsNAC6 (Nakashima et al., 2007).

TFs are powerful targets for genetic engineering in abiotic stress resistance in crop plants and many studies have been done in the last two decades on this topic.

Transcription factors are shown in ovals. Transcription factor-modifying enzymes are shown in circles. The small triangles correspond to post-translational modifications. Green squares with question marks represent putative MYC ICE1-like transcription factors that may activate CBF1/DREB1B and CBF2/DREB1C. The green boxes represent the *cis*-elements present in stress-responsive genes. The red dot corresponds to the sumoylation modification by SIZ1 of the ICE1 transcription factor. The dashed black line from SIZ1 to HOS1 represents competition for binding places on the ICE1 transcription factor. SIZ1 blocks the access of HOS1 to the ubiquitination sites on the ICE1. CBF4/DREB1D is a DRE *cis*-element binding factor that is ABA dependent.

#### 4. Drought stress transcriptional factors

The genome controls the regulation of the response to water deficit as well as the effectiveness of the response. Microarrays, largely performed using *Arabidopsis thaliana* as model plant, have been used to catalogue the many genes that are induced or repressed in

response to conditions that may lead to cellular water-deficit stress (Seki et al., 2002). These genes can be placed in at least four different functional groups: signal transduction, transcriptional regulation, cellular metabolism and transport and protection of cellular structures.

There are at least six different classes of TFs that participate in gene induction or repression in response to water deficit. Homeobox domain and NAC domain containing TFs are induced by multiple treatments that mimic water-deficit stress. Accumulation of proteins which have metabolic or structural functions promote adaptation to stress. One class of genes that could play a role in protection is called the late embryogenesis abundant (*Lea*) genes. The *Lea* genes are also developmentally programmed for expression in desiccating seeds. These genes encode small hydrophilic proteins that are predicted to protect proteins and membranes through chaperone-like functions. These proteins were thought to improve the performance of rice plants by protecting cell membranes from injury under abiotic stress (Chandra et al., 2004).

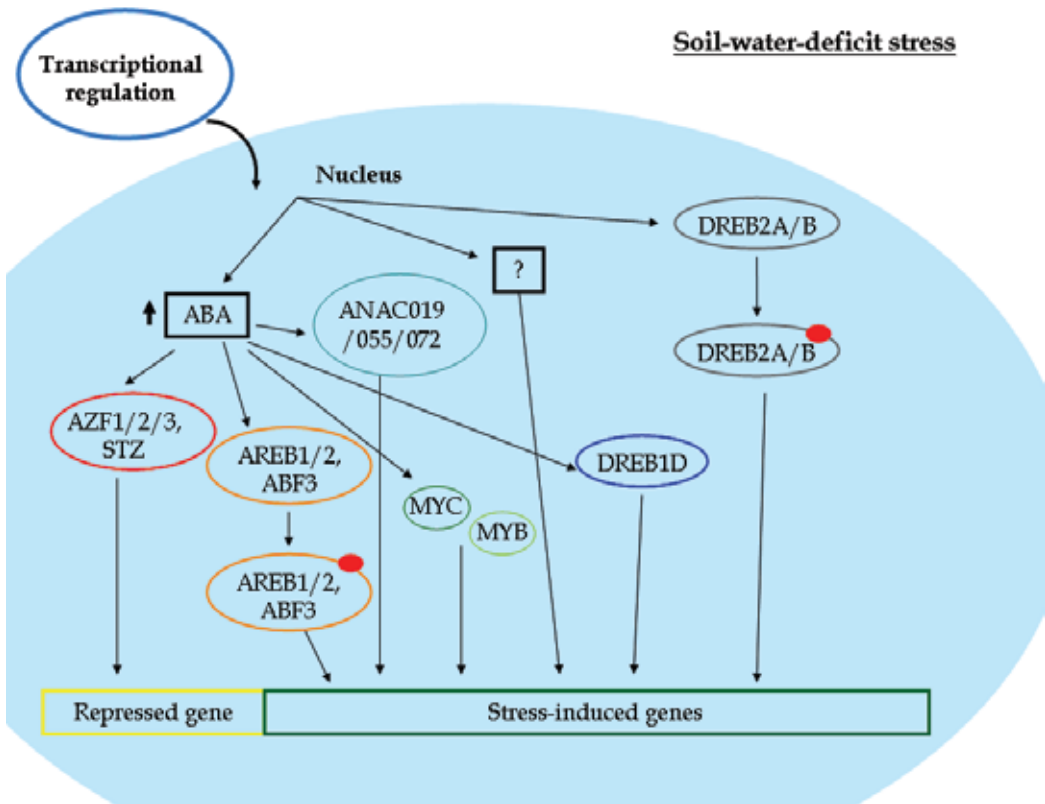
#### 4.1 Gene regulation and transcriptional factors in water deficit

A recent review (Shinozaki & Yamaguchi-Shinozaki, 2007) on analysis of gene expression during drought stress response in plants show and summarize the functions of some genes in both stress response and tolerance. Microarray analysis performed on wheat genome, showed that among 300 unique single expressed sequences tag (ESTs), the 30% of genes were significantly up-regulated and the 18% were down-regulated under drought stress (Way et al., 2005).

Potential functions of approximately 130 genes of *A. thaliana* up-regulated in water-deficit was reported by Bray (2002). These genes are involved in cellular response to drought stress by signalling events, detoxification and other functions. cDNA microarray analysis on 7000 Arabidopsis full-length cDNAs clarify relationship between rehydration-, proline- and water-treatment inducible genes. Among the 152 rehydration-inducible genes, 58 genes contained in their promoter regions the ACTCAT sequence involved in proline- and hypoosmolarity- inducible gene expression, suggesting that this motif is a major cis-activating element involved in rehydration-inducible gene expression (Oono et al., 2003).

Moreover, microarray analysis performed on two moderately drought-tolerant native Andean potato clones revealed that there was 1713 differentially expressed genes with 186 up-regulated involved in drought tolerance by inducing of osmotic adjustment, changes in carbohydrate metabolism, membrane modifications and cell rescue mechanisms, such as detoxification of oxygen radicals and protein stabilization (Schafleitner et al., 2007).

These recent study underline how the expression of genes in response to water deficit is complex and can be regulated at the transcriptional, post-transcriptional and translational levels. Two major transcriptional regulatory pathways of gene expression play an important role in response to water-deficit stress: the ABA-independent pathway and ABA-dependent pathway. The first is controlled largely by a family of TFs called dehydration response element binding protein (DREB), which contains a DNA binding motif originally identified in a flower patterning protein called APETALA2 (AP2) (Fig. 2), while transcription factor families known to be as the most responsive to ABA signalling under drought are NAC, AREB/ABF, and MYB.



The inset shows the different types of transcription factors involved in induction/repression of regulons.

Fig. 2. Classes of genes that are induced by water-deficit stress.

#### 4.1.1 ABA-independent pathway

DREB are important TFs which induce a set of abiotic stress-related genes and confer stress resistance to plants. The DREB TFs could be divided into two groups: DREB1, involved in signal transduction pathways under low temperature; DREB2, involved in signal transduction pathways under dehydration. They belong to the ethylene responsive element binding factors (ERF) family of TFs. ERF proteins are a sub-family of the AP2/ethylene responsive element binding protein (EREBP) TFs that is distinctive to plants. ERF proteins share a conserved 58–59 amino acid domain (the ERF domain) that binds to *cis*-elements, the GCC box, found in many pathogens related (PR) gene promoters conferring ethylene responsiveness (Gu et al., 2000), and to the C-repeat CRT/dehydration responsive element (DRE) motif involved in the expression of cold and dehydration responsive genes (Agarwal et al., 2006).

The DREB proteins contain an ERF/AP2DNA-binding domain quite conserved: amino acid alignment shows high sequence similarity in the nuclear localization signal at the N-terminal region and some similarity in the C-terminal acidic domain (Agarwal et al., 2006). Indeed, TFs containing ERF/AP2DNA-binding domain are widely found in many

plants such as *Arabidopsis* (Okamoto et al., 1997), tomato (Zhou et al., 1997), tobacco (Ohme-Takagi & Shinshi, 1995), rice (Sasaki et al., 1994; Weigel, 1995) and maize (Moose & Sisco, 1996).

Another ABA-independent pathway was identified after the observation that Early Responsive to Dehydration Stress 1 (ERD1) gene transcripts accumulated before any increase of ABA in response to dehydration and high salinity (Nakashima et al., 1997). Promoter analysis of ERD1 revealed TFs belonging to the NAC family and zinc finger homeodomain (ZF-HD) as essential to the activation of the ERD1 gene (Tran et al., 2007). The increased drought tolerance may be due both to the reduced transpiration rate (increased stomatal closure) and to an increased ABA sensitivity.

Many genes (e.g. Aquaporin, ERD10, ERD13 and ERF) already described as being involved in plant response to water stress are down-regulated in drought stress (Cominelli et al., 2005). A member of the *A. thaliana* family of R2R3-MYB TFs, AtMYB61, is also specifically expressed in guard cells in a consistent manner, being involved in the regulation of stomatal aperture (Liang et al., 2005).

The strong induction of Stress Responsive -NAC1 (SNAC1) gene expression by drought in guard cells suggests an effect in stomatal closure (Hu et al., 2006). It has been reported that modulation of transcription plays an important role in controlling guard cell activity. Recently two MYB-type TFs were identified as regulators of stomatal movements.

#### 4.1.2 ABA-dependent pathway

ABA-dependent gene induction during water deficit is controlled by at least five different classes of TFs. The ABA response element (ABRE) with the consensus ACGTGG/TC is bound by basic Leucine Zipper Domain (bZIP-type) TFs (Fig. 2). Three *Arabidopsis* bZIP TFs (AREB1/ABF2, AREB2/ABF4, and ABF3) are expressed in response to water-deficit stress and ABA treatment. Activation of the TFs requires ABA accumulation and the induction of an ABA-responsive protein kinase which activates the TF through phosphorylation.

Other TFs are also involved in ABA regulation of gene expression during cellular water deficit. Three genes encoding a class of TFs that is unique to plants, the NAC domain proteins ANAC019, ANAC055, and ANAC072 are induced by water deficit and ABA treatment. The NAC domain is a 60 bp DNA binding domain that is predicted to form a helix-turn-helix motif.

MYB, MYC and homeodomain TFs, and a family of transcriptional repressors (Cys2/His2-type zinc-finger proteins) are also involved in the ABA response to water deficit. Expression of the drought-inducible gene Responsive to Dehydration 22 (RD22) from *Arabidopsis* was found to be induced by ABA. The promoter region of RD22 contains MYC (CANNTG) and MYB (C/TAACNA/G) *cis*-element recognition sites. MYC and MYB TFs only accumulate after an increase of ABA concentration. Over-expression of these TFs result in enhanced sensitivity to ABA and drought tolerance (Abe et al., 2003).

### 5. Transcriptional factor involved in response to flooding stress

Flooding and submergence are two conditions that cannot be tolerated by most plants for periods of time longer than a few days. These stresses lead to anoxic conditions in the root system. At a critical oxygen pressure, mitochondrial respiration that provides the energy for growth in the photosynthetically inactive roots will decrease, then cease and the cells will die (Bray, 2004).

Recent reviews on gene expression analysis performed by microarray tools reported as the expression of several transcription factors, such as heat shock factors, ethylene response-binding proteins, MADS-box proteins, AP2 domain, leucine zipper, zinc finger and WRKY factors, increases in response to various regimes of oxygen deprivation in Arabidopsis and rice (Loreti et al., 2005; Lasanthi-Kudahettige et al., 2007).

Recently Licausi et al. (2010), using a qRT-PCR platform (Czechowski et al., 2002; Scheible et al., 2004; Morcuende et al., 2007; Osuna et al., 2007; Barrero et al., 2009), have identified TFs that are differentially expressed by hypoxic conditions. Among the TFs that have been characterized, members of the AP2/ERF-type family are the most commonly represented in the set of up-regulated TFs, followed by Zinc-finger and basic helix-loop-helix (bHLH-type) TFs, while TFs belonging to the bHLH family are the most commonly represented in the set of down-regulated TFs, together with members from the bZIP and MYB families.

*In silico* experiments and *trans*-activation assays shown that some TFs active in flooding stress are able to regulate the expression of hypoxia responsive genes. Particularly, five hypoxia-induced TFs (At4g29190; LBD41, At3g02550;HRE1, At1g72360; At1g69570; At5g66980) from different TF families [Zinc Finger, Ligand Binding Domain (LBD) or Lateral Organ Boundary Domain, ERF, DNA binding with one finger (DOF), ARF] showed this ability (Licausi et al., 2010).

Accumulation of ROS is a common consequence of biotic and abiotic stresses, including oxygen deprivation. There is evidence of redox-sensitive TFs, at least one of which might be involved in the adaptive response to low oxygen. ZAT12, a putative zinc finger-containing TF, is recognized as a component in the oxidative stress response signalling network of Arabidopsis (Rizhsky et al., 2004), promotes expression of other TFs and the upregulation of cytosolic ascorbate peroxidase 1, a key enzyme in the removal of H<sub>2</sub>O<sub>2</sub>.

Advances have been made in molecular analyses of cDNAs and genes involved in the anaerobic response. Huq and Hodges (2000) reported early activation of a rice (*Oryza sativa* L.) gene by anoxia, the *ai1* (anaerobically inducible early) gene. This gene encodes for a putative protein that shows short stretches of similarities to functionally interesting proteins (e. g. DNA binding proteins and nitric oxide synthase), indicating its putative involvement in signalling.

## 6. Salinity stress

High salinity is a critical environmental factor that inimically affects large areas of cultivated land. Plant growth, physiological and metabolic processes are affected, resulting in significant reductions in global crop productivity (Magome et al., 2008; Zhang et al., 2009). Exposure to high levels of NaCl not only affects plant water relations but also creates ionic stress in the form of cellular accumulation of Cl<sup>-</sup> and, in particular, Na<sup>+</sup> ions. Salt stress also changes the homeostasis of other ions such as Ca<sup>2+</sup>, K<sup>+</sup>, and NO<sup>3-</sup>.

Salt accumulation can modify plant cell plasma membrane lipid and protein composition, cause ion imbalance and hyperosmotic stress and eventually disturb normal growth and development (Fujii & Zhu 2009; López-Pérez et al., 2009).

In general, high NaCl concentrations affect plant physiology and metabolism at different levels (water deficit, ion toxicity, nutrient imbalance, and oxidative stress; Vinocur & Altman, 2005), and at least two main responses can be expected: a rapid protective response together with a long term adaptation response. During initial exposure to salinity, plants experience water stress, which in turn reduces leaf expansion. During long-term exposure to

salinity, plants experience ionic stress, which can lead to premature senescence of adult leaves, and thus a reduction in the photosynthetic area available to support continued growth (Cramer & Nowak, 1992).

Salt tolerance determinants are categorized either as effectors that directly modulate stress etiology or attenuate stress effects, or as regulatory molecules that are involved in stress perception, signal transduction, or modulation of effector function. Genomics studies are focused on gene expression analysis following exposure of plants to high salinity, using salt shock experiments to mimic stresses that affect hydration and ion homeostasis.

The stress-responsive genes can be classified into two classes, i.e. early and delayed response genes (Sairam & Tyagi, 2004). The former are induced quickly and transiently, while the latter are activated more slowly and their expression is sustained. The early response genes encode transcription factors that activate downstream delayed response genes (Zhu, 2002).

When microarray expression profiles of wild type plants, a T-DNA insertion knockout mutant of *AtNHX1* (*nhx1*), and a rescued line (*NHX1::nhx1*) exposed to both short (12 h and 48 h) and long (one and two weeks) durations of a non-lethal salt stress were investigated, 147 transcripts showed both salt responsiveness and a significant influence of *AtNHX1*. Fifty-seven of these genes showed differential regulation across all salt treatments, while the rest were regulated as a result of a particular duration.

A large number of genes from a variety of biochemical pathways participate in responses conferring salt tolerance. These pathways include notably those involved in: signal transduction; carbon metabolism and energy production; oxidative stress protection; uptake, exclusion, transport and compartmentalization of sodium ions; modifications of structural components of cell walls and membranes.

Several genes have been identified as functional components in the plant response to salt stress, including those encoding detoxifying enzymes like glutathione peroxidase (Roxas et al., 1997),  $\text{Na}^+/\text{H}^+$  antiporter *AtNHX1* (Apse et al., 1999), osmolytes such as glycine-betaine and LEA (late embryogenesis abundant protein) (Xu et al., 1996), flavoprotein *AtHAL3* (Espinosa-Ruiz et al., 1999), signal mediator  $\text{Ca}^{2+}$ /calmodulin-dependent protein phosphatase (Pardo et al., 1998) and transcription factor *Atfin1* (Bastola et al., 1998). Analyses of complete transcriptomes suggest that systems like synthesis of osmolytes and ion transporters and regulation of transcriptional and translational machineries have distinct roles in salt-stress response. In particular, induction of transcripts of specific TFs, RNA-binding proteins, ribosomal genes and translation initiation and elongation factors has been reported to be important during salt stress (Sahi et al., 2006).

Since not many stress-specific consensus sequences were identified in promoters of stress specific genes to activate or repress transcription, transcription factors must be located in the nucleus, bind DNA and interact with the basal transcription apparatus. Transcription factors involved in stress responses include DRE-related binding factors, leucine zipper DNA-binding proteins, putative zinc finger proteins, myb proteins, bZIP/HD-ZIPs, and AP2/EREBP (Chen et al., 2002; Seki et al., 2002), interact with promoters of osmotic-regulated genes (Abe et al., 1997; Liu et al., 1998; Hasegawa et al., 2000 a-b). Particularly, AP2/ERF domain proteins include the DREB or CBF proteins binding to dehydration response elements (DRE) or C-repeats. A major transcriptional regulatory system is represented by DRE/C-repeat promoter sequences in stress-activated genes and DREBs/CBF factors that control stress gene expression (Stockinger et al., 1997; Liu et al.,



1998). Several stress-inducible genes such as rd29A, Cor6.6, Cor15a and Kin1 are target genes of DREBs/CBFs in Arabidopsis and contain DRE/C-repeat sequences in their promoters.

Moreover, basic region leucine zipper (bZIP) proteins contain a DNA binding domain rich in basic residues that bind to an ACGT core sequence. One bZIP subfamily has been linked genetically to an ABA response: ABI5 and its homologs, the ABRE binding factors (ABFs/AREBs). ABRE binding factors (ABFs)/ABA-responsive element binding (AREBs) proteins respond at the transcriptional and post-transcriptional level to dehydration and salt stress (Choi et al., 2000; Uno et al., 2000).

Other regulatory intermediates that modulate plant salt stress responses include SOS3 (Ca<sup>2+</sup>-binding protein), SOS2 (Suc nonfermenting- like) kinase, Ca<sup>2+</sup>-dependent protein kinases, and mitogen-activated protein kinases (Halfter et al., 2000). Genetic and physiological data indicate that SOS3, SOS2, and SOS1 are components of a signal pathway that regulates ion homeostasis and salt tolerance and their functions are Ca<sup>2+</sup> dependent. In particular, SOS1, encoding a plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter, plays a critical role in sodium extrusion and in controlling long-distance Na<sup>+</sup> transport from the root to shoot (Liu & Zhu, 1998). This antiporter forms one component in a mechanism based on sensing of the salt stress that involves an increase of cytosolic [Ca<sup>2+</sup>] and reversible phosphorylation with SOS1 acting in concert with SOS2 and SOS3 (Shi et al., 2000). SOS2 encodes a Suc non-fermenting-like (SNF) kinase, and SOS3 encodes a Ca<sup>2+</sup>-binding protein with sequence similarity to the regulatory subunit of calcineurin and neuronal Ca<sup>2+</sup> sensors (Liu & Zhu, 1998; Liu et al., 2000). In yeast, co-expression of SOS1, SOS2, and SOS3 increases the salt tolerance of transformed yeast cells much more than expression of one or two SOS proteins (Shi et al., 2000), suggesting that the full activity of SOS1 depends on the SOS2/SOS3 complex.

Several studies have shown that reactive oxygen species (ROS) and oxidative stress may be mediating at least some of the toxic effects of NaCl on legumes (Jungklang et al., 2004) and other vascular plants (Attia et al., 2008). ROS are predominantly generated in the chloroplast by direct transfer of excitation energy from chlorophyll to produce singlet oxygen, or by univalent oxygen reduction at photosystem I, in the Mehler reaction (Allen, 1995) and to some extent in mitochondria. ROS have the potential to interact non-specifically with many cellular components, triggering peroxidative reactions and causing significant damage to proteins, lipids, and nucleic acids. To cope with ROS and to maintain redox homeostasis, living organisms evolved antioxidant defense systems, comprised of enzymatic and non-enzymatic components, which normally maintain ROS balance within the cell. Major nonenzymatic antioxidants include ascorbate (vitamin C) and glutathione in plants, although tocopherol (vitamin E), flavonoids, alkaloids, and carotenoids can also act as antioxidants.

Intracellular ROS can also influence the ROS induced MAPK signal pathway through inhibition of phosphatases or downstream transcription factors (Mittler et al., 2004) (Fig. 3).

## 7. Chilling and cold stress: Gene regulation and transcriptional factor

Cold stress prevents the expression of full genetic potential of plants owing to its direct inhibition of metabolic reactions and, indirectly, through cold-induced osmotic (chilling-induced inhibition of water uptake and freezing-induced cellular dehydration), oxidative and other stresses. Cold stress, which includes chilling (<20°C) and/or freezing (<0°C) temperatures, adversely affects the growth and development of plants. Chilling and freezing

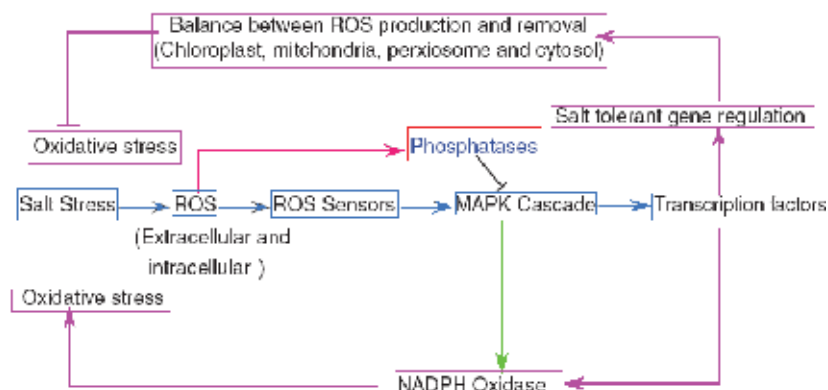


Fig. 3. ROS signal transduction pathway under salt stress.

are stresses that show different effects on plants: the first leads to slow biochemical reactions, such as enzyme and membrane transport activities; the second leads to ice crystal formation that can cause the disruption of cell membrane system (Chinnusamy et al., 2007).

A large number of studies have used a transcriptional profiling approach to identify genes in *Arabidopsis* that respond to cold (4°C) and chilling (13°C) temperatures. Results have shown that plants respond to low temperatures by altering mRNA levels of a large number of genes belonging to different and independent pathways. The quantitative and qualitative difference in transcriptional response to low temperature suggests the presence in higher plants of different molecular mechanisms to cold-stress response (Zhu & Provart, 2003).

The cold induction of genes involved in calcium signalling, lipid signalling or encoding receptor-like protein kinases are also affected by the *ice1* mutation (Lee et al., 2005).

Controlled proteolysis of transcriptional regulators also plays an important role in shaping the cold-responsive transcriptome in plants.

TFs that bind to the DRE/CRT are named DREB1/CTR-binding factor (CBF) and DREB2. Cold stress induces the expression of AP<sub>2</sub>/ERF family TFs, that is, CBFs, which can bind to *cis*-elements in the promoters of COR genes and activate their expression (Fig. 4). CBFs regulate the expression of genes involved in phosphoinositide metabolism, transcription, osmolyte biosynthesis, ROS detoxification, membrane transport, hormone metabolism and signalling and many others with known or presumed cellular protective functions (Fowler et al., 2002; Maruyama et al., 2004; Lee et al., 2005).

The first isolated cDNAs encoding DRE binding proteins were DREB1A and DREB2A (Liu et al., 1998) from *Arabidopsis* and then, DREB genes have been isolated from a wide variety of plants. In wheat and barley, a number of CBF homologs have been mapped to low temperature QTLs, *Fr-2* chromosomal region (Skinner et al., 2005; Vágújfalvi et al., 2005; Miller et al., 2006). Thus, it is clear that the DREB1/CBF regulon is ubiquitous within higher plants.

Expression of *DREB1* genes was extensively investigated in various crops with regard to different abiotic stresses. It was found that the expression of *AtDREB1* gene is induced by cold, but not by dehydration, or high salt stress (Liu et al., 1998; Shinwari et al., 1998). Similarly, CBF genes also showed high expression in response to low temperature treatment and its transcript was detectable after 30 min of exposure to 4°C, and showed maximum expression at 1 h (Medina et al., 1999). Indeed, CBF regulon could be sub-regulated by cold-responsive transcription factor genes RAP2.1 and RAP2.7 as shown by microarray analysis of transgenic *Arabidopsis* plants ectopically expressing CBFs (Fowler et al., 2002).

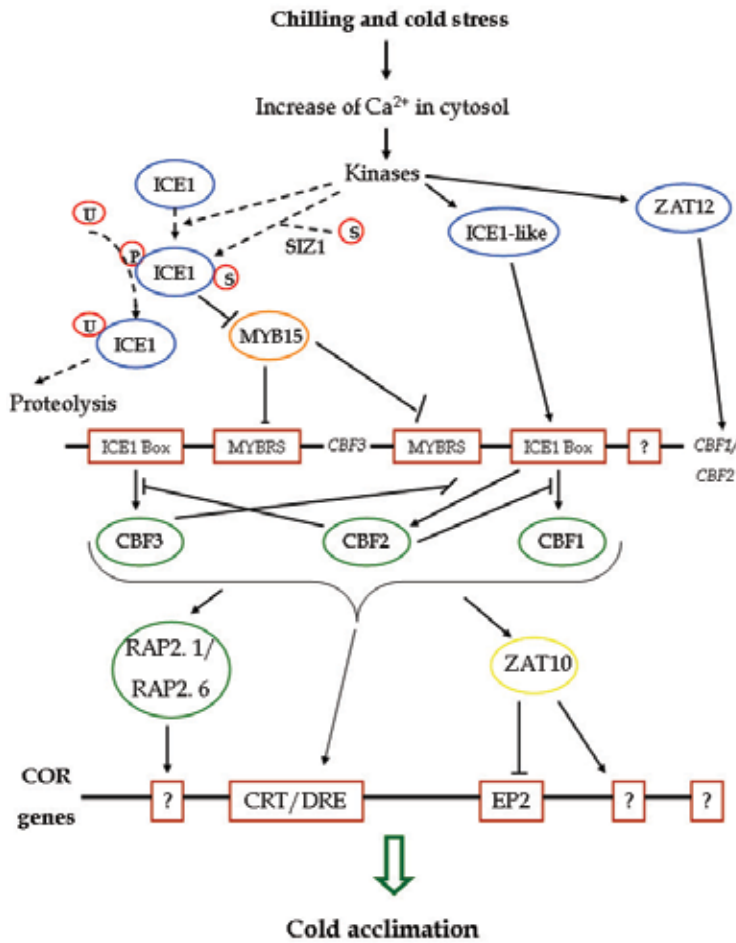


Fig. 4. Cold-responsive transcriptional network in Arabidopsis. CBFs regulate the expression of COR genes that confer cold tolerance. CBFs might cross-regulate the each other's transcription. CBFs induce the expression of ZAT10 which might downregulate the expression of COR genes. Constitutive expressed ICE1 is activated through sumoylation and phosphorylation induced by cold stress. ICE1 activated induce the transcription of CBFs and repress MYB15. The expression of CBFs is negatively regulated by MYB15 and ZAT12. HOS1 mediates the ubiquitination and proteolysis of ICE1, thus negatively regulates CBF regulons. Lines ending with bar indicate negative regulation; question mark (?) indicate unknown *cis*-elements; broken arrows indicate post-translational regulation; solid arrows indicate activation; lines ending with bar indicate negative regulation.

In Arabidopsis, ICE1 (Inducer of CBF Expression1), a MYC-type bHLH TF, can bind to MYC recognition elements in the CBF3 promoter and is important for the expression of CBF3 during cold acclimation. ICE1 is constitutively expressed and localized in the nucleus, but it induces expression of CBFs only under cold stress (Fig. 4). This suggests that cold stress-

induced post-translational modification is necessary for ICE1 to activate downstream genes in plants (Chinnusamy et al., 2003).

Two important post-translational protein modifications are the ubiquitination and the sumoylation. Ubiquitination is mediated by High Expression of Osmotically Responsive1 (HOS1). For *HOS1* encodes for a RING finger ubiquitin E3 ligase that physically interacts with ICE1 and mediates the ubiquitination of ICE1 to regulate negatively the expression of ICE1 target genes (Fig. 4) and is thus critical for the de-sensitization of plant cells to cold stress (Dong et al. 2006). Sumoylation is induced by SUMO (Small Ubiquitin-related Modifier) proteins that are conjugated to proteins substrates in a process dependent on SUMO E3 ligases. Sumoylation might protect target proteins from proteasomal degradation preventing the ubiquitination (Ulrich, 2005).

## 8. Heavy metal accumulation and metal stress

Uptake of excess metal ions is toxic to most plants. Phytotoxicity of heavy metals can be attributed to symplastic accumulation of heavy metals, particularly in the plasmatic compartments of the cells, such as the cytosol and chloroplast stroma (Brune et al., 1995). Metal-induced changes in development are the result of either a direct and immediate impairment of metabolism (Van Assche & Clijsters, 1990) or signalling processes that initiate adaptive or toxicity responses that need to be considered as active processes of the organism (Jonak et al., 2004). The detoxification of heavy metals by plants is achieved by uptake and translocation, sequestration into the vacuole and metabolization, including oxidation, reduction or hydrolysis and conjugation with glucose, glytanyl cysteine syntase (GSH) or amino acids (Salt et al., 1998; Meagher, 2000; Dietz & Schnoor, 2001).

So, in order to determine genes involved in response to heavy metal, recently, several studies, based on use of *A. thaliana* as model plant, performed the analysis of global gene expression after exposure to salts of lead (Pb) and cadmium (Cd). The analysis revealed 65 and 338 up- and down-regulated genes by Cd and 19 and 76 by Pb (Kovalchuk et al., 2005). Particularly, it was found that ABC transporters were differentially regulated after Cd treatments, suggesting for some plant ABC transponders a key role in glutathione-Cd or phytochelatin-Cd complex transport both into cellular compartments and outside of the cell (Bovet et al., 2005).

Subsequently studies performed on *Arabidopsis*, using microarray tools, demonstrated that exist a complex regulatory network which differentially modulates gene expression in a tissue-specific manner. Responses observed in roots included the induction of genes involved in sulphur assimilation-reduction and glutathione metabolism. Therefore, it was suggested that plants activate the sulphur assimilation pathway by increasing transcription of related genes to provide an enhanced supply of glutathione for phytochelatin biosynthesis (Fig. 5).

Non specific defense mechanisms include accumulation of osmolytes, antioxidants, aminoacids and changes in hormonal balances.

The significance of glutathione and the metal-induced phytochelatin (PCs) in heavy metal tolerance has been summarized intensely in excellent reviews (Rauser, 1995, 1999; Hall, 2002). Depletion of glutathione appears to be a major mechanism in short-term heavy metal toxicity and in accordance with this hypothesis, a good correlation between glutathione contents and tolerance index was observed with 10 pea genotypes differing in Cd sensitivity (Metwally et al., 2005).

In roots, after Cd exposure, three categories of genes were identified from transcriptome analysis: (1) common responses conserved across species; (2) metallophyte-specific responses representing candidate genes for Cd hypertolerance; (3) specific responses to Cd (Weber et al., 2006).

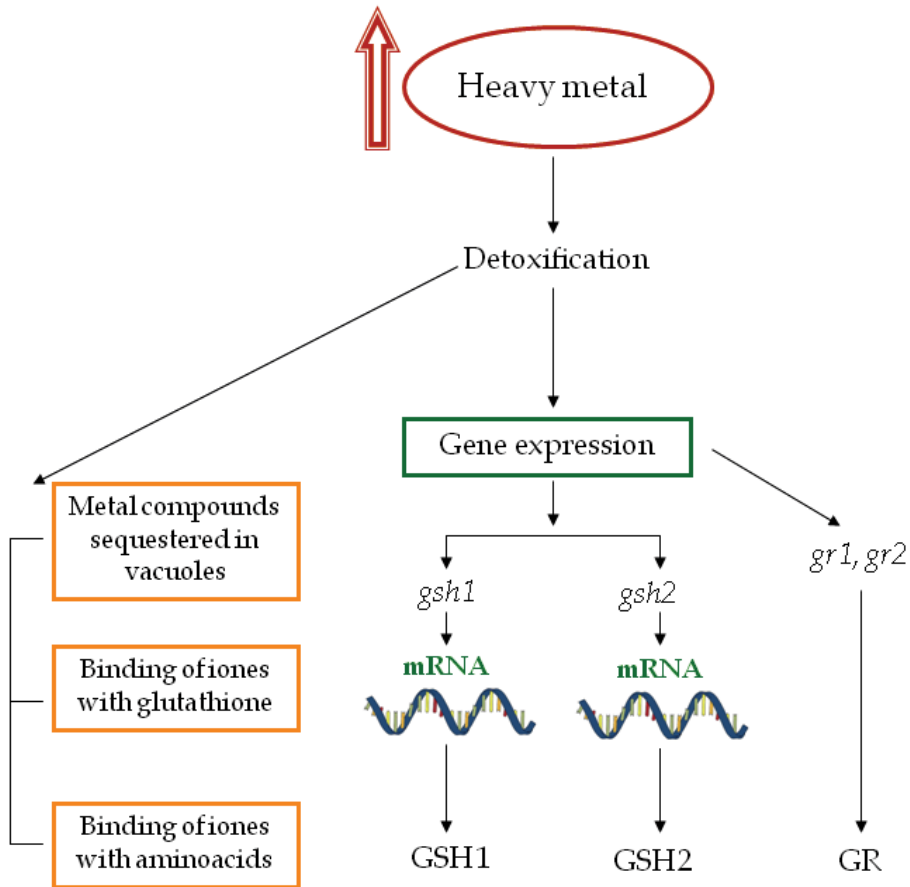


Fig. 5. Response of plant cell to toxic levels of heavy metals. The synthesis of phytochelatin (PCs) accompanies with decrease in cell glutathione pool and increase in the activities of glutathione synthetase (GSH1), glutathione synthetase (GSH2) and glutathione reductase (GR). The elevated activities of GSH1, GSH2 and GR is correlated with enhanced expression of corresponding genes *gsh1*, *gsh2*, *gr1* and *gr2*.

In leaves, instead, was reported an early induction of several genes encoding enzymes involved in the biosynthesis of phenylpropanoids (Herbette et al., 2006).

## 9. High light stress

Light plays a critical role in regulating plant growth and development through the modulation of expression levels of light-responsive genes that regulate developmental and metabolic processes. Light signals are perceived through at least four distinct families

of photoreceptors, which include phytochromes (Phy), cryptochromes, phototropins and unidentified ultraviolet B (UVB) photoreceptor(s). For each developmental response, more than one photoreceptor can contribute to the perception of light signals, indicating that signal integration points for different light signals must exist in transcriptional hierarchies. Light can modulate photoreceptor activity by inducing changes that alter their cellular localization. The best characterized light receptor is Phy, which exists in two photochemically interconvertible forms, Pr and Pfr, and is encoded by a small family of genes in angiosperms. Phytochromes are synthesized in the inactive Pr form, that absorbs red light, (660 nm), and are activated on light absorption by conversion to the biologically active Pfr form, that absorbs far-red light (730 nm). The photoconversion of phytochromes results in their translocation from the cytoplasm into the nucleus, which is crucial for allowing them to interact with transducers in initiating downstream transcriptional cascades (Quail, 2002).

The responses of plants to light are complex: seed germination, seedlings photomorphogenesis, chloroplast development and orientation, photodinesis, stem growth, pigment biosynthesis, flowering and senescence (Kendrick & Kronenberg, 1994). Collectively these processes are known as photomorphogenesis.

Besides excess light, a range of abiotic environmental conditions such as O<sub>3</sub>, salt, toxic metals, and temperature can induce increased production of ROS by limiting the ability of a plant to utilize light energy through photosynthesis (Shinozaki & Yamaguchi-Shinozaki, 2000). Exposure of a plant to light exceeding what is utilized in photochemistry leads to inactivation of photosynthetic functions and the production of reactive oxygen species (ROS) such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide (O<sub>2</sub><sup>-</sup>), hydroxyl radicals, and singlet oxygen (<sup>1</sup>O<sub>2</sub>; Niyogi, 1999). Indeed, high light drove change in the redox potential of plastoquinone (PQ) regulating the expression of two cytosolic peroxidases during HL stress (Karpinski et al., 1999). Furthermore, the redox state of PQ has been shown to be involved in the expression of chloroplast encoded genes (Pfannschmidt et al., 1999).

Classical genetic and molecular approaches have identified various regulators downstream of photoreceptors. Many of these encode TFs, as well as kinases, phosphatases and degradation-pathway proteins. Although some of these regulators are specific for light quality, others regulate signal transduction networks in response to various light signals, representing potential signal integration points. Several basic post-translational mechanisms are involved in regulating TF activities and the subcellular localization in response to light. The phosphorylation of TFs is a common modification that can influence their ability to bind to promoters. For example, the level of G-Box Binding Factor 1 (GBF1) is constant, but its affinity for the G-box is modulated by its phosphorylation status: its phosphorylation by nuclear Casein Kinase II (CKII) enables G-box binding (Klimczak et al., 1995).

In the dark, some TFs that positively regulate gene expression in response to light, such as Long After Farred Light 1 (LAF1), are ubiquitinated by Constitutive Photomorphogenic 1 (COP1), a ring-finger-type ubiquitin E3 ligase. In darkness, COP1 acts as E3 ligase in the nucleus, targeting TFs like Long Hypocotyl5 (HY5) and LAF1 to degradation via the 26S proteasome. Upon exposure to light, COP1 migrates from the nucleus to the cytosol. The study by Ulm and coworkers (2004) established that HY5, a bZIP transcription factor that is one of the key regulators of cryptochrome and phytochrome controlled photomorphogenesis, is an important component of the UVB-induced signalling network. UVB promotes rapid transcriptional activation of HY5 (and its interacting partner Long

Hypocotyl5-Like [HYH]) independently of all known photoreceptors, and loss of HY5 results in the impairment of the transcriptional induction of a subset of UVB-responsive genes. Taken together, these observations demonstrate that UVB up-regulates HY5 transcription by yet-unknown signalling pathway (s), and that the signalling cascades that mediate responses to visible light and long-wavelength UVB (300–320 nm) use shared components. Additional studies suggested that HY5 also regulates the transcription of several photosynthesis-related genes, such as the ribulose biphosphate carboxylase small subunit (RbcS1A) (Lee et al., 2007). Given that HY5 appears to regulate the expression of several Arabidopsis genes known to respond to abiotic stress conditions (e.g. CBF1, DREB2A, RD20 and MYB59) (Lee et al., 2007), it is inferred that HY5 could also be involved in the regulation of photosynthesis by adverse environmental conditions.

In vitro analysis showed that HY5 directly binds to the promoters of several light-inducible genes (Hiltbrunner et al., 2006) and a recent chromatin immuno-precipitation analysis in combination with a whole-genome tiling microarray revealed that HY5 binds directly to a large number of genomic sites, mainly at the promoter regions of annotated genes. HY5 interacts specifically with the G-box (CACGTG) and is required for normal control by light of promoters bearing this sequence (Lee et al., 2007).

Recently, some review showed as DNA *cis*-elements responsible for light regulated transcription are located within 5' upstream sequences.

The evolution of regulatory sequences, which determine where, when, and the level at which genes are transcribed, has been largely neglected. In the case of the photosynthesis-associated nuclear genes (PhANGs) from higher plants, interesting evolutionary aspects of the molecular mechanisms by which transcription is activated by light receptors (e.g. phytochrome) could be addressed through the comparative analysis of promoter sequences. For instance, why does light profoundly affect transcription of PhANGs in monocotyledonous and dicotyledonous plants, while PhANG promoters in conifers, ferns, and mosses are either light insensitive or, at most, weakly photoresponsive (Mukai et al., 1992).

Light-responsive Transcription Factors (TFs) have been identified through screens for light-responsive *cis*-element (LRE)-binding proteins and through genetic analyses of mutants that are deficient in their response to specific types of light. A combination of various methods has been used to identify these LREs. Such analyses have been successfully performed in identifying *cis*-acting elements involved in the light responsiveness of PhANGs, such as the G-box and I-box elements from *rbcS* genes (Giuliano et al., 1988) and the GATA motifs of *Lhcb1* genes (Gidoni et al., 1989; Millar et al., 1994).

Although many LREs and their binding proteins have been identified, no single element is found in all light-regulated promoters, suggesting a complex light-regulation network and a lack of a universal switch (Jiao et al., 2007). Sequence heterogeneity of regulatory elements may be functionally overcome if multiprotein regulatory complexes facilitate binding to imperfect target sites (Miner et al. 1991). The individual elements found within a multipartite *cis*-regulatory region are termed phylogenetic footprints (PFs); they share high conservation over a segment of 6 contiguous base pairs in alignments of orthologous upstream sequences and represent potential binding sites for transcription factors (Gumucio et al., 1993).

The “phylogenetic-structural method” is based on the search of “homologous” (rather than “similar”) DNA sequences of a functionally characterized promoter. Two sequences are homologous when they share common ancestry, regardless of the degree of similarity between them (Doolittle et al., 1987).

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# Plant Metabolomics: A Characterisation of Plant Responses to Abiotic Stresses

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## 1. Introduction

As with all organisms, plants thrive within a range of environmental conditions that are optimal for their growth and development. They must, however, respond and adapt to conditions that deviate from the optimal, such as low/high temperature, dehydration, high salinity, oxidative stress, heavy metals and nutrient deficiency; these deviations are often responsible for losses in productivity and for spatial (geographical) and temporal (growing season) limitations in the cultivation of crops.

Although plants and animals share some responsive mechanisms to unfavourable environmental conditions, plants, as sessile organisms, have developed highly sophisticated and efficient strategies of response.

Because of the great interest for both basic and applied research, many scientific endeavours have long addressed the understanding of the mechanisms underlying the stress response and the identification of the specific genes/metabolites that are responsible for tolerance phenotypes. In recent years, the “omics” approaches have allowed high-throughput analyses of the changes that are induced by environmental stresses, confirming data previously obtained with targeted analysis and extending the scope of investigation.

It is noteworthy that the metabolomic changes that have been observed in plants subjected to stress conditions depend on different causes; therefore, they have different significance and are expected to differently correlate with tolerance/sensitivity phenotypes. Namely, changes in the metabolome composition due to adverse environmental conditions may depend on i) the stability and catalytic activity of enzymes involved in the production/degradation of specific metabolites, ii) the production of abnormal compounds (or abnormal concentrations of normal compounds) as a result of cell damage, iii) the adjustment of concentration of some metabolites to restore homeostasis and normal metabolic fluxes and iv) the synthesis and/or accumulation of compounds involved in mediating tolerance mechanisms.

The main goal of studying metabolic changes during stress responses is to identify metabolites belonging to the (iii) and (iv) groups that are responsible for stress tolerance.

Upon exposure to osmotic stress as a result of low temperature, drought and high salinity, plants accumulate a range of osmolytes with the primary function of turgor maintenance.

The solutes accumulated vary among species and include sugars (i.e., sucrose, glucose, fructose and trehalose), polyols, betaines and amino acids, such as proline (Shulaev et al., 2008; Smirnov, 1998). Many compounds are known to play a role as osmoprotectants, acting as low molecular weight chaperones, stabilising the photosystem II complex, protecting the structure of enzymes and proteins, maintaining membrane integrity and scavenging the reactive oxygen species (ROS). Examples of these molecules are glycine betaine, proline and mannitol (Chen & Murata, 2008; Szabados & Savourè, 2010). Other compounds act as chelating agents (sequestering toxic metals and ions), redesigners of lipids (optimising the structure and fluidity in membranes), energy sources and/or signalling molecules (Alcázar et al., 2010; Valluru & Van den Ende, 2008).

Although the involvement in tolerance phenotypes for some metabolites is inferred on the basis of their increase under stress and of their physico-chemical and biological properties, it may be very difficult to assign a specific function.

For some compounds, such as proline and glycine betaine, the exogenous application of the molecule or the enhancement of their biosynthesis through the ectopic expression of a rate-limiting gene has resulted in a stress tolerance improvement (Chen & Murata, 2008; Kishor et al., 1995; Quan et al., 2004; Szabados & Savourè, 2010). Moreover, another transgenic approach has included the overexpression of transcription factors involved in stress-specific gene regulation, such as DREB or MYB factors, in particular, those regulating the synthesis of osmoprotectants (Gosal et al., 2009). However, even if genetic engineering offers a good tool for a substantial improvement in a desired trait within a short time, it must be considered that most of the transgenic lines obtained thus far have not been field-tested (Ashraf, 2010).

The possibility of monitoring a complete set of metabolites could largely improve the understanding of the adaptation mechanisms. This systematic study defined “metabolomics” is intended to provide an integrated view of the functional status of an organism, significantly contributing to the study of stress biology in plants. Depending on the question addressed, specific approaches or their combination can be used in metabolomic investigations: metabolic fingerprinting, metabolic profiling and targeted analysis. A variety of analytical techniques, such as GC-MS, LC-DAD-MS, FT-IR and NMR, are successfully employed for metabolic fingerprinting and profiling, whereas targeted analysis is performed using both the above-mentioned techniques (integrated with the use of spiking experiments or *in vivo* labelling) and the more traditional biochemical analyses. The huge volumes of data generated by these approaches require advanced multivariate statistical analysis (supervised or unsupervised) to increase the knowledge base. Moreover, in the last years, metabolomics data handling has been improved because of the development of publicly available bioinformatic tools and databases.

Here, we review the recent progress in this field, highlighting the advantages and limitations of the above-mentioned approaches and techniques. We will focus on metabolite changes induced by abiotic stresses and discuss the meaning of specific and non-specific responses to different stresses. Moreover, a comparison of metabolite profiling among species and/or cultivars differing in their stress tolerance, as well as the metabolic content of wild type plants versus mutants or transgenics, will be reported to highlight qualitative and/or quantitative differences correlating with the phenotypes.

The differences in the metabolite content can also represent good predictors for stress tolerance phenotypes both in screening of varieties and in plant breeding programs.

Finally, we will discuss the potentiality of the global analysis of data obtained with different “omics” approaches, such as an integrated metabolome, transcriptome and proteome analysis, as a valuable strategy to attain a holistic view of mechanisms sustaining stress tolerance in plants.

## 2. Analytical techniques

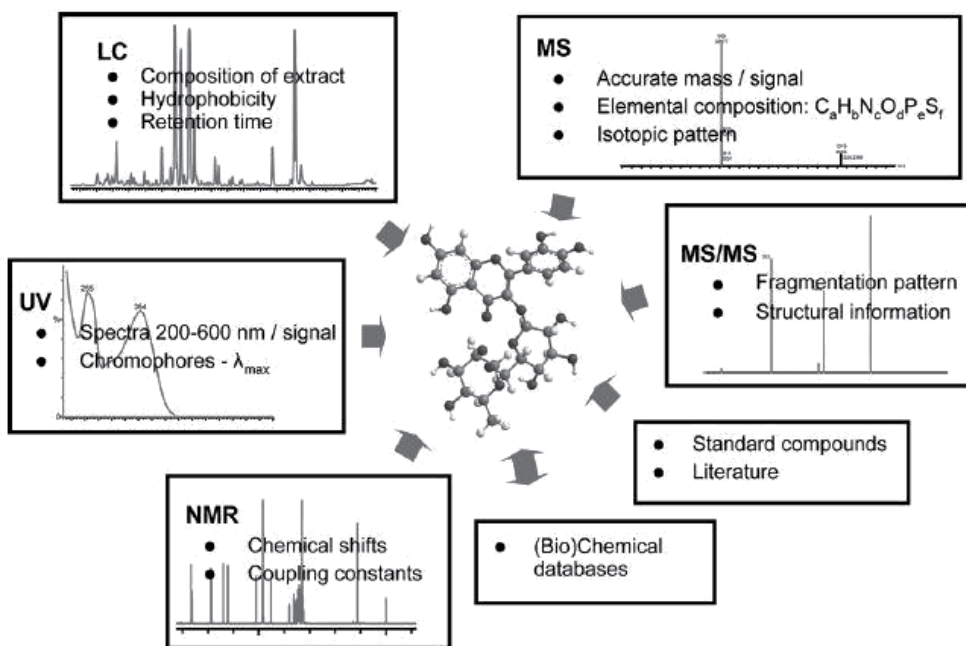
It has been estimated that hundreds of thousands of different metabolites are present in plants, with various chemical structures and, for many of them, with well-established bioactivities (Verpoorte, 1998). The analysis, chemical characterisation and quantification of these metabolites usually involves a multidisciplinary approach, based on different analytical techniques.

Metabolomics studies have been applied to different fields (Krastanov, 2010), ranging from environmental science, food science, human safety and plant biology (Bundy et al., 2008; Cevallos-Cevallos et al., 2009; Fukushima et al., 2009).

The aim of the metabolomic approach is to identify a much larger possible number of metabolites to better understand the biological system under investigation. Recently, (Dettmer et al., 2007) several terms have been used for metabolomics related definitions, such as metabolic profiling, metabolic fingerprinting and metabolic footprinting. These three approaches are fully integrated into the metabolomic investigations.

Metabolite identification is a real challenge, where many factors play a relevant role, including the analytical tool used, the sample preparation, the bio-computational tool for the data mining and the quality of the acquired data. Sample preparation is the most underestimated problematic part of the metabolomics analysis; a wide chemical diversity of compounds is present with a very high range of concentrations that could be present simultaneously. Appropriate extraction procedures need to be evaluated to obtain the maximum number of chemical components within the same sample. In this respect, the chemical classes of compounds could require specific separation processes involving solvents with different polarity. Furthermore, especially when NMR analysis is performed, the presence of buffered solutions (to control small shifts of NMR signals due to different pH values) or deuterated solvents is required. Detailed sample preparation procedures can be found in a recent review (Schripsema, 2010). Extraction procedures can be followed by chromatographic techniques, including TLC, HPLC, UPLC, HILIC (Hydrophilic Interaction Liquid Chromatography) (Bajad & Shulaev, 2011) and GC to eliminate possible contaminants and to obtain selected fractions. The commonly accepted analytical platforms to investigate plant metabolome are MS- and NMR-based systems, and, even more frequently, these two approaches have been combined to address the identification of metabolites in complex extracts (Moco et al., 2007). In Scheme 1, a pictorial diagram of different platforms used in metabolite identification is represented. MS-based approaches are often limited by separation and derivatisation protocols, as well as the detection capability, which usually allows single metabolite detection and quantification. Furthermore, the physico-chemical properties of metabolites (e.g., volatility, low ionisability, lack of chromophores) could limit the determination; in such cases, only limited metabolic profiling can be performed. Other techniques, such as NMR and all of its technical modifications, do not require any derivatisation and limited (liquid state) or no extraction procedures of the sample (solid state), thus, allowing for the identification and quantification of different kinds of metabolites from the same sample in the shortest time.

This technique is partially limited by the relatively low sensibility when compared to MS spectrometry (the detection limit in the sub-microgram region at 14.1 T). As a matter of fact, with the advent of new ultra-high field magnets (1 GHz is now commercially available) and cooled probes, NMR methods have experienced a dramatic increase in sensibility and, thus, have become a valid alternative to mass spectrometry, reaching almost the same sensitivity (ppb). Moreover, the advent of NMR microprobes, with active volume as low as 1.5  $\mu\text{L}$ , have provided new possibilities for analysing molecules in very low volumes, increasing concentration of the analyte without compromising the Signal/Noise ratio.



Scheme 1. Example of possible analytical technologies and databases that can be used to identify rutin. (Reproduced with permission from Moco et al., 2007).

The development of the so-called “hyphenated techniques” take advantage of the separation and detection processes performed continuously in a single step; these techniques range from the basic LC-MS and GC-MS approaches recently reviewed by T’Kindt et al. (2009). It has been demonstrated that combination of high-end analytical technologies facilitates the structure elucidation process of small mass molecules present in minute quantities in valuable samples. In this respect, several applications have been recently developed, such as LC-SPE-NMR or the more efficient LC-SPE-NMR-MS set up which overcomes the limitation of direct coupling between LC and NMR (Schlotterbeck & Ceccarelli, 2009; Yang et al., 2009; Van Beek et al., 2009). The instrumental setup of these techniques implies the physical joint of chromatographic-based instruments to spectroscopic detection-based instrumentations, making these systems not easily affordable to standard research laboratories. Nevertheless, improvements have been obtained either with the introduction of reproducible strategies for metabolite identifications or with the exchange of identifications among laboratories. The introduction of the concept of Mass Spectral Tag (MST), defined as ensemble of properties

(molecular mass to charge ratio, chromatographic retention index and the induced mass fragmentation pattern) (Kopka, 2006), enhanced the GC-MS data exchange and then the identification of compounds. The term “hyphenated techniques”, first introduced by Hirschfeld et al. (1980), has experienced a progressive evolution among differently combined techniques selected to tackle challenging problems. The theoretical application of multiple hyphenation steps, usually called “hyphernation” (e.g., LC-DAD UV-NMR/MS-MS method), is technically very difficult and not applicable due to the high cost. These approaches have been reviewed and discussed previously (Wilson & Brinkman, 2003).

## 2.1 Hyphenated chromatographic techniques

Chromatographic techniques, usually adopted to select chemically equivalent compounds, are essentially based on two different phases: liquid and gas. With recent technological improvements (e.g., long narrow bore capillary columns, capillary columns), the liquid phase techniques can reach sensitivity magnitudes on the order of nano-g. In the gas phase, sensitivity is largely affected by the ionisation source conditions because ion production is the basic requirement for this type of analysis. The detection limit can be as low as a few ng/L.

Based on the different mobile phases adopted in the chromatographic techniques, an arsenal of different methods has grown during recent years, and all of them took the advantage of the combination of techniques, due to the fact that both quantification and identification are important in metabolomic research. Techniques, such as HPLC-MS, GC-MS, CE-UV and HPTLC, or multi-combined techniques, such as LC-MS-NMR, GC-IT-MS-MS and LC-MS-MDF, are adopted almost routinely in current plants metabolomic studies (Abou-Donia et al., 2007; Gotti et al., 2006; Llop et al., 2010). Recently, Berkov et al. (2011) developed and validated a new GC-MS method for the rapid determination of galanthamine in *Leucojum aestivum*, a study that also focused on the determination of the origin of the plant. This method, with the aid of Principal Component Analysis (PCA), was rather informative (metabolomic based), providing information not only on the galanthamine content but also on alkaloid profiles; these data could be successfully correlated with the plant species, plant organs and the geographical origin of the plant.

## 2.2 Hyphenated spectroscopic techniques

The possible combination of a high performance chromatographic technique with a high characterisation technique is probably one of the best possible approaches for the quantification and characterisation of metabolite composition. As a matter of fact, hyphenated LC-MS, HPLC-MS, GC-MS and HILIC-LC-MS have been largely adopted in plant metabolite profiling (Allwood et al., 2009; Allwood & Goodacre, 2010; Cubbon et al., 2010) for their performance for selectivity and sensitivity in targeted analysis, enabling the detection of very low abundant and/or volatile compounds. In contrast, NMR hyphenated techniques take the advantage of a non-targeted analysis performed in a quantitative fashion to detect most of the highly abundant primary and secondary metabolites, with relevant structural information. A comparison between MS and NMR techniques have been reviewed by Krishnan et al. (2005). More recently Dai et al. (2010a, 2010b) successfully applied and combined NMR and LC-DAD-MS analysis to investigate the metabolic variations of three cultivars of *Salvia miltiorrhiza* Bunge (SMB), as well as changes induced by water depletion. The combination of these two analytical techniques has allowed the

detection of both the primary and secondary metabolites content. In particular, the authors have found that the metabolome of SMB is dominated by 28 primary metabolites (sugars, amino acids and carboxylic acids) and 4 secondary metabolites (polyphenols) (Figs 1 and 2).

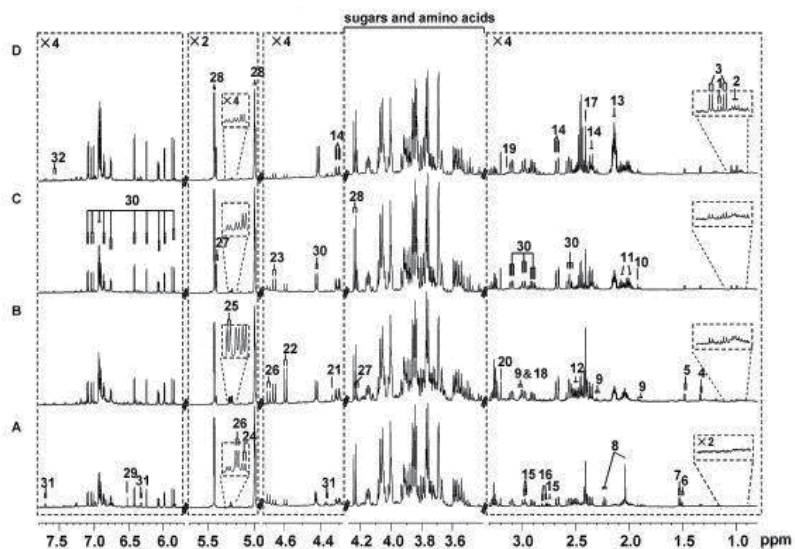


Fig. 1.  $^1\text{H}$  NMR spectra (600 MHz) of *Salvia milthiorrhiza* Bunge extracts from four different geographical origins: A) Zhongjiang, Sichuan B) Wuhan, Hubei C) Anding, Hebei D) Nanyang, Henan (Reproduced with permission from Dai et al., 2010a).

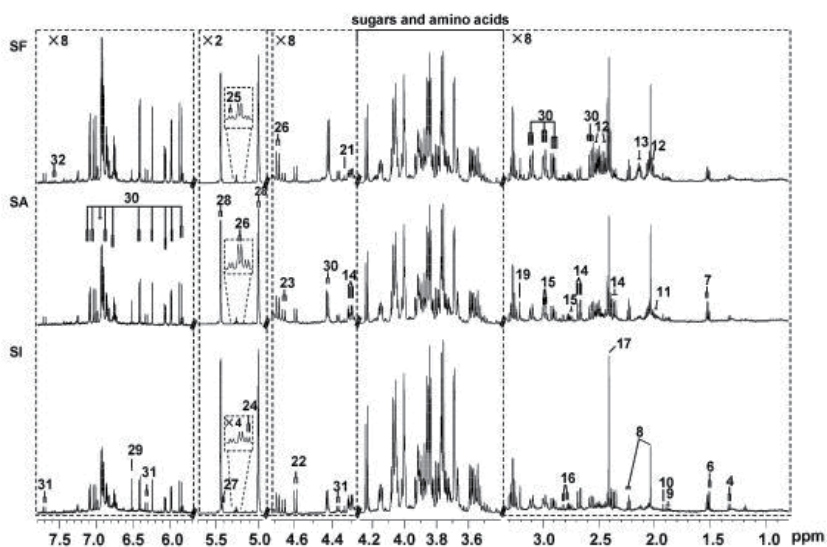


Fig. 2.  $^1\text{H}$  NMR spectra (600 MHz) of three different cultivars from *Salvia milthiorrhiza* Bunge: SF) Folium SA) Sativa SI) Silcestris (Reproduced with permission from Dai et al., 2010a).

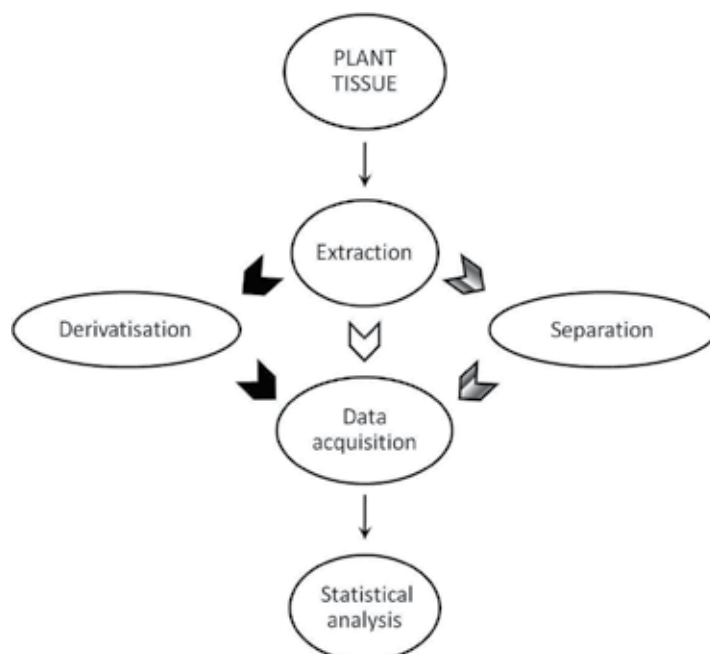
The systematic analysis of the metabolite composition of these three cultivars of SMB, grown in four geographical areas, allowed the assessment of differences among ecotypes and growing-location effects for the same cultivars.

### 3. Data treatment

#### 3.1 Chemometrics

The increased specificity and sensitivity of the analytical tools has offered the feasibility of obtaining a wide range of information with a single experiment. This technological breakthrough has allowed large dataset collections, enabling the possibility to evaluate similarities/differences among samples not possible in earlier studies. This approach, known under the general term “metabolomics,” properly refers to the collection of small molecules that can be found in a cell, organ, or organism. The metabolomic approach can be created by the following two different schools of thought: i) the chemometric approach, in which the chemical compounds are not identified, but their spectral patterns are statistically analysed to identify relevant spectral features that could differentiate samples and ii) the targeted or comprehensive profiling approach, in which the aim is firstly to identify and quantify most of the chemical compounds and secondly to perform statistical methods to identify relevant biomarkers. In Scheme 2, a pictorial representation of the flowchart used for metabolite identification is represented.

The term “chemometrics” is largely accepted today as a general statistical approach coupled to analytical techniques. The statistical approaches could be represented into two different classes: monivariate statistical analysis and multivariate statistical analysis.



Scheme 2. Schematic representation of the processes in metabolite analysis. Filled, dashed and empty arrows indicate the routes for GC-MS, chromatographic (LC/HPLC/CE) and NMR based metabolomics, respectively.

### 3.1.1 Monivariate statistical analysis

Significant amounts of data are obtained by measuring many variables on an ensemble of chemical samples or by recording many signals from an industrial process to track its behaviour. A data collection task, whether in science, business, or engineering, typically involves many measurements made on several samples. The unavoidable data variability has traditionally been analysed using one or two variables at a time. However, to discover the relationships among all of the samples and variables efficiently, all of the data must be processed simultaneously. Chemometrics is intended to extract information in multivariate chemical data, using the tools of statistics and mathematics. It is typically used for three primary purposes: to explore the patterns of similarities in the data, to track the properties of materials on a continuous basis, and to prepare and use multivariate classification models. In general, the algorithms applied have demonstrated significant capacity in analysing and modelling a wide variety of data types for an even more diverse set of applications. Different mathematical methods can be used to explore experimental data, based on the different possible targets; this phase provides information about statistical parameters of each variable and correlations among variables, reducing the data dimensionality. Among the possible systems, the analysis of variance, ANOVA (Miller & Miller, 1993), is used to select the variables that are most significant in the sample differentiation. This univariate statistical technique is used for testing the null hypothesis when two or more samples are drawn from the same population; high values of the F-test suggest that the null hypothesis can be discarded. This technique is no longer used for large data sets (especially in the case of spectroscopic data). The extension of ANOVA is called "multivariate data analysis" (MANOVA), and it is used whenever more than one correlated variable is concerned and they cannot be simply combined. MANOVA selects discriminant variables with high indices of reliability.

### 3.1.2 Multivariate statistical analysis

Unlike monivariate methods, where only one variable is considered, in multivariate statistical analysis, correlations among more variables are concerned. Multivariate data analysis is frequently used to address the following aspects: i) data overview ii) classification and or discrimination among groups of observations and iii) regression modelling between two blocks of data (X and Y). These applications reflect the main stages of multivariate analysis. One of the aims of this technique is to reduce the system dimensionality. Among the so-called "compression techniques", PCA (Geladi & Kowalski, 1986; Jackson, 1991) is widely used and recognised as the main "unsupervised" technique for the primary analysis of data. This method finds linear combinations of the variables in the original data, called PCs, which are orthogonally related and describe the major trends in the data. When the minimum meaningful number of PCs has been found, by means of loadings and score matrices, the original data matrix can be rebuilt. Inspection of the loadings gives indications on how the PCs are obtained from the original variables and how much the variable has in common with that PC. Scores show how the observations are clustered together on the basis of their variables.

Another compression technique, member of the so-called "classification methods", is the cluster analysis (CA) (Romesburg, 1984), that is applied to evaluate similarities and clusters among samples. This approach based on "similarities" or "classification" methods can also be split into hierarchical or non-hierarchical approaches. Commonly,



two types of clustering are used: K-Mean and Tree Clustering, named TCA. These classification methods are without *a priori* hypotheses in finding meaningful groups, and the result is often used for further statistical analysis. Dendrograms are usually adopted as graphical representation tools to visualise the data clustering. The same representation could be used to visualise the results of Hierarchical Clustering Analysis (HCA), included in the so called "segmentation methods", which group samples in dataset by their similarities according to their distances. These distances can be measured by different methods: Euclidean, Manhattan distances or correlations.

Another clustering method is the K-mean, which uses a fixed number (K) of groups. In this case, a metric distance should be defined to govern the clustering but the way of making groups is different. Several other clustering algorithms exist, essentially consisting of small theme variations, but none of them is best for a specific problem. As a matter of fact, these clustering methods are no longer used because more sophisticated and precise grouping methods have been developed.

Discriminant analysis (DA), also called "discriminant function analysis", performs sample classification with a *a priori* hypothesis. This hypothesis could be based on a previously determined TCA or other CA protocols. The natural extension of DA is the "multiple discriminant analysis" (MDA), which sometimes is named "discriminant factor analysis" or "canonical discriminant analysis" (CDA). Among these type of analyses, "linear discriminant analysis" (LDA) has been largely used to enforce differences among samples classes.

Another classification method is called "quadratic discriminant analysis" (QDA) (Frank & Friedman, 1989) and consists of an extension of LDA. Another method, named "regularised discriminant analysis" (RDA), works better with various class distribution and in the case of high-dimensional data, is a compromise between LDA and QDA. More recently, "independent component analysis" (ICA) has been developed for the analysis of signals from complex mixtures (Comon, 1994). In this approach, the coefficients of the linear expansion of the data vectors must be mutually independent; this requires higher order statistics in determining the ICA expansion and some non-linearities must also be used in the learning phase, thus, resulting in a more meaningful data representation with respect to PCA. "Generalised discriminant analysis" (GDA) (McLachland, 1992) is used to determine whether a given classification of samples into a group is appropriate. Therefore, each sample is assigned to a group and a model is searched and computed to maximise the classification. The general aim is to find out a mathematical model with high predictive capacity for a variable obtained from known values derived from the ensemble of independent variables; these types of protocols are called "regression methods." The simplest model describes the Y variable that is linearly dependent on the X variable; this casual dependence is a linear regression. Science often involves controllable variables (factor or predictor variables) to explain, to regulate, or to predict the behaviour of other variables (response variables). When factors are few, not significantly redundant (collinear), and show a correct relationship to the responses, the multiple regression can be the proper means to turn data into information. When spectroscopic data are considered, factors (variables) can be hundreds and highly collinear; the responses are components that need to be predicted for future samples. In these cases, Partial Least Squares Projections to Latent Structures (PLS) (Wold et al., 1984) is used to create multivariate calibration models with predictive capacity. In principle, multiple linear regression can be used with a large number of factors. However, if this number is bigger than the number of observations, the model will fail to predict a new data set because of problems with overfitting. In such cases, there can be only a few

underlying or latent factors that account for most of the variation in the response. The origin of PLS acronym can be explained by considering the general idea of PLS, which is to extract these latent factors accounting for the largest manifest factor variation possible, while optimally modelling the response. In PLS, factors are used to predict responses in the population, which is achieved indirectly by extracting the latent factors, T and U, from factors and responses, respectively. The extracted factors, T (X scores), are used to predict the Y scores, U, and then to build predictions for the responses. In PLS, the X and Y scores are chosen so that the relationship between successive scores is as strong as possible. Currently, several linear and nonlinear multivariate classification methods exist: the choice implies the evaluation of discriminatory power against the ability to interpret the meaning of class differences. In this respect, Soft Independent Modelling of Class Analogy (SIMCA) (Wold, 1976) is a well-established method for multivariate classification; disjoint PCA is used for fitting each class and it is largely used, even though it does not give easily accessible class difference information, thus, hampering the quality of interpretation of the classification model. PLS discriminant analysis (PLS-DA) has largely been used for explaining differences among overall class properties that become progressively more complicated with an increasing number of classes. The relatively new orthogonal PLS-DA (OPLS-DA) (Bylesjö et al., 2006) approach has been demonstrated to be the most revealing of the generated models. OPLS-DA was obtained as an extension of the PLS method featuring an orthogonal signal correction (OSC) filter (Trygg & Wold, 2002). In other words, compared to PLS-DA, OPLS-DA effectively separates predictive from non-predictive (orthogonal) loadings variation, which is particularly enforced when a two-class model is concerned.

#### 4. Abiotic stresses

During recent decades, most studies investigating the complex cascade of events occurring in plants upon exposure to abiotic stresses have been mainly focused on the gene expression level. Through the application of transcriptomics (in addition to forward and reverse genetics) hundred of genes have been linked with environmental stress responses and regulatory networks of gene expression have been delineated.

Relatively less is known about changes at the metabolomic level, but in the last few years the global metabolite analysis of plant stress response is representing a very rapidly expanding research field.

Here we will review a selection of recent publications, describing results obtained both in the model plant, *Arabidopsis thaliana*, and in several crop species, focusing on the following main abiotic stresses: low- and high-temperature, drought, high salinity and oxidative stress.

##### 4.1 Temperature stress

Temperature is one of the most crucial environmental factors determining plant growth and development. Plants are subjected to continuous diurnal and seasonal temperature fluctuations, with consequences depending on whether these deviations from the optimal values remain within a natural temperature range for each species or whether extremes of this range are reached. However, the temperature range of survival in some species can be extended through “cold acclimation” or “acquired thermotolerance”, the adaptive processes whereby plants increase in chilling/freezing or in heat tolerance in response to a prior low non-freezing or high temperature exposure, respectively. These highly complex inducible

mechanisms are accomplished by extensive reprogramming of the plant transcriptome, proteome and metabolome.

#### 4.1.1 Low-temperature stress response in Arabidopsis

Metabolic changes in response to low temperatures (chilling and freezing) have been extensively characterised in the model plant, *A. thaliana*.

In one of the first studies aimed to explore (on a large scale) the Arabidopsis metabolome variations occurring in response to low temperature, Cook et al. (2004) compared the stress-induced global changes in two Arabidopsis ecotypes, differing for their acclimation ability (Ws-2: high acclimation and Cvi-1: low acclimation). They reported that, out of 434 metabolites monitored in cold-acclimated plants, 75% and 62% increased their amount in Ws-2 and in Cvi-1, respectively; most of the changes (91%) observed in the Cvi-1 plants significantly overlapped with those occurring in Ws-2 plants. Moreover, 114 metabolites showed a fivefold or higher increase in Ws-2, compared to only 47 metabolites in Cvi-1. Altogether, these findings suggested that the ability to acclimate may depend on and correlate with the magnitude of cold-induced global changes in the metabolome (Cook et al., 2004). However, Hannah et al. (2006), through a comparison of nine different Arabidopsis accessions, found that the extent of cold-driven metabolic responses did not simply correlate with the cold-acclimation capacity.

Several of the cold-induced metabolites, such as sugars, proline and polyamines, identified through global analysis have been previously reported to accumulate under cold stress in Arabidopsis and other species in studies utilising targeted analysis (Guy et al., 2008). However, the power of metabolite profiling with respect to targeted analysis consists of the identification of changes in the amount of metabolites not yet known to be involved in the process being considered (i.e., cold acclimation).

To elucidate the role of the CBF pathway in the reconfiguration of the low-temperature metabolome, Cook et al. (2004) also compared the metabolic profiles of Ws-2 wild-type (wt) plants and transgenic lines ectopically expressing the transcription factor CBF3. In non-acclimated transgenic lines, they found an increase in almost 80% of the metabolites that were cold-induced in wt (90% if metabolites induced more than fivefold were considered), thus, confirming a prominent role of the CBF pathway in the cold response.

More recently, Maruyama et al. (2009) reported analogous studies, with results that were only partially in agreement with those of Cook et al. (2004). Namely, Maruyama and co-workers compared the metabolite profile of wt Arabidopsis plants grown under control or stress conditions (cold and drought) and transgenic plants ectopically expressing DREB1A/CBF3 or an active form of DREB2A. The overexpression of DREB1A/CBF3 has been previously shown to confer a higher tolerance to both freezing and dehydration, whereas the overexpression of DREB2A significantly improved the tolerance to dehydration but not to freezing in transgenic plants.

Maruyama and co-workers found that DREB1A/CBF3 ectopic expression resulted in an increased amount of 37 metabolites, 33 of which also accumulated in cold-treated wt plants. Because cold induced the accumulation of 155 metabolites in these experimental conditions, the CBF pathway appeared to be involved in the increase of only 21% of the cold-induced metabolites (in comparison to 80%, as reported by Cook et al., 2004). In DREB2A transgenic plants (that are dehydration, but not freezing, tolerant), the level of 28 metabolites increased, 17 of which were also positively affected by DREB1A/CBF3.

Seventeen metabolites (including myo-inositol, galactinol, raffinose, sucrose and 13 unknowns) were found to increase both in cold-exposed wt plants and DREB1A/CBF3-overexpressing plants but not in DREB2A-overexpressing plants. Thus, these compounds were considered as possible candidates that play an active role in the cold response. However, the level of the 4 known and 7 of the 13 unknown metabolites also significantly increased in dehydration-exposed wt plants.

Interestingly, gene expression data obtained by a microarray analysis of the different lines and growth conditions were found to be in agreement with the metabolic data; in particular, the expression levels of genes involved in carbohydrate metabolism positively correlated with the accumulation of specific sugars and sugar alcohols under stress conditions (Maruyama et al., 2009).

Vannini et al. (2004) analysed the low temperature stress response of wt and transgenic Arabidopsis plants ectopically expressing *Osmyb4*, a rice gene involved in cold responses. The non-acclimated *OsMyb4*-transgenic plants exhibited a degree of tolerance to both chilling and freezing comparable to that developed by wt plants after cold acclimation. Subsequently, through both targeted and profiling analyses, Mattana et al. (2005a) compared the changes in the metabolic content of wt and *OsMyb4*-transgenic plants during a ten-day-long cold experiment. They correlated the better tolerance of transgenic plants to a higher content of several metabolites (proline, sucrose, glucose, fructose, glycine betaine, alanine and sinapoyl malate) that were present in the transgenics prior to the stress treatment and that may prepare plants to face the stress. Moreover, during cold treatment, the degree of tolerance of the transformed plants further increased; the amount of the above-mentioned metabolites raised in both wt and transgenic plants, but it was always higher in the transgenic lines than in wt during the time course of the experiment (Mattana et al., 2005a). Furthermore, the increased metabolic contents in transformed plants were consistent with the global changes observed in the mRNA population (Mattana et al., 2005a; Vannini et al., 2004, 2006).

Kaplan et al. (2004), in a time course experiment, found that the amount of 311 out of the 497 low- $M_r$  polar compounds that were detected was affected by cold exposure. The authors showed that changes in the metabolite contents were evenly distributed across all of the temporal stages of the cold-response: early, intermediate and late (corresponding to 1-4 hours, 12-24 hours and 48-96 hours, respectively), with either sustained or transient increase or decrease. These results indicated that acclimation is a long-term dynamic process.

This idea was strengthened by the finding (Gray & Heath, 2005) that the metabolic profile of Arabidopsis leaves that were shifted to low temperature was constantly changing (at least up to 49 days, the maximum cold-treatment period evaluated by the authors), whereas leaves that had developed at low temperature exhibited a stable metabolite composition.

The profile of the shifted leaves became more distinct from the profile of the untreated ones the longer the shifted leaves stayed at low temperature. However, the profile remained different from the profile of leaves that had developed at 4 °C. Therefore, the authors suggested the existence of two distinct metabolic networks in response to cold stress: one that is environmentally modulated and another that is developmentally modulated at low temperature. The authors hypothesised that the same might be true also in response to any other environmental stimulus.

Although many quantitative and qualitative differences among the results reported by several authors do exist, one of the aspects that is consistent in all of the studies on cold acclimation is the crucial role of carbohydrate metabolism.

Its prominent function in the cold response was confirmed by the comparative metabolite analysis conducted by Hannah et al. (2006). As we have already mentioned, these authors analysed nine different *Arabidopsis* accessions, originating from Scandinavia to the Cape Verde Islands and differing in their freezing tolerance, combining transcript and metabolite profiling. They showed that the global changes of transcripts, but not of metabolites, correlated with the cold acclimation ability. However, the accumulation of individual metabolites, including several carbohydrates (i.e., glucose, fructose, sucrose and raffinose), correlated significantly with freezing tolerance. Although the important role of soluble sugars and particularly of raffinose has been highlighted by many studies, it has been demonstrated that raffinose accumulation alone is neither necessary nor sufficient for cold acclimation (Zuther et al., 2004).

An important role for carbohydrate has also been reported in relation to heterosis for freezing tolerance (Korn et al., 2008, 2010; Rohde et al., 2004). A significant heterosis effect on leaf-freezing tolerance was first observed by Rohde et al. (2004) in the F<sub>1</sub> progeny resulting from reciprocal crosses between the accessions Columbia-0 (Col) and C24, where Col plants are more freezing-tolerant than C24 plants, in both non-acclimated and acclimated conditions. In this case, among the soluble sugars measured, only raffinose showed a strong correlation with the leaf-freezing tolerance.

Korn et al. (2008) extended this study to the analysis of 24 F<sub>1</sub> hybrid lines, generated by reciprocal crosses of either Col or C24 accessions with six other parental accessions, widely differing in freezing tolerance. The degree of heterosis for freezing tolerance depended on the analysed cross (with C24 showing a better combining ability than Col) and was genetically unrelated to the heterosis for biomass production. Through a targeted analysis, they found that freezing tolerance in acclimated and non-acclimated plants correlated with the content of sugars (glucose, fructose, sucrose and raffinose), flavonols and proline. Very interestingly, heterosis for freezing tolerance correlated with heterosis for flavonols and sugars accumulation, whereas the proline content exhibited no correlation with heterotic effects in freezing tolerance (Korn et al., 2008).

More recently, the same research group (Korn et al., 2010) used global metabolic profiling to discover metabolite combinations able to predict freezing tolerance and its heterosis. They identified several compatible solutes as crucial predictors for both phenotypes, in particular, metabolites belonging to the raffinose biosynthetic pathway and other yet unidentified compounds, in addition to some TCA cycle intermediates that specifically contributed only to predict the heterotic phenotype.

The approach used by Korn et al. (2010), aimed to identify groups of metabolites, instead of individual metabolites, that together possess a predictive potential, seems to be well suited to analyse a redundant cellular protection system, such as that represented by compatible solutes, where single compounds might act non-specifically and substitute for each other with compensatory mechanisms (Panikulangara et al., 2004; Zuther et al., 2004).

#### **4.1.2 High-temperature stress response in *Arabidopsis***

In comparison with the numerous works conducted on the metabolic changes induced by low temperature, only a few non-targeted metabolomic studies have been carried out on plants subjected to heat stress.

Kaplan et al. (2004; see section 4.1.1) performed a global metabolite profiling analysis by GC-MS to identify similarities and differences in temporal metabolite responses associated with

the induction of acquired thermotolerance in response to heat shock (HS) and acquired freezing tolerance in response to cold shock (CS).

One of the most prominent differences between the plant responses to high and low temperature was represented by the temporal dynamics of the induced metabolic changes. Indeed, whereas during cold exposure, changes were evenly distributed along the time course of the treatment, most of the heat-induced metabolic alterations occurred within the first 30 minutes (Gray & Heath, 2005; Kaplan et al., 2004).

Out of the 497 low- $M_r$  polar compounds detected, 143 were affected by HS; among them, several pyruvate- and oxaloacetate-derived amino acids, fumarate and malate (oxaloacetate precursors), some amine-containing metabolites ( $\beta$ -alanine, GABA and putrescine) and several carbohydrates (including maltose, sucrose, raffinose, galactinol and myo-inositol) were found to be coordinately increased (Kaplan et al., 2004).

Because 311 metabolites or mass spectral tags were altered in response to CS, it appeared that cold shock influenced metabolism more profoundly than heat shock. Moreover, a very large proportion of the HS metabolite response was shared with the CS response (with only a very small fraction being HS specific); in contrast, the majority of metabolites (60%) that were responsive to cold shock were CS-specific.

Ninety-three metabolites showed a common response between the two thermal stresses; among these, pyruvate- and oxaloacetate-derived amino acids, polyamines and several carbohydrates (including fructose, sucrose, myo-inositol-phosphate, galactinol and raffinose) were increased in their content under both HS and CS. Several of these molecules are known to be either compatible solutes or precursors for secondary metabolites with properties of protection against pathogens.

Concerning the role of raffinose in the heat stress response, Panikulangara et al. (2004) determined the content of this sugar in *Arabidopsis* wt plants, in transgenic plants overexpressing the major heat-shock transcription factor HSF3 and in two *Galactinol synthase1* (*GolS1*) T-DNA knockout mutants. Galactinol synthase is a key enzyme in the biosynthetic pathway of the raffinose family oligosaccharides (RFOs): raffinose, stachyose and verbascose. The expression of *GolS1* was heat-inducible in wt plants, constitutively up-regulated in HSF3-overexpressing transgenic plants and almost completely inhibited in the T-DNA insertion mutants. Wild-type plants showed a basal level of raffinose under non-stress conditions, that was increased following heat stress; in transgenic lines, a constitutive strong accumulation was observed that was further induced after heat stress; on the contrary, only a basal level without any increase after heat stress was detectable in the knockout mutants. Surprisingly, when the heat tolerance phenotype was investigated, no differences between the wt and knockout mutant lines were detected.

These results, together with those obtained by Zuther et al. (2004; see section 4.1.1.) about a lack of difference in the cold tolerance phenotype among *Arabidopsis* lines accumulating very different levels of raffinose, led to the unexpected conclusion that altering the raffinose content (using T-DNA inactivated or transgenic plants) did not affect the stress temperature tolerance phenotype.

One possible explanation is the presence of feedback and/or compensative mechanisms, whereby alterations of raffinose levels are accompanied by changes in the amount of other sugars/metabolites important in temperature stress tolerance. This hypothesis was supported by the increased amount of galactinol under cold acclimation found in the raffinose synthase knockout mutant (Zuther et al., 2004).

An increased level of the signalling molecule, salicylic acid (SA), was also observed by Kaplan et al. (2004) during both heat and cold stresses, although with different time courses. This finding suggested that SA, already known to play a key role in systemic acquired resistance to pathogens, could also function as an early signalling molecule in temperature stress responses. Actually, comparing wt and mutant plants accumulating different SA amounts, a correlation between pre-stress levels of endogenous SA and both basal and acquired thermotolerance was demonstrated (Clarke et al., 2004; Larkindale et al., 2005). Clarke and co-workers also have suggested an involvement of jasmonates in conferring basal thermotolerance to Arabidopsis plants.

#### 4.1.3 Temperature stress response in crop species

Two rice genotypes, contrasting in chilling tolerance, were investigated for their response to cold, as well as to salt and osmotic stress (Morsy et al., 2007). Targeted metabolite analysis revealed that the two genotypes responded differently to the different stresses. Unexpectedly, the chilling-tolerant (CT) genotype was found to be more sensitive to drought and especially to salt stress than the chilling-sensitive (CS) one.

Differences in stress tolerance matched with, and may depend on, differences in metabolite accumulation between the two cultivars: indeed, under cold stress, CT accumulated galactose and raffinose, whereas these sugars decreased in CS. Conversely, CS specifically accumulated higher levels of osmoprotectants, such as mannitol and trehalose under salt and drought conditions, respectively. The endogenous content of oxidative products and the activities of some antioxidative enzymes were also measured, leading the authors to hypothesise on the presence of a more efficient ROS scavenging metabolism in CT genotype during chilling stress (Morsy et al., 2007).

In addition to the studies in transgenic Arabidopsis mentioned above, Mattana et al. (2005b) performed similar studies on stress tolerance response in wt and *Osm4*-expressing transgenic plants in several species, using a constitutive (pCaMV35S) or a stress-inducible (pCOR15a) promoter.

The authors reported that the increase in Myb4-driven cold tolerance in maize, apple and *Osteospermum* well correlated with the increased concentration of sugars and proline. The transgenic maize plants grown under control conditions did not show any difference in metabolite concentration with respect to the wild-type, as the *Osm4* gene expression in maize was under the pCOR15a stress-inducible promoter. However, a 6-day cold-treatment increased the sugar (fructose, sucrose and glucose) and proline contents in both wt and transgenic maize plants, with the concentration being significantly higher for all of them in the *Osm4*-transgenic plants.

The use of the pCaMV35S constitutive promoter to drive *Osm4* expression in *Osteospermum* and apple transgenic plants resulted in increased sugars and proline concentration prior to the stress exposure (Mattana et al., 2005b).

In a further investigation, the authors reported the first metabolic profile of the *Osteospermum* species under control and stress growth conditions, thus, confirming and extending the previous observations on Myb4-driven cold tolerance and metabolic changes (Laura et al., 2010). Namely, in the PCA score plot, *Osteospermum* samples distributed according to genotype and treatments: control and 2-day cold-treated plants (wt and transgenics) clustered together, whereas 10-day cold-treated and freezing-treated plants separated into different regions based on genotype. The samples separation correlated with different amounts of sucrose (higher in transgenic plants), inuline, glucose, fructose and

amino acids (higher in wt) that accumulated under stress conditions (Laura et al., 2010). Determination of proline content and proline/amino acids ratio confirmed previous reported results on a higher concentration in *Osmyb4*-transgenic plants and highlighted that differences between accumulation in the two genotypes was boosted during the cold stress treatment (Laura et al., 2010).

In transgenic apple, in agreement with the previously described overlap between cold and heat stress responses, *Osmyb4* was found to ameliorate the tolerance to both cold and heat stress (Mattana et al., 2005b).

In a more detailed analysis, the authors confirmed that the observed tolerance in transgenic apple plants may be driven by the higher content of sugars (glucose, sucrose and fructose) and proline present in plants grown under control conditions. In particular, the cold treatment amplified the differences of metabolites accumulation between wt and transgenics. Moreover, the *Osmyb4*-overexpressing plants showed an improved tolerance phenotype towards drought stress (Pasquali et al., 2008).

A common characteristic displayed by all of the *Myb4*-transgenic plants under cold treatment was the increase in the proline content, whereas wt plants showed an increase in free amino acids (Laura et al., 2010; Mattana et al., 2005a; Pasquali et al., 2008). This result underlined the importance of proline accumulation during the stress, in agreement with the several roles proposed for this amino acid during abiotic stress (Verbruggen & Hermans, 2008).

To identify the metabolites associated with differential heat tolerance in two perennial grass species, Du et al. (2010) performed a metabolite profile analysis of C4 warm-season bermudagrass (a tolerant species) and C3 cool-season Kentucky bluegrass (a susceptible species), grown under optimum temperature conditions or subjected to short-term and long-term heat stress. All of measured physiological parameters confirmed that bermudagrass exhibited better heat tolerance than Kentucky bluegrass. The metabolic profile analysis revealed differences in the accumulation of many metabolites, depending both on species and growth conditions. In particular, bermudagrass accumulated a higher content of most of the metabolites identified (including organic acids, amino acids, sugars and sugar alcohols) in comparison to Kentucky bluegrass, especially following long-term heat stress.

## 4.2 Drought stress

In addition to extreme temperatures, drought is one of the major constraints for plant productivity worldwide. Timing and severity of water deficit may vary a lot, ranging from long drought seasons (when the water supply by rain is lower than the demand) to short periods without rain at all.

Whether exposed to mild or severe drought conditions, plants exhibit a range of specific responses, aimed to reduce water loss and/or to optimise water uptake. Among the earliest responses, reduction in vegetative growth, stomatal closure and a decrease in the rate of photosynthesis are observed. Osmotic adjustment, that is the active accumulation of solutes in response to drought, resulting in reduced osmotic potential and contributing to maintain cell turgor, represents an important adaptation mechanism to water deficit in several plants.

Drought triggers the production of the phytohormone abscisic acid (ABA) and the occurrence of both ABA-dependent and ABA-independent pathways involved in plant drought response has been well described (Yamaguchi-Shinozaki & Shinozaki, 2006). Many



drought-inducible genes have been identified so far in many species and the complex gene networks are becoming to be elucidated. More recently, the global metabolic changes induced by water deficit have also been addressed by several authors.

#### 4.2.1 Drought stress response in Arabidopsis

The global transcriptional and metabolic changes induced in Arabidopsis by drought and their dependence on ABA-mediated or not mediated pathways have been investigated in an integrated analysis, comparing wt and a T-DNA-tagged NCED3 knockout mutant (*nc3-2*, impaired in the dehydration-inducible ABA biosynthesis) under control and water deficit conditions (Urano et al., 2009). The metabolic analysis indicated that drought strongly affected the metabolic profile, influencing the level of 82 metabolites in the wt (61 increased and 21 decreased) and 78 metabolites in the mutant (46 increased and 32 decreased). The authors classified the changes in the profiles of metabolites into categories based both on the timing (early, middle and late phase) and the trend of variations throughout the time-course experiment (transient or stable).

Comparison of the wt and mutant metabolic profiles highlighted that, among a total of 64 dehydration-increased metabolites, 16 were regulated by ABA-dependent pathways, including some amino acids, ethanolamine, glucose and fructose, 35 were regulated by ABA-independent pathways, such as raffinose and galactinol, metabolites belonging to TCA cycle and GABA shunt, and 13 were regulated by both ABA-dependent and ABA-independent pathways, including proline, agmatine, methionine, lysine, saccharopine and phenylalanine.

The metabolic analysis performed by the authors revealed that most of the drought-induced amino acids showed global correlation with each other, whereas the sugar groups did not show any correlation with the amino acid groups, thus, indicating a response to drought stress through completely different metabolic networks.

The authors also reported the integrated analysis of drought-induced transcriptome and metabolome changes.

The relationship between temperature and drought responses has been widely documented (Yamaguchi-Shinozaki & Shinozaki, 2006); mutations affecting the tolerance to both stresses, as well as transgenic plants with increased tolerance to both stresses, have been described (Bouchabke-Coussa et al., 2008; Kasuga et al., 1999; Mattana et al., 2005a).

An emblematic example of the overlap between the temperature and drought stress responses is represented by the *eskimo1* (*esk1*) mutant phenotype. Although *esk1* was originally isolated as a freezing-tolerant mutant in the absence of cold acclimation, able to constitutively accumulate high amounts of proline (Xin & Browse, 1998), genes regulated by the ESK1 protein showed a larger overlap with genes regulated by osmotic, salt and ABA treatment than with genes regulated by cold acclimation or belonging to the CBF/DREB pathway (Xin et al., 2007).

Lugan et al. (2009), comparing global metabolic profiles of wt and *esk1* plants grown under control and three different stress conditions (cold, salinity or dehydration), found that the mutant constitutively mimicked the phenotypic traits of wt abiotic stress response, in terms of development, osmotic status and metabolic profile. Despite some discrepancies, the changes at the metabolomic level were consistent with changes observed in the transcriptome, previously described by Xin and co-workers (2007).

A more detailed comparison between mutant and stressed wt metabolomes indicated that *esk1* was closer to drought-stressed than cold-acclimated wt plants. Indeed, the mutant

accumulated melibiose, raffinose, galactinol, proline, galactose, fructose and GABA (all associated with the three considered stress conditions), but not other metabolites, such as glutamine, trehalose and sucrose, involved in the wt cold response. Therefore, the authors suggested that the freezing tolerance exhibited by the *esk1* mutant was a side effect of a constitutive acclimation to dehydration. Based on transcriptome analysis, a major role of ESK1 in the plant response to water shortage and in the whole-plant water economy was also suggested by Bouchabke-Coussa et al. (2008).

The improved tolerance to multiple stresses as a consequence of the altered expression, in mutant or transgenic lines, of a gene normally activated in response to a specific stress, depends on the overlap at the molecular level of the response to different stresses. The rationale of this overlap is evident considering the two aspects of abiotic stress effects on plant cells: i) some primary cellular damages may be shared by several stresses, as for example the water deficiency caused not only by drought, but also by salinity, freezing and hypoxia (damaged roots being unable to transport water to the aerial parts) and ii) different primary effects may induce a common secondary stress (and, therefore, common secondary damages), such as oxidative stress, derived from the ROS production following the impaired photosynthetic ability in most of the suboptimal growth conditions (for a review on the specific and unspecific responses to cold and drought stress, see Beck et al., 2007).

In *Arabidopsis* plants subjected to cold or drought treatment, Mattana et al. (2005a) observed similar metabolic changes, such as an increase of proline, sugars and amino acids, although with a different time-course accumulation between the two stress conditions. In the same paper, the authors also reported the increased tolerance to both stresses of plants ectopically expressing the rice gene *Osmyb4*. Under stress conditions, the *Osmyb4* action seemed to amplify the changes in metabolites observed in wt, maintaining the difference in the timing of accumulation between the two stresses.

We have already cited the studies performed by Maruyama and co-workers (2009) on wt and *Arabidopsis* transgenic plants overexpressing DREB1A/CBF3 or DREB2A, the former conferring tolerance both to freezing and dehydration and the latter only to dehydration in transgenic plants. Indeed, in agreement with these tolerance phenotypes, on the basis of the metabolic profile data (in particular, the accumulation of arginosuccinate, fumarate, malate and several unknown metabolites), DREB2A transgenic plants clustered with drought-treated, but not with cold-treated wt plants.

We have also discussed that DREB1A/CBF3 transgenic plants and cold acclimated wt plants shared the accumulation of 17 metabolites (see section 4.1.1.), which were not affected in DREB2A transgenic plants and, therefore, were presumed to be involved in freezing tolerance. In contrast, as both transgenic lines showed strong drought tolerance, it was proposed that this phenotype did not depend on metabolites shared by DREB2A transgenic plants and drought-treated plants, but on those metabolites whose accumulation was increased in both transgenic lines (Maruyama et al., 2009). The comparison of global transcriptomes performed by the authors led to analogous conclusions.

Because of the possible simultaneous occurrence in nature of drought and high temperature conditions, Rizhsky et al. (2004) performed an analysis of the molecular and metabolic response of *Arabidopsis* plants to these stresses, considered either individually or in combination. The metabolic profile of plants subjected to both stresses was more similar to that of drought-treated plants than to that of heat-treated plants, with accumulation of high levels of sugars, such as sucrose, maltose and gulose. However, double stress-treated plants also accumulated sugars that are specific of heat-treated plants (i.e., fucose and melibiose)

and did not accumulate other metabolites typical of the drought response, the most remarkable example being proline. The authors suggested that proline might be toxic during a combination of drought and high temperature stress. A parallel transcriptome profiling of the same samples highlighted a similar preferential overlapping of the double-stressed plants with the drought-stressed ones.

#### 4.2.2 Drought stress response in crop species

Many wild species are more tolerant to unfavourable environmental conditions than their relative cultivated crops, suggesting that crossing between wild species and elite cultivars could lead to an improvement of stress adaptation in modern crops.

On the basis of this observation, Semel et al. (2007) compared the metabolic profile of tomato fruit pericarp from irrigated and non-irrigated field grown plants, belonging either to the cultivated tomato, *Solanum lycopersicum* (cv. M82), or to its interspecific hybrid with *Solanum pennellii*. A total of 72 identified metabolites were detected, and the variance due to the genotype and the environment was evaluated.

Under irrigated field conditions, the metabolite composition of the elite cultivar and the F1 hybrid strongly differed, with a significantly higher content of several amino acids in M82 and a higher level of the majority of fatty and organic acids in the F1 hybrid. The two genotypes were also quite distinct with regard to the contents of sugars, sugar phosphates, sugar alcohols and other metabolites, with most of them (i.e., fructose, glucose, maltose, sucrose, trehalose and myo-inositol) being present at higher levels in the F1 hybrid and only a few of them (putrescine and fructose-6-phosphate) more abundant in M82 (Semel et al., 2007).

In the cultivated tomato, M82, the stress strongly affected the content of many metabolites, with large increases in several amino acids (including proline,  $\beta$ -alanine, GABA, glutamate and glycine), fatty and organic acids (including TCA cycle intermediates), as well as sugars and sugar derivatives. Whereas a change in the content and/or a role in response to water stress had largely been documented for some of these solutes (e.g., proline and some sugars), the increase of other compounds was considered more intriguing, such as for branched amino acids, TCA cycle intermediates or gentiobiose. It is noteworthy that a signalling role during tomato fruit development had been proposed for this latter molecule.

On the contrary, the F1 hybrid metabolic profile was not significantly affected by the experimental growth conditions, possibly because of the “constitutive” elevated concentration of many of the metabolites known to be involved in drought stress response.

The statistical analysis performed on the entire dataset supported the analytical results. Indeed, PCA clearly discriminated M82 from the F1 hybrid as well as irrigated from non-irrigated M82, but was unable to discriminate irrigated from non-irrigated F1 samples. Similarly, Hierarchical Clustering Analysis (HCA) revealed a strong influence of the genotype and a lower influence of the environment on the metabolic profiles (Semel et al., 2007).

Vannini et al. (2007) analysed the drought-tolerant phenotype and targeted metabolite accumulation in wt and transgenic tomato plants overexpressing the rice *Osmyb4* gene either under a constitutive (pCaMV35S) or a stress-inducible (pCOR15a) promoter. They found that the ameliorated tolerance of the transgenic lines was associated with a higher accumulation of sugars (sucrose, fructose and glucose) and proline (measured as percentage of total amino acid content).

Following drought treatment, the content of these molecules increased in both wt and transgenic plants, with the levels observed in transgenics always higher than those observed

in wt. The Myb4-constitutively expressing plants accumulated a higher content of free sugars even under control conditions. As expected, because of the use of a stress-inducible promoter, no significant difference in the concentration of any analysed metabolite was observed between wt and pCOR15a-Myb4 plants under control conditions. However, under water deficit conditions, the concentration of these compounds was found to be significantly higher in pCOR15a-Myb4 transgenic lines than in wt and to be comparable to that found in pCaMV35S-Myb4 transgenic plants (Vannini et al., 2007).

Dai et al. (2010b) have performed a systematic characterisation of the metabolic changes induced by water depletion in the roots of the medicinal plant, *Salvia miltiorrhiza* Bunge, comparing the results obtained using two metabolite analysis techniques ( $^1\text{H-NMR}$  and LC-DAD-MS) and four extraction methods based on different solvents (see also section 2.2). As phytomedicines are usually either air-dried or sun-dried for the purposes of transportation, storage or pharmacological requirements, the effect of these two different drying processes on the metabolite composition was also investigated and compared with a freeze-drying process, taken as the control.

$^1\text{H-NMR}$  analysis revealed the presence of both primary and secondary metabolites, whereas LC-DAD-MS detected 44 secondary metabolites, among which 5 polyphenolic acids, genipin, umbelliferone and tormentic acid had not been previously described in this plant.

Both approaches revealed distinct metabolite profiles of the extracts obtained from the different drying treatments and the PCA score plots generated from both data series could discriminate the samples depending on the drying process.

However, the two approaches detected different metabolic changes following the two drying processes. Among the primary metabolites detected by  $^1\text{H-NMR}$ , an increase in proline, alanine and succinate accompanied by a decrease in *n*-butanol and lactate was observed in both the air-dried and sun-dried samples; in contrast, an increase in the content of sucrose and glutamine was observed only in air-dried roots, whereas an increase in leucine, melibiose and raffinose was found only in sun-dried roots. Different effects of air- and sun-drying processes were also highlighted on secondary metabolism by the LC-DAD-MS method, with air-drying enhancing the biosynthesis of oligomeric caffeic acids and tanshinones and sun-drying promoting the biosynthesis of tanshinones but inhibiting that of polyphenolic acids. The differences in metabolite content variations between the two sample groups was suggested to be attributable to the different drying speed of the two methods and to the concomitant occurrence of light and thermal stresses in sun-, but not in air-dried roots (Dai et al., 2010b). This study has the merit of showing the effectiveness of the combination of two different analytical approaches ( $^1\text{H-NMR}$  and LC-DAD-MS) and of highlighting the importance to carefully consider and optimise the extraction method when metabolomic analyses are performed.

To assess the association of osmotic adjustment (OA) with drought tolerance, seed yield and specific metabolites accumulation, a recent study was conducted on three different castor (*Ricinus communis* L.) hybrids and their respective parents, grown under irrigated and non-irrigated field conditions (Babita et al., 2010). The authors reported that genotypes with a greater OA also had higher leaf Relative Water Content (RWC) and maintained higher leaf water potential under water deficit; moreover, a positive relationship existed between OA and total seed yield under drought stress conditions. The high-OA genotypes accumulated significantly higher amounts of proline, total soluble sugars, total free amino acids and potassium, with sugars representing the major contributors to OA (Babita et al., 2010).

Three cotton near-isogenic lines (NILs), obtained via marker-assisted selection from the elite cultivars of the two species *Gossypium barbadense* (GB) cv. F-177 and *Gossypium hirsutum* (GH) cv. Siv'on, have been characterised for their metabolic and mineral compositions and compared to their parental genotypes (Levi et al., 2011). Plants were field-grown under well-watered and water-limited conditions and comparative analysis was performed between i) GB and GH genotypes ii) the two water regimes and iii) each NIL and its recipient parent. The HCA, based on either 27 metabolites or 5 minerals, clearly distinguished between GB and GH genotypes. Within each species, in most but not all of the cases, the irrigation treatments had a more pronounced effect on the clustering than the genotypes. Comparisons between plants grown under well-watered and water-limited conditions for each genotype showed different trends in the various solutes. On the basis of the previously reported improved drought tolerance of the NILs versus their recipient parents, the authors focused their attention on those metabolites whose amount increased under stress in one or more of the NILs. In particular, an increase in aspartic acid, citric acid, malic acid, threonic acid, alanine, glycerol and myo-inositol among metabolites as well as in potassium, magnesium and calcium among minerals was suggested to contribute to the ameliorated adaptation to drought of these NILs (Levi et al., 2011).

#### 4.2.3 Resurrection plants

Whereas the term “drought tolerance” refers to the ability of plants to survive a moderate dehydration (down to  $\sim 0.3 \text{ g H}_2\text{O g}^{-1}$  dry weight), the capacity to tolerate further dehydration (down to an absolute water content of  $0.1 \text{ g H}_2\text{O g}^{-1}$  dry weight) is referred to as “desiccation tolerance” (Moore et al., 2009). This term also includes the ability of plants to rehydrate successfully and to regain normal metabolism and growth within several hours of rewatering.

Although desiccation is part of the normal developmental program of seeds in most higher plants, only a few species possess desiccation-tolerant vegetative tissues. These include the individual members of different angiosperm families and are termed “resurrection plants” (Moore et al., 2009).

Such species have been extensively studied in attempts to identify the mechanisms associated with their remarkable tolerance and with the aim of using the obtained knowledge to improve drought tolerance in economically important crop species.

Many different approaches have been employed in these studies, focusing on molecular, biochemical, metabolic, ultrastructural and physiological aspects of such a complex trait (Moore et al., 2009).

Among the plethora of data obtained, the identification of several upstream-acting genes, such as those encoding transcription factors and small regulatory RNA molecules, are of particular interest (Moore et al., 2009).

With regard to metabolites involved in desiccation tolerance, the importance of antioxidants, such as phenolic acids and polyphenols (galloylquinic acid) has been highlighted. Namely, a correlation between the galloylquinic acid content/composition and the maximum desiccation period that different populations of *Myrothamnus flabellifolius* can survive has been reported. These molecules have been suggested to act as a “reservoir”, able to determine the length of the desiccation period that a plant can suffer before its viability is irreversibly compromised (Moore et al., 2005).

As in other species, in resurrection plants dehydration leads to an increase in the content of proline and of soluble carbohydrates (i.e., sucrose, trehalose, raffinose and glucose).

Moreover, the localisation of glucose and sucrose in plant tissues was reported in accordance with their possible function as cellular protectants during water stress (Martinelli, 2008).

Despite all of the results achieved in elucidating single aspects of the desiccation tolerance phenomenon, from gene regulation to metabolic adjustment or macromolecular stability, the secrets of resurrection plants still remain to be discovered. A holistic comprehension of how the identified individual factors interact spatially and temporally and the identification of (if it exists) the master switch is still lacking. Consistently, the ectopic expression of *Craterostigma plantagineum* transcription factors-encoding genes in Arabidopsis, tobacco and desiccation-sensitive callus tissue from *C. plantagineum* itself has led to inconsistent results: either improved drought tolerance or no effects on the phenotype, or even unexpected side effects, such as ABA insensitivity (Moore et al., 2009).

### 4.3 Salt stress

The increased salinisation of arable land, due to both natural processes and agricultural practises, is expected to have a dramatic negative impact on soil fertility in the next decades, resulting in a high percentage of land loss by the middle of the century. Most of the economically important crop species are very sensitive to high salt concentration in the soil. High salinity engenders both hyper-osmotic stress (caused by the reduction of water availability due to the reduced water potential) and hyper-ionic stress (caused by the toxic effects of the accumulated ions). Plants are thus subjected to dehydration, ion toxicity, nutritional deficiencies and oxidative stress, with the main negative effects being the disruption of ionic equilibrium, the inhibition of cell division and expansion, and the reduction in photosynthesis and growth. Plant acclimation responses include ion exclusion and tissue tolerance, osmotic adjustment and several molecular and biochemical changes, with both conserved and divergent metabolic responses among different species (D.H. Sanchez et al., 2008).

#### 4.3.1 Salt stress response in Arabidopsis

Kim et al. (2007) have reported a detailed analysis of metabolic changes occurring during a time-course experiment (up to 72 hours) on salt-stressed Arabidopsis cell cultures. PCA and Batch Learning Self-Organising Mapping analysis (BL-SOM) revealed a coordinated induction of several pathways at different time points. Namely, short-term responses included the induction of the methylation cycle (for the supply of methyl groups), of the phenylpropanoid pathway (for lignin production) and of glycine betaine biosynthesis, whereas long-term response was characterised by the co-induction of glycolysis and sucrose metabolism and the co-reduction of the methylation cycle. In particular, metabolites that transiently increased in the short-term period included S-adenosyl-L-methionine (SAM), ethanolamine, cysteine and aromatic amino acids. Twenty-four hours after salt treatment, a decrease in SAM and the aromatic amino acid content and an increase in glycerol, inositol and S-adenosyl-L-homocysteine (SAH) were observed. As a consequence, the methylation index SAM/SAH increased as a short-term response to salt stress and constantly decreased after 12 hours of salt stress. Finally, long-term stressed cells abundantly accumulated sucrose and lactate (Kim et al., 2007).

The metabolic response to high salinity stress was also addressed in a time-course experiment by Kempa et al. (2008). These authors investigated the ABA involvement in the

complex re-adjustment of carbohydrate metabolism during salt stress, by exploring the temporal dynamics of the *Arabidopsis* metabolome in response to high soil salinity (up to 5 days) or to ABA treatment (up to 3 days). Comparison of the salt- and ABA-induced metabolic changes in an Independent Component Analysis (ICA) revealed both common and distinct metabolic responses, indicating the existence of ABA-dependent and ABA-independent pathways. Notably, both high salt and ABA treatments led to depletion of starch and increase in maltose levels, suggesting a role of this hormone in triggering stress-induced starch mobilisation.

The authors also addressed the question of whether a correlation exists between changes in specific metabolite levels and changes in the expression levels of genes encoding the corresponding metabolic enzymes and found such a correlation in several, but not all, of the pathways examined.

As plant hormones play a crucial role in responses to various environmental stresses, studies on the effects of hormone treatments on intracellular metabolites have also to be mentioned here. One of such studies was performed by Okamoto et al. (2009), who investigated by NMR the metabolic profiling of *Arabidopsis* T87 cultured cells following various hormone treatments (ABA, salicylic acid [SA], auxin and brassinosteroid). Moreover, as ABA and SA are known to mediate abiotic and biotic stress responses and to act antagonistically each other, the authors also monitored the dynamic metabolic changes in cells treated with ABA and SA simultaneously or successively for different time periods. Based on their data, the authors suggested that ABA and SA do not have simple antagonistic effects but that they cross-talk at the metabolite levels in a much more complex manner.

The single and combinatorial effect of salinity stress and elevated CO<sub>2</sub>, two environmental conditions that are expected individually to affect plant growth in opposite directions, has also been investigated (Kanani et al., 2010). The authors found that, while the transcriptional responses to the salinity and to the combined stresses were very similar, this was not the case for the metabolic responses, thus, representing an example of “inconsistency” between these two levels of plant response. In particular, the combination of the two perturbations had a milder effect on the metabolic physiology than the salinity stress alone. This suggested a beneficial role of elevated CO<sub>2</sub> on salt-stressed plants at the metabolic level, at least within the experimental timeframe (30 hours), probably due to the provided additional resources in the presence of elevated CO<sub>2</sub> concentration.

#### **4.3.2 Salt stress response: *Thellungiella* vs. *Arabidopsis***

*Thellungiella halophila* (also known as *T. salsuginea*), a *Brassicaceae* species closely related to *Arabidopsis*, displays “extremophile” characteristics represented by a remarkable tolerance to a variety of abiotic stresses, namely high salinity, water-deficit and freezing. Studies have taken advantage of the high nucleotide sequence identity between *Thellungiella* and *Arabidopsis*, utilising tools developed for the model species to investigate the transcriptome of the halophyte species.

Gong and co-workers (2005) investigated the salinity stress adaptation competence of *Thellungiella*. To identify pathways relevant for the stress adaptation phenotype of *Thellungiella*, they compared the transcript and metabolite profiles of the two species, grown under both optimal and salt-stressed conditions. In addition to stress responses shared by the two species, three *Thellungiella*-specific response categories were defined: i) additional

pathways that are stress-activated in *Thellungiella* but not activated in *Arabidopsis*, ii) genes with a significantly higher pre-stress intensity in *Thellungiella* and iii) novel stress-relevant genes whose homologs are not stress-responsive in *Arabidopsis*.

At the metabolic level, changes in *Arabidopsis* plants subjected to 150 mM NaCl for 24 hours were mainly represented by an increase, with respect to control plants, of proline, sucrose and an unknown compound (putative complex sugar). Drastic differences distinguished the two species, the most relevant being a higher amount in *Thellungiella* of sugars and sugar alcohols, both under control and salinity growth conditions. Under salt stress, *Thellungiella* also accumulated higher levels of proline, glutamic acid, malic acid, succinic acid, whereas in both control and stress conditions, *Arabidopsis* showed a higher accumulation of fumaric acid and mannitol.

Metabolome data, together with transcriptome results, have pointed towards the presence of a stress anticipatory strategy in *Thellungiella* as responsible for its “extremophile” characteristics.

More recently, *Arabidopsis* and *Thellungiella* responses to salinity and osmotic stress have been compared with an analogous approach by Lugan et al. (2010).

The authors found that, apart from a few differences in raffinose and secondary metabolites, salt stress affected the same metabolic pathways in the two species, the main differences being quantitative. *Thellungiella* had a higher concentration of many stress-related metabolites than *Arabidopsis*, independent of the growth conditions. It also contained less water and showed a higher ability to lose water following stress, without any detrimental effect, which could contribute to maintaining a water potential gradient between the soil and plants in water-limiting conditions.

PCA analysis sharply separated the samples, both depending on the species and on the environmental conditions, the genetic background being the main contributor to the metabolome variations. The species-dependent differences appeared to relate partially to the stress anticipatory strategy that has been hypothesised for *Thellungiella*; indeed, 42 of the 58 metabolites that were more abundant, and 19 of the 34 metabolites that were less abundant, in *Thellungiella* under the control growth conditions, were found to increase and decrease, respectively, in *Arabidopsis* under stress treatment. Therefore, the *Arabidopsis* metabolic response to salt seemed to, at least partially, mimic the constitutive status of *Thellungiella*.

A very original contribution to the metabolomic analysis approach that was provided by this study is represented by the idea of considering the metabolome of each species as a single “virtual molecule”, the physicochemical properties of which are the weighted averages of the properties of the individual metabolites.

Therefore, based on this idea, the significant differences between the two species can be summarised as follows: i) under both standard and stressed conditions, the *Thellungiella* metabolome was more soluble, polar, massive and reduced than the *Arabidopsis* metabolome; ii) osmotic and salinity stresses changed the metabolome biophysical properties in a different way, depending on the stress and on the species; iii) both stresses induced more dramatic changes in *Arabidopsis* than in *Thellungiella*; iv) in *Arabidopsis*, salt affected the metabolome biophysical properties more than osmotic treatment and v) in *Thellungiella*, water stress induced more dramatic changes than salt stress (Lugan et al., 2010).

#### 4.3.3 Salt stress response in crop species

One of the first applications of metabolomic analysis to the plant response to salt stress was reported by Johnson et al. (2003) on tomato fruits. Extracts from two varieties



differing in their salt tolerance were analysed using FT-IR spectroscopy coupled with chemometric techniques.

Whereas the unsupervised method, PCA, was not able to discriminate between the control and salt-treated fruits for either variety, the supervised method, Discriminant Function Analysis (DFA), classified the untreated and salt-treated samples of both varieties. The application of Genetic Algorithms (GAs) enabled the identification of key regions within the FT-IR spectra important for this discrimination, corresponding to nitrile-containing compounds and amino radicals.

Analyses of both gene expression and metabolite profiles were performed to elucidate the mechanisms responsible for the ability of a salt-tolerant tree species, *Populus euphratica*, grown in one of its natural habitats, a saline semi-arid area (the Ein Advat valley, located in the Negev desert in Israel) to acclimate to high salinity (Brosché et al., 2005). Leaf samples were collected from trees grown in four experimental sites in the valley, represented by three distinct areas that are characterised by a different degree of soil salinity, in addition to a non-saline well-irrigated area used as a control.

The accumulation of 22 selected metabolites in the leaves was examined by GC-MS. Trees growing in the most saline area, which accumulated more Na<sup>+</sup>, displayed a significantly higher concentration of the amino acids,  $\beta$ -alanine, valine and proline, whereas changes in stress-responsive carbohydrates and organic acids were of relatively limited extent, when compared to what is observed in *Arabidopsis*, and were statistically significant only for glycerol, glyceric acid and myo-inositol.

An interesting comparison between water deficit and salt stress has been described by Cramer et al. (2007). These authors monitored the early and late changes in the transcript and metabolite profiles induced in the vegetative tissues of grapevines (*Vitis vinifera*, cv. Cabernet Sauvignon) by long-term (16 days) water deficit and salinity stresses. Both stresses were gradually applied to the plants to better mimic field conditions. Moreover, the uniqueness of the experimental design was represented by the imposition of equivalent water potentials over time in the two stress treatments, thus, allowing the discrimination of the osmotic effects from the ion toxicity effects.

As expected, the relative abundance of several metabolites was altered by both stress conditions; however, at equivalent water potentials, water deficit had a more severe effect than salinity. Namely, among the key compounds involved in energy metabolism and osmotic adjustment, malate, proline and glucose were significantly higher in drought-treated than in salt-treated plants; moreover, only drought caused an increase in citrate and tartrate. With regard to inorganic molecules, a higher accumulation of sulphate, chloride and phosphate was observed under salinity than under drought stress. These differences in metabolite accumulation between the two growth conditions were correlated to differences observed in the transcript levels of genes involved in energy metabolism and nitrogen assimilation. Altogether, the data reported by Cramer et al. (2007) suggested a greater need for osmotic adjustment, ROS detoxification and photoinhibition amelioration in drought-treated than in salt-treated plants.

Another example of a multiple stress comparison is represented by the afore-mentioned results by Morsy and co-workers (2007; see section 4.1.3). These authors characterised two rice genotypes that contrasted in chilling tolerance for their response to water-deficit and high salinity stresses and found that, unexpectedly, the chilling-tolerant (CT) genotype was more sensitive than the chilling-sensitive (CS) one to both of the stresses. The high

accumulation of specific osmoprotectants, such as trehalose (under drought conditions) and mannitol (under salt conditions), observed in CS relative to CT, might account for its higher tolerance under these stresses.

Two rice cultivars (Arborio and Nipponbare) have been characterised by  $^1\text{H-NMR}$  analysis for their metabolic profiles under either osmotic or salt-stress conditions in *in vitro* experiments by Fumagalli et al. (2009). Nipponbare was found to be more tolerant to both stresses than Arborio, as shown by the percentage of inhibition on shoot and root growth. For both genotypes, PCA score plots clustered the samples into three distinct groups, depending on the growth conditions: untreated, osmotic treated (PEG 20%) and salt treated (NaCl 150 mM) seedlings. In comparison to control growth conditions, shoots of both cultivars accumulated a higher amount of glucose, glutamine and glutamate under both stress conditions; under salt stress, an increase in the content of sucrose, threonine, valine and lactate was also induced.

Although the two rice cultivars showed the same trend in metabolic changes during stress, they significantly differed in the relative amount of some metabolites, namely in the sucrose/glucose ratio and in the glutamate/total amino acids and glutamine/total amino acids ratios. These results suggested that both sugar and glutamine-glutamate metabolism were differentially regulated in the two cultivars in response to abiotic stresses.

More recently, Widodo et al. (2009) conducted an analysis of the metabolic responses to salinity stress in barley, a species of particular interest for metabolomic studies among cereals, as it is characterised by a higher  $\text{Na}^+$  tissue tolerance (i.e., the capacity of accumulation of high concentrations of  $\text{Na}^+$  in leaves) in comparison to rice and wheat.

Two barley cultivars differing in their salt tolerance, Sahara (more tolerant) and Clipper (more susceptible), were compared for their metabolic profiles under normal or saline conditions in a time-course experiment (24 hours, 3 and 5 weeks). The PCA of the leaf metabolites separated the samples belonging to the two cultivars grown in any conditions, the distance increasing with the time of the experiment for both control and treated samples. In both cultivars, a clear separation between short-term (24-h) and long-term responses (3 and 5 weeks) was also evident. Indeed, after 24 hours of salt treatment, only a few changes in metabolite concentrations were detected, whereas after long-term exposure (3 and 5 weeks) a greater number of metabolic changes and a larger magnitude of these changes were observed in both cultivars.

The authors suggested that, with the exception of proline, the observed accumulation of several amino acids in Clipper leaves after long-term salt exposure might correlate, as reported for other species, with slower growth and/or leaf necrosis, thus, being an indicator of general stress and cell damage rather than part of an adaptive response to salinity.

On the contrary, the specific accumulation in Sahara leaves of organic acids (including TCA cycle intermediates), sugars, polyols and other compounds, already known to be involved in cellular protection, may actually have a functional role in establishing the salt-tolerant phenotype of the cultivar (Widodo et al., 2009).

An interesting aspect of stress response is represented by the observation that mycorrhizal plants exposed to osmotic constraints generally perform better than nonmycorrhizal plants. Most of the knowledge on the improved stress protection comes from plants interacting with arbuscular mycorrhizas (AMs), whereas relatively little information is available on molecular and physiological mechanisms underlying the enhancement of stress tolerance in host plants by ectomycorrhizas (EMs).

Luo et al. (2009) have investigated the transcriptional and metabolic profiles in EM and non-EM roots of gray poplar (*Populus x canescens*) under control or excess-salinity conditions. The mycelia of the fungus *Paxillus involutus* were used for mycorrhizal inoculation. Unstressed EM roots accumulated osmolytes, such as soluble carbohydrates, sugar alcohols and free amino acids, at a higher extent than non-EM roots. Moreover, sugars of both major and minor pathways were more abundant in EM than non-EM roots also under stress conditions. Conversely, there were no significant differences in the amino acid content between stressed non-EM roots and both unstressed and stressed EM-roots. In agreement with the metabolic data, a microarray analysis indicated a constitutive activation of stress-related genes in control EM-roots, that are activated by salt stress in non-EM roots. Altogether, the data of Luo et al. (2009) indicated a stronger induction of defence pathways and metabolites in EM roots than in non-EM roots exposed to excess salinity, suggesting that the fungus *P. involutus* was able to prime the poplar plants for increased stress tolerance.

#### 4.4 Oxidative stress response

A common consequence of most abiotic stresses is an increased production of reactive oxygen species (ROS), which are highly toxic and cause damage to proteins, lipids, carbohydrates, chlorophyll and DNA, thus, resulting in oxidative stress (Gill & Tuteja, 2010). ROS are mainly by-products of processes, such as photosynthetic or respiratory electron transport. Under normal growth conditions, there is an equilibrium between the production and the scavenging of ROS, but abiotic stress factors may disturb this equilibrium, leading to a sudden increase in intracellular levels of ROS. Most of the studies on this topic have been performed on ROS-scavenging enzymatic antioxidants, which represent the initial defence mechanism, whereas fewer studies have been reported about the direct consequences of oxidative stress on the plant metabolome.

Baxter et al. (2007), using the redox-active quinone menadione (MD), induced oxidative stress in *Arabidopsis* cell suspension cultures and characterised the dynamics of metabolic responses by following changes in metabolite abundance and in <sup>13</sup>C-labeling kinetics. A total of 23 metabolites out of the 50 analysed were significantly affected (16 decreasing and 7 increasing). The integrated evaluation of such metabolic changes (an increase in hexose and triose phosphates, gluconate, ribose, a decrease in malate and some amino acids) indicated a dramatic inhibition of the TCA cycle and a diversion of carbon into the oxidative pentose phosphate pathway (OPPP). The decrease of ascorbate (one of the principal cell antioxidant molecules), concomitant with the accumulation of threonate (an ascorbate breakdown product), indicated a prolonged severe oxidative stress with a failure to recycle the oxidised ascorbate entirely (Baxter et al., 2007).

Analogous studies performed on *Arabidopsis* roots from hydroponically-grown plants highlighted similar metabolic changes in short-term menadione responses (30 minutes), whereas after longer oxidative stress (2 and 6 hours), changes observed in *Arabidopsis* roots and cultured cells clearly differed (Lehmann et al., 2009). In menadione-treated roots, among 56 identified polar metabolites, 33 were significantly affected within the first 30 minutes, and 39 were altered in at least two time points. The early changes, analogous to the observations in cell cultures, consisted of a decrease in the TCA cycle metabolites and associated amino acids and an increase in the OPPP intermediates Ribose 5-P and Ribulose 5-P and some glycolytic intermediates. As the time course proceeded, the amount of many

metabolites (i.e., TCA cycle intermediates and some amino acids) returned to normal values and further increased in the roots, in contrast to the response of cultured cells, in which most metabolites remained depressed throughout the time course. A major difference in the response of cells in culture and roots was in glycolysis: whereas in cultured cells a sustained increase in hexose 6-phosphates and a transient increase in 3-PGA were observed, in roots a significant decrease in hexose 6-phosphates and a linear increase in pyruvate were found. Moreover, the following variations in metabolites that can prevent oxidative damage were reported in menadione-treated roots: an increased abundance of proline (with a concomitant decrease in its precursor, glutamate), changes in polyamine metabolism with a decrease in putrescine, and accumulation of some methionine-derived aliphatic glucosinolates (Baxter et al., 2007; Lehmann et al., 2009).

An enhanced tolerance to menadione-induced oxidative stress was displayed by rice cultured cells overexpressing the Arabidopsis *Bax Inhibitor-1* (*AtBI-1*) gene (Ishikawa et al., 2010). *Bax Inhibitor-1* is an endoplasmic reticulum membrane protein, acting as a cell death suppression factor, that is widely conserved in animals and higher plants.

Using Capillary Electrophoresis-Mass Spectrometry (CE-MS), the authors investigated the metabolic responses to cell death-inducible oxidative stress. The control rice cells showed a shift in carbon flow from the central pathway to the OPPP, probably due to an increased requirement for NADPH as reducing power, in agreement with data obtained in roots and cultured cells of Arabidopsis (Baxter et al., 2007; Lehmann et al., 2009). However, despite the depression of carbon metabolism in the central pathway, a marked accumulation of most amino acids derived from PEP, pyruvate and oxaloacetate was found in MD-treated rice cells. This observation was inconsistent with results obtained in MD-treated Arabidopsis, in which decreased levels of several amino acids correlated with decreases in their precursors (Baxter et al., 2007; Lehmann et al., 2009). *AtBI-1* overexpression did not produce any significant effect on primary metabolism in non-stressed cultured cells. However, clear differences between *AtBI-1* overexpressing and control cells were found following a 24 h exposure to stress (but not at earlier time points), mainly in some metabolic pathways, i.e., glycolysis, amino acids of the glutamate and aspartate families, and components of redox and energy metabolism. These results suggested that tolerance to oxidative stress conferred by the *AtBI-1* factor was due to a higher capacity of metabolic acclimation, with a recovery of metabolite composition that was depleted during the early response.

Oxidative stress and programmed cell death may be induced both in natural and cultivated plants, including forest trees, by ozone ( $O_3$ ) exposure. In the past few years the increase in tropospheric ozone concentration has become one of the most serious environmental stress factors, that negatively affect plant growth, development and productivity. Ozone is a photochemically generated air pollutant, that can enter the intercellular space of leaves through the stomata, react with water and spontaneously generate ROS. Depending on the severity of the stress ( $O_3$  concentration and length of exposure) and on the susceptibility of the plants (varying with age and genotypes), damage symptoms may range from visible chlorosis and necrosis in the leaves to inhibition of photosynthesis and reduced yield. The ozone effects have been studied with two main approaches, by exposing plants either to a high-dose of  $O_3$  for a short period (acute ozone exposure) or to a weaker dose for a longer period (chronic ozone exposure), which represents a more realistic stress condition. The plant responses to this atmospheric pollutant have been recently investigated through “omics” tools in different

species, such as *Arabidopsis*, rice and birch (Cho et al., 2008; D'Haese et al., 2006; Kontunen-Soppela et al., 2007; Li et al., 2006; Ludwikow & Sadowski, 2008).

Cho et al. (2008) performed a systematic analysis of rice seedling molecular responses, using parallel transcriptomics, proteomics and metabolomics approaches, thus, providing a global view of signalling and metabolic pathways involved in rice response to O<sub>3</sub> exposure. CE-MS based metabolomic profiling revealed an increase in the content of several amino acids, GABA, glutathione and sakuranetin, a main rice secondary metabolite. The integration of the outputs from all these different approaches allowed the authors to indicate glutamate, GABA and glutamate dehydrogenase as possible biomarkers for O<sub>3</sub> damages in rice.

A long-term ozone exposure experiment was conducted in realistic open field conditions by Kontunen-Soppela et al. (2007), to compare O<sub>3</sub>-induced leaf metabolome changes in two genotypes of white birch (*Betula pendula* Roth) differing in their ozone sensitivity. Among 339 low molecular weight metabolites, ozone enrichment led to increased concentrations of phenolic compounds (such as chlorogenic acid and quercetin glycosides) and lipophilic compounds related to leaf cuticular wax formation. On the contrary, decreases in concentrations of many carbohydrates and chlorophyll-related compounds were induced by elevated ozone.

#### 4.5 Plant stress response and circadian clock

A very interesting aspect that has been more recently addressed is the interaction between the endogenous circadian clock and the transcriptional and metabolic reprogramming that occurs during the plant stress responses.

A role for the circadian clock in cold stress responses has been demonstrated (Nakamichi et al., 2009) and a large overlap between cold- and circadian-regulated genes has been observed (Bieniawska et al., 2008). These authors reported that diurnal- and circadian-regulated genes were responsible for the majority of the substantial variation observed between different experiments carried out to characterise the cold-responsive transcriptome in *Arabidopsis*. That is, genes identified as cold-responsive were dependent on the time of day the experiment was performed and a control at normal temperature did not correct for this effect, contrary to what is currently assumed.

Espinoza et al., (2010) have investigated the role of diurnal and/or circadian regulation in metabolic cold-induced responses, performing an integrated analysis of both transcripts and metabolites. Their findings also underlined the importance of understanding cold acclimation in the correct day-night context. Furthermore, they observed that a mutant with a disruption in the circadian clock was more sensitive to freezing and impaired in its cold acclimation capacity. This finding was in agreement with data reported in *Populus* on a reduced ability to cold acclimate of transgenic lines where the expression of some clock-component homologs genes had been down-regulated by RNA interference (Ibáñez et al., 2010). On the contrary, an *Arabidopsis* triple mutant for other clock-component genes has been reported to have an increased freezing tolerance, associated with a higher accumulation of the compatible solutes, proline and raffinose (Nakamichi et al., 2009). The reason for these contrasting phenotypes of different clock mutants remains to be elucidated. It must be mentioned that in *Arabidopsis*, the time-of-day has also been shown to influence the transcriptome alterations following drought exposure (Wilkins et al., 2010).

The circadian clock seems to function as a central coordinator of plant metabolism, to maintain homeostasis by determining the levels of both primary and secondary metabolites

and also to allow plants to anticipate future environmental stresses, such as drought at midday and cold at midnight. In addition, a feedback mechanism is brought about by metabolic and stress cues on the central oscillator itself (A. Sanchez et al., 2011).

It is noteworthy that a functional link between the circadian clock and plant immunity has also been reported very recently (W. Wang et al., 2011), with a remarkable and intriguing example of a plant tuning its immune response against a pathogen. Arabidopsis defence genes involved in the response to an oomycete pathogen were found to be under the control of a central circadian regulator (the *cca1* gene), thus, allowing plants to anticipate infection at dawn (when the pathogen disperses the spores) through a maximal expression of the relevant genes at the time of day when attack is most likely (W. Wang et al., 2011).

Thus, the circadian clock and the response to both abiotic and biotic stresses appear to be firmly interconnected in plants. Furthermore, the integration of the circadian clock with the stress signalling pathways might have played a crucial role in the development of plant adaptation to their environments during evolution.

## 5. Integration of “omics” results

The availability of high-density microarray and next generation sequencing technologies has opened the route to carry out whole genome transcriptome (WGT) analyses in a high-throughput manner.

Likewise, high throughput LC-MS approaches enable the rapid identification of large sets of proteins and of their post-translational modifications (Huang & Xu, 2008).

In fact, plant acclimation to abiotic stress conditions is associated with profound changes in proteome composition. Since proteins are directly involved in plant stress response, proteomics studies can significantly contribute to unravel the possible relationships between protein abundance and plant stress acclimation (Kosovà et al., 2011)

Post-translational modifications (PTMs) are also involved in the regulation of a wide range of cellular responses to abiotic stress stimuli and greatly affect protein structure, activity and stability. Several hundred PTMs have been described in the literature and the advent of high-throughput quantitative proteomics technologies has allowed the systematic identification of the PTMs (phosphorylation, S-nitrosylation, ubiquitylation, SUMOylation, glycosylation) and the determination of their functional relevance in the context of regulation and response to abiotic stress (Ytterberg & Jensen, 2010).

These global analyses were in numerous cases coupled to metabolomic approaches (see above), reinforcing the tight link between changes in specific transcriptional patterns of candidate gene and/or specific proteomic patterns to the production of metabolites (both primary and secondary).

The WGT technologies enable to precisely pinpoint the classes of genes under transcriptional control (down/up-regulation) and to define not only responses at the gene level but also at that of “network” (Yamaguchi-Shinozaki et al., 2006; Swindell et al., 2007).

In fact, as described for the metabolomic approaches, the characterisation of the whole transcriptome enables researchers to identify the whole cascades of target genes from the transcriptional factors to the effector genes (whose expressions is dependent upon that of specific transcriptional factors).

The transcriptome analyses allow to define co-regulatory pathways that often underlie the concerted up/down-regulation of large sets of genes involved in the same regulatory

and/or biosynthetic pathways (Krouk et al., 2010). This is of high relevance to define the genetic components of specific or common plant responses to abiotic stress conditions.

The advent of high-throughput sequencing technologies has markedly accelerated the generation of whole transcriptome data and the capability of capturing changes in expression also of rare transcripts. It is now possible to globally define transcription start sites, polyadenylation signals, alternative splice sites and generate quantitative data on gene transcript accumulation in single tissues or cell types (L. Wang et al., 2010). These deep-sequencing technologies (also called Next Generation Sequencing Technologies) are thus paving the way for global genome transcriptomics and will undoubtedly lead to novel insights into plant abiotic stress responses. However, several challenges exist to making this technology broadly accessible to the plant research community, including the current need for a computationally intensive analysis of large data sets.

## 6. Conclusion

Metabolome analysis has become an invaluable tool to study plant metabolic changes that occur in response to abiotic stresses. This approach has already enabled to identify a large number of metabolites whose accumulation is affected by exposure to stress conditions. However, despite the many progresses that have been achieved in this field, much work is still required to identify novel metabolites and pathways not yet linked to stress response and tolerance and to decipher the extensive coordination and interaction among the various metabolic pathways.

To better understand the role of stress-associated metabolites in abiotic stress response, it has to be taken into account that metabolites not only have functional roles in stress tolerance but also act as signalling molecules. In most of the studies, the production, increase or depletion of metabolites are mainly regarded as the final, downstream response of the plant cell to the external stimuli. However, the question should be addressed whether the changes in metabolic networks that are observed are driven by alterations in gene expression, or whether the transcriptome changes are responding to a specific metabolic perturbation. In addition to hormones or other canonical mediators, such as sucrose and glucose, many other small molecules may play a crucial role in signalling pathways; it seems likely that only a subset of the metabolites with a mediator function in the regulation of transcription in response to stresses has been identified so far.

To this purpose, it is essential to consider the temporal dynamics of the response, through an integration of the "omics" data obtained at different time points during stress exposure. But even this "snapshots"-based approach, consisting in the comparison among different samples taken at different time points, has been recently considered as a rather static approach and its usefulness for obtaining a comprehensive and global information on stress-induced molecular changes has been questioned. More dynamic approaches, such as fluxomics (Wiechert et al., 2007), aimed to follow the flux of metabolites through pathways, are being currently developed and might reveal to be much more informative.

To elucidate the function of a single compound, it is also important to be aware that compensatory mechanisms are commonplace and that a change in the content of a single metabolite may have no effects on the phenotype, because of compensative modulation of other components of the same family of compounds.

The original approach proposed by Lukan et al. (2010), considering the metabolome as a single "virtual molecule" whose physicochemical properties are the weighted averages of

the properties of individual metabolites, also appears to be quite promising both to investigate the global metabolic strategies of a species to maintain cell function under stress and to evaluate differences among species.

In addition, further efforts to make stress treatment conditions more relevant to plant growth outside of the lab are required. Because plants are often subjected to a combination of multiple adverse conditions rather than to individual stresses (a common example being represented by the simultaneous occurrence of heat and drought in the field), tolerance to multiple abiotic stresses is an important breeding target in crops. Studies performed comparing single or combined stresses have already demonstrated that metabolic responses may be quite different and these results have to be considered in identifying strategies to improve stress tolerance, either by breeding or by transgenics approaches.

Moreover, as relatively little is known about the molecular mechanisms that underlie the acclimation of plants at a long-term realistic exposure to specific stressors, the focus has to move from how plants survive “acute” (sudden and short-term) stress conditions to how plants respond to “chronic” (long-term), sub-optimal growing conditions.

Another aspect to be considered is that, besides classical stress factors, plants also have to cope with emerging stressors (such as tropospheric ozone and anthropogenic stressors), which were not previously met by species during evolutionary times.

The recent findings on a firm interconnection between the plant circadian clock and the response to both abiotic and biotic stresses also emphasize the importance of having a diurnal perspective when plant stress responses are characterized and of investigating stress response in the correct day-night context.

Another major challenge is the elucidation of epigenetic regulation mechanisms, including changes in nucleosome distribution, histone modification, DNA methylation, and non-protein-coding RNAs (npcRNAs), which also play important roles in abiotic stress gene networks (Urano et al., 2010).

The integration of the -omics approaches, that have markedly increased our understanding of global plant systems in response to stress conditions, is likely to enable researchers to reconstruct the whole cascade of cellular events leading to rapid responses and adaptation to the various abiotic stress stimuli.

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# The Importance of Genetic Diversity to Manage Abiotic Stress

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## 1. Introduction

Before the first half of Twentieth Century the biggest concern of the agricultural technology was the environmental adaptation instead of the development of plant varieties and livestock breeds with improved resistance or tolerance to biotic and abiotic stresses. Consequently, after decades of human intervention, the agriculture shift from a low impact activity to became a threat for environmental conservation. Besides that, over the years this model of "Green Revolution" proven to be ineffective in increase the yield continuously, since the genetic source of variability used in breeding programs became narrow and limited.

Afterwards, breeders began to change their strategies for developing new plant varieties and animal stocks. They started to realize and reflect on the value contained in the biodiversity and the genetic treasure available in landraces, exotic and wild species that evolved with the goal of be more adapted to their environment. Nowadays, researchers all around world are working hard to collect and preserve plant and animal samples for germplasm conservation because most of the wild life remains in continuous risk of extinction at natural environments. The main prompters of it are the agricultural activity itself, the rising land occupation by people and the heavy and growing industrial activity.

With the advance and deployment of new technologies, mainly in the field of the modern biotechnology, a new frontier was opened to the development and improvement of plants and animals. Nowadays is increasing the adoption of commercial transgenic organisms by farms and breeders. For some species as maize, cotton and soybean, most of the world production is already transgenic and the expectation is this kind of technology is going to be adopted for many of the species regularly used by man and replace the conventional crops. With the development of transgenic organisms, the insuperable sexual barriers considered before as a problem to hybridize different species are not longer a concern for plant and animal breeders. Now, this new technology allowed us the use of the whole gene pool available in the nature to improve plant and animal genotypes.

The natural environmental in many occasions can be hostile to living organisms. For species that not evolved to a specific and restricted condition, the chances to stay alive without human intervention are very small. Unfortunately, most of the cultivated species are not properly adapted to handle itself against several kinds of stress commonly found during its

cultivation. The most restrictive factors faced by agriculture are the drought stress, attack of pests and diseases, poor soil fertility, soil salinity, excessive heat and cold, flooding, soil acidity and aluminum toxicity among many other types of stresses. Several wild species get used to these stresses because they are exposed to them for thousands of years, evolving to be low or even unaffected by them.

On centers of genetic diversity the chances to finding wild variants of species with traits of rusticity and resistance are greater than in any other place of Earth. The accurate knowledge, characterization and conservation of these variants in its diversity centers are crucial steps for the success of breeding programs whose aim nowadays is mainly focused in adaptability and improvement for marginal environments.

Besides that, the new tools of the modern biotechnology such as whole genome sequencing and large scale transcriptomes (microarrays) make the identification of genes involved with valuable traits easier and faster. A new perspective is emerging for the knowledge and use of biodiversity and the most incredible aspect of this new scientific field is the opportunity to rescue resistance genes from other living sources if it is not available in the target germplasm.

Wild species and exotic relatives of crop plants as well as landraces contain valuable genes that are of immense genetic value in crop improvement programs. However, several species and variants of living beings are vanished from the Earth surface at daily basis in a rate never seen before. Nowadays the genetic diversity conservation is the biggest concern of humankind due its essential role in agriculture, medicine, industry and forestry and today it is a priority issue in many government's agenda. The dramatic fact is the gene pool available in wild species remains poorly known and unexplored in its numerous opportunities. Therefore, the main challenge is to preserve most of this richness for next generations.

## 2. Population growth and genetic diversity

The term abiotic stress was first used to describe the negative impacts of non-living factors on organisms in a given environment. In recent decades the use of this term has become constant and its effects are considered more and more concerning due the intense human activity as a result of the rapid world population growth (Fig. 1).

However, due to increasing global awareness and the prompt actions of governments in few countries, nowadays the rate of population growth in the world is a decreasing slope. In 2009 this growth rate was 1.2% while in 40 years ago it was 2.1% (Fig. 2). But the situation still worrying and it is far from the ideal. Many other countries must join these actions to ensure a sustainable world and decrease the pressure over the natural resources.

Many developing countries keep up with the rate of growth at a very accelerate pace. Thus, the prediction for population growth is worrisome. With the current rates, the predictions indicate that world population will be over 10 billion people around the year of 2100 (Fig. 3) increasing the negative effects on natural resources and changing the earth's climatic balance. The deleterious effects of this massive population growth can be already observed in the environment.

The recent and rapid increase in human population overload the need for food production. The "Industrial Revolution" that occurred from the 18<sup>th</sup> to the 19<sup>th</sup> centuries, also revolutionized food production in 1960s changing from traditional farmsteads that grew a variety of crops on smaller scale to the new industrial farms focused on growing massive amounts of a single product. This agricultural revolution became known as "Green Revolution".

Consequently, the lands needed for food production moved from small farms to huge and continuous fields that enormously increased the demand for natural resources like soil and water. Thus, to make room for this new agricultural industry the destruction of natural environments has increased considerably since 1960.

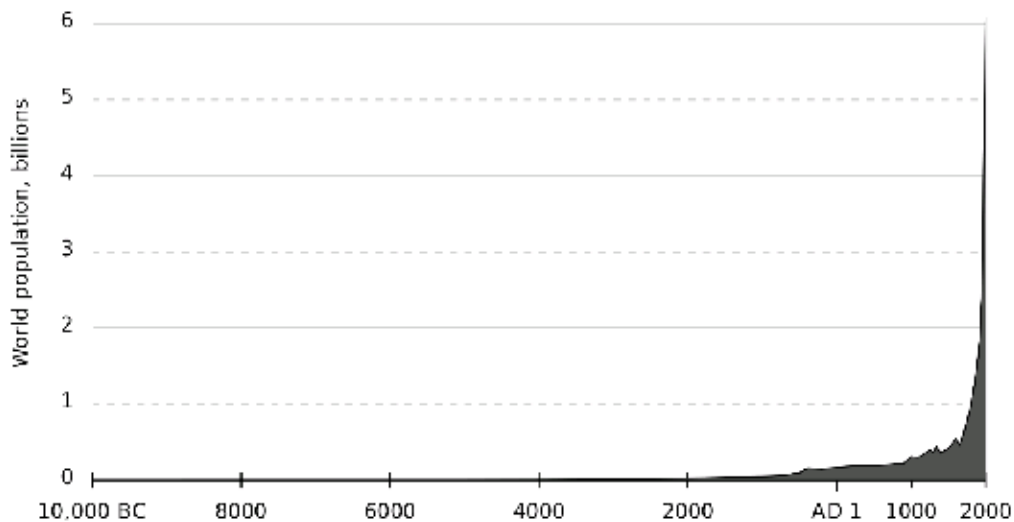


Fig. 1. World population curve from 10,000 BC to 2000 AD.



Fig. 2. World population growth rate. Percentage change of resident population compared to previous year. Data source: World Bank Development Indicators (April 26, 2011).

However, while the current agriculture is carried out in large areas increasing the natural resource demands, in the last 50 years they were not the only villain. Population growth and migration to urban centers have exacerbated the impact of cities on the environment. In 2007 for the first time in human history, the earth's population was more urban than rural, according to scientists from North Carolina State University and the University of Georgia

(DALLAS, May 25, 2007, <http://www.sciencedaily.com/releases/2007/05/070525000642.htm>).

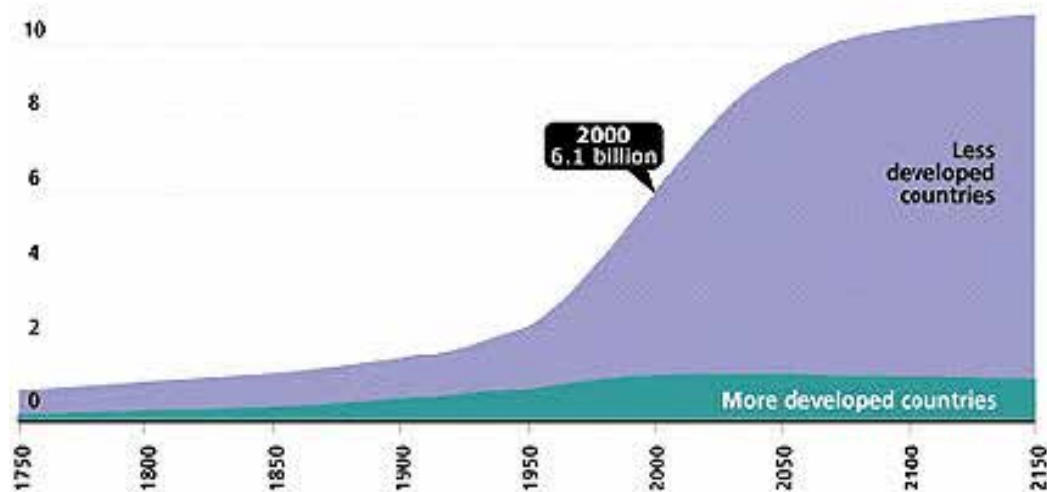


Fig. 3. World population growth (in billions) from 1750 to 2150. Data source: United Nations, World Populations Prospects, The 1998 Revision; and estimates by Population Reference Bureau.

In 1950 cities as São Paulo in Brazil, Mexico City in Mexico and Bombay in India, had population of 2.33, 2.88 e 2.86 millions of habitants, respectively. In 2010 these same cities recorded population numbers of 20.26, 19.46 and 20.04 millions, representing a stunning growth of 869.5%, 675.7% and 700.7%, respectively, in a short period of six decades (United Nations, Department of Economic and Social Affairs, World Urbanization Prospects: The 2009 Revision, <http://esa.un.org/unpd/wup/index.htm>).

One of the main reasons for this dramatic urban growth is the migration from rural lands to urban areas. Small and traditional farms are left behind because they have become uncompetitive and non-profit when compared to the current agribusiness industry.

There are many kinds of abiotic stress and they can be originated from natural sources or triggered by human activity. The most common examples are extreme temperatures, drought, flood, high winds, soil salinity, mineral deficiency and toxicity, soil and water poisoning as well as other natural disasters such as tornadoes, wildfires, volcanic activities and earthquakes. All of them may affect plant growth and reproduction at different severity levels.

According to recent data from research carried out by several scientific teams worldwide, agriculture and livestock have played a considerable role in the environmental changing, including global warming due greenhouse gas emission, land degradation and desertification, air and water pollution and intense loss of biodiversity (FAO, <http://www.fao.org/ag/magazine/0612sp1.htm>).

Deforestation, desertification, use of fossil fuels are examples of anthropogenic sources of devastation that aggravates abiotic stress effects. The agriculture production, massive land use and biomass burning together contribute to 22.5% of the green house gas emissions, 46.6% of methane production and 88% of the nitrous oxide (Fig. 4).

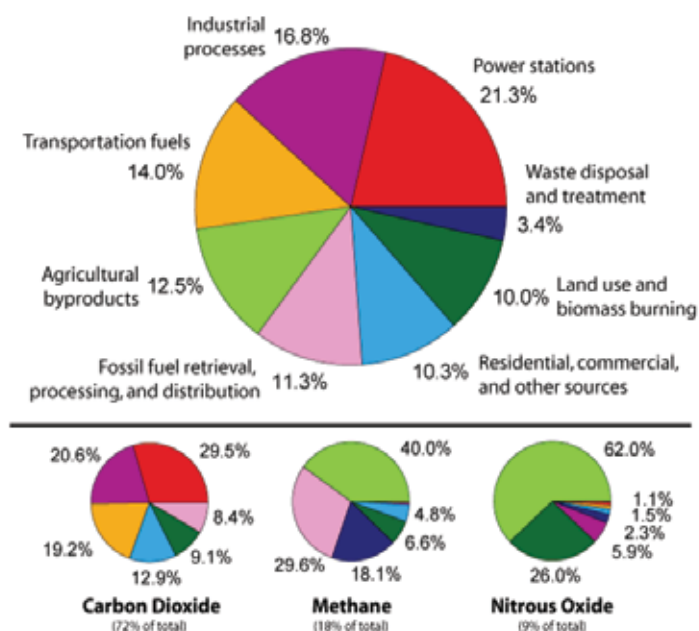


Fig. 4. Annual greenhouse gas emissions by sector. Data source: Kedar Karki. Effect of Climate change in agriculture and livestock production (<http://pt.scribd.com/doc/3323459/Effect-of-Climat-change-in-agriculture-and-ivestock-production>).

The main victims of the increasing abiotic stress are the fauna and flora closely followed by the poor populations living in marginal areas. Although in a natural context all living organisms are frequently exposed to any kind of abiotic stress during their life, the natural evolution plays a fundamental role to adapt and adjust these organisms to adverse conditions in a process that evolves in a continuous and slow way. However, today what we observe is that the majority of wild species are not able to overcome as quickly as necessary to adapt to abrupt changes that are occurring in the environment. Therefore, scientists are estimating that the number of species extinction is happening in a rate 1000 folds faster than would occur if the anthropic interference was not so intense.

The society and many Governments began to realize that the time for change is right now. Agriculture has undergone a huge shift from the paradigm introduced by the "Green Revolution" which advocated the radical environment intervention to make it friendly to agriculture and now it is beginning to be replaced by the concept of sustainable agriculture where crops and livestock must be designed by breeders having as goal the better adaptation to environment, and consequently, a better performance against biotic and abiotic stresses.

The major challenge, however, results from the complex nature of abiotic stress-tolerance traits and the difficulty in dissecting them into manageable genetic components feasible to be modified by molecular approaches. In crop breeding, advances in molecular biology and genomics have had a large impact on the speed of identification and characterization of genes and genetic regions associated with quantitative and qualitative traits. Marker-assisted selection through the use of high-throughput marker systems are currently being

used extensively in breeding programs to improve selection efficiency, accuracy and to direct focus towards traits of great importance for adaptation (Sutton, 2009).

Association between gene activity and response to abiotic stress is the major challenge involving functional genomics research and plant and animal breeding. New tools and approaches as genetic modification, gene knockout, RNA interference, genomics, proteomics, metabolomics and metagenomics have allowed new insights in this field and many advances in the role of genetics controlling complex traits such as those involved with response to abiotic stress.

The genetic variability accumulated in various plant species and involved with increase of production and adaptation to less favorable environments is being used by humankind since beginning of agriculture by selection of seeds collected from the best genotypes. Although in the “Green Revolution” the main focus of the genetic improvement has been converged to large and uniform productions, currently features such as disease resistance, nutritional quality and abiotic stress tolerance have assumed a prominent space in the attention of modern breeding programs.

Moreover, genetic engineering has proven to be a strong ally to the conventional plant breeding, transposing the sexual barriers among species and allowing the expansion of search for desirable agronomic traits virtually to all living beings. It also make the breeding process less random since specific traits might be handled without fear of genetic dragging (i.e. the inheritance of negative traits genetically linked to desirable traits).

Below we will detail some of the main abiotic stress and how molecular biology and genetic engineering can be used to alter cellular metabolism towards the genetic improvement to abiotic stress tolerance.

### 3. Types of abiotic stress

#### 3.1 Metal toxicity

The compartmentalization of toxic ions in the vacuole is a strategy widely used by plants when exposed to various types of stress. Thus the cytoplasmic sites are protected from possible toxic effects of ions such as Al, Mn, Zn and Cd. Tobacco plants expressing the gene CAX2 from Arabidopsis, which encodes an exchanger in  $[Ca^{2+}] / [H^+]$ , showed higher tolerance to Mn, possibly due to the fact that this protein also acts in transport of  $Mn^{2+}$  and  $Cd^{2+}$  to the vacuole (Hirschi et al., 2000). Arabidopsis plants overexpressing ZAT gene showed no sign of stress when exposed to 0.2 mM zinc, concentrations that cause chlorosis and inhibits the growth of untransformed plants (Zaal et al., 1999). The ZAT gene encodes a probable carrier, which transfers  $Zn^{2+}$  from the cytoplasm to the vacuole.

The toxicity of  $Al^{3+}$  ion is an important factor limiting plant growth in acid soils, such as savannahs in Africa and cerrado in Brazil. Many plant species exude organic acids in response to toxic levels of Al. De la Fuente et al. (1997), altered the balance of organic acids in tobacco plants and papaya, through overexpression of a gene that encodes a citrate synthase of *Pseudomonas aeruginosa*. This disturbance in metabolism caused an increase in citrate exudation by root cells. By assessing the growth of transgenic plants in a solution containing toxic levels of Al, the authors observed a significantly better performance in these plants than that observed in wild plants. This is probably due to the chelating action of citrate over the ions of  $Al^{3+}$ , preventing the toxic effects of this ion.

Cançado et al. (2008) working with root tips of two contrasting Al tolerance maize lines (Fig. 5) identified several genes whose responses were altered by the stress triggered by Al.



The toxicity promoted by this metal is one of the major limiting factors to plant growth in acid soils. The most dramatic symptom of Al toxicity is the inhibition of root growth and consequently, the water uptake and mineral nutrition in plants affected by Al is completely unbalanced. Cançado et al. (2008) used an approach where more than two thousands genes were evaluated simultaneously. Many genes showed up-regulated expression while another showed down-regulated expression when the maize plants were exposed to Al stress.



Fig. 5. Root growth of two aluminum-contrasting maize genotypes, Cat 100-6 aluminum tolerant and S1587-17 aluminum sensitive, after five days in nutrient solution with aluminum and without aluminum (control). Source: Cançado et al. (2008).

As example of up-regulated the authors found genes encoding proteins previously identified in several other works as induced by aluminum stress, such as phenyl ammonia-lyase, chitinase, Bowman-Birk proteinase inhibitor, and wali7. Most of these genes were up-regulated only in the tolerant maize genotype, strongly indicating a putative role of these genes in the trait of aluminum tolerance in this genotype. The use of maize transgenic plants overexpressing or silencing aluminum-stress responsive genes in the root tips may be a useful tool to clarify the role of these genes in the aluminum tolerance.

Cançado et al. (2005) studying a glutathione S transferase (GST) gene in the same maize lines cited above also observed that this gene showed nucleotide differences between both genotypes that reflected in differences in the amino acid composition of the proteins encoded by these genes in each maize line. Modeling of the tertiary structure of the GSTs in each line evidenced that there were different not only in composition but as well in the spatial folding of the catalytic site.

### 3.2 Salt stress

Salt stress causes large drops in productivity due to excess  $\text{Na}^+$ . The principal strategies employed to minimize the effects of toxic salt is the uptake blocking of  $\text{Na}^+$  or sequestration of these ions in the intracellular vacuole in addition to the synthesis of molecules osmoprotectant such as sucrose, proline, betaine and trehalose that allow osmotic adjustment, stabilizing some macromolecules and maintaining the integrity of the membrane (Garcia et al., 1997).

Apse et al. (1999), scanning the genome project data from *Arabidopsis thaliana*, found the AtNHX1 gene, homologous to yeast Nhx1 gene, which encodes a protein that acts transporting sodium ions into the vacuole. The authors observed that overexpressing AtNHX1 gene in *A. thaliana* the exchange rate between  $\text{Na}^+$  and  $\text{H}^+$  in the vacuoles was much greater than that observed in wild plants. Associated with that, the authors verified that the transgenic plants showed normal development when kept in a nutrient solution containing 200 mM NaCl, while wild plants had their growth strongly inhibited, with reduced leaf size and chlorosis.

In another successful example, Prasad et al. (2000) transformed *Brassica juncea* with the *codA* gene from *Arthrobacter globiformis*, which encodes the enzyme choline oxidase involved in the synthesis of glycine-betaine, an osmoprotectant. The transgenic plants when exposed to toxic concentrations of NaCl, showed better performance than the wild plants.

One consequence of various types of stress, such as saline, is the production of reactive oxygen species, which interact with various cellular components. Overexpression of enzymes involved in detoxification of compounds generated by oxidative stress has been employed to obtain plants with high performance against various types of stress. Roxas et al. (1997) produced tobacco plants overexpressing genes that encode a glutathione S-transferase and glutathione peroxidase. These plants showed some performance improvement in environments containing toxic concentrations of salts compared to normal environments.

### 3.3 Temperature

High temperatures affect the photosynthetic capacity of plants, with clear effects on agricultural yield. There are several indications that the average temperatures of Earth surface is increasing, perhaps as a result of the greenhouse effect. Thus, the production of plants with improved performance against high temperatures is a research field that arouses great attention worldwide.

Recently, Murakami et al. (2000) modified the composition of fatty acids in thylakoid membranes of chloroplasts from tobacco plants. Expressing the anti sense gene that encodes a desaturase of omega-3 there was a decrease in the proportion of unsaturated lipids, increasing the thylakoid membrane fluidity. The transgenic plants showed better performance when exposed to 36 °C and 40 °C while the rate of photosynthesis was greater than that observed in wild plants.

The manipulation of the synthesis of glycine-betaine using the gene code of *Arthrobacter globiformis* in *Arabidopsis* plants also produced encouraging results regarding the tolerance to heat and cold. Alia et al. (1998) obtained plants with high levels of choline oxidase, which induced the accumulation of glycine-betaine. These plants had higher heat tolerance during seed germination and early stages of seedling development. These same plants also showed increased tolerance to cold (Sakamoto et al., 2000). Thus, manipulation of osmoprotector levels is a strategy that can improve the performance of plants against various types of abiotic stresses.

Similar to the example of the protection observed with respect to salt stress, the expression of genes encoding enzymes that combat oxidative stress also has beneficial effects on plants exposed to low temperatures. In maize plants, overexpression of a gene for superoxide dismutase in chloroplasts from tobacco increased the metabolism capacity of superoxide radical ( $O_2^-$ ), which is toxic to cells (Van Breusegem et al., 1999). Thus there is an improvement in cold tolerance.

### 3.4 Phosphorus deficiency

Phosphorus deficiency is a major factor limiting agricultural productivity. To meet the needs of P it is necessary high investment in fertilizer application, which in turn leads risk of environmental pollution.

A response often observed in plants growing in P deprivation is the exudation of organic acids. López-Bucio et al. (2000) assessed the effects of P deficiency in transgenic plants with increased tolerance to Al (De la Fuente et al., 1997), which exuded higher citrate levels due to overexpression of the gene citrate synthase. The transgenic plants when grown in alkaline soil with low P availability had larger leaf area and dry weights of fruits and they were taller than wild plants. This performance is associated with higher levels of P intracellular. Supposedly, citrate increases the availability of P by moving it into insoluble complexes.

Another alternative for expanding the ability to uptake phosphate is the overexpression of proteins that act transporting P to the interior of root cells. Mitsukawa et al. (1997) expressed the gene PHT1 of *Arabidopsis* in cell cultures of tobacco. When transferred to medium without P for 4 days, the transgenic cells showed increase of 42% in the fresh weight compared to control cells, probably due to increased ability to acquire P (up to 3 times higher than in control cells).

## 4. The genomics as a tool for discovery of genes related to abiotic stress

Recent technological advances of the apparatus of sequencing and bioinformatics have allowed the discovery of a huge number of genes in a very short time. Genes associated to agronomic traits of interest are being sequenced by genome projects like ESTs (expressed sequence tags). These projects identify expressed genes in different tissues and stages of plant development.

Togheter with the ability to discover new genes, the technology for simultaneous analysis of thousands of genes have also evolved, with the possibility of access the expression profile of a myriad genes by using cDNA arrays, consisting of cDNA clones neatly set on supports of glass or nylon. The microarrays are arrays of genes in high-density platform, usually set on glass holder (Schena et al., 1995) while the macroarrays have lower density of genes, normally spotted on nylon membranes (Dezprez et al., 1998). These DNA arrangements can be hybridized with different cDNA probes representing the messengers RNA of distinct populations of cells. By comparing the signals of each gene with the different probes it is possible to evaluate the expression pattern of thousands of genes simultaneously (Fig. 6).

The large scale technology for gene expression study allowed researchers assess which genes are activated by plants in response to various types of stress, as demonstrated in the response of *Arabidopsis* plants to a deficiency of nitrate (Wang et al., 2000). The authors evaluated 5524 genes and identified 40 induced genes, which encode transcription factors, enzymes of several metabolic pathways and proteins without known function.

Nogueira et al. (2003) have studied the response of sugarcane to abiotic stresses, using nylon membranes containing clones of sugarcane ESTs. These membranes were hybridized with probes obtained by reverse transcription of total RNA from treated and control plants. The methodology allowed the identification of several genes up-regulated by low temperatures in sugarcane.

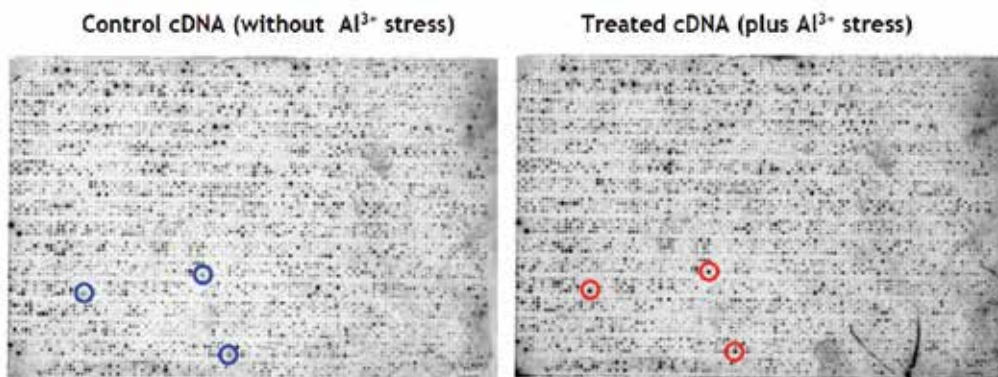


Fig. 6. Two filter arrays (macroarrays) hybridized with radioactive labelled probes (cDNA) obtained from two samples of maize root tips: one growing in a control treatment and another growing in presence of toxic aluminum. The circled spots indicate cDNAs whose expression was up-regulated by aluminum stress (Cançado et al., 2008).

The technology of DNA arrays have an important contribution during the identification of new genes that may be employed for producing transgenic plants as well as molecular markers in classic breeding programs.

## 5. Gene diversity and biodiversity

Diversity is a fundamental foundation to promote sustainable and environmentally friendly agriculture and livestock. In this case the concept of diversity is wide and it goes from number of species used by mankind, through the large number of varieties and breeds within each specie and finally, reaching the genetic richness inside the genome of each organism.

Although the DNA molecule was already known as the molecule responsible to storage the genetic inheritance, until the late 80s very little was known about the role of genes. With the fast technological progress observed in the biotechnological research since then, much was disclosure and new opportunities of improve the adaptation to abiotic stress began to emerge.

The vast concept of biodiversity can be defined as the conjunct of all existing living organisms, that inhabit the biosphere, with their taxonomic and ecological characteristics, their genetic variation, without considering the chemical and physical factors of the environment. Therefore, not only the differences between species but also the differences within the same species aggregates value to the biodiversity (Sandes & DiBiasi, 2000; Varella et al., 1998). Thus, greater is the biological variation and the abundance of species in a given location, greater is going to be the biodiversity and vice versa.

In biodiversity there is an hierarchical relationship between the different levels, starting in the gene through the individual, then through species and so on into specie populations,

ecosystems and finally reaching the most complex network level of interaction between living organisms with the surrounding environment.

Some predictions indicate that a number of different living organisms that inhabit the Earth diverge from 5 to 100 million, but until now has only identified around 1.7 millions (Wilson, 1999). This large variation in species number is due to the myriad of microorganisms, insects and fungi that colonize the most diverse places on Earth and which still remains unknown. Recently, a group of NASA researchers in the U.S. discovered a new specie of bacteria that use Arsenic in its DNA molecule instead of Phosphorus, breaking the paradigm that the DNA molecule has the same composition in all living beings (Wolfe-Simon et al., 2010). This fact illustrates how our knowledge about this subject still in its childhood. The awful truth is that we don't even know exactly how many species there are in flora and fauna of Earth. At general, the biodiversity is larger in tropical lands decreasing with the rising of latitude and altitude, being richer in places where rainfalls are more abundant and poorest in arid regions.

Since their evolutionary climb up to the present day of modern civilization, man has used as food only 1% of the nearly 300,000 known species of higher plants. At present, only 0.1% of existing species are used, with only 15 of them being responsible for about 90% of all food (Goodman, 1990). More than 60% of food production that reach consumers in all world are based on four main products: potatoes, rice, wheat and corn (Diniz & Ferreira, 2000).

Conservation of genetic resources available in biodiversity, as well as the knowledge of genes role, is important not only for the nations that harbor this biodiversity but also for the humanity, because it can ensuring its maintenance and food security in the future. Genetic resources are invaluable because have an incalculable potential for use and application now and in the future generations, holding ecological, genetic, social, economic, scientific, educational, cultural, recreational and aesthetic value (Varella et al., 1998). However the complexity of their benefits is still little known and measured by mankind.

Anthropogenic interference on natural ecosystems is currently the main cause for the biodiversity reduction and poverty is one of the most destructive forces in tropical lands. According to Mendonça-Hagler (2001), the emergence and extinction of species are natural phenomena that occur on defined geologic ages. However, if the destruction of ecosystems and habitats continues at its current pace, considering that there are 100 millions of different species on Earth, currently it is estimated that at least ten thousands species go extinct every year (considering an extinction rate of 0.01%/year). This rate is higher or lower depending on location. In countries like the US, where much of the biodiversity has already been extinguished the rate today is lower, but in places like Malaysia and the Amazon basin, the rate may be much higher than in other places on Earth due the high abundance of threatened species that still have this kind of environmental as natural habitat. In fact, the lack of knowledge about species richness is so great that it is difficult to accurately measure the rate of species loss.

Considering mainly the agriculture, the big concern about biodiversity reduction is the loss of genetic diversity also known as "genetic erosion". Many commercially cultivated species have narrowed their genetic basis due to intense process of yield improvement that reduced the number of genotypes useful for breeding programs. Most of genetic variability in plants is preserved in wild relatives that still present in the centers of diversity. Nevertheless, most of these centers of diversity are being reduced or even vanished from nature, forcing researchers to preserve a small fraction of this genetic variability in germplasm collections

maintained artificially. Wild relatives have always been useful for breeders, through the hybridization process using gene pools from native and exotic species and transferring them to cultivated species, improving for example, resistance to disease or pest attack.

With the biotechnology evolution and adoption by agriculture, today is possible to transfer specifically one gene involved with the characteristic of interest directly to the genome of a host organism, which for example can be a variety or hybrid of a commercial plant.

## 6. Using biotechnology to alleviate abiotic stress in plants

Unlike animals, plants are inherently immovable and consequently throughout its evolution they needed evolve intricate ways to response quickly and efficiently to effects of biotic and abiotic stress. The genetic code of each plant was assembled and refined by the environment surrounding to give the exact response to each stress in a very cordenate and efficient way. Of course, each location with its specific peculiarities prompted the evolution process to promote the most appropriate combination of genes involved with the expression of the best traits, making plants able to handle with specific adversities of its environment.

Thus, the best chances to dealing with the harmful effects of abiotic stress are prospecting adequate genes available in genetic diversity of living beings. Since the dawn of agriculture, man has already improved the plant species, even if it was carried out in an unconscious way. However, the limitations imposed by reproductive barriers between species prevented breeders to exploit the potential of the gene pool available in nature and thus hindering the development of new varieties by preventing the use of genes most useful for dealing with rough conditions. For many centuries the solution was to modify the environment in an attempt to make it more suitable for growing plants. However, with the development of recombinant DNA technology about 40 years ago, a new perspective was created for plant breeding.

The first transgenic plants were created in the early 1980s by four groups working independently at Washington University and Monsanto Company both in St. Louis, Missouri; the Rijksuniversiteit in Ghent, Belgium; and the University of Wisconsin. On the same day in January 1983, the first three groups announced at a conference in Miami, Florida, that they had inserted bacterial genes into plants. The fourth group announced at a conference in Los Angeles, California, in April 1983 that they had inserted a plant gene from one species into another species.

The Washington University group, headed by Mary-Dell Chilton, had produced cells of *Nicotiana plumbaginifolia*, a close relative of ordinary tobacco, that were resistant to the antibiotic kanamycin (Bevan et al., 1983). Jeff Schell and Marc Van Montagu, working in Belgium, had produced tobacco plants that were resistant to kanamycin and to methotrexate, a drug used to treat cancer and rheumatoid arthritis (Herrera-Estrella al., 1983). Robert Fraley, Stephen Rogers, and Robert Horsch at Monsanto had produced petunia plants that were resistant to kanamycin (Fraley et al, 1983). The Wisconsin group, headed by John Kemp and Timothy Hall, had successfully inserted a bean gene into a sunflower plant.

From this point, the transgenic technology provides the tools to make even more distant “crosses” among plants. The use of this technology to improve resistance and tolerance to biotic and abiotic stress was quickly seen as an attractive target for commercial applications. The genetic engineering for developing stress tolerant plants, based on the introgression of genes previously identified as responsive to abiotic stress might assume the major role towards the development of improved plant cultivars.

Plants can be genetically transformed to be tolerant to several kinds of stress such as salinity, drought, heat, flooding and metal toxicity. Most of these stress inductors promote the response of several genes in a very coordinate way, therefore the simultaneous or cumulative genetic transformation with multiple genes seen to be more suitable to this kind of stress. In some cases not only the gene assumes important role but also their promoters that allow the fine control of the expression level, the most adequate timing and targeting the correct tissue, making this technology work in a very precise way and promoting the optimal functionality of these introduced genes.

## 7. Conclusions and perspectives

The large number of reports clearly shows that manipulation of metabolic pathways using genetic engineering is a strategy that improves the performance of plants against abiotic stress. However, in most cases the impact of transgene does not confer high levels of tolerance.

There is a possibility to increase the tolerance through of the expressing simultaneously transgenes involved in several metabolic pathway. Alternatively, may be used transcription factors that regulate the expression of several defense genes at the same time, as described by Kasuga et al. (1999). These authors induced the expression of the transcription factor CBF1, which induced the expression of several genes normally activated in response to cold in *Arabidopsis*. The transgenic plants had higher tolerance not only at low temperatures, but also to drought and salinity. Accordingly, genomic technologies have a central role in the discovery of genes that regulate the intricate defense networks activated by plants in response to several types of environmental challenges.

With the improvement of plant genetic transformation technologies, certainly those genes previously evaluated mainly in tobacco and *Arabidopsis* will be also transferred to agronomic crops such as corn, wheat, soybeans and rice. Still, it is necessary to emphasize that only trials in field conditions will accurately assess the degree of protection offered by transgenes.

Finally, the joining of new molecular technologies and the experience and methods of classical geneticists will increase production and reduce costs, with an impact certainly stronger than those observed during the "Green Revolution".

Actually, this approach might be applied for almost every case of abiotic stress and even to biotic stress, helping to elucidate the genetic mechanisms behind the behavior difference observed in plants of the same specie when exposed to the same kind of stress. Indeed not only the genes but also the nucleotide sequences involved in the expression control such transcription factors, promoters, enhancers among many other ways of gene regulation are very important in these cases.

Evolutionary events such as gene duplication are very important to create the genetic buffer that allows plants to experience mutation events and create new versions of genes without risk of loss of functionality in vital metabolic pathways. Mobile genetic elements known as retrotransposons are a huge source of genetic variability and they are observed in most of the living organisms in a very variable number. In plants they played a very important role during the genome evolution and they are still important to keep this process going on. Recent discoveries about the mechanism of gene silencing ruled by RNA interference explains how plants can fight against viral infection and genetic disturbances promoted by uncontrolled gene expression, working as a very fine-tuning process inside the cell.

Plants are unable to dislocate in its own environmental therefore they had evolve to solve abiotic and biotic stress with internal mechanisms of tolerance and resistance. Consequently, the gene must evolve to perform and adapt quickly to environmental changes.

## 8. Acknowledgments

The author would like to thank the financial support from Minas Gerais Research Foundation (FAPEMIG), São Paulo Research Foundation (FAPESP), Coordination for the Improvement of Higher Education Personnel (CAPES), Funding Agency of Studies and Projects (FINEP) and Brazilian Agricultural Research Corporation (EMBRAPA) during the development of some results presented in this chapter.

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# Emission and Function of Volatile Organic Compounds in Response to Abiotic Stress

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## 1. Introduction

Plants accumulate a diverse array of natural products, which are thought to be involved in their interactions with the environment. These chemicals function in plant communications with microbes, animals, and even other plants, as well as protecting the plant from ultraviolet radiation and oxidants. Some compounds may attract beneficial insects or microbes, whereas others kill or repel herbivorous. Many of these compounds have been referred to as “secondary metabolites” to distinguish them from the “primary metabolites” required for the growth of all plants (Theis & Lerdau, 2003). These secondary metabolites, however, are likely to be essential for successful competition or reproduction.

More than 100,000 chemical products are known to be produced by plants and at least 1,700 of these are known to be volatiles (Dicke & Loreto, 2010). Some of the volatiles considered in this chapter are shown in figure 1. **Volatile organic compounds (VOCs)** are defined as any organic compound with vapor pressures high enough under normal conditions to be vaporized into the atmosphere (Dicke & Loreto, 2010). The importance of these compounds can be deduced by the considerable amount of photoassimilated carbon released back into the atmosphere as VOCs (Holopainen, 2004). In fact, it has been estimated that the emission of VOCs by terrestrial plants accounts for the 36% of the whole photosynthates (Kesselmeier et al., 2002). This emission, therefore, substantially reduces the amount of available carbon and consequently affects plant physiology and productivity. Why plants, under stress conditions, where carbon availability is a crucial limiting resource, lose such a relevant amount of assimilated carbon?

VOCs are involved in a range of ecological functions, including indirect plant defense against insects, pollinator attraction, plant-plant communication, plant-pathogen interactions,

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### Note:

This chapter has been written in cooperation with the students of the course “Ecosystems and Environmental Stress Physiology” of the ERASMUS MUNDUS - International Master in Horticultural Sciences (IMaHS: [www.imahs.unibo.it](http://www.imahs.unibo.it)). Dr. Francesco Spinelli, the tenured professor of the course, was responsible for the whole chapter structure and for the direction and supervision of the manuscript. Dr. Antonio Cellini contributed to the section on nitric oxide. Livia Marchetti (role of light stress on VOCs induction), Chiara Piovene (jasmonates) and Karthik Mudigere Nagesh (drought driven emission of VOCs) were all students of this master course.

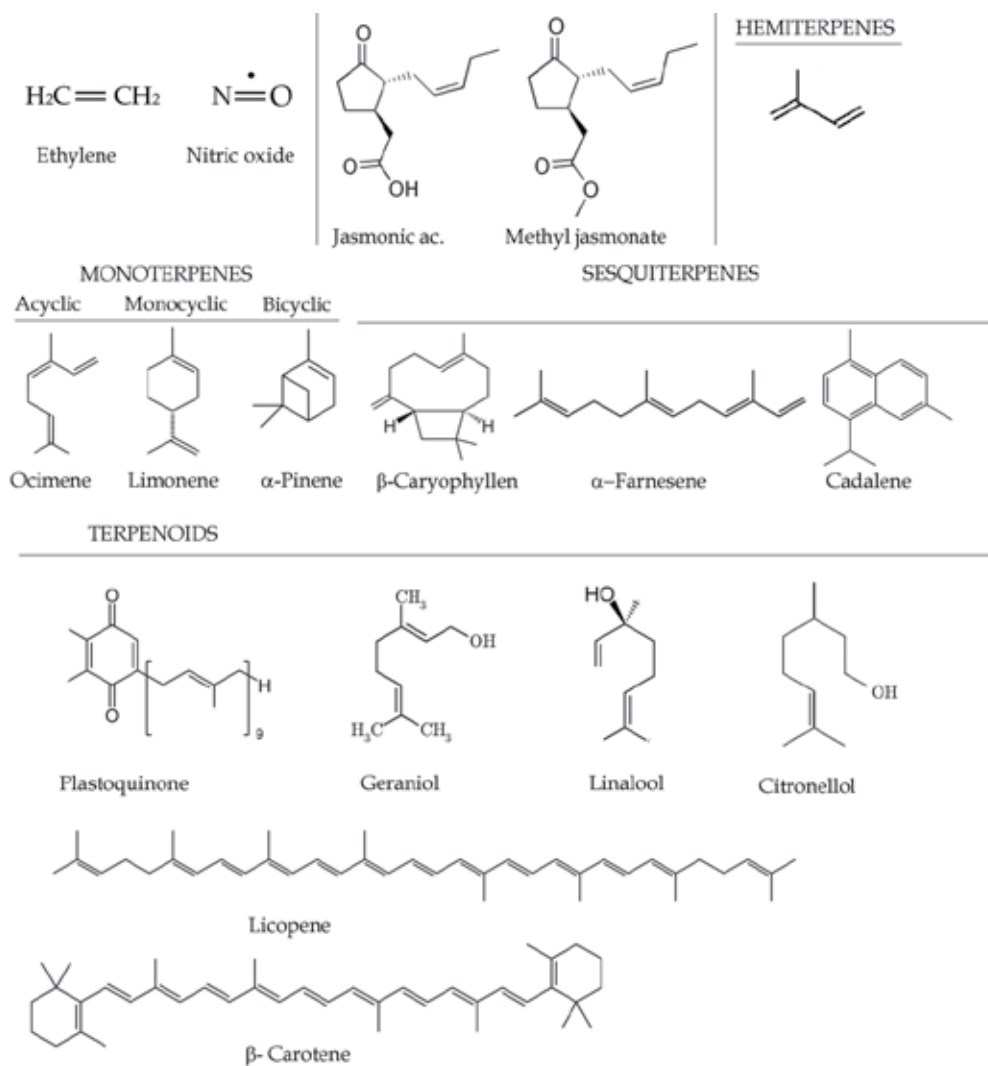


Fig 1. Chemical diversity of the different VOCs, and related compounds, present in the plant. The low molecular weight compounds (i.e. NO, ET, JA, MJA, ISOPRENE) usually act as stress signals. Isoprene, NO and the majority of the other compounds may also directly act as antioxidants.

reactive oxygen species removal, thermo-tolerance and environmental stress adaptation. Their evolution is quite complex and it is affected by interactions of plants with biotic and abiotic factors in constantly changing environments, at local and global level.

**Stored VOCs** may be volatilized into the atmosphere by healthy unwounded plants depending on their concentration and physiochemical properties (Niinemets et al., 2004). While many plants contain large amounts of stored VOCs, others do not synthesize and emit them until an environmental *stimulus* is perceived (Paré et al., 2005). **Induced VOCs** (IVOCs) may be emitted hours or days after a stress, both from the stressed sites as well as systemically from undamaged plant leaves (Paré & Tumlinson, 1997). Most of the

constitutive VOCs normally released from healthy intact plants become inducible volatiles after foliar damage (Wiens, 1991; Vuorinen, 2004). In contrast to the constitutive VOCs, the novel IVOCs are produced only after biotic and abiotic inductions. The advantage of novel IVOCs is that they are *de novo* synthesized only when needed and therefore they optimize carbon usage and do not reduce plant fitness (Dicke, 2000).

The VOCs are important infochemicals and their role in shaping the biotic interactions is well known. However, inducible VOCs are also emitted in response to abiotic stress perception and they may play a role in stress adaptation or response. Environmental stress such as physical damage, nutrient deficiency, salinity, drought and ozone exposure hamper the IVOCs emission. **Stress in plants could be defined as any change in growth conditions that disrupts metabolic homeostasis and requires an adjustment of metabolic pathways in a process that is usually referred to as acclimation (Shulaev et al., 2008).**

Plants have an extraordinarily diverse suite of protective mechanisms against abiotic stresses, since they must be capable of coping with a variety of changes in light intensity, temperature, moisture and other abiotic factors in their environments. When these factors shift out of a certain range, plants are subjected to stress; this can lead to decreased growth rate, reduced reproduction and even death (Vickers et al., 2009). Moreover, these stresses are rarely experienced singularly, but they often occur in combination.

The most common response to stresses is the production of excess reactive oxygen species (ROS), substances continuously produced in plants in normal conditions. Abiotic stress factors can perturb the equilibrium between production and scavenging of ROS, causing an excessive production of these components that lead to a direct damage to plant cells through oxidation of biological components (nucleic acids, proteins and lipids) and can instigate chain reactions resulting in accumulation of more ROS and initiation of programmed cell death (Apel & Hirt, 2004). Well known example of environmental stresses leading to direct ROS accumulation are light excess, which causes photoinhibition and damages to photosynthetic reaction centers, and high temperature stress, which denatures proteins and causes lipid peroxidation. In addition, these two stresses are often coincident thus amplifying one the effect of the other. In this chapter, it will be exposed how the ROS accumulation is the common mechanism underlying the volatile emission in response to both biotic and abiotic stresses.

There is a broad diversity of known IVOCs, including alkenes, alkanes, carboxylic acids, nitrogen-containing compounds and alcohols, but the dominating compounds tend to be isoprene, terpenes and C<sub>6</sub> green leaf volatiles (GLVs) (Holopainen & Gershenzon, 2010). The emission of VOCs from plants varies extensively depending on the species, organs, development stage and environmental conditions (Holopainen & Gershenzon, 2010). GLVs are produced *via* the lipoxygenase (LOX) pathway, and they can account for more 50% of the emissions from damaged plant parts. Chemically, GLVs are mostly saturated or monounsaturated aldehydes, alcohols and esters, and they can have different configurational isomers with different sensory properties (Ruther, 2000). GLVs are typically released only from damaged plant organelles within 1–2 seconds of the mechanical damage occurring (Fall et al., 1999), but some GLVs are released from younger undamaged leaves of herbivore damaged plants, indicating that the LOX pathway can be activated in intact leaves.

The high diversity of IVOCs suggests that plants are capable of disseminating information to their environment by using IVOCs and that plants can actively change the growth conditions using reactive IVOCs (reviewed by Holopainen, 2004).

The present chapter will review the current knowledge on the emission and function of IVOCs in response to abiotic stress. The review will also focus on the role of ethylene, nitric oxide, jasmonic acid and derivatives (i.e. methyl jasmonate), isoprene and terpenes.

## 2. Ethylene

Ethylene is the first gaseous hormone discovered in nature (Bleeker & Kende, 2000). Ethylene is a gaseous alkene and it is the simplest in structure among all the plant hormones. This 2-carbon olefin is a powerful elicitor of morphological changes during all stages of the plant life cycle from development, to fruit ripening and senescence. **In addition, a variety of stresses such as wounding, pathogen attack, flooding, drought, hypoxia, temperature shifts, physical loads and noxious chemicals (i.e. ozone and sulfur dioxide) induce ethylene production** (Yang & Hoffman 1984; Abeles et al., 1992; Bleeker & Kende 2000, Tschardt et al., 2001, Overmyer et al., 2003; Vahala et al., 2003). Furthermore, there is extensive crosstalk between the ethylene response pathway and other signaling networks (Johnson & Ecker 1998) (Fig. 2).

This complex and multifaceted regulatory network has not yet been completely elucidated. Nonetheless it is well known that ET induces diverse effects in plants throughout their life cycle from seed germination, floral differentiation to senescence. In order to achieve the myriad of effects it elicits in numerous physiological process, ET response must be precisely regulated at multiple levels, from hormone synthesis and perception to signal transduction and transcriptional regulation. However, even if ET has a central role in many fundamental processes, some evidences suggest that ET acts more as a potentiator or enhancer and it is not strictly required for survival. Failure to perceive ethylene is apparently not essential for survival in the laboratory setting, but ethylene signaling undoubtedly contributes to the hardiness of plants in the wild (Johnson & Ecker 1998).

**Ethylene also gave an example of plant-to-plant signalling**, probably unrelated to a role in defence: normally, the leaves of wild-type tobacco plants tend to stop growing as they approach neighbouring tobacco plants, this may stop them wasting energy producing leaves that would be shaded from useful light.

Plants in the vegetative growth phase, flowers, and immature fruits produce barely detectable levels of ethylene until they are subjected to stress or undergo maturation events, after which ethylene production accelerates in a spatially and temporally specific pattern (Johnson & Ecker 1998). All vascular plants analyzed to date synthesize ethylene via the Yang cycle, wherein S-adenosyl methionine is diverted from alternative fates to make the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang & Hoffman, 1984) (fig. 3).

Expression of the enzyme catalyzing this reaction, ACC synthase, is induced by stimuli that lead to increased ethylene production, suggesting that ACC synthase activity is rate-limiting for ethylene synthesis (fig. 3). ACC can be readily converted to ethylene, CO<sub>2</sub>, and HCN by ACC oxidase, or it can be conjugated to a malonyl or glutamyl group to limit its availability for ethylene production. ACS is encoded by a multigene family, and different members show distinct patterns of expression during growth and development, and in response to various external cues. In addition to this transcriptional control, the stability of the ACS protein is also highly regulated (Argueso et al., 2007). Although control of ethylene production is largely attributed to ACC synthase, the differential expression patterns of ACC oxidase (ACO) genes suggest that oxidases contribute to regulation of ethylene production as well. Periods of ACO induction correlate with ethylene-regulated events in several instances, including senescence, fruit ripening, and wounding.

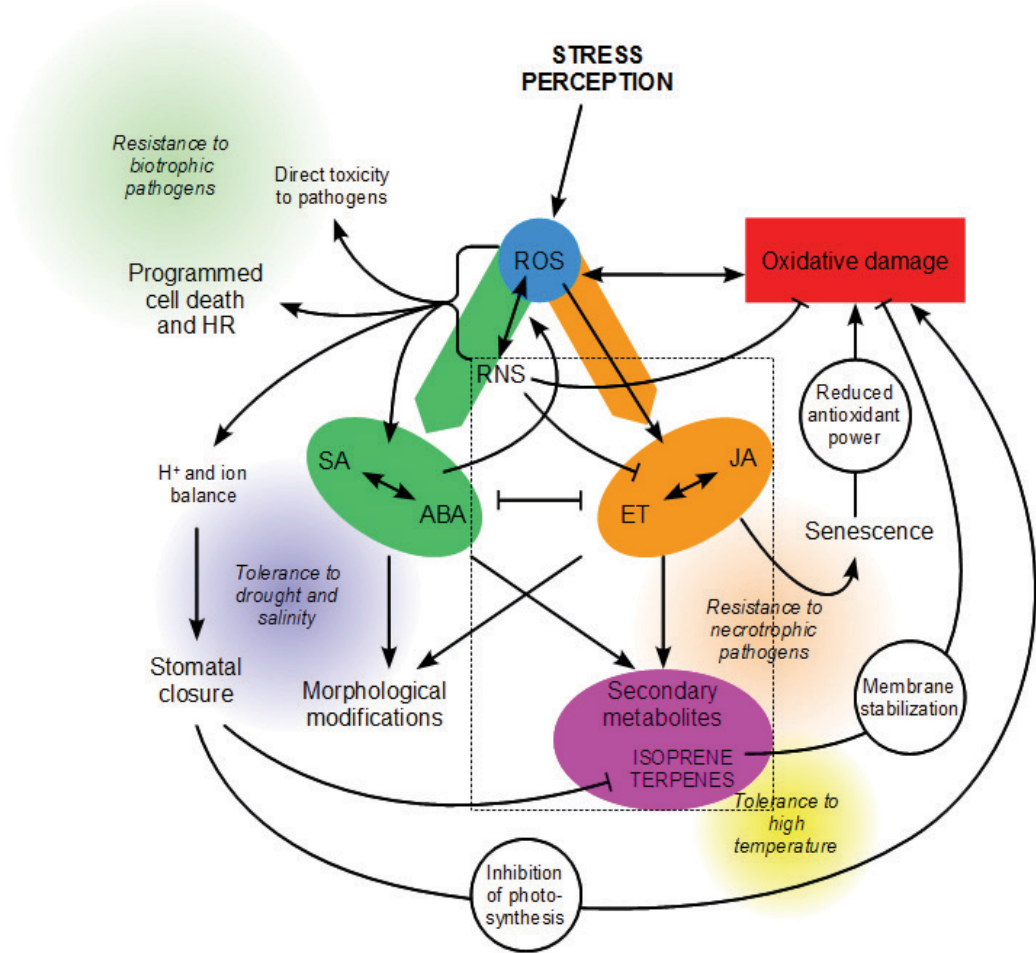


Fig. 2. Overview of the signalling events and reactions following the perception of stress. As a primary consequence, an oxidative burst occurs, due to the production of Reactive Oxygen Species (ROS). This early event causes oxidative damage and trigger the signal cascades leading to stress tolerance. The plant reacts to the ROS accumulation through two possible ways. On one side (green path), the interplay of ROS and Reactive Nitrogen Species (RNS) allows the induction of programmed cell death, the modulation of ion fluxes (including  $\text{Ca}^{2+}$ ), and the direct killing of noncompatible pathogens. Salicylic (SA) and abscisic acid (ABA) are long-ranged hormones mediating these responses in feedback with ROS and RNS. Several stressing agents, both abiotic and biotic, stimulate this pathway. A distinct and partially antagonistic signal cascade (orange path) involves the production of ethylene (ET) and jasmonates (JA). These hormones are required for the resistance to necrotrophic pathogens. Notably, the production of isoprene and terpenoids is stimulated by JA, and contributes to thermotolerance and stress mitigation. Apart from their role as signal molecules, isoprenoids can also act as quenching molecules of ROS. The volatile messengers in the described processed, namely NO (the precursor of RNS), ET, JA, isoprene and terpenoids are evidenced in the dashed frame.

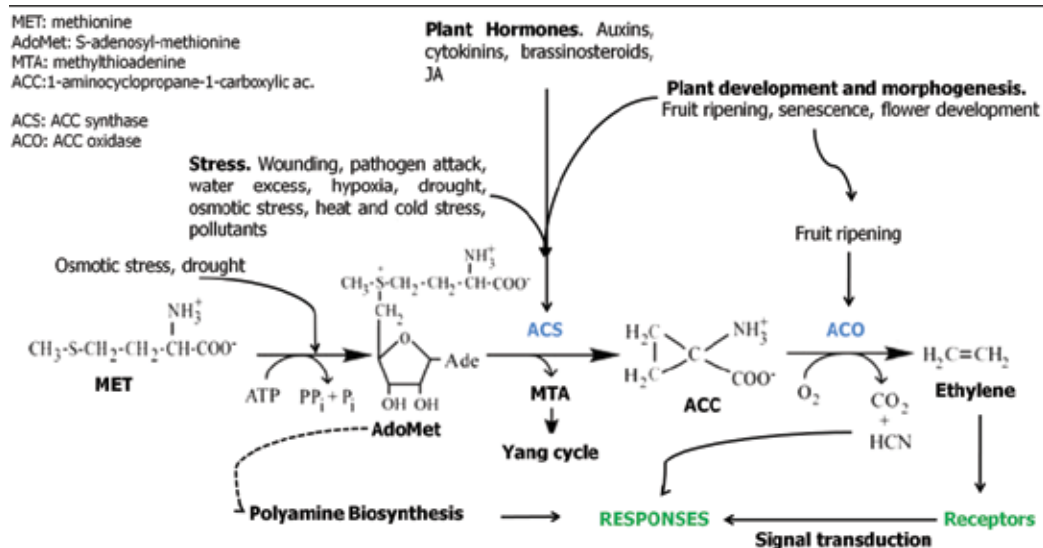


Fig. 3. The ethylene biosynthetic pathway and signalling showing the different enzymes involved in the process. The diverse *stimuli* promoting ET synthesis are also reported. In some plants, not only ACC and ACO are affected, but also AdoMet synthase. Finally, stress ethylene may also relate to stress symptoms through hydrogen cyanide, a by-product of ACC oxidase. Polyamine biosynthesis that start from AdoMet may interact with ethylene biosynthesis and responses to stress (After Argueso et al., 2007)

**Ethylene emission increases under drought stress** (McKeon et al. 1982). In this kind of stress ethylene may act as a signal molecule, but its possible protective role is still unclear. The literature collectively suggests that the production of ethylene in water-stressed plants depends on the rapidity of the decrease of plant water potential, the duration of the stress and the recovery conditions. In addition, plants that have previously experienced mild drought, can be hardened by former stress episodes (Morgan & Drew, 1997). One of the first reactions to water deficit is the stomata closure that is regulated by the hormone abscisic acid (ABA). Water stress induces the root synthesis of ABA which in turn induces ACC synthesis, and conversion of the ACC to ethylene in leaves (Morgan & Drew, 1997). Stomata closure prevent further lack of water from the plant thus avoiding wilting and more severe damages. Ethylene seems to antagonize this effect. In fact, ACC application or the use of the *Arabidopsis* ethylene overproducing mutant *eto1* leads to decreased stomata closure after ABA application, indicating that ethylene inhibits ABA-induced response (Tanaka et al., 2006). In addition, inhibition of ethylene synthesis in wheat inhibits chlorophyll loss associated with drought-induced senescence (Beltrano et al., 1999).

**Ethylene is also induced in osmotic stress**, and, again, its role is not well understood. ACS activity in tomato cells is increased after osmotic shock (Felix et al., 2000). Transgenic tobacco plants overexpressing an ethylene receptor show increased salt sensitivity compared to wild-type plants (Cao et al., 2006). On the other hand, overexpression of the ethylene-responsive transcription factor increased salt tolerance (Huang et al., 2004 - Reviewed by Argueso et al., 2007).

**Drought stress is often accompanied by high temperature which can additionally promote ethylene production.** Very high temperatures often occur at the edge of tree canopy under the sun. The temperature of an individual plant cell can change much more rapidly than other



factors that cause stress (e.g. water levels or salt levels). Thus, plants have evolved strategies for preventing damage caused by rapid changes in temperature and for repairing what damage is unavoidable. **Heat stress results in the production of specific families of proteins known as heat shock proteins (HSPs)** (Howarth & Ougham, 1993). Despite the ubiquitous nature of the heat shock response, little is known about how the plant senses an increase in temperature or the signaling pathways resulting in HSPs. **There is considerable evidence that oxidative stress induces pathways resulting in accumulation of some HSPs** (Dat et al., 1998). Even under optimal conditions, dangerous active oxygen species are synthesized at very high rates from electron transport chains involved in respiration and photosynthesis (Noctor & Foyer, 1998). Once damage is done to the photosystems by extreme temperature and/or light conditions, the production of these potentially damaging molecules increases, and these are the likely cause of the light-dependent, heat-induced oxidative damage (Noctor & Foyer, 1998). Given that high temperature, light excess and drought often occur simultaneously, their damages to the plants are usually cumulative. **High temperature can induce ethylene emission, up to a limit (about 35°C) after which production is inhibited (Abeles et al, 1992).** In *Arabidopsis*, calcium, abscisic acid (ABA), ethylene, and salicylic acid are involved in the protection against heat-induced oxidative damage. In fact, exogenous applications of SA, ACC and ABA protect plants from heat-induced oxidative damage. In addition, the ethylene-insensitive mutant *etr-1*, the ABA-insensitive mutant *abi-1*, and a transgenic line expressing *nahG* (consequently inhibited in SA production) showed increased susceptibility to heat (Larkindale & Knight, 2002).

**Ethylene may also be involved in the regulation of plant responses to low oxygen conditions, or hypoxia.** Hypoxia primarily occurs when the soil is flooded or water logged. Ethylene is highly induced when roots face a low oxygen environment. In these conditions, in *Arabidopsis*, the mRNA levels of a number of genes of the ACS family (i.e. ACS2, ACS6, ACS7, and ACS9) are upregulated (Peng et al., 2005). Under conditions of oxygen deficiency, ethylene is implicated in the triggering of a large number of responses that assist plants in avoiding stress. Among these responses are the accelerated elongation of submerged stems and leaf petioles, promotion of adventitious rooting on stems at or above the water line, leaf epinasty and hypertrophy of lenticels on stems and woody roots. One of the effects of hypoxia is the formation of aerenchymatic tissues. This kind of tissue is produced by programmed cell death that leave empty spaces inside the roots facilitating the gas diffusion. Ethylene may work as a signal regulating programmed cell death and lysigenous aerenchyma formation. A possible explanation reside on a production of cyanide. This toxic compound is produced in the ethylene biosynthetic pathway in stoichiometrically equivalent amounts to ethylene. It has been suggested that HCN may have a role in cell death in a variety of circumstances where ethylene synthesis is strongly stimulated. Induction of aerenchyma formation by ethylene is closely associated with increases in activity of cellulase (He et al, 1994), and also with expression of a putative cell wall metabolism gene, a xyloglucan endo-transglycosylase (XET) (Saab & Sachs 1996). It seems likely that cellulase, XET, and other potentially wall-degrading enzymes contribute to the rapid dissolution of dead cortical cells to give rise to aerenchyma.

**Pollutants such as heavy metals or O<sub>3</sub> also induce ethylene emission.** For example, lithium ion induces ACS gene expression and activity in various plant species (Tsuchisaka & Theologis 2004).

Ozone (O<sub>3</sub>) is presently a major phytotoxic air pollutant, and **exposure of plants to O<sub>3</sub>, or to other pollutants such as SO<sub>x</sub>, or HF, or NO<sub>x</sub>, leads to stress ethylene production.** It has been recognized for many years that there is a broad correlation between stress ethylene formation and sensitivity of different species and varieties to O<sub>3</sub> (see Wang et al, 1990).

Experimental application of high levels of ozone to plants induces a burst of ethylene (Mehlhorn & Wellburn 1987), promoting ozone-induced cell death (Overmyer et al., 2003) through a mechanism that might involve the biosynthesis and accumulation of salicylic acid (SA) (Ogawa et al., 2005) and suppression of the cell-protective action of JA (Tuominen et al., 2004). In *Arabidopsis*, ozone treatment elevates the steady-state level of ACS6 gene, one of the numerous genes of the ACS family (Vahala et al., 1998). Suppression of ACS activity in plants increases tolerance to oxidative stress and diminishes the damage caused by ozone treatment (Sinn et al., 2004).

**Finally, ethylene is stimulated by cold damages.** However, it is still not clear whether additional ethylene production is only a symptom of injury, or it has special significance in relation to acclimation or tolerance of chilling stress. As a generalization, greater stress ethylene production is indicative of greater chilling sensitivity (Morgan & Drew, 1997).

Ethylene seems to have a role as a switch by reducing the production of constitutive defence compounds after herbivore damage and stimulating the production of jasmonic acid and IVOCs (Kahl et al., 2000). Intriguingly, JA has been found to be conjugated to ACC in *Arabidopsis* plants, suggesting that JA-ACC conjugates could be involved in the co-regulation and crosstalk between JA- and ethylene-dependent pathways in plants (Staswick & Tiryaki 2004). Three endogenous plant signalling molecules: salicylic acid (SA), jasmonic acid (JA) and ethylene regulate plant defences in response to microbial attack (Dong, 1998). There is a growing body of literature that reports that the NO, JA, SA and ET defence signalling pathways do not work independently but rather influence each other through a complex network of regulatory interactions (fig.2).

A greater understanding of the NO, SA, JA and ET signalling pathways and their reciprocal modulation should provide insight into the mechanisms underlying the activation and regulation of plant responses to biotic and abiotic stress. While the SA and JA signalling pathways are mutually antagonistic, several studies provide evidence for positive interactions between the JA and ET signalling pathways. Both JA and ET signalling are required for the expression of the defence-related genes even when applied exogenously. This regulatory cross-talk may have evolved to allow plants to fine-tune the induction of their defences in response to different plant pathogens (reviewed by Kunkel & Brooks, 2002). NO generally counteracts the effects of ethylene by delaying senescence, flowering and ripening (Leshem & Wills, 1998). Methionine adenosyltransferase and S-adenosyl homocysteine hydrolase, required for ethylene biosynthesis, are inactivated by S-nitrosylation and nitration, respectively (Lindermayr *et al*, 2006). It is reported in a wide variety of fruits, both climacteric and non climacteric, that NO emission is higher before ripening, whereas it drops along with the ethylene increase during maturation or senescence; nitrous oxide (N<sub>2</sub>O) fumigations were successfully employed to extend the postharvest life of crops (Leshem & Wills, 1998).

### 3. Nitric oxide (NO)

**Nitrogen monoxide (more commonly named nitric oxide, NO) is one of the chemical signals shared by all the kingdoms,** and it can therefore be involved in interorganism communications. However, NO is also abundantly originated by antropic activities, such as fossil fuel combustion, nitrogen fertilization and it has long been considered only as a pollutant. The formation of photochemical smog involves NO, that drives the synthesis of ozone, either photochemically in presence of O<sub>2</sub>, or by oxidizing volatile compounds (Pinto et al., 2010; Lindroth, 2010). This

process increases the amount of ozone in the troposphere thus affecting the composition of cuticular waxes and volatile emissions and impacting on the regulation of ecological relations (insects and host plants, predators and parasitoids) (Lindroth, 2010).

**Since early 1990s, NO has been characterized as an actively produced physiological regulator in plants.** Nonetheless, its metabolism is not easy to study, because of several, uncommon biochemical features of the NO molecule. In fact, unlike most signalling molecules, NO is a small, ubiquitous, unstable radical gas, freely diffusible both in aqueous and lipidic media. The unpaired electron is delocalized between the N and O atoms in an anti-bond  $\pi$  molecular orbital. This fact grants the NO molecule a relatively high stability, and in the same time explains its typical reactivity. Other compounds biochemically related to NO, and possibly mediating its effects, are collectively named reactive nitrogen species (RNS) and include  $N_2O$ ,  $NO_2$ ,  $N_2O_3$  and peroxyxynitrite.

As a radical molecule, NO is highly reactive to other radicals. Due to its lipophilic nature, NO is supposed to quench effectively the lipid peroxidation chain reaction. The reaction with superoxide ( $O_2^{\cdot-}$ ) is nearly diffusion-limited, and yields peroxyxynitrite ( $ONOO^{\cdot}$ ). Peroxyxynitrite deserves a mention for its high reactivity and its powerful oxidizing (nitrating) activity toward phenolic compounds, such as tyrosine. In physiological conditions, very little superoxide is produced and not removed by superoxide dismutase: therefore, the occurrence of tyrosine nitration is proposed as a stress marker. Protonation of peroxyxynitrite yields the highly reactive nitrogen dioxide and hydroxyl radicals, further contributing to oxidative stress and disruption of cell structures. An overview of NO chemistry is shown in fig. 4.

NO is produced in plant in a number of possible ways such as the non-enzymatic reduction of  $NO_2^-$  to NO and the dismutation of nitrous acid to NO (Stöhr & Ullrich, 2002; Bethke et al., 2004), the NO synthase-like activity ("NOS-like activity") (del Río et al., 2004) and the enzymatic biosynthesis mediated by nitrate reductase (Yamasaki, 2005). Many other biochemical routes leading to production of NO have been described. Polyamines, salicylhydroxamic acid, hydroxylamine, hydroxyurea and hydroxyarginine were shown to act as NO precursors, possibly in association with reactive oxygen species (Arasimowicz-Jelonek et al., 2009; Rümer et al., 2009; Tun et al., 2006);  $NO_2$  can be photooxidized to NO by carotenoids. Among the enzymatic candidates for NO production, heme-proteins like peroxidases, cytochrome P450, hemoglobin and catalase, have been proposed. Xanthine oxidase, according to  $O_2$  availability, is reported to produce either  $O_2^{\cdot-}$  or NO (del Río et al., 2004).

Considering the role of **reactive oxygen species** in signalling, and how NO reacts with them, **NO can be considered a mediator of ROS signalling.** Plant cell death, for example induced by salicylic acid in the hypersensitive response, requires definite rates between  $H_2O_2$  and NO (Delledonne et al., 2001). If superoxide is not converted to  $H_2O_2$ , peroxyxynitrite will be formed, which has no effect in plant cell death, but may be toxic for an invading pathogen. In general, the causal relations may not be clear, since NO is reported both to promote ROS synthesis (stimulating the plasma membrane NADPH oxidase) (Zhang et al., 2007) and to act downstreams of  $H_2O_2$  (e. g. in ABA stomata closure signal) (Bright et al., 2006).

The effects of  $H_2O_2$  and NO are partially overlapping, for what concerns the regulation of ion channels (notably  $Ca^{2+}$ ), the activation of  $H^+$ :ATPase and  $H^+$ :pyrophosphatase, and second messenger cascade of the phospholipase products.

**Most of the NO-modulated genes in *Arabidopsis thaliana* respond to abiotic or biotic stress conditions, and are involved in signal transduction, cell death, defence,**

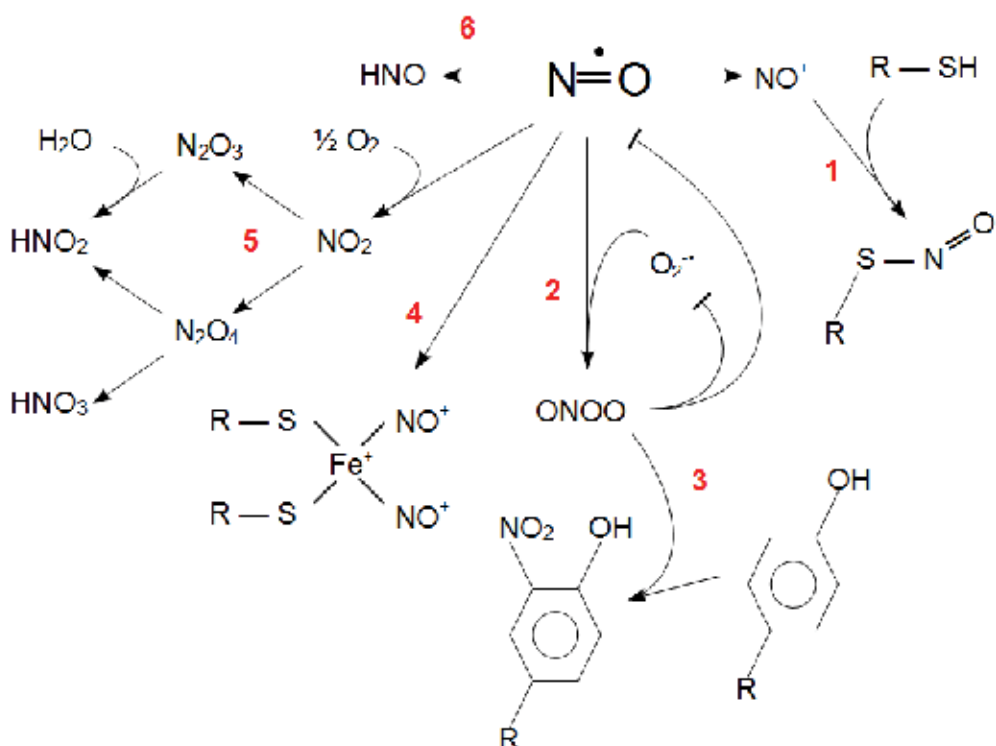


Fig. 4. Chemistry of NO. (1) Nitrosation of thiols (R-SH) by nitrosonium. (2) Production of peroxynitrite. (3) Nitration of a phenolic group. (4) Formation of a dinitrosyl-iron compound. (5) Oxidation reactions to nitric and nitrous acid. (6) Reduction to nitroxyl.

**generation or detoxification of ROS, photosynthetic processes, intracellular trafficking, and basic metabolism;** only a few among the classified ones can be directly linked to stress defence, but a dose-dependence to NO emerges for many genes, pointing to a form of signal specificity.

Apart from these functions, similarly to the traditional plant hormones, **NO has also a variety of effects on plant developments and morphogenesis.** For example, NO, acting downstream to auxins, mediates their effects on root architecture, promoting the formation of lateral roots against the elongation of the primary root (Correa-Aragunde et al., 2004) and root hair development (Lombardo et al., 2006). NO acts as a mediator in photoperiod sensing, delaying the floral transition (He et al., 2004). Along with other light-dependent responses, such as de-etiolation and internodal growth, NO affects seed germination overcoming light requirement, similarly to gibberellic acid, and possibly sharing the same cGMP signalling pathway (Beligni & Lamattina, 2000). While ABA is essential for the establishment and maintenance of dormancy, a NO donor renders arabidopsis seeds insensitive to ABA (Bethke et al., 2006).

**NO plays also a crucial role in the abiotic stress signaling and tolerance.** NO function in abiotic stress tolerance may be carried out by a signal cascade, or by direct detoxification. Plants improve their health status in response to many kinds of environmental stress, when exposed to low NO concentrations. Thus, **NO could be considered a generalized stress signal.** To prove this, several works were carried out adopting different species and tissues

and an array of stressing conditions (Corpas et al., 2008; Gould et al., 2003; Huang et al., 2004). As a result, increases in NO contents were observed in some, but not all the treatments, although its occurrence varies among species, and possibly on the basis of tissue-specific mechanisms. For example, **NO has a role in the plant response to drought and salinity stress.** One of the first responses to these stresses is the ABA-mediated stomata closure. A simplified model for the process, not accounting for self-amplification and feedback effects, is the cascade  $\text{H}_2\text{O}_2 \rightarrow \text{NO} \rightarrow \text{cGMP-cADPR} \rightarrow \text{release of Ca}^{2+}$ . The  $\text{K}^+$ -intaking channels are inactivated by  $\text{Ca}^{2+}$ , whereas the outwards  $\text{K}^+$  channels open in response to  $\text{H}_2\text{O}_2$ . As a result, the rise in water potential causes the guard cell to collapse and close the stoma (Bright et al., 2006). Salinity and osmotic stress share a common basis, so it is reasonable that plant adopt similar mechanisms to cope with them, consisting in the regulation of the water loss by transpiration, and of  $\text{Na}^+$  uptake, transport and redistribution. Proton concentration provides energy for  $\text{Na}^+$  sequestration in the apoplast or in the vacuole, carried out by a  $\text{H}^+:\text{Na}^+$  antiport; NO activates the  $\text{H}^+:\text{ATPase}$  and  $\text{H}^+:\text{pyrophosphatase}$  activities, in concert with  $\text{H}_2\text{O}_2$  and  $\text{Ca}^{2+}$  (Tanou et al., 2009; Zhang et al., 2006; Zhang et al., 2007; Zhao et al., 2007).

**NO is involved also in the response to hypoxia.** Seeds and roots face oxygen deprivation mainly when the soil is saturated with water. In such conditions, nitrite accumulation and NO production are often reported. NO lowers  $\text{O}_2$  consumption by repressing cytochrome C oxidase (COX), and shifts the metabolism toward fermentation, as a mechanism for hypoxic stress avoidance (Borisjuk et al., 2007). A class of plant stress-induced hemoglobins might help the de-repression of COX, or prevent PCD in the short term, allowing the development of adventitious root primordia (Dordas et al., 2003).

**The reaction to the metal ions excess also involves NO.** Metal ions, including heavy metal pollutants, can induce plant stress in several ways, such as the Fenton reaction (in which highly oxidizing ROS are produced), substitution of physiological metal cofactors, and effects on nucleus activity, hormonal signalling and photosynthetic apparatus. The oxidative stress symptoms can be reverted by NO (Laspina et al., 2005), or are associated to a reduced NO content (Rodríguez-Serrano et al., 2006; Tian et al., 2007). **NO might act by chelation of the metal ions, preventing them to react with sensitive targets.**

**Finally, NO has an important function also in the ecological interactions such as symbiosis and pathogenesis.** For example, NO regulated genes are crucial for the establishment of symbiotic root interactions between plants and host-specific bacteria, such as legumes/rhizobia or *Alnus firma*/*Frankia* spp. (Nagata et al., 2008; Perazzolli et al., 2006; Sasakura et al., 2006).

In addition, NO interplays with salicylic acid (SA), ethylene and jasmonate are known to act in the plant responses to invading pathogens. The reaction to avirulent pathogens required NO for the induction of phenylpropanoid metabolism genes, through the cGMP-cADPR pathway (Durner et al., 1998), and of hypersensitive response, together with  $\text{H}_2\text{O}_2$ . Furthermore, the production of peroxynitrite is suggested as a protective reaction aimed to killing the invader (Delledonne et al., 2001). The recognition of a potential pathogen causes a NO and ROS burst, which stimulates the synthesis of salicylic acid. In turn, SA promotes NO- and ROS-mediated redox signalling, in a positive feedback. Direct consequences of NO are the release of  $\text{Ca}^{2+}$  in the cytosol and the activation of protein-kinase cascades (Zottini et al., 2007). The programmed cell death is triggered by definite rates of NO and  $\text{H}_2\text{O}_2$ , whereas the absence of either one, or other RNS different from NO give no effect on cell viability (Delledonne et al., 2001).

#### 4. Jasmonates

Jasmonates group includes **jasmonic acid (JA)** and its esters, such as **methyl** and **n-propyl jasmonate** (respectively, MJ and PJ). The fragrant compound methyl jasmonate has been mostly studied for its role as a volatile signal in the regulation of pathogenesis-related gene expression (Farmer & Ryan, 1990; Farmer et al., 1992; Schweizer et al., 1997) and up-regulation of VOCs (Martin et al., 2003). **The role of JA in priming plant defences against pests and pathogens and its interactions with other signals and hormones (e.g. ET, SA, NO, IAA, ABA) has been widely studied** (fig. 2). The accumulation of jasmonic acid is caused by wounding, and it is followed by induction of a number of pathogenesis related genes (Schweizer et al., 1998).

Since jasmonates are derived from polyunsaturated C18 fatty acids, the corresponding signalling pathway has been referred to as the octadecanoid pathway (Sembdner & Parthier, 1993) which has been identified as one of the major signalling pathways in plant defence (Farmer & Ryan, 1990).

**The major functions of JA and its various metabolites is regulating plant responses to abiotic and biotic stresses as well as plant growth and development.**

Jasmonates have been shown to stimulate growth and development, including flower development, tuber formation, tendril coiling, nyctinastic movements, trichome formation and senescence, and it inhibits root and leaf growth and seed germination (Onkokesung 2010). Moreover, **JA levels in plants rapidly and transiently increase in response to wounding, water deficit, mechanical stimulation, elicitors and it also mediates some of the UV-induced defense responses** (Xiang 1998).

**Interestingly, the JA-dependent plant defenses which are activated in response to herbivores or pathogens are also triggered by abiotic stresses such as wounding, UV irradiation or O<sub>3</sub>. This observation suggests that the JA signal activates common, unspecific stress-defensive mechanisms. One of this mechanisms is the increase of glutathione (GSH) level** (Xiang 1998). GSH is an essential component of the glutathione-ascorbate cycle, which is the major metabolic pathway responsible for reduction and detoxification of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Noctor & Foyer, 1998). In addition, oligomers of GSH form the phytochelatins that chelate toxic heavy metals (Ha, 1999). Finally, GSH is used as substrate by glutaredoxins which are oxidoreductases involved in flower development, salicylic acid metabolism and plant defense signaling (Rouhier et al., 2008).

If JA is involved in the tolerance to the oxidative stress, how is this stress perceived and how does it stimulate the JA-mediated reaction? Oxidative species and O<sub>3</sub> react primarily with the plasma membrane, causing alterations in lipid composition and increasing the production of linoleic acid, which is the precursor of JA biosynthesis.

Thus, it is possible that reactive oxygen species (ROS) and, especially, O<sub>3</sub> directly trigger JA biosynthesis and that JA function as a signal in the cascade of plant reaction to oxidative stress-generating stimuli. In addition, JA synthesis and signaling are interlinked by a positive feedback loop whereby jasmonates stimulate their own synthesis (Sasaki et al., 2001; Acosta & Farmer, 2010).

Jasmonic acid is also involved in the response to wounding. JA biosynthesis is initiated by a wound-mediated release of  $\alpha$ -linolenic acid from chloroplastic membranes, followed by the activity of several chloroplast-located enzymes, including 13-lipoxygenase. The combination of JA deficiency and ET insensitivity resulted in a novel growth phenotype characterized by massive cell expansion around wounds, suggesting that both JA and ET may repress local growth after wounding and/or herbivore attack (Onkokesung 2010).

These evidences suggest that both JA and ET, while mediating inducible defenses, may also function as major switches between growth and defenses and the associated changes in resource allocations. Given that wounding and herbivory reduce leaf growth, both cell division and cell expansion could be possible targets of JA and ET action in preventing additional growth, putatively enhancing the accumulation of various defense metabolites used against herbivores. Exogenously applied JA inhibits tobacco BY-2 cell proliferation by arresting the cells in the G1 phase, thus suggesting that the JA and ET effects in primarily on cell division. Increasing endogenous JA levels *in vivo* caused by continuous wounding of *Arabidopsis* plants reduced their growth by suppressing mitosis in the young leaves (Zhang & Turner, 2008).

However, the leaf growth inhibition is also associated with a smaller leaf size as well as reduced fresh mass, but not dry mass, of leaves, suggesting that a decrease in cell expansion is additionally responsible for the observed growth differences (Onkokesung 2010).

**Finally, jasmonates also influence the emission of other stress-related IVOCS.** The biosynthesis and emission of the volatiles is, like many other chemical defenses, under the control of the octadecanoid signalling pathway, and is effectively stimulated by free jasmonic acid (Krumma et al., 1995). Treatment of plants with exogenous JA or MJ has been reported to induce volatile emission similar to herbivore induction, extrafloral nectar production, increased levels of endogenous secondary metabolites, increased VOCs-mediated attraction of predators and parasitoids, and enhanced parasitism rates of herbivores for a wide variety of plant species (Bruinsma et al., 2009).

The role of jasmonate in regulating other VOCs emission is confirmed in the tomato mutant *def-1*, which is deficient in inducing jasmonic acid accumulation upon wounding or herbivory. The application of exogenous JA restored the emission of volatile **methyl salicylate (MeSA)** and volatile terpenes by spider mite-infested *def-1* mutants increased significantly after the plants had been treated with exogenous JA (Ament et al., 2004).

## 5. Isoprene and terpenes<sup>1</sup>

The functional diversity of chemicals within plants is best demonstrated by terpenoids (Theis & Lerdau, 2003). More than 30,000 terpenoids have been identified (Buckingham 1998). Terpenoids, also known as isoprenoids, are functionally diverse, comprising both primary and secondary metabolites. These compounds include hormones, such as gibberellins and abscisic acid, electron carriers, such as plastoquinone and ubiquinone, terpene-derived compounds that form structural parts of membranes such as phytosterols, photosynthetic and photoprotective pigments such as carotenoids (Theis & Lerdau, 2003), the phytyl side chain of chlorophyll (Paiva, 2000), toxins, membrane-bound sugar carriers and heat stress resistance compounds (Sharkey & Singsaas, 1995). Volatile terpenoids are important compounds for plant biology and atmospheric chemistry because of their role in plant protection (e.g. in the protection of photosynthesis against thermal and oxidative stresses) and in direct and indirect defense against herbivores. Although the majority of volatiles emitted by damaged plants are GLVs, when the plants is intact, the emission of volatile isoprenoids is estimated to account for more than half of

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<sup>1</sup> Terpenes are derived biosynthetically from the polymerization isoprene units, whereas, terpenoids derive from chemical modification (e.g. oxidation, rearrangement) of the basic terpenes structure. In this chapter, a broad definition of terpenes which includes also terpenoids is used.

the total emission of VOCs and is constitutively ten times higher than other emissions (Loreto & Schnitzler, 2010).

All **isoprenoids** are produced from the precursor dimethylallyl diphosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP), which are synthesized by the deoxyxylulose-5-phosphate (DXP) pathway in the chloroplasts and by the mevalonate pathway in the cytoplasm (Lichtenthaler, 1999). Isoprenoids carbon skeletons are composed of five-carbon building blocks that may be assembled in a variety of formations and contain many different modifications. The five-carbon building blocks are added together to make 10-carbon, 15-carbon, and even 2000–500000 carbon chains, as in the case of rubber.

On the other hand, in the case of isoprene (2-methyl 1,3-butadiene), the five-carbon molecules are formed by the elimination of a phosphate group from dimethylallyl pyrophosphate (DMAPP), either in an acid-catalyzed or an enzyme-catalyzed reaction (Harley et al., 1996).

IPP can condense with one, two, or three molecules of DMAPP to form geranyl pyrophosphate (GPP) the 10-carbon precursor of **monoterpenes**, farnesyl pyrophosphate (FPP) the 15-carbon precursor of **sesquiterpenes**, or geranylgeranyl pyrophosphate (GGPP) the precursor of hundreds of **diterpenes**, including the growth hormone **gibberellic acid**. IPP or DMAPP can also serve as the source of the prenyl side of a number of natural products, including **cytokinins** (Paiva, 2000).

Two routes to IPP have been demonstrated in plants: the mevalonate (MVA) pathways and the recently discovered 2-deoxyxylulose 5-phosphate/2-methylerythritol 4-phosphate (MEP) pathway (Rohmer, 1993, Theis & Lerda, 2003; Degenhardt & Lincoln, 2006). The mevalonate pathway appears to function in the cytoplasm, whereas MEP pathway is responsible for most, and probably all, isoprenoids made in plastids (Lichtenthaler et al., 1997; Paiva, 2000; Dudareva et al., 2004), although studies are still in progress to localize the enzymes involved conclusively.

Terpenoids induce the expression of a number of **defence genes** (Arimura et al., 2000). Terpenes also act as band aids by sealing a **plant wound**. Once plant tissue is exposed to the air, the volatile terpene portion evaporates leaving a semi-hardened mass (Phillips & Croteau 1999). Concentrations of terpenoids are generally higher in reproductive structures and typically highest in the foliage during, and immediately following, flowering (Rapparini et al., 2001). Young leaves are more important to a plant than older leaves, and thus the highest levels of terpenoids are found in the young organs (Fischbach et al., 2002).

Finally, **terpenes emission is strongly increased by temperature increase**. This effect is due to the immediate stimulation of temperature on the activity of the enzymes that catalyze the synthesis of many VOCs (Loreto & Schnitzler, 2010).

## 6. Monoterpenes

Monoterpenes include many volatile flavours and aroma components (Sharkey & Yeh, 2001). Some compounds play a major role in plant defence being involved in plant-insect interactions as toxins and deterrents. Representative monoterpenes include the mildly antimicrobial menthol, aroma components citral and geraniol, insect repellent citronella, parasitoid wasp attractant linalool, and allelopathic camphor (Paiva, 2000). Monoterpenes non-storing species synthesize and emit these compounds in a light, temperature and damage-dependent way. Whereas, the storing species have a special structure for monoterpenes accumulation such as glandular cells on the leaf surface (Paiva, 2000;



Holopainen, 2004). In these species, emission of monoterpenes generally originates from pools of hydrocarbon stored in resin ducts, glands, or trichomes (Loreto et al., 1996). Monoterpenes emission is therefore dependent in large measure on their volatility (Lerdau, 1991) and on damage of leaves (Tingey et al., 1980; Tingey et al., 1991; Litvak et al., 1998). Only in a few cases, trees emit some monoterpenes as a result of *de novo* synthesis (Sharkey & Yeh, 2001): for example linalool is synthesized after herbivore damage and released systemically from the whole plant (Holopainen, 2004).

Monoterpenes, such as eucalyptol, linalool, camphor,  $\alpha$ -pinene,  $\beta$ -pinene,  $\alpha$ -terpineol, borneol and many others, are the principal components of plant volatile oils (Dorman & Deans, 2000, Candan et al., 2003). These are principally present in the aromatic plants (storing species). **Several works observed that volatile essential oils are involved in antimicrobial and antioxidant activity** (Candan et al 2003; Cimanga et al., 2002ab; Kuhnt et al., 1995; Setzer et al., 1999; Dorman & Deans, 2000).

Many SESQUITERPENES are typical fragrances emitted from flowers (Chen et al., 2003). A considerable amount of sesquiterpenes is also emitted from the herbivore-damaged foliage (Vuorinen et al., 2004), whereas in intact plants, the emission is lower (Wiens et al., 1991). **High levels of sesquiterpenes are produced after O<sub>3</sub> exposure.** The speed of sesquiterpenes release is related to the resistance of the plant thus demonstrating their role in O<sub>3</sub>-defence (Heiden et al., 1999). Sesquiterpenes are among the most studied class of **phytoalexins**. In the case of the phytoalexins and other toxins, the plant product is not only toxic to the microbial pathogen, but also potentially toxic to the plant cells. Many of the phytoalexin biosynthetic genes are not expressed until the plant senses the presence of the pathogen, when complex signal transduction mechanisms activate transcription of each of the biosynthetic steps (Paiva, 2000).

**ISOPRENE** (C<sub>5</sub>H<sub>8</sub>, 2-methyl 1,3-butadiene) is a natural product of many organisms (Sharkey, 1996). Sanadze and Kursunov discovered isoprene emission from plants in the 1950s (Sanadze & Kursanov, 1966).

**The global isoprene emission is now estimated to be about 500 Tg C yr<sup>-1</sup>, making it the dominant hydrocarbon that moves from plants to the air, roughly equal to the flux of methane to the atmosphere** (Guenther et al., 1995; Wang & Shallcross, 2000). Isoprene drains a considerable percentage of the carbon fixed through photosynthesis out of the pathway forming structural and storage sugars, especially in stressed leaves: in major emitting plant species such as oak and aspen its synthesis is typically 2% of photosynthesis at 30°C (Sharkey & Yeh, 2001).

Isoprene is a hemiterpene produced from DMAPP by the isoprene synthase (Silver & Fall, 1991). This enzyme has a relatively high pH optimum and a requirement for Mg<sup>2+</sup> (Schnitzler et al., 1996; Silver & Fall, 1995), consistent with its location inside chloroplasts (Mgaloblishvili et al., 1979; Wildermuth & Fall, 1996; Wildermuth & Fall, 1998). The energy cost of isoprene emission using the MVA pathway is 9 carbon atoms, 24 ATP, and 14 NADPH. However, the MEP pathway is more efficient than the MVA pathway in photosynthetic organisms, and isoprene emission based on the MEP pathway costs only 6 carbon atoms, 20 ATP, and 14 NADPH.

Therefore, the cost of isoprene production is substantial, and any benefits ascribed to its emission will have to be weighed against this cost in terms of carbon and energy. Isoprene emission is a common but not universal plant trait (Kesselmeier & Staudt, 1999). Nearly all plant species emit very low levels of isoprene, but only about one third of angiosperm

species tested emit isoprene at substantial rates (Hanson et al., 1999). Given the cost, plants that do not emit would out-compete those that do emit, unless isoprene emission provides a benefit that exceeds the cost of emission. What benefit, if any, do plants derive from isoprene emission? In other words, why do plants make isoprene? Some of the reasons suggested for isoprene emission include a leaf thermoprotector, a flowering hormone (Terry et al., 1995), an antioxidant (Zeidler et al., 1997), and a metabolite overflow to get rid of excess carbon (Logan et al., 2000; Wagner et al., 1999).

Although the isoprenoids are very well known, much less is known about biological isoprene production because isoprene is not an intermediate in isoprenoid production. Unlike monoterpenes, it is not stored within the leaf, but emitted through the stomata immediately upon its production. Thus, isoprene emission requires *de novo* synthesis (Fall & Monson, 1992; Loreto et al., 1998). For this reason, in plants, **isoprene synthesis is dependent on photosynthesis**. Fall and Wildermuth (1998) reported that changes in pH and  $Mg^{2+}$  that normally occur in thylakoids in response to light can cause an 11-fold stimulation in isoprene synthase activity. Therefore, the factors affecting photosynthesis, such as nitrogen deficiency, water stress, light and temperature excesses usually influence also isoprene emission.

Isoprene emission is very sensitive to temperature, although the temperature dependence of isoprene emission is different from that of photosynthesis. In *Quercus spp.*, an increase of temperature from  $\pm 30\text{ }^{\circ}\text{C}$  to  $\pm 40\text{ }^{\circ}\text{C}$  does not affect photosynthesis or slightly reduce it, whilst, it increases isoprene emission from typically 2% to 15% of carbon fixed by photosynthesis (Sharkey et al., 1996). On the contrary, photosynthesis is highly sensitive to  $\text{CO}_2$  but isoprene is relatively insensitive. Isoprene emission in  $\text{CO}_2$ -free air can be 50% of maximal rates; but if oxygen is also removed, isoprene emission stops. The interpretation of this phenomenon is that isoprene emission requires photosynthetic activity (Loreto & Sharkey, 1990).

**Isoprene emission can be also affected by nitrogen nutrition** (Harley et al., 1994; Litvak et al., 1996). Trees with low nitrogen availability had lower rates of isoprene emission than did trees with higher nitrogen nutrition. This effect interacted with light. Trees grown in sun or shade but with low nitrogen availability had similar, low rates of isoprene emission; but trees with high nitrogen availability emitted substantially more isoprene when grown in the sun than when grown in the shade. **On the other hand, isoprene emission seem to be only marginally effected by water-stress**. Drought directly affects stomatal conductance and produce diffusive and biochemical limitations of photosynthesis (Fall et al., 1999). Both the reduction of photosynthesis and the stomatal closure are expected to negatively impact on biogenic volatile organic compounds emission by altering the carbon supply into the MEP pathway and by increasing resistance to their emission (Fall. et al., 1999). However, isoprene emission changed little, even when photosynthesis is inactivated by a total and prolonged stomata closure (Tingey et al., 1981). In addition, upon rewatering, isoprene emission increased several fold above the pre-stress rate and stayed high for several weeks (Sharkey & Loreto, 1993). Nonetheless, the effect of drought, like other stressors on plant VOC emissions, can depend on the level of stress or damage caused to the plant by drought. Thus, severe drought might largely decrease emissions, whereas mild drought stress might increase emissions (reviewed by Peñuelas & Staudt, 2010). Similarly to isoprene, also terpenes and terpenoids are negatively affected by severe drought (Lerdau et al., 1994).

The role of isoprene in **thermotolerance** has been extensively studied. Since the 90s, several experiments indicated that isoprene has some effect on the temperature tolerance of the

photosynthesis (Sharkey & Singsaas, 1995; Seemann et al., 1984; Singsaas et al., 1997). Isoprene resulted to have a role mainly in the protection against short high-temperature episodes (Singsaas & Sharkey, 1998). As leaves can be subjected to dozens of high-temperature episodes each day, the increased recovery from each episode allowed by isoprene could become very important to the plant (Singsaas & Sharkey, 1998). The mechanism underlying the increased thermotolerance is still partially unknown and it has been attributed to the stabilization of membrane lipid bilayer by enhancing the hydrophobic interactions (Gounaris et al., 1984). In fact, the lipid membranes are particularly sensitive to exposure to high temperatures and often they get damaged or denatured unless protection mechanisms occur. Isoprene has been supposed to stabilize exclusively the thylakoid membranes of chloroplast in which it is formed (Sharkey, 1996; Sharkey & Yeh, 2001). However, no enhancement of stabilization by isoprene has been observed by using artificial membranes (Logan et al., 1999). Monoterpenes emitted in a light-dependent manner also provide this type of thermoprotection. Repeated cycles of high-temperature stress reduced the recovery in leaves without isoprene or monoterpene, although leaves with isoprene or monoterpene maintain high rates of photosynthesis, especially after repeated periods of high temperature (Loreto et al., 1998; Sharkey, 1996).

The thermotolerance hypothesis largely explains what plants may gain from isoprene emission, and the effect can be large compared to the cost of emission. This hypothesis suggests that plants suffering short high-temperature episodes, but not long term, constant high-temperature exposure, should emit isoprene. Trees often fit this description: the leaves at the tops of the canopy are exposed to full sun and they heat substantially up if the air is still (Ehleringer, 1991). On the other hand, desert plants, which are adapted to prolonged high temperatures, generally, do not emit isoprene. In fact, in desert plants, the leaves are absent or very small leaves so that the boundary layer is small and the leaves cannot heat up much above air temperature. A common mechanism to reduce the leaf temperature is transpiration that is regulated by stomata opening and air relative humidity. However, where the air relative humidity is very high, such as in humid tropical environments, transpiration is highly reduced and other mechanisms should account for the thermoprotection of leaves. In fact, tropical plants usually emit relatively more isoprene than most of the plants in temperate or cool climates (Keller & Lerdau, 1999).

Hanson et al. (1999) speculated that **isoprene emission might have been an important step in the evolution of land plants**. As plant progenitors started to stand up in the air, the low heat capacity of air caused plant temperatures to vary in a greater range than when the organisms were in water. Thus, the isoprene emission may have played a role in land colonization by the first terrestrial plants such as *Bryophyta*. Isoprene emission is, in fact, common in mosses but absent in algae (Hanson et al., 1999, reviewed by Sharkey & Yeh, 2001).

**Isoprene may play an important role also as antioxidant in leaves.** This idea is normally put forward on the basis of the rapid reaction of isoprene with ozone and hydroxyl radicals. Isoprene can dramatically reduce the damage caused by acute and short (3 h, 300 nL L<sup>-1</sup>) or relatively low and long (8h, 100 nL L<sup>-1</sup>) ozone treatments in leaves (Loreto et al., 2001; Loreto & Velikova, 2001). This effect may be perhaps related also to the membrane strengthening action of this compound. However, isoprene may also effectively react with ozone forming hydroxymethyl hydroperoxide and aggravating the ozone induced damage (Salter & Hewitt, 1992).

**Isoprene emission is a sensitive indicator of wound signals that can travel through plants** (Loreto & Sharkey, 1993). In a number of studies, wounding was inflicted by puncturing,

smashing, cutting and burning leaves. By wounding one leaf while monitoring isoprene emission from a different leaf, researchers could show the transmission of a signal. The time between wounding one leaf and a change in rate of isoprene emission of a different leaf was linearly related to the distance between the two leaves, allowing a calculation of travel rate of the signal. The signal travelled about  $2 \text{ mm s}^{-1}$ , which is likely to result from electrical signals travelling through the plant. The calcium chelator EGTA substantially delayed the wound signal effect on isoprene emission, indicating that the electrical signal may have caused calcium fluxes that ultimately affected isoprene emission.

## 7. Conclusions

The development and survival of all living things relies on the ability of organisms to perceive and respond to their environment. Responses to internal and external signals are frequently elicited by hormones, promoting changes in morphology to accommodate an ever-changing habitat. Figure 5 summarizes the VOCs emitted in response to different biotic and abiotic stresses.

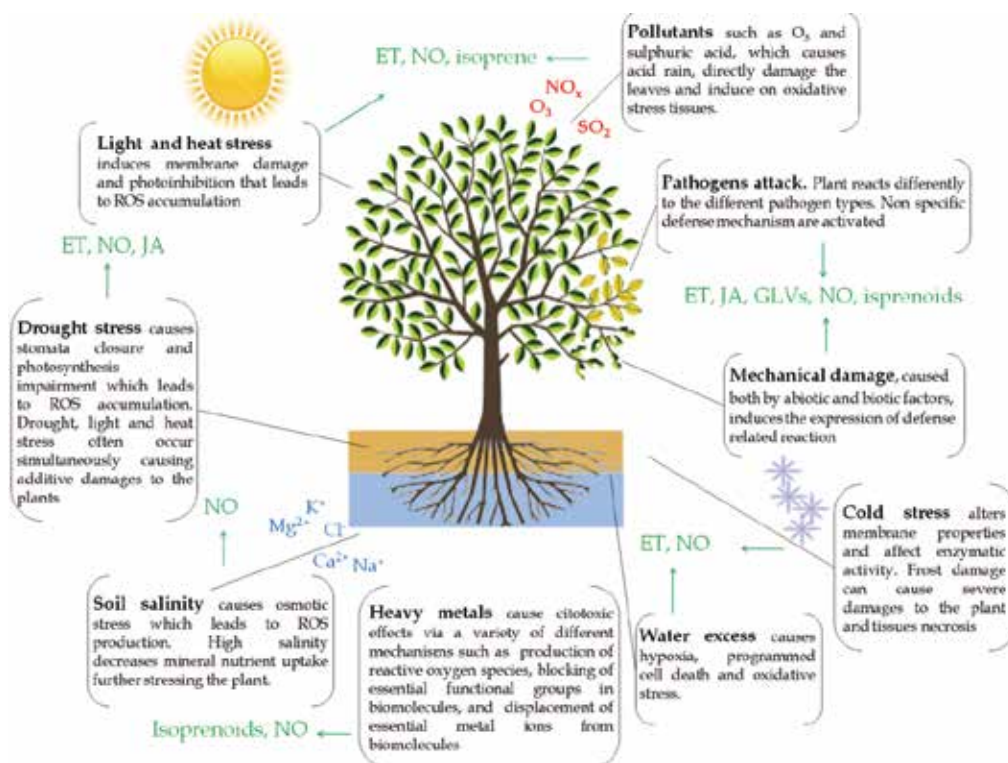


Fig. 5. All the abiotic stresses affecting plant trigger complexes responses aiming to increase the stress tolerance. The responses include the emission of VOCs. The VOCs produced in response to the different stresses are reported in green. A common mechanism links together the different stresses: all causes oxidative stress and hamper the production of reactive oxygen species (see also fig. 2). Excess light and heat, as well as exposure to oxidizing air pollutants, cause direct accumulation of ROS which crucially contributes to for initiate the stress-related signal cascades (see Fig. 2) - (After Vickers et al., 2009)

All kinds of stresses induce in the plants the production of ROS which will result in oxidative stress. Therefore, the complex network of reactions protecting the plant from stress is strictly linked with the response to oxidative stress. Changes in volatile emission under stress conditions supply evidence that VOCs are linked with the plant responses to stress. Their emissions often increase under abiotic stress conditions, particularly under leaf damage, water, heat and light stress (Vickers et al., 2009). Some of the volatiles, such as ethylene and NO, are primarily acting as stress messenger that allows the plant to trigger the stress induced defenses. On the other hand, other volatiles, such as isoprene and isoprenoids, and NO as well, play an important role in a direct protection against several of these stresses. The common mechanism underlying the protective effect of these VOCs is their general antioxidant role (Calfapietra et al., 2009).

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# Epigenetic Chromatin Regulators as Mediators of Abiotic Stress Responses in Cereals

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## 1. Introduction

Plants are constantly exposed to environmental changes and have to adapt to a multitude of abiotic and biotic stresses. Due to their sessile nature plants had to develop sophisticated ways to respond and adapt to a variety of external stress factors that would otherwise compromise proper development, reproductive success and ultimately survival.

Years of rigorous research have demonstrated that abiotic stress such as drought, high salinity, temperature extremes, UV irradiation and oxidative stress, affect various cellular processes in plants and induce alterations in gene expression programmes in order to activate the plants defense mechanisms to survival. Extensive studies based on forward genetic, reverse genetics, and biochemical investigations of individual loci as well as genome-wide approaches, especially in the model-plant *Arabidopsis*, have revealed a plethora of genes that are involved in abiotic stress response pathways and acquisition of stress tolerance. These include a wide range of stress-responsive genes encoding transcription factors and functional proteins whose transcription is altered during abiotic stress [1].

Growing evidence from recent studies has indicated that regulation of expression of stress-responsive genes is often accomplished by epigenetic mechanisms which modulate chromatin structure or regulate the level of mRNA accumulation at the postranscriptional level [2; 3; 4].

In eukaryotes nuclear DNA is organized in chromatin, a tightly packed higher order structure which permits genomic DNA to fit within the nucleus. The fundamental unit of chromatin is the nucleosome which is composed of 147 base pairs of DNA that is wrapped almost twice around an octamer of histone proteins. The octamer consists of two copies of each of histone H2A, H2B, H3 and H4. Chromatin higher-order structure switches between condensed and relaxed states and plays a crucial role in the epigenetic regulation of gene expression [5Kouzarides 2007]. Alterations in chromatin structure affect the accessibility of the transcriptional machinery (transcription factors, RNA polymerase) to nucleosomal DNA and determine the levels of gene expression in response to developmental and environmental stimuli.

Chromatin modulation is achieved by a variety of mechanisms including: DNA methylation catalyzed by DNA cytosine methyltransferases, histone post-translational modifications catalyzed by a wide range of enzymes specific for each modification, alterations in histone-DNA interactions which facilitate nucleosome sliding and are catalyzed by chromatin

remodeling complexes, histone variants, and small RNA related pathways (siRNAs and miRNAs) which act directly on chromatin and induce RNA-dependent DNA methylation (RdDM) [<sup>5</sup>Kouzarides et al, 2007; <sup>6</sup>Pfluger and Wagner, 2007; <sup>7</sup>Law and Jacobsen, 2010; <sup>8</sup>Chapman and Carrington, 2007; <sup>9</sup>Henderson and Jacobsen, 2007; <sup>10</sup>Kasschau et al., 2007; <sup>11</sup>Chinnusamy and Zhu; 2009]. In addition, small RNAs also regulate gene expression at the posttranscriptional level through mRNA degradation and/or translational inhibition [<sup>12</sup>Voinnet 2009; <sup>13</sup>Bartel 2009].

Research on the epigenetic regulation during plant development and in response to abiotic stress has focused on exploration of chromatin modulation at specific loci and the characterization of chromatin modifiers during development and under stress conditions [<sup>2</sup> <sup>3</sup>]. In recent years the advancement of -omics technologies [transcriptomics-microarrays/whole-genome tiling arrays, next generation sequencing (NGS), chromatin immunoprecipitation (ChIP) assays combined with sequencing technology (ChIP-seq), and bioinformatics tools] contributed greatly to these efforts and led to the transition from epigenetics (study of individual locus /small-scale) to epigenomics (study of whole epigenomes/global-scale) [reviewed in <sup>14</sup>Tsaftaris et al., in press]. Large-scale epigenomics studies have established the genome-wide profile of DNA methylations, histone modifications and small RNA patterns, in different developmental stages or under abiotic stress conditions, primarily in the model-plant Arabidopsis [<sup>15</sup>Cokus et al. 2008; <sup>16</sup>Lister et al., 2008; <sup>17</sup>Zhang et al., 2007; <sup>18</sup>Bernatavichute et al., 2008; <sup>19</sup>Zhang et al.,2009; <sup>20</sup>Yang et al., 2010; <sup>21</sup>Van Dijk et al., 2010; <sup>22</sup>Roudier et al., 2011] but also in the cereal model-plant Brachypodium [<sup>23</sup>Zhang et al., 2009b] and in agronomically important cereal crops like rice [<sup>24</sup>Li et al., 2008, <sup>25</sup>Sunkar et al., 2008; <sup>26</sup>He et al., 2010] maize [<sup>27</sup>Wang et al.,2009; <sup>28</sup>Wang et al., 2011] wheat [<sup>29</sup>Yao et al., 2010] and barley [<sup>30</sup>Schreiber et al., 2011]. Together, epigenetics and epigenomics studies have provided a wealth of information about epigenetic regulation in response to developmental and environmental stimuli, mostly in Arabidopsis. Recently, the availability of the rice and maize genomes and epigenomes provided the opportunity for exploring this exciting area in monocots as well, and data on epigenetic regulation in response to abiotic stress in cereals have started to come into sight.

In this review we summarize the current progress on epigenetic regulation in response to abiotic stresses such as drought, cold, and high salinity, in Arabidopsis, and present the emerging information on the epigenetic regulatory mechanisms induced upon abiotic stress in cereals such as rice, maize, wheat and barley. Expanding our understanding of the epigenetic regulation associated with abiotic stress responses in cereals of agronomic importance could have a significant impact in breeding for improved varieties with increased stress tolerance. In view of the global climate change where abiotic stresses are expected to increase dramatically, this undertaking would be of paramount importance.

## **2. Histone modifications in response to abiotic stress**

### **2.1 Gene activation and deactivation marks**

Histone post-translational modifications usually take place on histone tails protruding from nucleosomes, and include methylation, acetylation, phosphorylation, ubiquitination, biotinylation, and sumoylation on specific lysine, arginine, serine and threonine residues [<sup>31</sup>Zhang et al., 2007a; <sup>32</sup>Berger et al., 2007]. A complex pattern of site-specific combinations of histone modifications on different residues known as the 'epigenetic histone code' leads to specific chromatin states in response to intrinsic (developmental) and external



(environmental signals) which regulate transcriptional activity and are inherited by daughter cells [<sup>33</sup>Strahl and Allis 2000].

The best characterized histone modifications associated with the response of plants to abiotic stress are the histone acetylation/deacetylation and histone methylation/demethylation reversible modulations at individual loci [<sup>2</sup>; <sup>3</sup>Chinnusamy et al. 2008; Chinnusamy and Zhu 2009]. Histone acetylation carried out by histone acetyltransferases (HATs) is associated with gene activation, whereas histone deacetylation, performed by histone deacetylases (HDACs) is associated with gene silencing [<sup>34</sup>Chen and Tien, 2007]. Histone methylation/demethylation is catalyzed by specific histone methyltransferases (HMTs) and histone demethylases (HDMs), respectively. Tri-methylation of H3 at lysine 4 (H3K4me3) which is catalyzed by a specific histone methyltransferase of the Trithorax (TrxG) group leads to gene transcription, whereas trimethylation of H3 at lysine 27 (H3K27me3) by a specific methyltransferase of the Polycomb group (PcG), which antagonizes TrxG, leads to gene repression [<sup>35</sup>Avramova 2009; <sup>36</sup>Alvarez et al., 2010; <sup>37</sup>Pontvianne et al., 2009; <sup>38</sup>Liu et al., 2010; <sup>39</sup>Kapazoglou et al., in press].

Abiotic stress such as drought, cold, heat, high salinity, oxidative stress and UV irradiation, alter the histone acetylation and/or methylation pattern within the promoters or coding regions of genes, thereby causing gene activation or gene silencing. In addition, abiotic (and biotic) stress factors trigger the production of certain phytohormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), gibberellic acid (GA) and ethylene, which mediate the regulation of gene expression during the adaptive responses of plants to various abiotic stresses. It has been proposed that histone acetylation/deacetylation through the action of HATs and HDACs, and histone methylation/demethylation through the action of HMTs and HDMs, respectively, epigenetically regulates the responses to various stresses as well as the integration of hormonal signals controlling stress-responsive genes [<sup>2</sup>Chinnusamy et al. 2008; <sup>3</sup>Chinnusamy and Zhu 2009; <sup>11</sup>Chinnusamy and Zhu 2009].

Much research has been conducted in Arabidopsis on the effects of abiotic stress on histone modifications at specific chromatin loci. For example, ChIP assays detected histone modifications on the N-terminal tails of H3 in four drought-stress responsive genes, namely, *RESPONSIVE TO DEHYDRATION(RD)29A*, *RD29B*, *RD20* and *AP2 DOMAIN-CONTAINING TRANSCRIPTION FACTOR*, *Atg20880*. In particular, the histone activation marks H3K23ac and H3K27ac were enriched in the coding regions of *RD29B*, *RD20* and *Atg20880* in response to drought stress and these changes were associated with increased expression of these genes under dehydration conditions [<sup>40</sup>Kim et al., 2008]. Enrichment for H3K4me3 was also observed at *RD29A* and *Atg20880* chromatin and it occurred after full activation of these genes under conditions of drought. In another study, histone modifications were detected in two cold-responsive genes *COLD-REGULATED (COR)15A* and *ATGOLS3* (encoding galactinol synthase) during exposure to low temperature conditions [<sup>41</sup>Taji et al., 2002]. H3K27me3, a gene silencing mark, was found to be decreased on the chromatin of both genes and this reduction was associated with reduced expression under cold stress. Another report revealed that phosphorylation of histone H3 at serine 10, phosphoacetylation of H3 at serine 10 and lysine 14, and acetylation of histone H4 were enriched as a response to cold, high salinity, and exogenous ABA application, in Arabidopsis and tobacco cells. The induction of these histone modifications correlated with up-regulation of stress-responsive genes [<sup>42</sup>Sokol et al., 2007].

Histone modification alterations were also reported in cereals exposed to abiotic stress. Submergence of rice seedlings induced H3K4me3 and H3 acetylation in the 5' and 3' regulatory regions and coding regions of the *ALCOHOL DEHYDROGENASE 1 (ADH1)* and

*PYRUVATE DECARBOXYLASE (PDC1)* genes. These modifications correlated with upregulation of *ADH1* and *PDC1* and were restored to pre-stress levels after seedlings were reinstating to aeration, underlying the dynamic nature of histone methylation and acetylation modifications [43Tsuji et al., 2006]. In maize, exposure to UV irradiation resulted in increased H3 and H4 acetylation within the promoter and coding regions of UV-B-induced genes in a maize-UV-B-tolerant line, whereas such enrichment was not detected in a UV-B-sensitive maize line [44Casati et al., 2008].

Finally, genome-wide analysis using ChIP and deep sequencing (ChIP-Seq) unraveled the global epigenomic map of H3Kme1, H3K4me2 and H3K4me3 during drought stress and non-stress conditions, in *Arabidopsis*. The H3K4me1 and H3K4me2 were found to be more widely distributed than the H3K4me3 mark. Upon dehydration stress a substantial change in H3K4me3 abundance was observed, whereas there were only moderate changes in H3K4me1 and H3K4me2 levels. In addition, whereas for most transcribed genes the H3K4me3 mark was more prominent at the 5'-ends, for drought- and ABA-induced genes H3K4me3 had an atypically broader distribution profile [21van Dijk et al., 2010].

## 2.2 Histone modification enzymes

### *Histone acetyltransferases (HATs)*

Histone acetyltransferases (HATs) transfer an acetyl moiety to the  $\epsilon$ -amino group of highly conserved lysines in the N-terminal extensions of nucleosomal core histones, thereby neutralizing the positive charge of lysines and resulting in less affinity to the negatively charged DNA molecules. This results in relaxation of chromatin structure and subsequent transcriptional activation. HATs comprise a superfamily including the GNAT/MYST, CBP and TFII250 families and are often subunits of large protein complexes.

AtGCN5, a member of the GNAT/MYST subfamily, is the best studied HAT protein in *Arabidopsis* and plays a role in gene activation in response to environmental changes such as cold [45Vlachonasis et al., 2003]. AtGCN5 associates *in vitro* with the transcriptional co-activator proteins ADA2a and ADA2b. *ada2b* mutants were found to exhibit hypersensitivity to salt and abscisic acid and had altered responses to low temperature stress [46Hark et al., 2009]. Elongator, another histone acetyltransferase complex consisting of six subunits and highly conserved in eukaryotic organisms, was implicated in abiotic stress response. Mutations in the core subcomplex ABO1/ELP1 and ELP2, but not in the accessory subcomplex ELP4 and ELP6, increased ABA-induced stomatal closure. These mutants also displayed increased tolerance to oxidative stress [47Zhou et al., 2009]. A recent report showed that *ADA2b* positively regulates salt-induced gene expression by maintaining the locus-specific acetylation of histones H4 and H3b. ChIP assays demonstrated that the promoter and coding regions of *COR6.6* (*COLD RESPONSIVE 6.6*), *RAB18* (*RESPONSIVE TO ABA 18*), and *RD29b* genes had reduced levels of histone H3 and H4 acetylation in *ada2b-1* mutants relative to wild-type plants [48Kaldis et al., 2011].

Our group has identified *HAT* gene homologues from barley. Representative members of the GNAT/MYST family, namely *HvMYST*, *HvELP3* and *HvGCN5*, were isolated and gene expression was examined in different stages of seed development and in response to ABA treatment. Exposure of barley seedlings to exogenous ABA resulted in marked induction of all three *HAT* genes. *HvELP3* was the one mostly affected by the application of the hormone and had expression levels four times as much in the ABA-treated tissue than the untreated controls. *HvGCN5* and *HvMYST* were also up-regulated by approximately two-fold. These

data implied possible ABA-dependent regulation of barley histone acetyltransferases during seed development and abiotic stress response [49Papaefthimiou et al., 2010].

*Histone deacetylases (HDACs).*

Histone deacetylases (HDACs) reverse the effect of HATs by removing the acetyl group on histones resulting in condensed chromatin structure and gene silencing [34Chen and Tian, 2007]. Eukaryotic HDACs can be grouped into three major families based on their primary homology to the yeast HDACs: 1) the RPD3/HDA1 family, 2) the SIR2 family and 3) the plant specific family HD2 (50Pandey et al., 2002).

Sequence and phylogenetic analysis of the rice genome identified the respective three HDAC families in rice [51Fu et al., 2007]. HDA1 is further subdivided in four classes Class I, Class II and Class III, and Class IV, and HD2 in two classes HD2a and HD2b. In maize, 15 HDAC genes have been identified (10 HDA1, 1 SIR2, and 4 HD2-like and a number of HDA1 members have been biochemically characterized [52Lusser et al., 2001; 53Rossi et al., 2003; 54Varotto et al., 2003].

Functional analysis using silencing or overexpression transgenic lines in Arabidopsis has demonstrated that both *HDA1* and *HD2* genes are associated with the response to abiotic (as well as biotic stress). For example, *AtHDAC19* was proposed to mediate jasmonic acid (JA) and ethylene signaling during pathogen defense (55Tian et al. 2005; 56Zhou et al. 2005). Overexpression of *AtHDA19* resulted in reduced histone acetylation levels and upregulation of the stress-related genes *ERF1* (*Ethylene Response Factor-1*) and *PR* (*Pathogenesis Related*). Conversely, silencing of *AtHDA19* led to increased histone acetylation and downregulation of *ERF1* and *PR*. *AtHDA6*, another HDA1-Class I, was shown to be required for jasmonate response, senescence, and flowering. *AtHDA6* was induced by exogenous JA and ethylene [57Wu et al. 2008]. In addition, in *hda6* mutants and in HDA6-RNAi plants the Arabidopsis JA-responsive genes *PDF1.2*, *VSP2*, *JIN1*, and *ERF1* were downregulated, suggesting an indirect involvement of HDAC6 in JA-responsive gene regulation.

Histone modification changes that take place as a response to abiotic stresses are often found to be induced by phytohormones, such as ABA [2Chinnusamy et al., 2008]. ABA affects a wide range of processes in plants like germination, vegetative to reproductive transitions, seed development, seed dormancy and abiotic stress tolerance. For example, *AtHD2C*, belonging to the HD2 family was proposed to play a role in ABA signaling and abiotic stress, in Arabidopsis (58Sridha and Wu 2006). ABA treatment caused severe reduction in expression of *AtHD2C*, whereas overexpression of *AtHD2C* resulted in enhanced abiotic stress tolerance to salt and drought stress, as well as repression of several ABA-responsive genes and induction of others (Sridha and Wu 2006). *AtHOS15* encoding a protein similar to human transducing- $\beta$ -like protein (TBC), a component of a repressor protein complex involved in histone deacetylation, was reported to mediate ABA-dependent deacetylation in response to cold stress [59Zhu et al., 2008]. The expression of *AtHOS15* is increased by cold, high salinity, and ABA treatment and *hos15* mutants are hypersensitive to freezing stress. In addition *hos15* mutants displayed increased H4 acetylation levels and concurrent increase of *RD29A* expression levels, suggesting a role for *HOS15* in regulating chromatin acetylation levels and gene expression under abiotic stress.

Furthermore, in a recent report, *AtHDA6* was shown to be involved in modulating the levels of H3K9, 14 ac and H3K4me3 (gene activating marks) and of H3K9me2 (histone deactivation mark) in response to ABA and salt-stress [60Chen et al., 2010]. The *hdac6* mutant and RNAi HDAC6 lines were hypersensitive to ABA and salt stress, and the expression of ABA- and

abiotic stress-inducible genes, *ABI1*, *ABI2*, *KAT1*, *KAT2*, *DREB2A*, *RD29A*, *RD29B* was decreased when these plants were subjected to ABA or salt stress as compared to wild-type plants. Moreover, both ABA application and salt stress increased the gene activation marks, H3K9,14 ac and H3K4me3, in the promoter and coding regions of some of the stress-inducible genes mentioned above. However, such increase was not observed in the *hdac6* mutant lines. Together these observations indicate that *HDAC6* is required for ABA and stress-induced histone acetylation, and most likely functions indirectly by suppressing a repressor of histone acetylation. Ultimately, this leads to gene activation of stress-responsive genes and stress tolerance [60Chen et al., 2010].

Studies on *HDAC* genes in relation to stress and stress-related hormones have been recently reported in cereals as well. Expression analyses of 18 rice *HDAC* genes from HDA1, SIR2 and HD2 families demonstrated distinct spatial expression patterns and differential responses to environmental stresses and hormones [51Fu et al., 2007]. Cold, osmotic and salt stresses, and external application of hormones such as JA, ABA, and SA, increased the expression of certain *HDA1* genes, and reduce the expression of others (51Fu et al. 2007). For example two members of the rice HDA1-class I (*HDA 702* and *HDA705*) and one member of class II (*HDA 704*) were induced by exogenous JA application. Conversely, the expression of a member of class IV (*HDA 712*) was reduced after JA treatment.

Our group has identified and characterized gene members of both HDA1 and HD2 families from barley and examined their expression during barley development and in response to stress-related hormones, such as ABA and JA [61Demetriou et al., 2009; 62Demetriou et al., 2010]. Barley *HDA1* genes (one of each class, I, II, III, and IV, respectively) were induced upon JA treatment, in agreement with the expression of their rice homologues. In addition, both *HvHDAC2-1* and *HvHDAC2-2* of the barley HD2 family, were significantly induced at 6 and 24 h after exogenous application of seedlings with JA. On the other hand, *HvHDAC2-1* showed a marked induction at 24 h after ABA treatment, whereas *HvHDAC2-2* transcript levels declined at 6 h after ABA treatment and showed no significant difference in 24 h after ABA treatment [61Demetriou et al., 2009]. In rice, the two *HD2* homologues (*HDT701*) and (*HDT702*) were also induced upon treatment with JA (51Fu et al., 2007) in accord to their barley homologues. On the contrary, whereas both rice *HD2* homologues were repressed by ABA, barley *HvHDAC2-1* and *HvHDAC2-2* showed differential responses to ABA exposure. Interestingly, the *HD2c* gene of Arabidopsis is also repressed by ABA (58Sridha and Wu, 2006). Together these results suggest common functions for some *HDAC* homologues among species but also possible species-specific functional diversification, in response to stress.

#### *Histone methyltransferases(HMTs)/Histone demethylases (HDMs)*

The best characterized histone methyltransferase (HMTs) genes are the ones coding for the enzymes that perform the deposition of the H3K4me3 activation mark and H3K27me3 silencing mark, respectively. These have been intensively studied both in monocots and dicots and the results of these studies have been discussed in a number of reviews [35Avramova 2009; 36Alvarez et al., 2010; 37Pontvianne et al., 2009; 38Liu et al., 2010; 39Kapazoglou et al., in press]. The Polycomb group (PcG) complex with H3K27me3 activity plays a crucial role in various stages of development, such as flowering and seed development and is composed of four subunits. Two WD40 proteins, FERTILIZATION INDEPENDENT ENDOSPERM (FIE), and MULTICOPY SUPPRESSOR OF IRA1 (MS1) remain constant in all PcG complex variants. Depending on cell type and function the different PcG complexes contain one of the three homologues of the *Drosophila* E(Z) homologues, MEA, CURLY LEAF (CLF) or SWINGER (SWN), which possess the histone

methyltransferase activity, and one of the three homologues of the *Drosophila* Su(z)12 protein, EMBRYONIC FLOWER 2 (EMF2), FERTILIZATION INDEPENDENT SEED 2 (FIS2), and VERNALIZATION2 (VRN2), respectively. It was shown that Arabidopsis *msi1-cs* co-suppressor lines displayed increased tolerance to drought stress. In addition, the expression of stress- and ABA-responsive genes was up-regulated in *msi1-cs* lines suggesting that MSI1 suppresses stress-related genes in an ABA-dependent manner [63Alexandre et al., 2009]. A recent study implicated the Trithorax protein ATX1, performing trimethylation of H3 at lysine 4 (H3K4me3), in dehydration stress signaling both in an ABA-dependent and ABA-independent manner. *atx1* plants exhibited larger stomatal apertures, increased transpiration rates and decreased tolerance to dehydration stress. ATX1 was shown to be required for induction of *NCED* (a gene encoding a key enzyme in ABA biosynthesis) and H3K4me3, in response to dehydration stress. By inducing *NCED3* and consequently ABA synthesis, ATX1 exerted an effect on ABA-dependent gene expression, but it was also shown to regulate ABA-independent gene expression pathways [64Ding et al., 2011].

A recent study by our group characterized the PcG gene homologues from barley and examined their expression during seed development and in response to ABA treatment. The barley homologues, *HvE(Z)* and *HvFIE* were significantly induced at 24 hours after ABA exposure, about 4-fold and 10-fold, respectively, implying a role of PcG genes in ABA-mediated processes, such as seed development, seed dormancy, germination and abiotic stress response [65Kapazoglou et al., 2010]. Moreover, a gene encoding a trithorax-like H3K4 methyltransferase, *HvTX*, was also identified and characterized in barley by our group. *HvTX* transcript levels showed a marked increase by drought in a drought-tolerant barley cultivar [Papaefthimiou and Tsaftaris, in press66].

Histone demethylases were only recently discovered and their molecular and functional characterization is an area of active research [Kapazoglou et al., in press39]. In Arabidopsis, functional studies assigned a role for H3K4-specific demethylases as regulators of flowering time by deactivating the flowering repressor gene *FLC* and promoting flowering [67]. In rice, a *jmjC* domain-containing gene encoding a H3K9 demethylase, JM706, was found to be required for floral organ development[68]. Reports describing a putative role of HDMs in abiotic stress are anticipated. In the cereal crop barley, one putative plant-specific PKDM7 subfamily histone demethylase was characterised and was shown to be significantly induced by drought stress [69Papaefthimiou and Tsaftaris, in revision].

### 3. ATP-dependent chromatin remodeling factors

The SWI/SNF (switch/sucrose non-fermenting) is a multisubunit assembly with DNA-dependent ATPase activity that is implicated in alteration of chromatin structure and subsequent changes in gene expression [70Schwabish and Stuhl, 2007]. An SNF-type putative remodeling gene was shown to be expressed in a desiccation- and ABA-dependent manner in pea [71Rios et al., 2007]. AtCHR12, a SNF/Brahma (BRM)-type chromatin remodeling factor, has been implicated as a negative regulator in the temporary growth arrest caused by drought and heat stress, in Arabidopsis [72Mlynarova et al., 2007]. Overexpression of *AtCHR12* resulted in growth arrest of primary buds and reduced growth of primary stems under drought and heat stress. On the contrary, in *atchr12* knockout mutants growth arrest was decreased as compared to wild type plants under stress. In another report it was shown that SWI3B, a subunit of a SWI/SNF complex in Arabidopsis, interacts with HABI1, (a

phosphatase 2C), which is a negative regulator of ABA signaling [<sup>73</sup>Saez et al., 2008]. *swi3b* mutant seedlings exposed to external ABA exhibited reduced sensitivity to ABA-mediated inhibition of seed germination and growth and reduced expression of ABA-responsive genes like *RD29B* and *RAB18* [<sup>73</sup>Saez et al., 2008]. Furthermore, ChIP assays showed that the interaction of HAB1 with *RD29B* and *RAB18* promoters was abolished by ABA, suggesting that HAB1 modulates the ABA response through regulation of a SWI/SNF complex.

Molecular and functional characterization of chromatin remodeling factors in cereals is scarce. In one study it was shown that ChIP assays conducted with maize leaf nuclei, detected an enrichment for SWI2/SNF2 at target genes after UV-B treatment of maize plants, implying involvement of chromatin remodelling factors in abiotic stress responses [<sup>44</sup>Casati et al., 2008]. It is expected that by exploiting the data from the completed rice, maize and recently Brachypodium genomes, additional studies on chromatin remodeling and its association with abiotic stress in cereals will soon be reported.

#### 4. DNA methylation/demethylation

DNA methylation is a critical epigenetic modification which is established and maintained by multiple interacting cellular mechanisms. Cytosine methylation in plants is found predominately in a symmetrical CG dinucleotide site. However unlike animals, it also occurs at CHG and asymmetric CHH sites (where H is A, C, or T). A dynamic interplay between methylation and demethylation accomplished through specific enzymes, is critical for proper cellular regulation during plant development. DNA methylation is carried out by “de novo” and “maintenance” DNA methyltransferases (MTases), and in most cases results in gene silencing although the opposite has been also observed [<sup>7</sup>Law and Jacobsen, 2010; <sup>74</sup>Macarevich et al., 2008; <sup>75</sup>Shibuya 2009]. A number of reports have demonstrated that DNA methylation may be employed by plants to regulated gene expression as a response to abiotic stresses.

An early study in maize had shown that cold stress induced the expression of the *ZmM11* gene (a retrotransposon-like gene) and this correlated with reduction in nucleosomal DNA methylation [<sup>76</sup>Steward et al., 2002]. Studies of F1 hybrids and their parents in maize revealed that under dense planting (a stressful condition), parents accumulated more DNA methylation sites than their hybrids which resist to DNA methylation changes [<sup>77</sup>Kovacevic et al., 2005; <sup>78</sup>Tani et al., 2005; and reviewed in <sup>79</sup>Tsaftaris et al., 2008]. Another report in tobacco showed that a methyltransferase (*met1*) mutant, exhibited demethylation of genomic regions that were associated with the expression of a large number of drought-related genes [<sup>80</sup>Wada et al., 2004]. Moreover, tobacco plants exposed to high salt, cold and aluminum displayed changes in the methylation pattern of a gene encoding glycerophosphodiesterase-like protein (NtGPDL) and known to be induced in response to aluminum stress, as compared to nonstressed plants [<sup>81</sup>Choi and Sano, 2007]. CG sites within the coding region were selectively demethylated suggesting that abiotic stress caused gene activation by changing the DNA methylation status of the particular genomic locus. A recent study exploring the genome-wide DNA methylation status of two rice cultivars with different tolerance to drought, revealed significant differences in the methylation patterns between the two genomes [<sup>82</sup>Wang et al., 2011]. In particular, a drought-tolerant line DK151 and its drought-sensitive parent, IR64, were anaadaptationlyzed by methylation-sensitive amplified polymorphism analysis (MSAP) under drought stress and no stress conditions.

DNA methylation/demethylation changes were induced under drought conditions in a developmental and tissue specific manner and they accounted for 12.1% of the total site-specific methylation differences between the two lines. Notably 70% drought-induced methylation changes were reversed after recovery, and 29% remained unaltered. These observations suggest that DNA methylation changes play a role in the response of rice to dehydration conditions probably by activating or deactivating stress-responsive genes and leading to adaptation to drought conditions [82Wang et al., 2011]. MSAP was also used recently in wheat, to assess DNA methylation changes upon salt stress in two cultivars with different tolerance to salt. Upon high salinity conditions DNA methylation alterations were observed in both cultivars which might be associated with the response and adaptation of wheat to salt stress [83Zhong et al., 2009].

Unlike the well characterized histone modification enzymes HATs, HDACs and HMTs, little is known regarding DNA methyltransferases and demethylases in association to stress. Ten putative DNA methyltransferases were characterized in rice and their expression examined in different developmental stages and under abiotic stress. *OsCMT2* was found to be induced by cold and high salinity but not by drought. Conversely, *OsCMT3* showed approximately a six- and four-fold reduction in mRNA accumulation in rice seedlings subjected to high salt and dehydration conditions, respectively [84Sharma et al., 2009]. In a recent study, the gene encoding the Arabidopsis DNA glycosylase ROS1 (REPRESSOR OF SILENCING 1)-now known as DML3 (DEMETETER-LIKE protein 3) and involved in DNA demethylation-was indirectly implicated in the response to abiotic stress, as it was shown to be the target of the stress-responsive miRNA402 [85Kim et al., 2010].

## 5. Small RNAs

Four major types of small RNAs have been identified in plants, namely, micro RNAs (miRNAs), transacting small interfering RNAs (ta-siRNAs), natural-antisense siRNAs (nat-siRNAs), and heterochromatic (hc-RNAs) siRNAs. Hc-siRNAs direct methylation of DNA sequences complementary to the siRNAs in a process known as RNA-directed DNA methylation (RdDM) and lead to gene silencing [8Chapman and Carrington, 2007; 9Henderson and Jacobsen, 2007]. MiRNAs, ta-siRNAs, and nat-siRNAs function predominately at the post-transcriptional level through mRNA degradation and/or translational inhibition resulting in gene silencing, and miRNAs have been shown to also regulate gene expression through DNA methylation [86Wu et al., 2009; 87Khraiwesh et al., 2010].

Small RNAs have essential functions in many aspects of plant growth and development [Liu et al., 2005; 88Jones-Rhoades et al, 2006; 12Voinnet 2009; Mallory and Vaucheret, 2006; 89Chen, 2009]. Furthermore, small RNAs have been shown to play key roles in the regulation of phytohormone signaling and the response to a variety of abiotic stresses [90Sunkar and Zhu 2004; 91Sunkar et al., 2007; 92Voinnet 2008; Liu and Chen, 2009; 93Covarrubias and Reyes, 2010].

Locus-specific studies as well as large-scale transcriptome analyses have revealed numerous miRNAs that are conserved across species and are responsive to a broad spectrum of stresses. In the last several years the development of high-throughput sequencing technology has allowed for the discovery of ever more miRNAs including very low abundance or species-specific miRNAs. In this way a growing number of small RNAs has been detected that respond to abiotic (as well as biotic) stress both in dicots and monocots.

In *Arabidopsis*, stress-related miRNAs were first detected in a library generated from small RNAs from seedlings exposed to various stresses (<sup>94</sup>Sunkar and Zhu, 2004). For example miR393, miR397b, and miR402 were found to be induced upon cold, drought and high salinity conditions as well as by ABA treatment. Follow-up studies with miR402 showed that miR402 overexpressing plants displayed reduced transcripts of the DNA demethylase DML3, implying miRNA-guided control through down-regulation of a DNA demethylase [<sup>85</sup>Kim et al., 2010].

An siRNA derived from a pair of natural *cis*-antisense transcript composed of *PYRROLINE-5-CARBOXYLATE DEHYDROGENASE(P5CDH)* (sense), a stress-related gene, and *SRO5* (antisense), a gene of unknown function, generates two types of siRNAs, 24-nt siRNA and 21-nt siRNA. These were found to down-regulate *P5CDH* by sequential cleavage of *P5CDH* mRNA after salt treatment leading to accumulation of the osmoprotectant proline and increased tolerance to salt stress [<sup>95</sup>Borsani et al., 2005]. Stress- or ABA-inducible sense and antisense transcripts were also detected in the stress-inducible gene loci, *RD29A* and *CYP707A1* [<sup>96</sup>Matsui et al., 2008]. Transcriptome microarray analysis revealed numerous other miRNAs involved in abiotic stress both in *Arabidopsis* and poplar [<sup>97</sup>Liu et al., 2008; <sup>98</sup>Lu et al., 2008]. Conserved miRNAs, such as miR397 and miR169, were up-regulated in both species under cold conditions, and species-specific stress responsive miRNAs were also detected.

MiRNA responsiveness to various abiotic stress factors has been demonstrated in cereals such as rice, wheat, maize and the model-plant of cereals, *Brachypodium*. For example drought and high salinity stress were found to induce several miRNAs in rice as determined by microarray analysis [<sup>99</sup>Zhao et al., 2009]. MiR169g was shown to be up-regulated in rice roots and shoots upon dehydration. Interestingly, the promoter of the miR169g gene was found to contain two dehydration responsive elements (DRE). Similar to miR169g, the rice miR169n gene was found to be induced at conditions of high salinity. A *cis*-acting ABA responsive element (ABRE) resides within the promoter of rice miR169n implying an ABA-mediated response to stress [<sup>99</sup>Zhao et al., 2009]. Notably, both miRNAs target a transcription factor, NF-YA, that has been shown to be down-regulated upon drought conditions [<sup>100</sup>Stephenson et al., 2007]. Recently, genome-wide profiling of miRNAs in rice revealed 29 novel miRNAs that were differentially expressed (11 down-regulated miRNAs and eight up-regulated) under drought [<sup>25</sup>Sunkar et al., 2008; <sup>101</sup>Zhou et al., 2010].

<sup>102</sup>Kantar et al. (2010), identified 28 new miRNAs in barley, of which Hvu-MIR156, Hvu-MIR166, Hvu-MIR171, and Hvu-MIR408 were shown to be induced under dehydration conditions. Microarray analysis in maize demonstrated that 34 miRNAs from 13 plant miRNA families exhibited substantial changes in expression after drought treatment of seedlings [Wei et al., 2009<sup>103</sup>]. MiR474 which targets a gene encoding proline dehydrogenase (PDH), an enzyme involved in the degradation of proline, was found to be up-regulated upon dehydration conditions. Proline is known to accumulate in plants as a protective mechanism against drought stress. Upon drought stress miR474 transcripts were increased, whereas PDH accumulation was reduced, suggestive of a miR474-dependent mechanism in regulating proline content under drought conditions in maize. Conversely, the expression of other maize miRNAs such as miR168, miR528, and miR167 was decreased and this probably resulted in increased expression of their target genes *MAPK* (*MITOGEN ACTIVATED PROTEIN KINASE*), *POD* (*PEROXIDASE*), and *PLD* (*PHOSPHOLIPASE D*), respectively. Interestingly, these genes contain an ABA responsive element and are involved in the ABA-induced stomatal movement and antioxidant defense in maize [Wei et al., 2009<sup>103</sup>].



Cold stress has also been shown to have a significant effect in the expression of a number of different miRNAs in cereals. Microarray analysis identified 18 rice miRNAs that were differentially expressed upon cold treatment of rice seedlings [104Lv et al., 2010]. 12 miRNAs corresponding to 10 different families exhibited significant down-regulation and 6 miRNAs corresponding to five families exhibited substantial up-regulation under cold. Four down-regulated rice miRNAs (miR1435, miR1876, miR1320, miR1884) were not present in Arabidopsis implying species-specific miRNAs functions in the response to cold-stress. Six conserved families (miR156, miR166, miR169, miR171, miR319, miR444) are known to target genes encoding transcriptional factors such as homeodomain-leucine zipper proteins, scarecrow-like proteins, TCP family transcription factors and MADS-box proteins [Lu et al., 2008; Zhao et al., 2009]. The targets of rice miR319a/b and miR171a, were predicted to be the genes Os01g59660 and Os04g46860, respectively. Os01g59660 and Os04g46860 were induced by cold, whereas their cognate miRNAs were found to be down-regulated by cold. This inverse correlation between the expression of the miRNAs and their targets and the fact that the targets were validated by 5'RACE assays, strongly suggests miRNA-regulated responsiveness to cold stress [104Lv et al., 2010]. Interestingly, rice miR444 which is also down-regulated by cold-stress, targets two MADS-box proteins, MADS57 and MADS27 [Lu et al., 2008] which have been shown previously to be up-regulated under cold conditions [105Arora et al., 2007]. Most cold-responsive miRNAs were found to harbor cis-acting hormone-responsive elements in their 5'upstream regions, such as ABRE, and GARE (Gibberellin responsive element). For example, an ABRE element and two GARE elements were detected within the miR319 promoter implying ABA-mediated regulation of gene expression. In support to this a recent study showed that miR319 is down-regulated by ABA and up-regulated by GA, and a large number of other rice miRNAs are either induced or down-regulated by ABA and GA [106Liu et al., 2009].

High throughput sequencing technology using the Solexa platform, uncovered 129 putative novel miRNAs in the model plant Brachypodium. 25 of the novel miRNAs as well as 3 conserved miRNAs (miR169e, miR172b and miR397) displayed significant alterations in gene expression in response to cold stress [23Zhang et al., 2009]. A subset of the novel cold-responsive miRNAs was found to be monocot-specific and another subset Brachypodium-specific. MiR169e, miR172 and miR397 and six of the novel predicted miRNAs were up-regulated under cold, whereas 19 novel miRNAs were down-regulated. Interestingly, miR397 is predicted to target laccases, enzymes involved in lignin biosynthesis and cell wall structure maintenance.

A recent study described the identification of a set of miRNAs from wheat that responded to heat stress as well as to the biotic-stress conditions of powdery mildew infection [107Xin et al, 2010]. Furthermore, by interrogating the recently deep-sequenced small RNA transcriptome of bread wheat, Yao et al. 2010<sup>29</sup> identified a set of small non-coding RNAs with differential responses in a variety of stress conditions. For example siRNA 002061\_0636\_3054.1 shows down-regulation under conditions of increased heat, salinity and dehydration, whereas siRNA 005047\_0654\_19041.1 is substantially induced by cold.

SiRNAs have been also implicated in abiotic stress response in rice [108Yan et al., 2011]. Rice siR441 and siR446 accumulation was down-regulated by cold, drought, high salinity and by ABA treatment. Functional analysis showed that siR441 and siR446 knockdown mutants were more sensitive to drought, cold or salt treatment than the wild type, suggesting a role for siRNAs in rice tolerance to abiotic stress. The validated target of siR441 and siR446, *MAIF1*(encoding an F-box protein), was previously shown to be up regulated under abiotic

stress conditions. In addition, transgenic rice plants with decreased accumulation of siR441 and siR446 had the same phenotype as *MAIF1* overexpressing plants [108Yan et al., 2010]. Together these observations point to a role for rice siR441 and siR446 in abiotic stress response through regulation of *MAIF1*.

Genome-wide studies of intraspecific hybrids and their parents, in *Arabidopsis*, have revealed major differences in the 24-nt siRNA levels between the two genomes which resulted in alterations in global DNA methylation and gene expression [109Groszman et al., 2011]. Hybrid vigor is characterized by the superior performance of a hybrid over its parents in various traits, including stress tolerance, and this suggests that siRNA pathways may be associated with abiotic stress response in this phenomenon.

Finally, a recent report showed that siRNA biogenesis is crucial for protection against transgenerational retrotransposition under heat stress, in *Arabidopsis* [110Ito et al., 2011]. It is likely that such stress-related siRNA/retrotransposon effects will be revealed for cereal genomes as well.

## 6. Transgenerational stress memory

Adverse environmental conditions may induce changes in the epigenetic state of genes which can be inherited over successive generations and these could play a role in stress adaptation [111Paszkowski and Grossniklaus, in press].

Exposure to stress can result in changes in DNA methylation patterns and genome instability. Studies on *Arabidopsis* and *Pinus silvestris* growing in the vicinity of the Chernobyl reactor area suggested an association between increased global genome methylation with genome stability and stress tolerance in response to irradiation [112Kovalchuk et al., 2003; 1132004]. An association between transgenerational changes in DNA methylation and stress tolerance was also reported in the progeny of plants exposed to different abiotic stresses [114Boyko et al. 2010]. *Arabidopsis* plants were exposed to a wide spectrum of abiotic stresses including high salinity, UV-C, cold and heat as well as biotic stress. This resulted in higher homologous recombination frequency, increased global DNA methylation and higher stress tolerance in the untreated progeny. Moreover, in mutants defective in *DICER-like* genes, important for siRNA biosynthesis pathways, stress-induced homologous recombination frequency, DNA methylation and stress tolerance were impaired. These results suggested that stress-induced transgenerational responses require DNA methylation and the function of siRNA silencing pathways.

The significance of induced genome changes in adaptation was examined also in rice [115Akimoto et al., 2007]. Rice seeds were treated with 5-aza-deoxycytidine (inhibitor of cytosine methylation) and progeny after ten generations was screened to identify changes in DNA methylation by the MSAP and bisulfite assays. In one of the tested lines, line-2, DNA methylation was completely abolished in the gene coding region for the *Xa21G* gene encoding the Xa21-like protein. In wild type plants the *XA21G* promoter was methylated and there was no detectable expression of *Xa21G*, whereas in the line-2, *Xa21G* was expressed constitutively and the line was resistant to the pathogen race *Xanthomonas oryzae* pv. *oryzae*, race PR. These results suggested that DNA methylation can be stably inherited and maybe associated with the plants adaptation to stressful environments.

With the rapid progress in epigenetic research it is expected that further studies will emerge on the association of epigenetic states and transgenerational stress memory in more crop species.

## 7. Conclusions

Great progress in the research of epigenetic regulation in response to abiotic stress has been accomplished in the last several years, especially in the model plant *Arabidopsis*. Changes in histone modifications and changes in the expression of genes encoding histone modifying enzymes, as well as changes in DNA methylation patterns and the effect of small RNAs have been shown to play critical roles in the response to abiotic stress at a gene-specific and genome-wide level. Similar studies have been performed in cereals and a growing number of reports on the epigenetic regulation during cereal plant development and in response to abiotic stress have accumulated. However, plenty more efforts are still required in order to fully characterize and understand this process. The completion of the two cereal genomes, rice and maize, and of the cereal/grass-model plant *Brachypodium*, as well as the rapid progress in the sequencing of wheat and barley, will contribute significantly to this endeavor. The detailed study of both the genetic and epigenetic components of this complex process is necessary to comprehend the molecular aspects of the abiotic stress response. Furthermore, understanding the molecular mechanisms underlying the association of epigenetic regulation and transgenerational stress memory will help us in establishing the potential adaptive significance of this process and could have significant implications in agriculture. Considering that cereals represent approximately 50% of total caloric intake worldwide ([www.fao.org](http://www.fao.org)) and in view of the upcoming adverse changes of the global climate it is vital to delineate the molecular mechanisms by which such agronomically important crops manage to cope under conditions of stress. This could have important ramifications for agriculture as it would enable the generation of improved varieties with increased stress tolerance.

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# C<sub>4</sub> Plants Adaptation to High Levels of CO<sub>2</sub> and to Drought Environments

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## 1. Introduction

### 1.1 General features of the C<sub>4</sub> cycle

All plants use the Photosynthetic Carbon Reduction (PCR or Calvin-Benson) cycle for CO<sub>2</sub> fixation in which Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the first step producing a three-carbon compound, phosphoglycerate (3-PGA). For this reason this process is referred to as the C<sub>3</sub> cycle. Plants utilizing this pathway are often named as C<sub>3</sub> species. A major problem with the C<sub>3</sub> cycle is that the enzyme Rubisco catalyzes two competing reactions: carboxylation and oxygenation (Portis & Parry, 2007). The oxygenation reaction directs the flow of carbon through the photorespiratory pathway, and this can result in losses of between 25% and 30% of the carbon fixed. Environmental variables such as high temperature and drought can result in an increase in the oxygenase reaction. Therefore, reducing the Rubisco oxygenase reaction has the potential to increase carbon assimilation significantly and would represent a step change in photosynthesis (up to 100% depending on temperature; Long et al., 2006).

The C<sub>4</sub> photosynthesis is an adaptation of the C<sub>3</sub> pathway that overcomes the limitation of the photorespiration, improving photosynthetic efficiency and minimizing the water loss in hot, dry environments (Edwards & Walker, 1983). Generally, C<sub>4</sub> species originate from warmer climates than C<sub>3</sub> species (Sage & Monson, 1999). Most C<sub>4</sub> plants are native to the tropics and warm temperate zones with high light intensity and high temperature. Under these conditions, C<sub>4</sub> plants exhibit higher photosynthetic and growth rates due to gains in the water, carbon and nitrogen efficiency uses. Indeed, the highest known productivity in natural vegetation is for a C<sub>4</sub> perennial grass in the central Amazon, which achieves a net production of 100 t (dry matter) ha<sup>-1</sup> year<sup>-1</sup> (Piedade et al., 1991; Long, 1999). Some of the world's most productive crops and pasture, such as maize (*Zea mays*), sugar cane (*Saccharum officinarum*), sorghum (*Sorghum bicolor*), amaranth, paspalums (*Paspalum notatum* and *P. urvillei*), bermudagrass (*Cynodon dactylon*), blue grama (*Bouteloua gracilis*) and rhodes grass (*Chloris gayana*) are C<sub>4</sub> plants. In addition, the most troublesome weeds like nutgrass, crabgrass and barnyard, are also C<sub>4</sub> species. Although C<sub>4</sub> plants represent only a small portion of the world's plant species, accounting for only 3 % of the vascular plants, they contribute about 20% to the global primary productivity because of highly productive C<sub>4</sub>-grass-lands (Ehleringer et al., 1997). Approximately half of the ~10,000 grass and sedge species have C<sub>4</sub> photosynthesis, but fewer than 2,000 of the dicotyledonous species exhibit

$C_4$  photosynthesis. Given their disproportionate influence on global productivity,  $C_4$  plants have attracted much attention by the ecophysiological and ecosystem communities (Sage & Monson, 1999).

In  $C_4$  plants, the photorespiration is suppressed by elevating the  $CO_2$  concentration at the site of Rubisco through suppressing the oxygenase activity of the enzyme. This is achieved by a biochemical  $CO_2$  pump and relies on a spatial separation of the  $CO_2$  fixation and assimilation. In general, these species have a particular anatomy (Kranz anatomy), where mesophyll and bundle sheath cells cooperate to fix  $CO_2$  (Figure 1). Differentiation of these two cell types is essential for the operation of  $C_4$  photosynthesis, although special cases for the operation of the  $C_4$  cycle within only one type of photosynthetic cell have been found (Edwards et al., 2004; Lara et al., 2002; Lara & Andreo, 2005).

Basically, carboxylation of phosphoenolpyruvate (PEP) by the phosphoenolpyruvate carboxylase (PEP-carboxylase) produces four-carbon organic acids in the cytosol of mesophyll cells. These so-called  $C_4$  compounds are transported to the bundle sheath cells and decarboxylated to yield  $CO_2$  which is assimilated by Rubisco in the Photosynthetic Carbon Reduction (PCR) cycle (Hatch, 1987). The decarboxylation reaction also produces three-carbon organic acids ( $C_3$ ) that return to the mesophyll cells to regenerate PEP in a reaction catalyzed by the enzyme pyruvate orthophosphate dikinase (PPDK). This process called

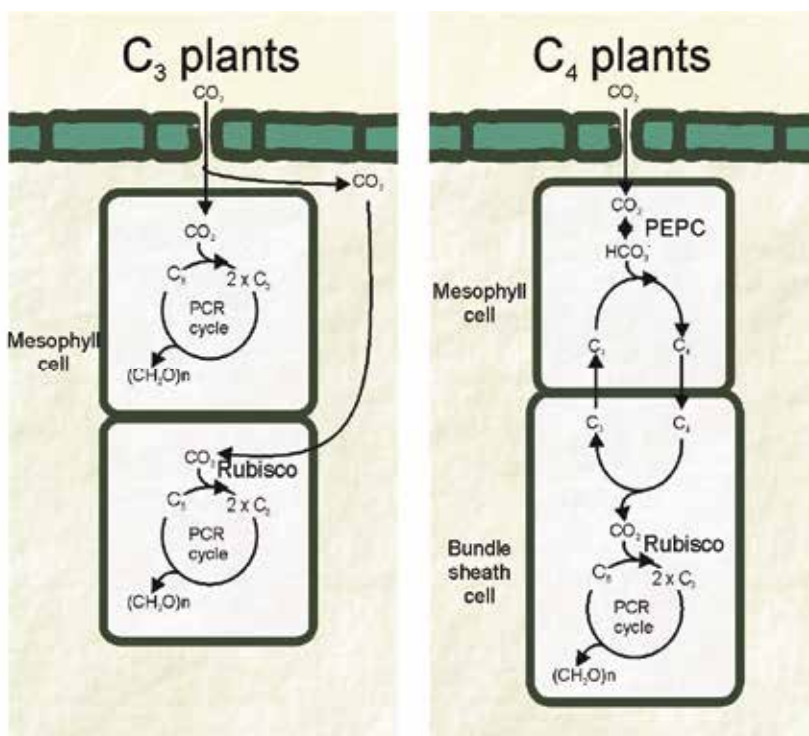


Fig. 1. Simplified scheme of carbon fixation pathways operating in  $C_3$  and  $C_4$  plants. Abbreviations:  $C_3$ , three-carbon organic acids;  $C_4$ , four-carbon organic acids;  $C_5$ , ribulose-1,5-bisphosphate; PCR, Photosynthetic Carbon Reduction Cycle; PEPC, phosphoenolpyruvate carboxylase; Rubisco, Ribulose-1,5-bisphosphate carboxylase/oxygenase.

*Hatch-Slack pathway*, after the first scientists that postulated the metabolic pathway. However, they used the name *C<sub>4</sub> dicarboxylic acid pathway of photosynthesis*. Due to current use, the name has been shortened to *C<sub>4</sub> photosynthesis*, *C<sub>4</sub> pathway*, *C<sub>4</sub> syndrome* or *C<sub>4</sub> metabolism*. The plants that perform this type of photosynthesis are then called *C<sub>4</sub> plants*.

This general scheme is common among the C<sub>4</sub> species; however, there are variations to this basic pathway that include diverse decarboxylation enzymes as well as different transported metabolites. Thus, the decarboxylation process occurs in three diverse ways, mainly using one of the following enzymes: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) or phosphoenolpyruvate carboxykinase (PEP-CK). Therefore, C<sub>4</sub> plants have been traditionally grouped into three biochemical subtypes depending on the major decarboxylase used (C<sub>4</sub>-NADP-ME subtype; C<sub>4</sub>-NAD-ME subtype or C<sub>4</sub>-PEP-CK subtype). Each C<sub>4</sub> subgroup possesses particular structural features, biochemistry and physiology, and also differences in the mechanism used to regenerate phosphoenolpyruvate (PEP), the substrate of PEP-carboxylase in mesophyll cells. Nevertheless, it is now becoming apparent that, in several cases, more than one decarboxylase operates at the same time (Drincovich et al., 2011).

### 1.2 C<sub>3</sub> vs C<sub>4</sub> species

C<sub>4</sub> species have evolved in a high CO<sub>2</sub> environment. This increases both their nitrogen and water use efficiency compared to C<sub>3</sub> species. C<sub>4</sub> plants have greater rates of CO<sub>2</sub> assimilation than C<sub>3</sub> species for a given leaf nitrogen when both parameters are expressed either on a mass or an area basis (Ghannoum et al., 2011). Although the range in leaf nitrogen content per unit areas is less in C<sub>4</sub> compared to C<sub>3</sub> plants, the range in leaf nitrogen concentration per unit dry mass is similar for both C<sub>4</sub> and C<sub>3</sub> species. Even though leaf nitrogen is invested into photosynthetic components into the same fraction in both C<sub>3</sub> and C<sub>4</sub> species, C<sub>4</sub> plants allocate less nitrogen to Rubisco protein and more to other soluble protein and thylakoids components. In C<sub>3</sub> plants, the photosynthetic enzyme Rubisco accounts for up to 30% of the leaf nitrogen content (Lawlor et al., 1989), but accounts for only 4–21% of leaf nitrogen in C<sub>4</sub> species (Evans & von Caemmerer, 2000; Sage et al., 1987). The lower nitrogen requirement of C<sub>4</sub> plants results from their CO<sub>2</sub>-concentrating mechanism, which raises the bundle sheath CO<sub>2</sub> concentration, saturating Rubisco in normal air and almost eliminating photorespiration. Without this mechanism, Rubisco in the C<sub>3</sub> photosynthetic pathway operates at only 25% of its capacity (Sage et al., 1987) and loses ca. 25% of fixed carbon to photorespiration (Ludwig & Calvin, 1971). To attain comparable photosynthetic rates to those in C<sub>4</sub> plants, C<sub>3</sub> leaves must therefore invest more heavily in Rubisco and have a greater nitrogen requirement. Because the Rubisco specificity for CO<sub>2</sub> decreases with increasing temperature (Long, 1991), this difference between the C<sub>3</sub> and C<sub>4</sub> photosynthetic nitrogen-use efficiency is greatest at high temperatures (Long, 1999). The high photosynthetic nitrogen-use efficiency of C<sub>4</sub> plants is partially offset by the nitrogen-requirement for CO<sub>2</sub>-concentrating mechanism enzymes, but the high maximum catalytic rate of PEP-carboxylase means that these account for only ca. 5% of leaf nitrogen (Long, 1999). Improved leaf and plant water use efficiency in C<sub>4</sub> plants is due to both higher photosynthetic rates per unit leaf area and lower stomatal conductance, with the greater CO<sub>2</sub> assimilation contributing to a major extent (Ghannoum et al., 2011).

The advantages of greater nitrogen use efficiency and water use efficiency of C<sub>4</sub> relative to C<sub>3</sub> photosynthesis are fully realized at high light and temperature, where oxygenase reaction of Rubisco is greatly increased. It is worth noting, although in C<sub>4</sub> plants energy loss

due to photorespiration is eliminated, and additional energy is required to operate the  $C_4$  cycle (2 ATPs per  $CO_2$  assimilated). In dim light, when photosynthesis is linearly dependent on the radiative flux, the rate of  $CO_2$  assimilation depends entirely on the energy requirements of carbon assimilation (Long, 1999). The additional ATP required for assimilation of one  $CO_2$  in  $C_4$  photosynthesis, compared with  $C_3$  photosynthesis, increases the energy requirement in  $C_4$  plants (Hatch, 1987). However, when the temperature of a  $C_3$  leaf exceeds ca. 25 °C, the amount of light energy diverted into photorespiratory metabolism in  $C_3$  photosynthesis exceeds the additional energy required for  $CO_2$  assimilation in  $C_4$  photosynthesis (Hatch, 1992; Long, 1999). This is the reason why at temperatures below ca. 25–28 °C,  $C_4$  photosynthesis is less efficient than  $C_3$  photosynthesis under light-limiting conditions. It is interesting to note, that while global distribution of  $C_4$  grasses is positively correlated with growing season temperature, the geographic distribution of the different  $C_4$  subtypes is strongly correlated with rainfall (Ghannoum et al., 2011).

On the contrary,  $C_4$  plants are rare to absent in cold environments. Although there are examples of plants with  $C_4$  metabolisms that show cold adaptation, they still require warm periods during the day in order to exist in cold habitats (Sage et al., 2011). In consequence,  $C_4$  species are poorly competitive against  $C_3$  plants in cold climates (Sage & McKown, 2006; Sage & Pearce, 2000). The mechanisms explaining the lower performance of  $C_4$  plants under cold conditions have not been clarified (Sage et al., 2011). Among early plausible explanations were the low quantum yield of the  $C_4$  relative to the  $C_3$  pathway (Ehleringer et al., 1997), and enzyme lability in the  $C_4$  cycle, most notably around PEP metabolism (PEP-carboxylase and pyruvate orthophosphate dikinase) (Matsuba et al., 1997). Both hypothesis are insufficient since maximum quantum yield differences do not relate to conditions under which the vast majority of daily carbon is assimilated and there cold-adapted  $C_4$  species that have cold stabled forms of PEP-carboxylase and pyruvate orthophosphate dikinase, and synthesize sufficient quantity to overcome any short term limitation (Du et al., 1999; Hamel & Simon, 2000; Sage et al., 2011). The current hypothesis is that  $C_4$  photosynthesis is limited by Rubisco capacity at low temperatures. Even in cold-tolerant  $C_4$  species, Rubisco capacity becomes limiting at low temperature and imposes a ceiling on photosynthetic rate below 20 °C (Kubien et al., 2003; Pittermann & Sage, 2000; Sage, 2002).

## 2. Climate change

According to the Intergovernmental Panel on Climate Change (IPCC), the current atmospheric  $CO_2$  level of 384  $\mu\text{mol l}^{-1}$  (800 Gt) is predicted to rise to 1000 Gt by the year 2050. Only this time humans are the drivers of these changes and not glacial-interglacial cycles. Human-caused increases in atmospheric  $CO_2$  concentration are thought to be largely responsible for recent increases in global mean surface temperatures and are projected to increase by 1.4 to over 5 °C by 2100 (Intergovernmental Panel on Climate Change, 2001, 2007). Increase in global average temperatures would further result in drastic shifts in the annual precipitation with a 20% reduction per year, and about 20% loss in soil moisture (Schiermeier, 2008). Regarding plants, higher atmospheric  $CO_2$  levels tend to reduce stomatal conductance and transpiration, thereby lowering latent heat loss and causing higher leaf temperatures (Bernacchi et al., 2007). Thus, in the future, plants will likely experience increases in acute heat and drought stress, which can impact ecosystem productivity (Cias et al., 2005) and biodiversity (Thomas et al., 2004). The sensitivity of photosynthesis to each of the environmental variables including high temperature, low

water availability, vapor pressure deficit and soil salinity, associated with the inevitable rise in atmospheric CO<sub>2</sub>, has not been well documented in assessing plant responses to the new changing environment (Reddy et al., 2010). How plant growth responds to the rising CO<sub>2</sub> concentration will not only affect ecosystem productivity in the future, but also the magnitude of C sequestration by plants and, consequently, the rate of CO<sub>2</sub> increase in the atmosphere. C<sub>4</sub> plants are directly affected by all major global change parameters, often in a manner that is distinct from that of C<sub>3</sub> plants. In the present chapter, we will focus on the effect of increased CO<sub>2</sub> and its relation to temperature and drought, on C<sub>4</sub> plants. Understanding how plants have and will respond to the rapid change in CO<sub>2</sub> concentration, together with developing knowledge about their capacity to adapt, is an essential initial step in understanding the full impact that the multiple interacting factors of global change (e.g. drought, temperature, ozone) will have on terrestrial ecosystems. These ecosystems produce services upon which we are dependent for food, fuel, fiber, clean air, and fresh water (Leakey et al., 2009).

### 3. The CO<sub>2</sub> response

In theory, increases in atmospheric levels of CO<sub>2</sub> above current levels can increase photosynthesis by decreasing photorespiration (fixation of O<sub>2</sub> rather than CO<sub>2</sub> by Rubisco), which increases with temperature and is higher in C<sub>3</sub> than C<sub>4</sub> and crassulacean acid metabolism (CAM) plants (Sage & Monson, 1999). In addition, rising CO<sub>2</sub> generally stimulates C<sub>3</sub> photosynthesis more than C<sub>4</sub>. Doubling of the current ambient CO<sub>2</sub> concentration stimulated the growth of C<sub>4</sub> plants to the tune of 10–20% whereas that in C<sub>3</sub> plants was about 40–45% (Ghannoum et al., 2000).

C<sub>3</sub> photosynthesis is known to operate at less than optimal CO<sub>2</sub> levels and can show dramatic increase in carbon assimilation, growth and yields. As Rubisco is substrate-limited by the current atmospheric CO<sub>2</sub> levels, this enzyme has the potential to respond to increases in CO<sub>2</sub> concentration; and have a metabolic control to alter the CO<sub>2</sub> flux during carbon assimilation (Bernacchi et al., 2003; Long et al., 2004). On the contrary, photosynthetic carbon assimilation in the C<sub>4</sub> species is saturated or almost CO<sub>2</sub>-saturated at a low ambient pCO<sub>2</sub>. The reason is that PEP-carboxylase utilizes HCO<sub>3</sub><sup>-</sup> as substrate rather than CO<sub>2</sub>; in consequence, the enzyme is insensitive to changes in the ratio of CO<sub>2</sub>: O<sub>2</sub> due to lack of binding of O<sub>2</sub> to the catalytic site of PEP-carboxylase. Therefore, if plants were grown under elevated CO<sub>2</sub>, carbon fixation would be little affected. This assumption that the inherent CO<sub>2</sub> concentrating mechanism in C<sub>4</sub> plants renders these plants insensitive to elevated CO<sub>2</sub> atmosphere is reflected in the lack of interest that it has been attributed to the study of the C<sub>4</sub> plants response to elevated CO<sub>2</sub> levels. To show this, Reddy et al. (2010) performed an exhaustive fifteen year- literature survey on the influence of elevated CO<sub>2</sub> among certain C<sub>3</sub>, C<sub>4</sub> and CAM species. The authors provided information for forty C<sub>3</sub> plants and for only two C<sub>4</sub> species and three CAM plants. Most of the C<sub>3</sub> plants presented a significant positive response to photosynthetic acclimation, *Sorghum* and *Panicum* (C<sub>4</sub> plants) exhibited negative response, whereas *Ananas*, *Agave* and *Kalanchoe* (CAM plants) showed positive responses to increased CO<sub>2</sub> concentration during growth. In view of this survey, it is then evident, that responses to elevated CO<sub>2</sub> have been little investigated in C<sub>4</sub> species. Moreover, conflicting reports on plant responses to elevated CO<sub>2</sub>, and several such differential photosynthetic responses, could be attributed to differences in experimental technologies, plant species used for the experiments, age of the plant as well as duration of the treatment (Sage, 2002).

Nevertheless,  $C_4$  species still exhibit positive responses (Fig. 2), particularly at elevated temperature and arid conditions where they are currently common and under nutrient-limited situations as well (Ghannoum et al., 2000; Sage & Kubien, 2003). High  $CO_2$  aggravates nitrogen limitations and in doing so may favor  $C_4$  species, which have greater photosynthetic nitrogen use efficiency (Sage & Kubien, 2003). On the other hand, elevated  $CO_2$  can also increase water use efficiency, in part by decreasing stomatal conductance and transpiration (Ainsworth et al., 2002). The irradiance is also a paramount factor; enhanced photosynthesis under elevated  $CO_2$  conditions was observed in  $C_4$  plants grown under high irradiance, while there was not much response when grown under low irradiance (Ghannoum et al., 2000).

Differences in the conductance of the bundle sheath cells to  $CO_2$  (varying with the decarboxylating subtype and also associated with changes in the ratio of Rubisco:PEP-carboxylase activity) were proposed to be responsible for different rates of  $CO_2$  leakage (Brown & Byrd, 1993; Ehleringer & Pearcy, 1983; Hattersley, 1982; Saliendra et al., 1996). Nevertheless, further studies showed that the stimulation of leaf photosynthesis at elevated  $CO_2$  was not associated with  $CO_2$  leak rates from the bundle sheath or with changes in the ratio of activities of PEP-carboxylase to Rubisco (Ziska et al., 1999).

Another aspect of plant metabolism which may vary under exposure to increased  $CO_2$  is the respiration. As highlighted by Reddy and colleagues (2010) in  $C_4$  plants little is known about the impact of elevated  $CO_2$  on the respiratory rates, which are reduced in  $C_3$  species and thus, probably contributing to increase biomass yield.

Neither  $C_3$  nor  $C_4$  species show acclimation responses that are directly linked to  $CO_2$  level. Instead, the  $CO_2$  effect on the photosynthetic biochemistry is largely mediated by carbohydrate accumulation in leaves under conditions where carbon sinks in the plant are also experiencing high carbon supply (Sage & McKown, 2006). The effectiveness with which increases in  $CO_2$  can be translated into growth benefits is depending in the sink-source balance and is affected by various plant and environmental factors. Depending on the growing conditions, these changes may or not conduct to increases in leaf area (Ghannoum et al., 2001; Leakey et al., 2006; Morison & Lawlor, 1999). For plants grown under optimal growth conditions and elevated  $CO_2$ , photosynthetic rates can be more than 50% higher than for plants grown under normal  $CO_2$  concentrations. This reduces to 40% higher for plants grown under the average of optimal and suboptimal conditions, and over the course of a full day, average photosynthetic enhancements under elevated  $CO_2$  are estimated to be about 30%. The 30% enhancement in photosynthesis is reported to increase relative growth rate by only about 10%. This discrepancy is probably due to enhanced carbohydrate availability exceeding many plants' ability to fully utilize it due to nutrient or inherent internal growth limitations. Consequently, growth responses to elevated  $CO_2$  increase with a plant's sink capacity and nutrient status (Kirschbaum, 2010).

### **3.1 Responses to increased $CO_2$ levels are dependent on other environmental factors**

#### **3.1.1 Increased $CO_2$ and drought**

Global circulation models have predicted that, together with increases in the  $CO_2$  concentration, in the future some regions will have increases in the frequency and severity of droughts.

Leaky et al. (2009) proposed that the potential for increased growth and yield of  $C_4$  plants at elevated  $CO_2$  concentrations relays on the decrease in water use and reduction of drought stress, and not by a direct effect of increased photosynthesis. In this respect, some  $C_4$  plants



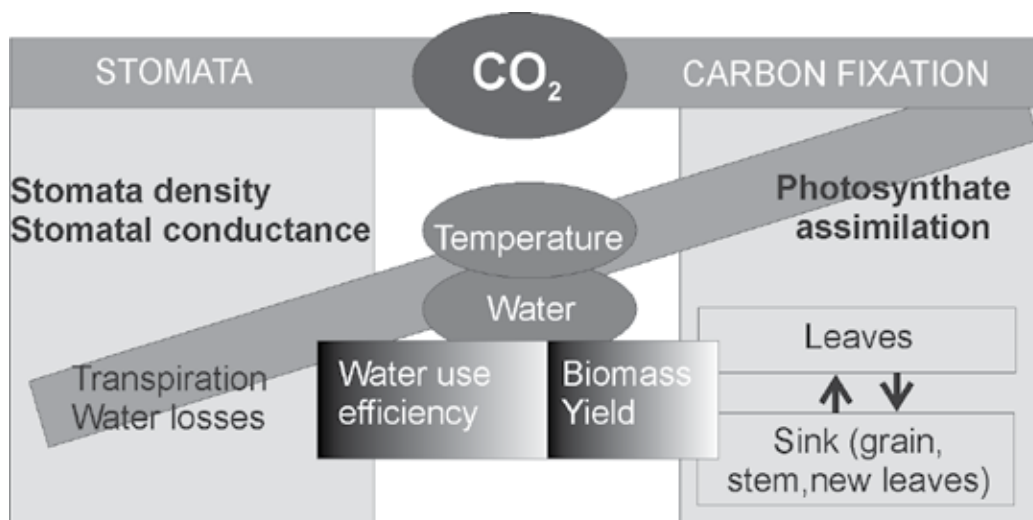


Fig. 2. Summary of the main factors involved in the response of plants to elevated CO<sub>2</sub>

grown under Free-Air Carbon dioxide Enrichment (FACE) exhibited increased photosynthetic rates only during drought or under the conditions of atmospheric vapor pressure deficits (Cousins, et al., 2002; Leakey et al., 2009). Elevated CO<sub>2</sub> reduced midday stomatal conductance of FACE-grown sorghum by 32% with irrigation and by 37% under drought stress (Wall et al., 2001). The effect of elevated CO<sub>2</sub> concentration on whole plant water use was smaller, but still significant (Conley et al., 2001). It is worth mentioning, that this indirect mechanism of enhanced carbon uptake by elevated CO<sub>2</sub> concentration is not unique to C<sub>4</sub> plants. Decreased stomatal conductance at elevated concentration of CO<sub>2</sub> in a C<sub>3</sub> soybean canopy also led to a significant reduction in canopy evapo-transpiration (Bernacchi et al., 2007). Therefore, interactive effects of CO<sub>2</sub> and water availability may alter the relative performance of C<sub>3</sub> and C<sub>4</sub> species. As stated before, at current CO<sub>2</sub> levels, C<sub>4</sub> species (particularly dicots) generally require less water than C<sub>3</sub> because of the higher CO<sub>2</sub> uptakes rates and greater stomatal resistance to water loss (Ehleringer et al., 1997). Under conditions of drought and elevated CO<sub>2</sub>, based on comparative studies using model C<sub>3</sub> and C<sub>4</sub> plants, Ward et al. (1999) postulated that C<sub>3</sub> species would be more competitive than C<sub>4</sub> species as results of decreased water loss through transpirations and higher CO<sub>2</sub> rates that would decrease the relative advantage of C<sub>4</sub> plants under drought conditions.

### 3.1.2 Increased CO<sub>2</sub> and temperature

Global increases in temperature and CO<sub>2</sub> may have interactive effects on photosynthesis. On one hand, negative effects of heat stress on plants are well known, since photosynthesis is thought to be among the most thermosensitive aspects of plant function. Both the light (electron transport) and dark (Calvin cycle) reactions of photosynthesis have thermolabile components, especially photosystem II (PSII) in the light reactions (Berry & Björkman, 1980; Heckathorn et al., 1998, 2002; Santarius 1975; Weis & Berry, 1988) and Rubisco activase in the Calvin cycle (Crafts-Brandner & Salvucci, 2002). Therefore, limiting processes controlling photosynthesis at elevated temperature could be either declining capacity of electron transport to regenerate ribulose-1,5-bisphosphate, or reductions in the capacity of Rubisco activase to maintain Rubisco in an active configuration (Sage et al., 2008).

Since, studies examining the effects of elevated CO<sub>2</sub> and increased growth temperature (typically 3–5 °C) had yield positive (Faria et al., 1996, 1999; Ferris et al., 1998; Huxman et al., 1998; Taub et al., 2000), negative (Bassow et al., 1994; Roden & Ball, 1996), and no effects (Coleman et al., 1991) on photosynthetic and plant tolerance to acute heat stress. Again, growing conditions and type of carbon assimilation pathways are need to be discriminated. General effects of elevated CO<sub>2</sub> on photosynthetic heat tolerance were recently investigated in a comparative study including C<sub>3</sub> and C<sub>4</sub> species and they can be summarized as follows: (i) in C<sub>3</sub> species, elevated CO<sub>2</sub> typically increases heat tolerance of photosynthesis, except for plants grown at supra-optimal growing temperature, then elevated CO<sub>2</sub> may provide no benefit or even decrease photosynthesis; (ii) in C<sub>4</sub> species, elevated CO<sub>2</sub> frequently decreases photosynthetic thermotolerance, at near-optimal growing temperature as well as supra-optimal growing temperature (Wang et al. 2008; Hamilton et al., 2008). Although both C<sub>3</sub> and C<sub>4</sub> plants experience reductions of similar magnitude in stomatal conductance with increasing CO<sub>2</sub> (e.g., 20%–50% with a doubling of CO<sub>2</sub>) (Sage, 1994; Reich et al., 2001; Wang et al., 2008), the lower stomatal conductance of C<sub>4</sub> plants at any given CO<sub>2</sub> level means lower average transpiration and higher leaf temperatures in C<sub>4</sub> plants, which may increase heat related damage in C<sub>4</sub> plants compared with C<sub>3</sub> plants in the same habitat. On the other hand, elevated CO<sub>2</sub> increases leaf size (Morison & Lawlor, 1999), and this should increase leaf temperatures during heat stress more in C<sub>3</sub> than C<sub>4</sub> species, given the greater average stimulation of growth in elevated CO<sub>2</sub> in C<sub>3</sub> species (Poorter & Navas, 2003).

### 3.1.3 Other considerations

Finally, to have a deeply understanding of the performance of C<sub>4</sub> plants under increased CO<sub>2</sub> conditions other factors besides water availability, soil nutrition and temperature, should be considered. One aspect to be included in the analysis should be pests and diseases.

Changes in the ratio of CO<sub>2</sub>/O<sub>2</sub> in the atmosphere affects plant metabolism in ways that ultimately influence the quality of leaves as a food resource for animals. To herbivores, the decreased leaf protein contents and increased carbon/nitrogen ratios common to all leaves under elevated atmospheric carbon dioxide imply a reduction in food quality. Stiling and Cornelissen (2007) analyzed plant-herbivore interactions using C<sub>3</sub> species and found that plants grown under elevated CO<sub>2</sub> usually had lower nutrient concentrations, which reduced the growth rate of herbivores feeding on that plant material. Contrasting C<sub>4</sub> and C<sub>3</sub> species, C<sub>4</sub> grasses are a less nutritious food resource than C<sub>3</sub> grasses, both in terms of reduced protein content and increased carbon/nitrogen ratios. The abundance of C<sub>3</sub> and C<sub>4</sub> plants (particularly grasses) are affected by atmospheric carbon dioxide. There is an indication that as C<sub>4</sub>-dominated ecosystems expanded 6–8 Ma b.p., there were significant species-level changes in mammalian grazers. Today there is evidence that mammalian herbivores differ in their preference for C<sub>3</sub> *versus* C<sub>4</sub> food resources, although the factors contributing to these patterns are not clear. Elevated carbon dioxide levels will likely alter food quality to grazers both in terms of fine-scale (protein content, carbon/nitrogen ratio) and coarse-scale (C<sub>3</sub> *versus* C<sub>4</sub>) changes (Ehleringer et al., 2002).

Regarding plant-plant interactions using C<sub>3</sub> species, Wang (2007) showed that the growth response of mixed-species communities to elevated CO<sub>2</sub> was less than the response of single-species populations. In addition, the relative importance of these and other factors should be established for C<sub>4</sub> species grown under elevated CO<sub>2</sub>.

#### 4. Conclusion

C<sub>4</sub> plants are directly affected by all major global change parameters, often in a manner that is distinct from that of C<sub>3</sub> plants. Although an ongoing effort has been dedicated to the study of the response of C<sub>4</sub> plants to CO<sub>2</sub> enrichment, the literature regarding the response of C<sub>4</sub> plants is still under-represented when comparing to that of C<sub>3</sub> species. An understanding of C<sub>4</sub> plants responses to ambient variables such as temperature, CO<sub>2</sub>, nutrients and water is essential for predictions of how agricultural and wild C<sub>4</sub> populations will respond to climate variations such as those predicted to occur with global climate change (Intergovernmental Panel on Climate Change, IPCC, 2001).

#### 5. Acknowledgment

This work was funded by a grant from Agencia Nacional de Promoción Científica y Tecnológica (PICT N° 2008-2164) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP N°0679). CSA and MVL and are members of the Researcher Career of CONICET.

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*Edited by Arun Shanker and B. Venkateswarlu*

World population is growing at an alarming rate and is anticipated to reach about six billion by the end of year 2050. On the other hand, agricultural productivity is not increasing at a required rate to keep up with the food demand. The reasons for this are water shortages, depleting soil fertility and mainly various abiotic stresses. The fast pace at which developments and novel findings that are recently taking place in the cutting edge areas of molecular biology and basic genetics, have reinforced and augmented the efficiency of science outputs in dealing with plant abiotic stresses.

In depth understanding of the stresses and their effects on plants is of paramount importance to evolve effective strategies to counter them. This book is broadly divided into sections on the stresses, their mechanisms and tolerance, genetics and adaptation, and focuses on the mechanistic aspects in addition to touching some adaptation features.

The chief objective of the book hence is to deliver state of the art information for comprehending the nature of abiotic stress in plants. We attempted here to present a judicious mixture of outlooks in order to interest workers in all areas of plant sciences.

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