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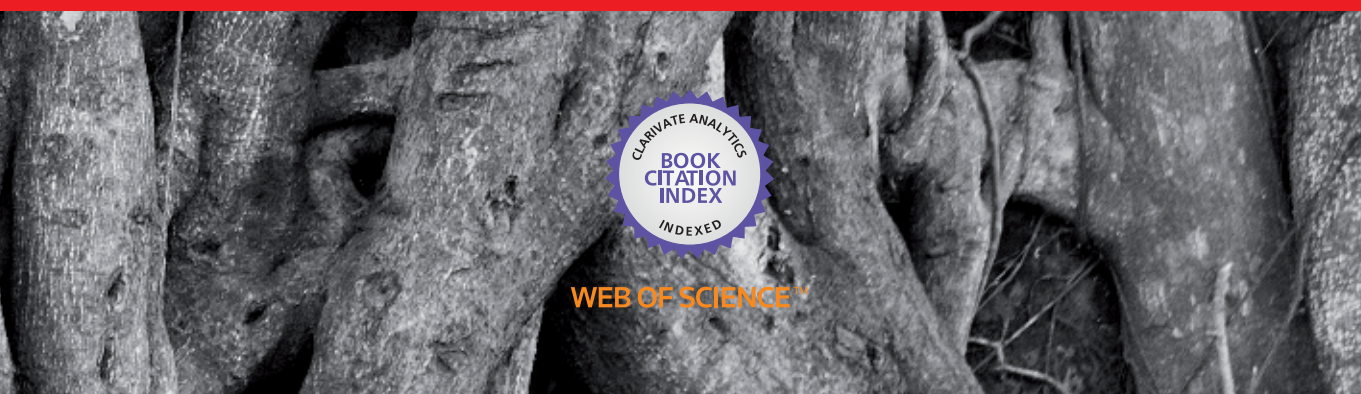
Abiotic Stress

Plant Responses and Applications in Agriculture

Edited by Kourosh Vahdati and Charles Leslie



ABIOTIC STRESS - PLANT RESPONSES AND APPLICATIONS IN AGRICULTURE

Edited by **Kourosh Vahdati**
and **Charles Leslie**

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Notice

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Meet the editors

Kourosh Vahdati received his Ph.D. in Horticultural Science, Fruit Breeding and Biotechnology, from the University of Tehran in 2002 and is now a professor at the University of Tehran specializing in walnut research, particularly genetic transformation and rootstock breeding for resistance to abiotic stresses. He was a visiting scholar at the University of California-Davis in 2000-2001 and returned in 2010-2011 as associate visiting professor and worked on tissue culture and genetic transformation of walnut. Dr. Vahdati teaches pomology, breeding and biotechnology of fruit and nut trees, supervises graduate students, has published more than 50 research articles and book chapters, and has served on the editorial boards of several journals. He also serves as Chairman of the ISHS Walnut Working Group and of the Iranian Center of Excellence for Walnut Improvement and Technology, and has made numerous contributions worldwide to the fields of walnut breeding and biotechnology.

Charles Leslie earned his B.S. in Zoology from Oregon State University in 1973 and his M.S. degree in Plant Physiology from the University of California – Davis in 1985. He worked in private sector forestry research as a wildlife biologist, entomologist and tissue culture specialist, has 25 years of experience at UC Davis in tissue culture, genetic engineering, and walnut breeding, and is current Director of the UC-Davis Walnut improvement Program. His research includes application of genomics to breeding walnut scion cultivars, enhancing efficiency of in vitro walnut propagation and clonal rootstock production, and developing hybrid walnut rootstocks with resistance to soil-borne disease, pathogens and abiotic stresses. He has published more than 60 journal articles and book chapters on these topics.

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Preface

Abiotic stresses are serious threats to agriculture and the environment which have been exacerbated in the current century by global warming and industrialization. According to FAO statistics, more than 800 million hectares of land throughout the world are currently salt-affected, including both saline and sodic soils equating to more than 6% of the world's total land area. Continuing salinization of arable land is expected to have overwhelming global impact, resulting in a 30% loss of agricultural land over the next 25 years and up to 50% loss by 2050. Overall, it has been estimated that the world is losing at least 3 ha of arable land every minute due to soil salinity. Some of the most serious effects of abiotic stresses occur in the arid and semiarid regions where low rainfall, high evaporation, native rocks, saline irrigation water, and poor water management all contribute in agricultural areas.

As stated in one of the chapters of this book, Kofi Annan has proposed a "Blue Revolution in Agriculture" as we enter the current millennium, an international initiative focusing on increasing our productivity per unit of water in order to achieve "More crop per drop". Efforts to improve the efficiency of agricultural water use while simultaneously reducing adverse environmental impacts will need to draw on results of extensive and diverse research in several areas. Over the last few decades there has been tremendous progress in understanding the molecular, biochemical, and physiological basis of stress tolerance in plants. As we move forward, emerging information and novel approaches must continuously be applied in a timely and effective manner by both the research and applied agricultural communities. One promising approach to improving the ability of plants to cope with abiotic stress is to combine utilization of the vast biodiversity of crop plants and their wild relatives with the rapidly emerging genetic and molecular techniques. Global programs, such as the Global Partnership Initiative for Plant Breeding Capacity Building (GIPB), aim to select and distribute seed crops and cultivars with tolerance to abiotic stresses in order to facilitate sustainable use of plant genetic resources for food and agriculture.

Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes in plants that adversely affect growth and productivity. A frequent result is protein dysfunction. Understanding the mechanisms of protein folding stability and how this knowledge can be utilized is one of the most challenging strategies for aiding organisms undergoing stress conditions. Stresses also affect the biosynthesis, concentration, transport, and storage of primary and secondary metabolites. As a more comprehensive view of these processes evolves, applications to reducing plant stress are emerging.

While much has been achieved in recent years in developing plants genetically engineered for resistance to herbicides, pests and diseases, production of plants engineered for tolerance to abiotic stress has not progressed as rapidly and applications in canola, rice and maize, for

example, have only recently begun to be commercialized. This is due largely to the more complex genetic mechanisms involved in tolerance to abiotic stresses. Additionally, under natural conditions plants can suffer from various stress combinations at different development stages and during different time periods. Many of the gene products differentially expressed under stress, such as dehydrins, enzymes for the synthesis of osmolytes, and enzymes for the removal of reactive oxygen species (ROS), protect plant cells from damage. The production of these functional proteins is widely regulated by specific transcription factors. Use of transcription factors is now under development as an additional biotechnological approach to improving plant response to abiotic stresses.

This book is not intended to cover all known abiotic stresses or every possible technique used to understand plant tolerance but instead to describe some of the widely used approaches to addressing such major abiotic stresses as drought, salinity, extreme temperature, cold, light, calcareous soils, excessive irradiation, ozone, ultraviolet radiation, and flooding, and to describe major or newly emerging techniques employed in understanding and improving plant tolerance. Among the strategies for plant stress survival, deep rooting, programmed cell death and accumulation of compatible osmolytes are presented in detail and comprehensive case studies of progress and directions in several agricultural crops such as apple, walnut, grape and wheat are included.

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Section 1

Mechanisms of Response and Adaptation

Chapter 1

Abiotic Stress Adaptation: Protein Folding Stability and Dynamics

Martina Ortbauer

Additional information is available at the end of the chapter

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1. Introduction

Abiotic stress is best defined as any factor exerted by the environment on the optimal functioning of an organism. Abiotic stresses like heat, cold, freezing, drought salinity, flooding or oxidizing agents usually cause protein dysfunction [1]. Protein folding stability is undoubtedly one of the most challenging problems in organisms undergoing stress conditions. Efficient protein repair systems and general protein stability facilitate survival upon sudden changes in the environment. As sessile organisms plants need to adopt quickly to overcome various environmental stresses during their lifespan. Recently, most emphasis is being directed towards an understanding of how plants recognize external conditions and initiate protective reactions such as mechanisms through which protein function is protected and maintained. Proteins are biological macromolecules involved in virtually every biological process in a living system. The roles played by proteins are varied and complex. Proteins are used for storage and transport of small molecules or ions and control the passage of molecules through the cell membranes essential for metabolic function [2]. Hormones, which transmit information and allow the regulation of complex cellular processes, are important regulators in responses to abiotic stress [3]. Enzymes act as catalysts and increase, with a remarkable specificity, the speed of chemical reactions essential to the organism's survival.

Protein function is dependent on its unique three-dimensional structure that is adopted by the initial folding of the polypeptide chains after translation. Encoded by DNA and synthesized on ribosomes as chains of hundreds of amino acids, each protein must find its characteristic and correct fold, rather than the countless alternatives, in order to function properly [4]. Folding into its native and active structure may involve one or more partially folded intermediate states (Figure 1). It is not surprising that stress induced alterations in the physiological conditions may change the folding process and give rise to protein misfolding and

aggregation [5]. Folded proteins are generally much less prone to aggregation and degradation but partially unfolded or intrinsically disordered regions of proteins can confer functional advantages, as they allow efficient interaction with binding partners and provide a mechanism for the regulation of cellular processes. Protein dynamics, meaning structural or conformational change with time, are an essential part of regulation of biological activity.

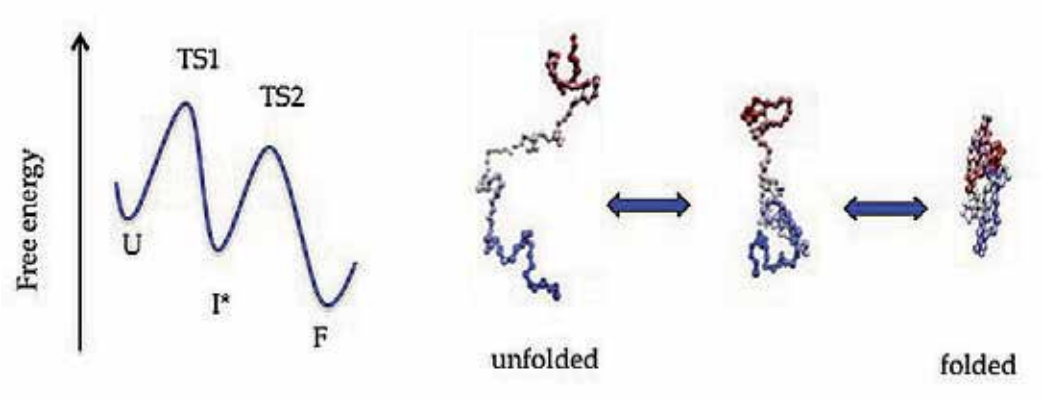

Figure 1. Protein folding involving a partially folded intermediate state. The two transition states (TS1, TS2) are separated by a metastable intermediate (I*), modified from [6, 7]. The driving force for protein folding is the search for lower free energy states, separated by free energy barriers. The free energy of a protein in solution is highly dependent on temperature, pressure and solvent conditions

Many cellular processes are coupled to protein folding and unfolding, a process that is highly sensitive to rapid changes in environmental conditions such as denaturant concentration, temperature or pH. In determining the conformational properties of proteins, it is therefore important to include solvent and co-solvent conditions.

Protein conformation and activity can differ markedly between diluted and crowded environments. The diverse and highly specific function of proteins is a consequence of their sophisticated, individual surface pattern regarding shape, charge and hydrophobicity that is a consequence of the three-dimensional structure of polypeptide chains. The stability of proteins results from a number of counteracting enthalpic and entropic contributions. Native states represent the most stable conformation under equilibrium. This does not necessarily mean that protein function is restricted to well-defined folded states. Internal dynamics play an important role in protein function. *In vivo* folding, catalytic function, transport and degradation of proteins all involve transitions between different conformations. Locally unfolded or disordered regions of a protein allow efficient interaction with binding partners and thus the regulation of cellular mechanisms. Identifying and defining the rules for protein folding and unfolding is fundamental for our understanding how living systems cope with abiotic stresses. Advanced experimental methods continue to be developed to elucidate the sheer complexity of protein folding and unfolding and the mechanisms of preserving functional folds under stress conditions.

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2. Protein folding and abiotic stress

A striking feature of protein folding is that the overall mechanism follows simple physical rules, but examination in finer detail reveals a much greater complexity [8]. The protein structure-function paradigm has been reassessed with the discovery of partially unfolded or intrinsically disordered proteins that are fully functional. These proteins are widely distributed in eukaryotes and fulfill crucial biological functions like transcriptional regulation, signal transduction [9], enzyme catalysis and protein ligand interactions. They contain native-like secondary structure elements but lack the tertiary interactions of folded proteins. One has to keep in mind that protein function is protected by stabilization of well-defined structural regions but is largely dependent on protein motion and dynamics. NMR dynamic experiments indicate that protein conformational exchange spans a variety of time scales ranging from picoseconds to milliseconds [10]. Complete description of protein function, that may involve motion, requires an understanding of the molecular dynamics [11]. Many proteins form partially folded intermediate states along their folding-pathway. To search for correlations between function, structure and dynamics, it is essential to include all states formed at equilibrium [12, 13] in order to characterize protein dynamics under unfavorable environmental conditions.

Protein conformations and interconversion between different states are largely modified by internal and external signals such as ligand binding, phosphorylation or cleavage, molecular recognition or environmental changes [14]. *In vivo*, protein folding occurs spontaneously, meaning that proteins permanently exchange between folded, partially folded, locally unfolded and unfolded states during the period from protein synthesis until their degradation. According to the energy landscape theory, the free energy barriers connecting these states are small [15], suggesting that minor perturbations *in vivo* can have significant effects on the populations of different states and hence protein function. Intermolecular forces that drive protein folding generally stabilize both folded and unfolded states, but an altered balance in these forces can result in aggregation or misfolding to non-functional proteins [16]. Protein unfolding, misfolding and aggregation are a common threat to the living cell, especially when undergoing abiotic stress. To cope with stress, plants have developed various mechanisms to facilitate protein folding and to suppress protein misfolding.

3. Stability versus flexibility - How to protect protein function?

Stabilizing proteins in their functional conformation is one of the great challenges in plant stress metabolism. Stress induced alterations in the structural and energy landscape of proteins affect and may inhibit both protein-ligand and protein-protein interactions. Small molecules typically bind proteins in small cavities, whereas proteins recognize large surface areas [17]. Thus, protein function is a balancing act between structural stability and the conformational flexibility needed for protein function. Protein stability results from stabilizing and destabilizing interactions of the polypeptide chains that slightly favor the folded state as compared to partially folded or unfolded states under physiological conditions (Figure 2).

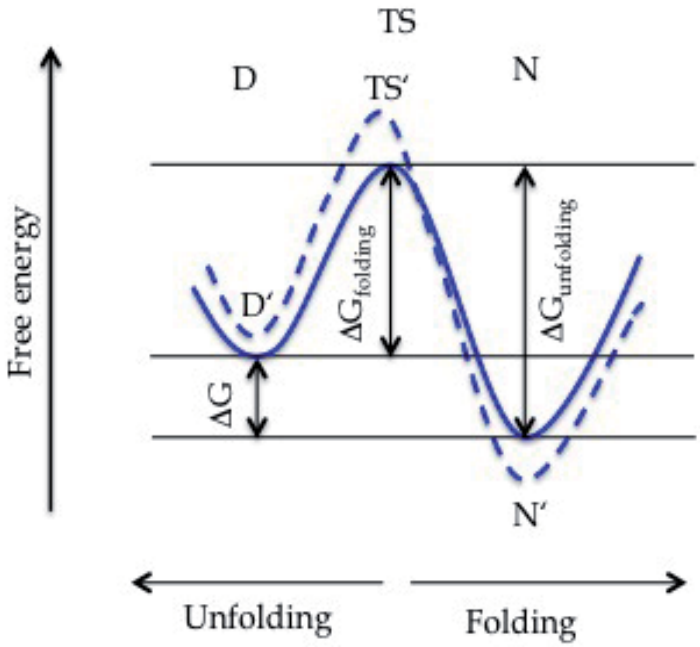

Figure 2. Free energy model for protein folding and unfolding. Stabilization of the native state can be achieved by destabilization of the denatured state (D') or a more stabilized native state (N'). The free energy barrier (TS') may also be affected

The difference in the free energy (ΔG) of different states as measured from the reversible transition from the native to the denatured state is small [18]. Externally forced conformational changes in the protein structure lead to a substantial decrease in its stability. The denaturation process causes conformational destabilization by exposing hydrophobic residues to the solvent, normally deeply buried in the interior of a folded protein. The burial of non polar surfaces and the hydrophobic force is considered as the main driving force for protein folding and stability [19] as proteins become thermally more stable upon decreasing hydration levels [20].

Evidence from proteins produced by hyperthermophil microorganisms, which are very thermostable and resistant to chemical denaturation, indicates that this resistance comes from lower protein flexibility and higher protein rigidity [21]. Thermostable proteins tend to be very rigid at mesophilic temperatures (10-45^{0}C), but allow for greater flexibility at high temperatures, which is essential for their function in their thermophilic environment. It is assumed that intrinsic stability due to increased protein rigidity is important for thermal stabilization, since thermal motion decreases rigidity and enhances flexibility.

3.1. Assisted folding under stress conditions

Molecular responses to abiotic stress are complex and highly dependent on the level and duration of stress and on the tissue and organ that is affected. Sensing of environmental

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changes and transduction of stress signals triggers activation of molecular response mechanisms [22]. A general response to environmental stress conditions is the onset of stress proteins that facilitate protein folding and protect proteins from misfolding and aggregation. The targets for these so-called chaperons (heat shock proteins HSP, late embryogenesis abundant LEA proteins) are partially unfolded or misfolded proteins with stretches of hydrophobic residues that are normally buried in the interior of the protein fold now exposed to the surface. Since aggregates of misfolded proteins can be very stable and the energy barriers towards the folded state can be of higher energy, chaperons assist the folding process by helping to overcome the energy barriers and to refold proteins from aggregates [23].

Transcription of many genes is up regulated under stress conditions. Among these genes, several code for stress-induced proteins that act to improve water movement through membranes (water channel proteins), detoxification enzymes or enzymes required for osmolyte biosynthesis [24]. Studies on plants reported that one of the initial responses to water deficit is the induction of osmolyte (Figure 3) production. Changes in protein expression levels are required to regulate osmolyte transport and distribution throughout the plant. The accumulation of low-molecular weight osmolytes (compatible solutes) is well known to protect macromolecular structure from stress-induced damage. Increased intracellular osmolyte concentrations on the other hand may affect protein structure and dynamics. Solvent and (co-)solvent conditions and protein solvent accessibility is of particular importance during stress periods because it influences ionic strength, pH values and affinity to certain molecular groups on the protein surface.

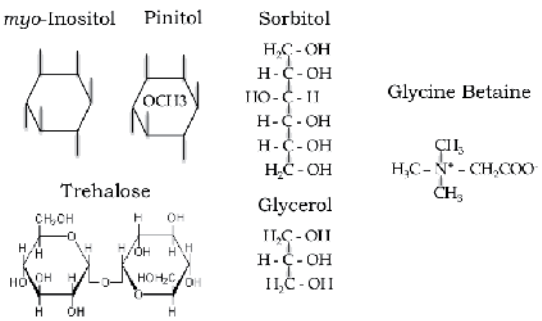

Figure 3. Examples of organic co-solvents (osmolytes): uncharged sugars, polyols and betaines

Accumulated osmolytes within the cells change the interaction of proteins with the solvent [25] by increasing (kosmotropic) or decreasing (chaotropic) the order of water. Kosmotropic, or so-called compensatory co-solvents are well-hydrated molecules with little tendency to aggregate, have no net charge, and strongly hydrogen-bond with water. They are preferentially solubilized within the bulk of water and preferentially excluded from the protein surface, which leads to a decrease in the water diffusion around the protein [26, 27]. Although molecules do not seem to directly interact with the protein surface, they modify protein stability by altering solvent properties. According to the "water structure hypothesis" chaotrop-

ic co-solvents increase the fraction of more dense species in the hydration water thereby destabilizing protein structure [28]. Molecules that stabilize the surface low-density water and increase the surface tension will stabilize the protein structure (kosmotropes).

Co-solvent effects that alter the water structure are not the sole driving force for increasing or decreasing protein stability. It also has to be considered that the interaction of a co-solvent with the protein surface may not be favorable and thus would destabilize a protein. Due to the fact that unfolded or denatured states comprise a higher solvent and co-solvent accessible surface area, the equilibrium tends to shift to the more compact folded state known as the "excluded volume effect". Among all the interactions that may stabilize or destabilize proteins, a main driving force for protein folding is "hydrophobic interactions". Hydrophobic forces will also be affected in the presence of co-solvents, partly depending on the ability of a solute to be excluded or incorporated in the hydration shell of a certain protein [29].

Increases in temperature, pressure or osmotic stress alter the properties of protein conformation and the hydration state. The free energy change resulting from folding or unfolding depends on the combined effects of the exposure of the interior and non-polar groups and their interaction with water, including changes in water-water interactions.

3.2. Dynamics in enzymatic activity

The ability to maintain protein performance under abiotic stress depends on intrinsic stability, chaperon activity, protein turnover and extrinsic stabilization through co-solvents (compatible solutes). Molecular motion as well as protein flexibility and dynamics is highly linked to enzymatic activity, which is clearly dependent on the particular environment of a protein [30].

Hydration status and temperature are the main factors that contribute to the catalytic mechanism. Hydration is necessary for enzyme catalytic function since dry enzymes are less functional, and below a threshold hydration level enzymes are inactive. Protein hydration may also be necessary for diffusion of substrate and product [31]. Temperature is a fundamental environmental stress, as flexibility and functionality of enzymes are highly temperature dependent. Low temperatures result in decreased catalytically activity, which is metabolically not favorable. Increases in the thermal energy will increase enzyme molecules that have the required energy for conformational changes into catalytically active enzymes, showing an increased catalytic rate (k_{cat}). High temperatures, on the other hand, can cause the structure to become so loose that substrates and co-factors can no longer bind [32]. Extreme temperatures cause complete denaturation. Osmolyte (glycerol, sorbitol, xylitol, glucose, fructose, saccharose, proline, glycine betaine, *myo*-Inositol, pinitol, quercitol) protection of enzymes against heat-induced loss of activity has been extensively studied *in vitro* [33-35]. The particular properties of a protein and the nature of the added osmolyte strongly influence protein thermal stability and enzyme activity. The ability to protect enzymes from heat induced activity loss varies between different osmolytes but preserving enzymes under heat stress seems to be a general feature for these osmolytes. Loss of enzymatic activity under high temperature treatment does occur but is always slower and at higher temperatures when compared to proteins without protective additives. Enzymatic activity tests demonstrate the

function of osmolytes in preventing heat induced activity loss. To get further insights into folding stability and dynamics of proteins under stress conditions, more detailed analysis and extended methods are needed.

3.3. Global conformational stability of proteins under stress

Circular dichroism (CD) spectroscopy has been introduced as a quick and valuable technique for examining the structure and stability of proteins in solution. CD is used for determining whether a protein is folded and for characterizing its secondary structure (alpha-helices, beta-strands) and some aspects of the tertiary structure (aromatic amino acids, disulfide bonds). Conformational changes during the acquisition of the native structure are measured in the near-UV (250-350) and far-UV (190-250). This technique has been used widely to determine the folding stability of proteins dependent on temperature, pH and under denaturant conditions [36, 37]. CD is a convenient tool to characterize the interactions between co-solvents and proteins and to find co-solvent conditions that increase the melting temperature or fully refold proteins after thermal unfolding. If the melting is fully reversible, the melting temperature is directly related to conformational stability, and the thermodynamics of protein folding can be extracted from the data [38].

CD studies have been employed to investigate how osmolytes such as glycerol, trehalose and *myo*-Inositol affect the global folding of native proteins and its thermal unfolding process. CD signals arising from protein chromophors reflect an average of the protein population. The resulting spectrum is a sum of individual spectra arising from secondary structure elements present in the protein sample (Figure 4).

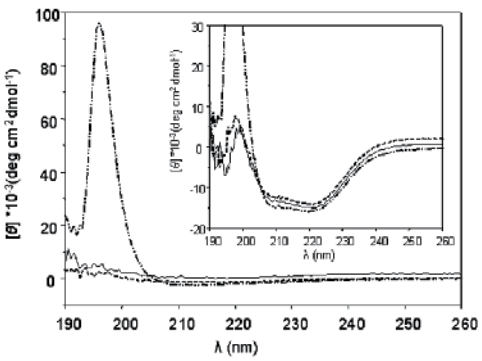

Figure 4. Circular dichroism spectra of Malate Dehydrogenase (insert) in 20mM NaP-buffer at pH 7.0. The far-UV spectrum recorded from 260 to 190nm at 20°C displays a typical α-helical protein with two negative maxima at 208 and 222nm. Addition of 0.4M glycerol (–), *myo*-Inositol (—) or trehalose (- -) did not change protein secondary structure and did not show self-absorbance in this spectral region [39]

Thermally induced protein unfolding was monitored in the far-UV region by gradually increasing the temperature in the protein sample. Thermal denaturation curves were monitored at a fixed wavelength of 222nm (Figure 5) and acquired data were fitted to a simple thermodynamic unfolding model. The melting temperature, T_m (midpoint transition temper-

ature) can be extracted from thermal denaturation curves, reflecting the global stability of the folded versus the unfolded protein. 0.4M glycerol, *myo*-Inositol and trehalose increased the melting temperature of malate dehydrogenase by 3 to 5°C as compared to proteins alone [39].

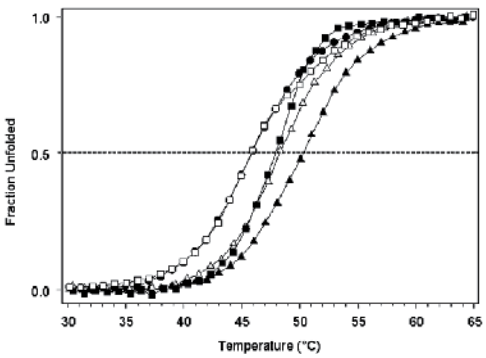

Figure 5. Thermally induced unfolding of Malate Dehydrogenase (•) in the presence of 0.4M glycerol (□), trehalose (▵), glucose (▪) and *myo*-Inositol (▴). The horizontal line indicates the midpoint transition temperature (T_m). Osmolytes and proteins were mixed to protein solution and equilibrated at room temperature prior to heating. Temperature profiles at 222nm were recorded for 1 °C increments in the temperature range 20–90 °C at a heating rate of 1 °C min^{-1} [39]. Thermal unfolding measurements were set up in quartz cuvettes, placed into a Peltier controlled sample holder unit connected to a temperature probe to provide an accurate temperature record. Thermal unfolding curves were analyzed using a sigmoidal curve function according to (Equation 1) [40]:

$$\theta_T = \left[\frac{(m_D \times T - b_D) - (m_N \times T - b_{N)}}{1 + (T / T_m)^{m_T}} + m_N \times T - b_N \right] \quad (1)$$

where θ_T is the ellipticity at temperature T, m_T is the slope of the curve within the transition region, and the inflection point of the curve the melting temperature T_m. At each temperature b_N and b_D can be extrapolated from the pre- and post-transition baselines, ($m_N \times T - b_N$) and ($m_D \times T - b_D$), respectively. The fraction of unfolded protein can be calculated by subtracting these baselines (Equation 2):

$$f_v = \frac{\theta_T - \theta_N}{\theta_v - \theta_N} = \frac{\theta_T - (m_N \times T - b_N)}{(m_D \times T - b_D) - (m_N \times T - b_N)} \quad (2)$$

The stabilization of protein global folds through naturally occurring osmolytes seems to be a general mechanism. Other studies also reported increases in the midpoint transition temperature (ΔT_m) of 2 to 18°C upon the addition of 0.1-2M glycerol, trehalose and sucrose measured on various proteins [41-43]. Additionally, all proteins studied in the presence of osmolytes showed a remarkably retention of secondary structure at T_m relative to proteins

alone. Retention of secondary structure in osmolyte solution was monitored even at temperatures where proteins were fully unfolded when heated without additives.

Studies on RnaseA previously showed that increases in ΔH_m by the addition of trehalose resulted in a lower ΔCp-vlaue (heat capacity change). [41]. The heat capacity change, ΔCp, is a very sensitive thermodynamic parameter that correlates with the amount of the protein surface that is exposed to the solvent [44]. A decrease in ΔCp upon the addition of osmolytes reflects a lower surface exposed area and/or decreased exposure of hydrophobic groups to the solvent. Decreases in ΔCp may also result in flattening of thermal unfolding curves, leading to conformational stability over a wider range of temperature. This has shown to be an effective strategy for many mesophilic proteins.

The thermal stability of a protein is determined by the response to thermal energy, concerning globally and locally unfolding and the ability to refold into its active conformation. Thermal unfolding was shown to be highly reversible for thermostable proteins of hyperthermophilic organisms. The far-UV CD spectrum of the native protein was identical to that after heat denaturing and re-cooling [45]. Many mesophilic proteins, however, aggregate or precipitate after thermal unfolding making the unfolding process irreversible. Finding co-solvent conditions that facilitate refolding is as important as increasing the melting temperature. Facilitated refolding was observed for ribonuclease that undergoes a reversible denaturation in the presence of trehalose [46].

Taken together, these results from CD measurements reveal that osmolytes stabilize protein global folds under heat by supporting retention of secondary structure elements and aid in refolding of thermally unfolded proteins.

4. New insights into molecular dynamics of protein folding and unfolding from Nuclear Magnetic Resonance (NMR) spectroscopy

Internal dynamics of proteins play an important role in their biological function. Proteins do not only exist in well-defined natively folded or fully unfolded states, but also in partially folded intermediate states. The conformational exchange between a folded state and partially folded states is highly sensitive to changes in the environment such as temperature, pH, solvent and co-solvent conditions. In the plant cell, proteins are predestinated to function in environments crowded by macromolecules, metabolites and other co-solvents that facilitate protein folding under non-stress and stress conditions [47]. By measuring protein dynamics, it is therefore important to include (co-)solvent conditions (Figure 6). High-osmolyte accumulation upon stress conditions induces changes in the protein environment. Variable protein folds may be affected slightly different according to their hydrophobic or hydrophilic surface properties, compactness, flexibility, hydrogen bonding patterns, excluded volume effects and the affinity of binding sites for co-solvents or the hydration water.

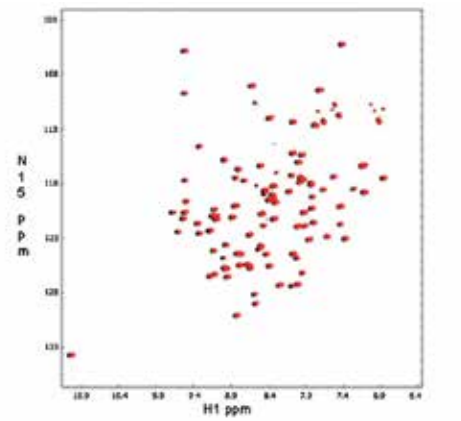

Figure 6. HSQC of the uniformly ^{15}N-labeled KID-binding (KIX) domain of CREB-binding protein (CBP), residues 586-672 (black). Overlaid is a spectrum of KIX in the presence of 0.4M *myo*-Inositol (red) showing the intactness of the three-dimensional protein folds upon the addition of an osmolyte. Spectra were acquired on a Varian Inova 800MHz spectrometer at 26.9°C

Nuclear Magnetic Resonance (NMR) spectroscopy has greatly contributed to understanding of protein folding by characterizing protein conformation at the level of individual amino acid residues. NMR techniques can be used to monitor temperature dependence of folding and unfolding in order to determine their thermodynamic properties, measure sensitivity to denaturants and address solvent and co-solvent accessibility. NMR experiments provide information at multiple sites within the protein, unlike spectroscopic techniques such as circular dichroism that provide nonspecific information about aromatic side chains and averaged properties of the polypeptide backbone. Heteronuclear NMR relaxation and relaxation dispersion experiments have emerged as powerful tool to study internal dynamics under a wide range of experimental conditions.

NMR relaxation experiments

Information about protein dynamics, extracted form heteronuclear NMR relaxation studies, is based on measurements of the longitudinal (T1) and transverse (T2) relaxation rate and the heteronuclear NOE, all sensitive for the motion of the N-H bond vector in the protein backbone [11]. Fast atomic motions on a picosecond to nanosecond (ps-ns) time scale are gained from the slower relaxation processes (R1, R2 and NOE) of nuclear spins, measured along the backbone and in the side chains using isotopically labeling (^{15}N).

Relaxation data (T1, T2, NOE) can be interpreted according to the "model free" formalism in terms of the internal motional correlation time and an order parameter (S_2) [48]. In the NMR experiment, order parameters (S^2) report on the refinement of the N-H bond vector. The value of S^2 varies from 0 (no motional restriction) to 1 (complete motional restriction) [49]. Backbone segments in highly flexible parts of the protein, not restricted in their motion, have low S^2 values, whereas rigid regions show typical high S^2 values. Main chain ^{15}N relaxation data can be analyzed to yield S^2 order parameters on a per residue basis (Figure 7).

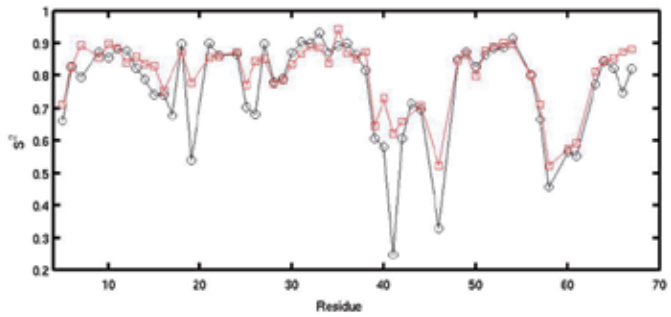

Figure 7. Comparison of N-H order parameters (S^2) of cold shock protein A (CspA) (red). Addition of 0.4M *myo*-Inositol (black) showed an overall increase in protein compactness by rigidification of former flexible parts of the protein (S^2=0 flexible, S^2=1 rigid)

Order parameter (S^2) for cold shock protein A (CspA) showed an overall increase in the presence of the model osmolyte *myo*-Inositol. Residues in very flexible parts of the protein that have low motional restrictions tend to become more rigid and motional restricted upon the addition of the *myo*-Inositol. The overall protein compactness increases in the presence of the osmolyte, most profoundly observed in protein regions with high locally structural fluctuations.

(CPMG)-type NMR relaxation dispersion experiments

NMR relaxation dispersion methods have been introduced enabling studies on protein folding under native conditions without the need for disturbing the equilibrium. Studying protein folding and unfolding requires a thoroughly view of all states including the native state, folding intermediates and the unfolded state [12] as it is increasingly recognized that even small proteins fold via intermediates. Because these intermediates are low populated and short-lived (in the order of ms), their structural characterization has been a difficult task. In NMR relaxation dispersion experiments conformational exchange between a native ground state and low populated partially folded states can be characterized even if states are not visible in NMR spectra [50].

CPMG (Carr-Purcell-Meiboom-Gill)-type NMR relaxation dispersion techniques have been employed to investigate the site-specific conformational exchange processes of proteins on a microsecond-to-millisecond time scale that is highly sensitive to solvent and co-solvent conditions. These experiments are particular useful for simple two state exchange processes, providing information about the kinetics of the exchange process, the relative populations and structural features of invisible states in terms of NMR chemical shifts [51, 52]. Residues that undergo conformational exchange on the μs-ms time scale contribute to the effective transverse ^{15}N relaxation rates ($R_{2,eff}$). By measuring the increased contribution, R_{ex}, to the effective transverse relaxation rate as a function of CPMG pulse spacing relaxation, typical non-flat dispersion profiles are obtained (Figure 8).

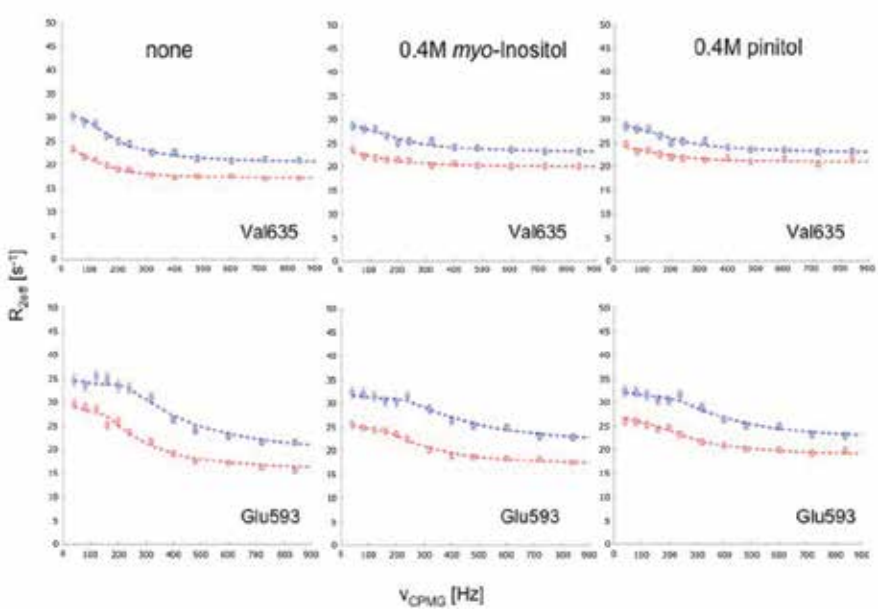

Figure 8. Typical ^{15}N relaxation dispersion profiles for KIX displaying residues Glu593 and Val635, recorded at static magnetic field strengths of 11.7T (red) and 18.8T (blue) at 26.9°C and in the presence of 0.4M *myo*-Inositol and 0.4M pinitol. Error bars represent uncertainties in relaxation rates. CPMG radio frequency field strengths, ν_{CPMG}, ranged from 40 to 960 Hz, relaxation delays were 50ms. Spectra were collected as series of two-dimensional data sets. Duplicate data sets were recorded at selected ν_{CPMG} values for error analysis. Peak intensities observed from ^{1}H-^{15}N spectra were converted into effective relaxation rates ($R_{2.eff}$) and uncertainties in relaxation rates were calculated from repeat experiments. $R_{2.eff}$ were calculated by numerical modeling of magnetization evolution during the CPMG sequences. Fit curves were obtained by combining the dispersion of all residues in a collective fit to a two-state process

^{15}N single quantum relaxation dispersion experiments were performed to characterize alterations in the two-site conformational exchange of the KID-binding (KIX) domain of CREB-binding protein (CBP) in the presence of osmolytes under native conditions. Conformational exchange of KIX ^{15}N backbone resonances has been shown to be in the intermediate to slow time regime. CPMG-type relaxation dispersion data showed that under non-denaturing conditions, KIX permanently exchanges between its folded (native) ground state (G) and a partially unfolded high-energy state (E) that is populated to 3±0.2% at 26.9°C and pH 5.5. Relaxation dispersion experiments were performed for KIX and in the presence of 0.4M osmolytes (pinitol, *myo*-Inositol, quebrachitol, quercitol), operating at static magnetic field strengths of 11.7 and 18.8T at 26.9°C. ^{15}N relaxation dispersion profiles were fit for each site individually (G↔E) to yield site-specific values of G→E and E→G rate constants (k_{GE} and k_{EG}) and differences in resonance frequencies between G and E states $|\Delta\omega_{fit}|$. Dispersion profiles of all sites were then fit to a global two-site model assuming uniform values for k_{GE} (k_u) and k_{EG} (k_f), but specific values for $|\Delta\omega_{fit}|$ (Table1). Dispersion profiles ($R_{2.eff}/V_{CPMG}$) are dependent on k_f and k_u rate constants or the population of the unfolded state p_E and the exchange rate constant ($k_{ex} = k_f + k_u$) and on chemical shift differences between the folded and unfolded state $|\Delta\omega|$ [53].

sample	k_f (s^{-1})	k_u (s^{-1})	p_E (%)
none	574,4	16,7	2,8
myo-Inositol	871,5	12,3	1,4
pinitol	935,9	12,7	1,3
quebrachitol	653,8	10,8	1,6
quercitol	661,8	13,0	1,9

Table 1. Two-site conformational exchange parameters of KIX. The response of $R_{2,eff}$ to ν_{CPMG} can be fitted to extract exchange parameters. A two-site exchange model (G⟷E) was fit to ^{15}N relaxation dispersion data, yielding site-specific values of G→E and E→G rate constants (k_u and k_f). k_f and k_u are the first order rate constants for folding and unfolding transitions, calculated from global fits of ^{15}N backbone relaxation experiments.

The two-site conformational exchange of KIX between its natively folded ground state and a partially unfolded high-energy state, that represents the equilibrium analog of a folding intermediate [54], was shown to be highly sensitive to the addition of osmolytes. NMR data showed that the composition of these two states differed between the protein in buffer alone and the osmolyte containing sample. Addition of 0.4M pinitol led to a decrease of more than 50% in the population of the partially unfolded state (p_E). Accordingly, the first order rate constant for folding (k_f) increased from 574.4s^{-1} to 935.9s^{-1} in the presence of pinitol, while the rate constant for unfolding (k_u) decreased (from 16.7s^{-1} to 12.7s^{-1}). These data provide evidence that even under native conditions osmolytes shift the folding equilibrium towards the folded state. NMR relaxation experiments revealed that osmolytes play an important role on the structure of the folding intermediate, which is the main determinant for protein folding and dynamics. Even though intermediate states are extremely short-lived (in the order of ms), osmolytes greatly influence these states. A decrease in the population of the partially folded state is associated with a destabilization of this state relative to the folded state in the osmolyte containing sample. The interaction of the osmolyte with the protein surface is not favorable and therefore osmolytes are preferentially excluded from the protein surface. Osmolytes indirectly act by changing the properties of water surrounding the protein and hence modify protein-solvent interactions by altering the specific arrangement of the hydrophobic and hydrophilic residues. Folded states are relatively favored over (partially) unfolded states due to their compact structure and smaller surface exposed (solvent accessible) area, leading to a net stabilization of the folded state even under native conditions. Accumulation of high amounts of osmolytes does not seem to be useful under non-stress conditions as they influence protein conformation and dynamics, but they confer great advances to enhance protein stability under stress conditions by counteracting the forces driving protein unfolding. Compact folded conformations are generally less prone to unfolding, misfolding and aggregation that lead to loss of protein function. Increased conformational stability through osmolytes on the other hand allows for greater protein flexibility under elevated temperatures, since thermal motion decreases rigidity and enhances flexibility. This mechanism greatly contributes to preserve protein function under stress conditions in plants.

5. Biotechnology approaches for improved abiotic stress tolerance in plants

Abiotic stress is one of the major causes of crop loss worldwide and restricts certain areas from productive agriculture and even less severe stress makes plants more susceptible to diseases and pests. As sessile organisms plants are exposed to various stresses during their lifespan. With increased understanding of the mechanisms of protein stabilization, advances have been made in genetically engineering more tolerant crop plants.

5.1. Genetically engineering overproduction of osmolytes

Progress is being made in genetically modifying plants to accumulate high amounts of osmolytes with the aim to enhance stress tolerance in plants. Transgenic plants have successfully been engineered to accumulate metabolites such as proline, mannitol, glycine betaine and trehalose, which resulted in increased tolerance to various stresses [55-57]. In addition to lowering the osmotic potential and assisting in osmotic adjustment, osmolytes act as hydroxyl radical scavengers and protect macromolecular structure. The accumulation of such metabolites in response to various stresses is a widely distributed phenomenon in the plant kingdom. Some important crop plants, however, are non-accumulators. Genetically introducing mannitol, sorbitol, trehalose or *myo*-Inositol production in tobacco, *Arabidopsis* and rice, all species that do not synthesize these compounds naturally, produced enhanced tolerance to salt and drought stress [58-60].

Recently, it has been shown that overexpression of rice (*Oryza sativa*) choline monooxygenase (OsCMO), the first enzyme in glycine betaine biosynthesis, enhances glycine betaine synthesis in transgenic tobacco plants and resulted in elevated tolerance to salt stress [61]. Although rice has been considered as typical non-accumulator of glycine betaine, this study revealed that the rice containing ortholog of CMO was fully functional in tobacco species. Enhanced tolerance toward salinity, heavy metal, oxidative stress and cold stress was also reported for transgenic tobacco plants when overexpressing rice cystathionine β-synthase [62] or cold regulated protein CbCOR15b transferred form *Capsella bursa-pastoris* [63]. Numerous reports show that introducing and enhancing abiotic stress tolerance by the transfer of one or more stress responsive genes between species would be an effective strategy to enhance performance of crop plants in less-productive agricultural areas.

Another strategy for osmolyte overproduction and enhaced plant growth relies on site-directed mutagenesis. Δ^1-Pyrroline-5-carboxylase synthase (P5CS), which is feedback inhibited by proline, has been mutated by site-directed mutagenesis, resulting in enzymes that were no longer inhibited. Plants expressing the mutated enzyme had twice the proline levels of WT-plants and exhibited increased tolerance to salt stress [64].

5.2. Protein engineering

Protein engineering approaches are being developed for the selection of protein mutations that increase protein stability. New stabilization strategies are based on random mutagene-

sis and high-throughput screening for thermostability-improving mutations, functional screening or comparison of homologous proteins. Some proteins have been successfully stabilized by the introduction of structural elements from thermophilic and hyperthermophilic homologues [65]. However, the mechanisms underlying thermostability are diverse. Much research has been focused on understanding the stabilization of the hydrophobic core and internal structural elements of proteins [66, 67]. Recent research has also revealed that protein surfaces have a strong influence on stability and, therefore, have to be taken into consideration. Surface residues are generally more flexible and the protein surface structure is less motional restricted than the compact core. Mutations in the protein surface are therefore supposed to largely affect protein stability and can be introduced to enhance protein stability. Much attention is paid to protein surface salt bridges, as it is known that surface salt bridges become more favorable with increasing temperature and hyperthermophilic proteins tend to have more salt bridges than their mesophilic homologues. Emphasis is made to investigate the contribution of surface salt bridges to enhanced protein stability under stress conditions.

Information from the protein biochemistry field will direct us toward an understanding of the rules for protein folding stability and dynamics with the goal to improve protein stability and stress tolerance in plants.

6. Conclusion

Abiotic stresses like desiccation, flooding, high salinity or extreme temperatures are common threats to plants and the optimal function of their metabolism. Protein conformation and stability is dramatically affected by sudden changes in the environment, giving rise to protein unfolding, misfolding and aggregation. Finding the rules for protein folding and unfolding that lead to conformational stability is a matter of ongoing research. Folded states represent the most stable forms under native conditions, but partially folded states that allow for efficient interaction with binding partners are of fundamental importance in biological activity. Studying protein stability under stress conditions has to take protein dynamics, meaning conformational changes of proteins with time, into consideration. Advances have been made in methods to study the conformational exchange in proteins and their folding stability under varying experimental conditions. Nuclear magnetic resonance spectroscopy techniques have been introduced to study the interconversion between folded and partially folded intermediate states. These short-lived, partially folded, states are extremely important for biological activity and play a major role in the energy landscape of proteins. NMR relaxation dispersion experiments revealed that such low populated intermediate folding states are strongly affected by solvent and co-solvent conditions. One of the early onsets of the stress response in plants is the accumulation of osmolytes that serve for osmotic adjustment and protect proteins by maintaining water at the protein surface where it is most needed. NMR dynamic measurements revealed that addition of osmolytes (*myo*-Inositol, pinitol, quebrachitol and quercitol) lead to a decreased population of the partially folded state by shifting the folding equilibrium towards the folded ensembles. Although osmolytes do not

directly interact with the protein surface, they alter protein surface properties by changing the water structure and hydrophobic interactions, thereby stabilizing the folded states relative to unfolded states. Even under native conditions, osmolytes were shown to favor the compact folded structure over partially folded structures, consequently leading to alterations in the dynamics of these two states. Thermodynamic considerations assume that osmolytes act by raising the chemical potential of the partially unfolded state relative to the folded state, thereby increasing the (positive) Gibbs energy difference (ΔG) between folded and unfolded assemblies, thus favoring the folded state with the respect to the unfolded state. By stabilizing compact folded states over unfolded structures even under non-stress conditions, osmolyte accumulation exhibits a great potential to counteract the forces that lead to stress induced protein unfolding. High osmolyte accumulation in plants may not be useful under non-stress conditions as they tend to decrease protein globally and locally flexibility and increase protein overall rigidity. Increased rigidity and overall compactness, however, confer great advances under stress conditions. Compact structures are less prone to unfolding, misfolding, aggregation and degradation. Lower structural flexibility under ambient temperatures allows for greater flexibility under elevated temperatures since thermal motion decreases rigidity and enhances flexibility, which is essential for protein function under stress conditions. Osmolyte production seems to be very effective strategy to adopt plants quickly and with a remarkable plasticity to various changes in their environment. High osmolyte accumulation serves to suppress protein unfolding and misfolding, enhances protein folding stability and facilitate the protein refolding process after complete denaturation. These lessions that we learned from plants and new insights from the protein biochemistry field are taken together for genetically engineering of more tolerant crop plants with the ultimate goal to improve yields in less productive agricultural land.

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Chapter 2

Abiotic Stress in Plants and Metabolic Responses

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Additional information is available at the end of the chapter

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1. Introduction

The vast metabolic diversity observed in plants is the direct result of continuous evolutionary processes. There are more than 200,000 known plant secondary metabolites, representing a vast reservoir of diverse functions. When the environment is adverse and plant growth is affected, metabolism is profoundly involved in signaling, physiological regulation, and defense responses. At the same time, in feedback, abiotic stresses affect the biosynthesis, concentration, transport, and storage of primary and secondary metabolites. Metabolic adjustments in response to abiotic stressors involve fine adjustments in amino acid, carbohydrate, and amine metabolic pathways. Proper activation of early metabolic responses helps cells restore chemical and energetic imbalances imposed by the stress and is crucial to acclimation and survival. Time-series experiments have revealed that metabolic activities respond to stress more quickly than transcriptional activities do. In order to study and map all the simultaneous metabolic responses and, more importantly, to link these responses to a specific abiotic stress, integrative and comprehensive analyses are required. Metabolomics is the systematic approach through which qualitative and quantitative analysis of a large number of metabolites is increasing our knowledge of how complex metabolic networks interact and how they are dynamically modified under stress adaptation and tolerance processes. A vast amount of research has been done using metabolomic approaches to (*i*) characterize metabolic responses to abiotic stress, (*ii*) to discover novel genes and annotate gene function, and, (*iii*) more recently, to identify metabolic quantitative trait loci. The integration of the collected metabolic data concerning abiotic stress responses is helping in the identification of tolerance traits that may be transferable to cultivated crop species. In this review, the diverse metabolic responses identified in plants so far are discussed. We also include recent advances in the study of plant metabolomes and metabolic fluxes with a focus on abiotic stress-tolerance trait interactions.

2. Abiotic stresses and the impact on agriculture

Today, in a world of 7 billion people, agriculture is facing great challenges to ensure a sufficient food supply while maintaining high productivity and quality standards. In addition to an ever increasing demographic demand, alterations in weather patterns due to changes in climate are impacting crop productivity globally. Warming and shifts in rainfall patterns caused an historically high $10.3 billion in crop insurance payments to cover agriculture losses in 2011 in the U.S. [1]. Unfavorable climate (resulting in abiotic stresses) not only causes changes in agro-ecological conditions, but indirectly affects growth and distribution of incomes, and thus increasing the demand for agricultural production [2]. Adverse climatic factors, such as water scarcity (drought), extreme temperatures (heat, freezing), photon irradiance, and contamination of soils by high ion concentration (salt, metals), are the major growth stressors that significantly limit productivity and quality of crop species worldwide. As has been pointed out, current achievements in crop production have been associated with management practices that have degraded the land and water systems [3]. Soil and water salinity problems exist in crop lands in China, India, the United States, Argentina, Sudan, and many other countries in Western and Central Asia. Globally, an estimated 34 million irrigated hectares are salinized [4], and the global cost of irrigation-induced salinity is equivalent to an estimated US$11 billion per year [5].

A promising strategy to cope with adverse scenario is to take advantage of the flexibility that biodiversity (genes, species, ecosystems) offers and increase the ability of crop plants to adapt to abiotic stresses. The Food and Agricultural Organization (FAO) of the United Nations promotes the use of adapted plants and the selection and propagation of crop varieties adapted or resistant to adverse conditions [6]. Global programs, such as the Global Partnership Initiative for Plant Breeding Capacity Building (GIPB), aim to select and distribute crops and cultivars with tolerance to abiotic stresses for sustainable use of plant genetic resources for food and agriculture [7].

3. Plant responses to abiotic stress

Through the history of evolution, plants have developed a wide variety of highly sophisticated and efficient mechanisms to sense, respond, and adapt to a wide range of environmental changes. When in adverse or limiting growth conditions, plants respond by activating tolerance mechanisms at multiple levels of organization (molecular, tissue, anatomical, and morphological), by adjusting the membrane system and the cell wall architecture, by altering the cell cycle and rate of cell division, and by metabolic tuning [8]. At a molecular level, many genes are induced or repressed by abiotic stress, involving a precise regulation of extensive stress-gene networks [9-11]. Products of those genes may function in stress response and tolerance at the cellular level. Proteins involved in biosynthesis of osmoprotectant compounds, detoxification enzyme systems, proteases, transporters, and chaperones are among the multiple protein functions triggered as a first line of direct protection from stress. In addition,

activation of regulatory proteins (*e.g.*, transcription factors, protein phosphatases, and kinases) and signaling molecules are essential in the concomitant regulation of signal transduction and stress-responsive gene expression [12, 13]. Early plant response mechanisms prevent or alleviate cellular damage caused by the stress and re-establish homeostatic conditions and allow continuation of growth [14]. Equilibrium recovery of the energetic, osmotic, and redox imbalances imposed by the stressor are the first targets of plant immediate responses.

Observed tolerance responses towards abiotic stress in plants are generally composed of stress-specific response mechanisms and also more general adaptive responses that confer strategic advantages in adverse conditions. General response mechanisms related to central pathways are involved in energy maintenance and include calcium signal cascades [15, 16], reactive oxygen species scavenging/signaling elements [17, 18], and energy deprivation (energy sensor protein kinase, SnRK1) signaling [19]. Induction of these central pathways is observed during plant acclimation towards different types of stress. For example, protein kinase SnRK1is a central metabolic regulator of the expression of genes related to energy-depleting conditions, but this kinase also becomes active when plants face different types of abiotic stress such as drought, salt, flooding, or nutrient depravation [20-24]. SnRK1 kinases modify the expression of over 1000 stress-responsive genes allowing the re-establishment of homeostasis by repressing energy consuming processes, thus promoting stress tolerance[24, 25]. The optimization of cellular energy resources during stress is essential for plant acclimation; energetically expensive processes are partially arrested, such as reproductive activities, translation, and some biosynthetic pathways. For example, nitrogen and carbon assimilation are impaired in maize during salt stress and potassium-deficiency stress; the synthesis of free amino acids, chlorophyll, and protein are also affected [26-28]. Once energy-expensive processes are curtailed, energy resources can be redirected to activate protective mechanisms. This is exemplified by the decrease in *de novo* protein synthesis in *Brassica napus* seedlings, *Glycine max, Lotus japonicas*, and *Medicago truncatula* during heat stress accompanied by an increased translation of heat shock proteins [29, 30].

4. Metabolic adjustments during stressing conditions: Osmolyte accumulation

A common defensive mechanism activated in plants exposed to stressing conditions is the production and accumulation of compatible solutes. The chemical nature of these small molecular weight organic osmoprotectants is diverse; these molecules include amino acids (asparagine, proline, serine), amines (polyamines and glycinebetaine), and γ-amino-N-butyric acid (GABA). Furthermore, carbohydrates, including fructose, sucrose, trehalose, raffinose, and polyols (myo-inositol, D-pinitol) [12, 31], as well as pools of anti-oxidants such as glutathione (GSH) and ascorbate [32, 33], accumulate in response to osmotic stress. Common characteristics of these diverse solutes are a high level of solubility in the cellular milieu and lack of inhibition of enzyme activities even at high concentrations. Accumulation of compatible solutes in response to stress is not only observed in plants, it is a defense mechanism triggered in animal cells, bacteria, and marine algae, indicative of an evolutionarily conserved trait [34,

35]. Scavenging of reactive oxygen species (ROS) to restore redox metabolism, preservation of cellular turgor by restitution of osmotic balance, and associated protection and stabilization of proteins and cellular structures are among the multiple protective functions of compatible osmoprotectants during environmental stress [36-38].

A large amount of research has been done on the beneficial effects of compatible solutes on plant tolerance to environmental stress. Correlation between amino acid accumulation (mainly proline) and stress tolerance was described in the mid-1960s in Bermuda grass during water stress [39]. Since then, extensive work has proven that proline serves as an osmoprotectant, a cryoprotectant, a signaling molecule, a protein structure stabilizer, and an ROS scavenger in response to stresses that cause dehydration; including salinity, freezing, heavy metals, and drought (low water potential) [40, 41]. Proline oxidation may also provide energy to sustain metabolically demanding programs of plant reproduction, once the stress has passed [42].

Proline metabolism and its regulation are processes well characterized in plants. Proline is synthesized from glutamate in the cytoplasm or chloroplasts: Δ-1-pyrroline-5-carboxylate synthetase (P5CS) reduces glutamate to glutamate semialdehyde (GSA). Then GSA spontaneously cyclizes into pyrroline-5-carboxylate (P5C), which is further reduced by P5C reductase (P5CR) to proline. Conversely, proline is catabolized within the mitochondrial matrix by action of proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH) to glutamate. In an alternative pathway, proline can be synthesized from ornithine in a pathway involving ornithine δ-aminotransferase (OAT). Core enzymes P5CS, P5C, P5CR, ProDH, and OAT are responsible for maintaining the balance between biosynthesis and catabolism of proline. Regulation comes at transcriptional level of genes encoding the key enzymes. Transcriptional up-regulation of genes for P5CS and P5C to increase proline synthesis from glutamate and down-regulation of genes for P5CR and ProDH to arrest proline catabolism is observed during dehydration/osmotic stress [43]. Also, post-translational regulation of core enzymes is closely associated with proline levels and environmental signals. For example, the *Arabidopsis* P5CS1 enzyme is subjected to feedback inhibition by proline, controlling the carbon influx into the biosynthetic pathway [44, 45]. Considering that proline accumulation is associated with stress tolerance, that core enzymes regulate proline biosynthesis, and that these core enzymes are likely rate-limiting steps for its accumulation, logic dictates that overexpression of biosynthetic proline enzymes might increase the levels of the compatible solute and thus improve the tolerance in plants against abiotic stress. Several studies have tested this by overexpressing genes for P5CS or P5C enzymes in different plant species, reporting the expected rise in proline levels and the associated resistance to dehydration, salinity, or freezing [46-53]. Furthermore, deletion of genes coding ProDH [54] or P5CDH [55, 56], expression of a feedback-insensitive P5CS [45], or the overexpression of OAT [57, 58] increase the cellular levels of proline and osmoprotection to some abiotic stresses.

Comparable extensive work has been done for other compatible solutes such as γ-aminobutyric acid [59], glycine betaine [60], trehalose [61], mannitol, and sorbitol [36]; these solutes are efficient protectors against some abiotic stressors. Metabolic pathways for biosynthesis and catabolism of compatible solutes, their regulation, participant enzymes, and compartmentalization are well characterized in most important plant species. This knowledge has led to

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strategies for improvement of plant tolerance involving the accumulation of those protective osmolytes in plants by expression of core biosynthetic enzymes or their improved derivatives, expression of related transporters, and deletion of osmolyte-consuming enzymes. These numerous studies have provided evidence that enhanced accumulation of compatible solutes correlates with reinforcement of plant resistance to adverse growth conditions.

5. Plant metabolomics and applications

The traditional approach of enhancing the accumulation of a specific compounds in response to a determined stimulus, as done with compatible solutes, have resulted in some degree of tolerance in plants, and also demonstrates that the ability to redirect nutrients to imperative processes and the induction of adequate metabolic adjustments are crucial for plant survival during conditions of stress. However, this is a sectioned view of how plants regulate their entire metabolism in response to stressing conditions. In order to achieve a more comprehensive understanding, we must consider that plant metabolism is an intricate network of interconnected reactions. Plants have a high degree of subcellular compartmentation, a vast repertory of metabolites, and developmental stage strongly influences metabolism. Therefore, metabolic responses are complex and dynamic and involve the modification of more than one metabolite. Also, accumulation of a specific compound is not an absolute requirement indicative of a tolerance trait; adjustment of the flux through a certain metabolic pathway might be enough to contribute to stress tolerance [62]. Recently, it has been reported that plants modulate stoichiometry and metabolism in a flexible manner in order to maintain optimal fitness in mechanisms of storage, defense, and reproduction under varying conditions of temperature and water availability [63]. Furthermore, time-series experiments in *Arabidopsis thaliana* plants subjected to temperature and/or light alterations revealed that time-resolved metabolic activities respond more quickly than transcriptional activities do [64].

Traditional molecular approaches for tracing metabolic phenotypes in plants responding to abiotic stress have identified and manipulated specific genes or groups of genes in plant models. These have primarily been genes involved in early responses or in down-stream assembly of the response reaction. With the application of new powerful tools of molecular biology and bioinformatics, large collections of genes have been subjected to complete analysis. To arrive at a complete and comprehensive knowledge of physiology in the plant response to abiotic stress, researchers are embracing ionomic profiling, transcriptomic, proteomic and metabolomic analysis. A deep dissection of the biochemical pathways in plants facing stressing conditions requires integrative and comprehensive analyses in order to identify all the simultaneous metabolic responses and, more importantly, to be able to link these responses to specific abiotic stress. In this sense, metabolomics could contribute significantly to the study of metabolic responses to stress in plants by identifying diverse metabolites, such as the by-products of stress metabolism, stress signal transduction molecules, and molecules that are part of the acclimation response [65].

The metabolome is the entirety of small molecules present in an organism and can be regarded as the ultimate expression of its genotype in response to environmental changes. Metabolomics

is gaining importance in plant research in both basic and applied contexts. Metabolomic studies have already shown how detailed information gained from chemical composition can help us to understand the various physiological and biochemical changes occurring in the plants and their influence on the phenotype. The analytical measurement of several hundreds to thousands of metabolites is becoming a standard laboratory technique with the advent of "hyphenated" analytical platforms of separation methods and various detection systems. Separation methods include gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE). Different types of mass spectrometry (MS), nuclear magnetic resonance (NMR), and ultraviolet light spectroscopy (UV/VIS) devices are utilized for detection. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) is a specialized technique often used in direct infusion (DI) mode for metabolomics analyses, as its high mass accuracy allows a separation solely based on this parameter. Each methodology offers advantages and disadvantages, and the method of choice will depend on the type of sample and metabolites to be determined, and the combination of analytical platforms [66].

GC and MS were the first pair of techniques to be combined, delivering high robustness and reproducibility. GC-MS remains one of the most widely used methods for obtaining metabolomic data because of its ease of use, excellent separation power, and its reproducibility. The main drawback of GC-MS is that only thermally stable volatile metabolites, or non-volatile compounds that can be chemically altered to make them volatile, can be detected [67, 68]. NMR spectroscopy is a fingerprinting technique that offers several advantages over high-throughput metabolite analyses, such as relatively simple sample preparation and the non-destructive analysis of samples. NMR can detect different classes of metabolites in a sample, regardless of their size, charge, volatility, or stability with excellent resolution and reproducibility [69]. Labeling of metabolites with isotopes and subsequent NMR analysis is also useful for metabolic flux analysis and fluxomics as it allows tracking the selective signal enhancement of isotopologues [70]. Recent advances with high-throughput approaches using ultra-high-field FT-ICR-MS alone or in combination with other tools of 'first pass' metabolome analysis as electrospray ionization mass spectrometry (ESI-MS) are expected to make inventory of the entire metabolome in a single sample possible in the near future [71, 72].

In metabolomics, the implicit objective is to identify and quantify all possible metabolites in a cellular system under defined states of stress conditions (biotic or abiotic) over a particular time scale in order to characterize accurately the metabolic profile [73]. But metabolome studies have some analytical limitations. It is important to have in mind that from the total amount of metabolites in a sample, only an informative portion can be reliably identified and quantified. In addition, metabolic networks in multicellular eukaryotes, specifically in plants, are challenging because of the large size of the metabolome, extensive secondary metabolism, and the considerable variation in tissue-specific metabolic activity [74]. Therefore, experimental design and sample preparation need to be done with great care because environmental and experimental variation confer noticeable impact on the resulting metabolic profiles. This has been demonstrated in legumes in which a high proportion of nutritional and metabolic changes depend on non-controllable environmental variables [75].

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Metabolomic analyses have been applied to the functional identification of unknown genes through metabolic profiling of plants in which some genes are up- or down-regulated, the discovery of biomarkers associated with disease phenotypes, the safety assessment of genetically modified organisms (GMOs), the characterization of plant metabolites of nutritional importance and significance in human health, and the discovery of compounds involved in plant resistance to biotic and abiotic stresses [76]. Metabolic profiles can be used as signatures for assessing the genetic variation among different cultivars or species of the same genotype at different growth stages and environments. The metabolite profile represents phenotypic information; this means that qualitative and quantitative metabolic measurements can be related to the genotypes of the plants to differentiate closely related individuals [77, 78]. Once the identification of individual metabolites is available, connections among metabolites can be established, and then metabolic profiles can be used to infer mechanisms of defense. Metabolic profiles will guide tailoring of genotypes for acceptable performance under adverse growth conditions and will be of help in design and development of crop plant cultivars best suited to sustainable agriculture [79, 80]. Metabolomics tools have been used to evaluate the impact of the genotype and the environment on the quality of plant growth in the study of interpecific hybrids between *Jacobaea aquatica* and *J. vulgaris* (common weeds native to Northern Eurasia). An NMR-based metabolomics profiling approach was used to correlate the expression of high and low concentrations of particular compounds, including phenylpropanoids and sugars, with results of quantification of genetically controlled differences between major primary and secondary metabolites [81]. In melon (*Cucumis melo* L.), metabolomic and elemental profiling of fruit quality were found to be affected by genotype and environment [82].

6. Plant metabolomics and drought stress

The variable and often insufficient rainfalls in extended areas of rain-fed agriculture, the unsustainable groundwater use for irrigated agriculture worldwide, and the fast-growing demands for urban water are putting extreme pressure on global food crop production. The demand for water to sustain the agriculture systems in many countries will continue to increase as a result of growing populations [83]. This progressively worsening water scarcity is imposing hydric stress on both rain-fed and irrigated crops. Water deficiency stress induces a wide range of physiological and biochemical alterations in plants; arrestment of cell growth and photosynthesis and enhanced respiration are among the early affects. Genome expression is extensively remodeled, activating and repressing a variety of genes with diverse functions [11, 84]. Sensing water deficit and activation of defense mechanisms comes through chemical signals in which abscisic acid (ABA) plays a central role. ABA accumulates in tissues of plants subjected to hydric stress and promotes transpiration reduction via stomatal closure. Through this mechanism, plants minimize water losses and diminish stress injury. ABA regulates expression of many stress-responsive genes, including the late embryogenesis abundant (LEA) proteins, leading to a reinforcement of drought stress tolerance in plants [85]. Many questions

remain unresolved concerning hydric stress-plant metabolic response: How does drought stress perturb metabolism in crop plants? How does hydric stress affect the metabolism of wild plants? What modern strategies of "omics" could be exploited to support future programs of crop breeding to lead to a more sustainable agriculture?

As previously described, one of the main mechanisms by which plants cope with water deficits is osmotic adjustment. These adjustments maintain a positive cell turgor via the active accumulation of compatible solutes. Traditionally, the analysis of metabolic responses to drought stress was limited to analysis of one or two classes of compounds considered as "role players" in the development of tolerance. Application of metabolomic approaches is providing a less biased perspective of metabolic profiles of response and also is aiding in the discovery of novel metabolic phenotypes. Unbiased GC-MS metabolomic profiling in *Eucalyptus* showed that drought stress alters a larger number of leaf metabolites than the previously reported in targeted analysis. Accumulation of shikimic acid and two cyclohexanepentol stereoisomers in response to drought stress was described for the first time in *Eucalyptus*. Also, the magnitude of metabolic adjustments in response to water stress correlates with the sensitivity/tolerant phenotype observed; drought affected around 30-40% of measured metabolites in *Eucalyptus dumosa* (a drought-sensitive specie) compared to 10-15% in *Eucalyptus pauciflora* (a drought-tolerant specie) [86]. Similarly, critical differences in the metabolic responses were observed when drought-tolerant (NA5009RG) and drought-sensitive (DM50048) soybean cultivars were analyzed by ^{1}H NMR-based metabolomics. Interestingly, no enhanced accumulation of the traditional osmoprotectants, such as proline, soluble sugars as sucrose or myo-inositol, organic acids or other amino acids (except for aspartate), were detected in the leaves of either genotype during water stress. In contrast, levels of 2-oxoglutaric acid, pinitol, and allantoin were affected differentially in the genotypes when drought was imposed, suggesting possible roles as osmoprotectants [87]. In contrast to soybean, levels of amino acids, including proline, tryptophan, leucine, isoleucine, and valine, were increased under drought stress in three different cultivars of wheat (*Triticum aestivum*) analyzed for 103 metabolites in a targeted GC-MS approach [88]. Metabolic adjustments in response to adverse conditions are transient and depend on the severity of the stress. In a 17-day time course experiment in maize (*Zea mays*) subjected to drought stress, GC-MS metabolic analysis revealed changes in concentrations of 28 metabolites. Accumulation of soluble carbohydrates, proline and eight other amino acids, shikimate, serine, glycine, and aconitase, was accompanied by the decrement of leaf starch, malate, fumarate, 2-oxoglutarate, and seven amino acids during the drought treatment course. However, as the water potential became more negative, between the 8th and 10th days, the changes in some metabolites were more dramatic, demonstrating their dependence on stress severity [89].

Accumulation of compatible solutes is an evolutionary conserved trait in bacteria, plants, animal cells, and marine algae. A recent GC-MS metabolomic analysis confirmed that the moss *Physcomitrella patens* also triggers compatible solute accumulation in response to drought stress. After two weeks of physiological drought stress, 26 metabolites were differentially affected in gametophores, including altrose, maltitol, L-proline, maltose, isomaltose, and butyric acid, comparable to metabolic adjustments previously reported in stressed *Arabidop-*

sis leaves. More interesting is the recent report of a new compound, annotated as EITTMS_N12C_ATHR_2988.6_1135EC44, with no previously mass spectra matching record, accumulated specifically in response to drought stress in this moss [90].

7. Plant metabolomics and salinity stress

A current problem for crop plants worldwide, which will become more critical in the future, is salt stress imposed by salinity in soils due to poor practices in irrigation and over-fertilization, among other causes. Salt stress induces abscisic acid synthesis; abscisic acid transported to guard cells closes stomata, resulting in decreased photosynthesis, photo-inhibition, and oxidative stress. This causes an immediate inhibition of cell expansion, visible as general plant growth inhibition, accelerated development, and senescence [91]. To cope with salt stress plants implement strategies that include lowering of rates of photosynthesis, stomatal conductance, and transpiration [92]. Sodium ion, by its similar chemical nature to potassium ion, competes with and inhibits the potassium uptake by the root. Potassium deficiency results in growth inhibition because this ion is involved in the capacitance of a plethora of enzyme activities in addition to its participation in maintaining membrane potential and cell turgor [91].

The metabolic perturbation in plants exposed to salinity involves a broad spectrum of metabolic pathways and both primary and secondary metabolism. For example, in a proteomic study in foxtail millet (cv. Prasad), 29 proteins were significantly up- or down-regulated due to NaCl stress, with great impact on primary metabolism. These proteins were classified into nine functional categories: cell wall biogenesis (lignin biosynthesis), among these were caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase; photosynthesis and energy metabolism, which included proteins like cytochrome P450 71D9, phytochrome 1, photosystem I reaction center subunit IV B, and ATP synthase F1 sector subunit beta, among others; nitrogen metabolism, proteins like glutamine synthetase root isozyme 4, ferredoxin-dependent glutamate synthase, chloroplast precursor (Fd-GOGAT), and urease; carbohydrate metabolism, proteins such as UDP-glucose 4-epimerase GEPI42 (galactowaldenase) and beta-amylase; and lipid metabolism including isovaleryl-CoA dehydrogenase 2 and aldehyde dehydrogenase [93].

Studies using metabolomic tools in plant models and plant crops have shown that the physiology in salt stress courses through a complex metabolic response including different systematic mechanisms, time-course changes, and salt-dose dependence. The biochemical changes involve metabolic pathways that fulfill crucial functions in the plant adaptation to salt stressing conditions. Time-course metabolite profiling in cell cultures of *A. thaliana* exposed to salt stress demonstrates that glycerol and inositol are abundant 24 h after salt stress exposure, whereas lactate and sucrose accumulate 48 h later. The methylation cycle, the phenylpropanoid pathway, and glycine betaine biosynthesis exhibit induction as a short-term response to salinity stress, whereas glycolysis and sucrose metabolism and reduction in methylation are long-term responses. Long-term salt exposure also causes a reduction in the metabolites that

were initially responsive [94]. In tobacco plants treated with various doses of salt, 1 day of treatment with 50 mM NaCl induced accumulation of sucrose, and to a lesser extent glucose and fructose, through gluconeogenesis. Further stress (500 mM NaCl for another day) led to elevation of proline and even higher elevation in sucrose levels compared to the lower dose; at the same time, glucose and fructose levels decreased as transamination-related metabolites (asparagine, glutamine, and GABA) did. These data suggest that sugar and proline biosynthesis pathways are metabolic mechanisms for control of salt stress over one- to two-day periods (short-term). Proline continues to be observed at high levels at later stages (3 to 7 days under highly stressing concentrations of 500 mM NaCl) and sucrose decreases (although it remains at high levels compared to control). There are also significant elevations in levels of asparagine, valine, isoleucine, tryptophan, myo-inositol, uracil, and allantoin, and reductions in glucose, fructose, glutamine, GABA, malate, fumarate, choline, uridine, hypoxantine, nicotine, N-methylnicotinamide, and formate [95]. Similarly, in maize plants stressed with salt solutions ranging in concentration from 50 to 150 mM NaCl, the metabolic profile of the shoot extracts changes most dramatically compared to controls in the plants exposed to the highest salt concentration [96].

Another complexity in the metabolic perturbations in salt-stressed plants consists of tissue-specific response differences. In maize plants exposed to 50-150 mM NaCl saline solution, levels of sucrose and alanine were increased and levels of glucose decreased in roots and shoots. Other osmoprotectants exhibited differentiated behavior: GABA, malic acid, and succinate levels increased in roots, while glutamate, asparagine and glycine betaine were at higher concentrations in shoots. There were decreased levels of acetoacetate in roots and of malic acid and *trans*-aconitic acid in shoots. A progressive metabolic response was more evident in shoots than in roots [96].

In comparative ionomics and metabolite profiling of related *Lotus* species (*Lotus corniculatus*, *L. tenuis*, and *L. creticus*) under salt stress, the extremophile *L. creticus* (adapted to highly saline coastal regions) exhibits better survival after long-term exposure to salinity and is more efficient at excluding Cl^- from shoot tissue than the two cultivated glycophytes *L. corniculatus* and *L. tenuis* (grassland forage species). Sodium ion levels are higher in the extremophile than the cultivars under both control conditions and salt stress. In *L. creticus*, a differential homeostasis of Cl^-, Na^+, and K^+ is accompanied by distinct nutritional changes compared to the glycophytes *L. corniculatus* and *L. tenuis*. Magnesium and iron levels increase in *L. creticus* after salt treatment, but levels of potassium, manganese, zinc, and calcium do not. In non-stressed control plants, 41 metabolites are found at lower levels in *L. creticus* than in the two glycophytes, and 10 metabolites are at higher levels in *L. creticus*. These data demonstrate that each of these species has a distinct basal metabolic profile and that these profiles do not show a concordance with salt stress or salt tolerance. In salt stress conditions, 48 metabolites show similar changes in all species, either increasing or decreasing, with increased levels the amino acids proline, serine, threonine, glycine, and phenylalanine; the sugars sucrose and fructose, myo-inositol and other unidentified metabolites; and with decreased levels of organic acids such as citric, succinic, fumaric, erythronic, glycolic, and aconitic acid, including ethanolamine and putrescine, among others. Of note is that more than half of the metabolites affected by salt

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treatment are common among the three species, and only one-third of responsive metabolites in *L. creticus* are not shared with the glycophytes. Interestingly, the changes in the pool sizes of these metabolites are only marginal [97]. A few changes in the metabolic profile are extremophile-specific, but most salt-elicited changes in metabolism are similar. Other studies in glycophytes under salt stress indicate that organic acids and intermediates of the citric acid cycle tend to decrease [98]. Also in genus *Lotus*, model species (*L. japonicus*, *L. filicaulis*, and *L. burttii*) and cultivated species (*L. corniculatus*, *L. glaber*, and *L. uliginosus*) exhibit consistent negative correlation in the Cl^- levels in the shoots and tolerance to salinity, but metabolic profiles diverge amongst genotypes; asparagine levels are higher in the more tolerant genotypes. These results support the conclusion that Cl^-exclusion from the shoots represents a key physiological mechanism for salt tolerance in legumes; moreover, an increased level of the osmoprotectant asparagine is typical [99]. In *L. japonicus*, which has a robust metabolic response to salt stress, levels of proline and serine, polyolsononitol and pinitol, and myo-inositol increase [75].

All these studies demonstrate that the metabolic plant response to salinity stress is variable depending on the genus and species and even the cultivar under consideration. Differential metabolic rearrangements are in intimate correlation with genetic backgrounds. Furthermore, the plant physiology in salt stress with time proceeds through a complex metabolic response including different systematic mechanisms and changes. Inside a salt-stressed plant as a biological unit, different tissues respond differentially and in some cases the responses are even contrasting. From comparative ionomics studies, it is evident also that under salinity stress, differential homeostasis of ions as Cl^-, Na^+, and K^+ is correlated with distinct nutritional changes in extremophile and glycophyte species, even inside the same genus. Noticeable differences exist between plant species in the way they react to surpass the osmotic pressure imposed by high soil salt content through mechanisms such as tolerance, efficiency in salt exclusion, changes in nutrient homeostasis, and osmotic adjustment. From the aforementioned studies, metabolic markers in the response to high salinity in plants include glycine betaine, sucrose, asparagine, GABA, malic acid, aspartic acid, and *trans*-aconitic acid. In legumes, increases in levels of the amino acids asparagine, proline, and serine are notable as are increases in polyolsononitol, pinitol, and myo-inositol [75].

8. Plant metabolomics and oxidative stress

An increase in intracellular levels of ROS is a common consequence of adverse growth conditions. An imbalance between ROS synthesis and scavenging is caused in a manner independent of the nature of the stress; it is induced by both biotic and abiotic types of stress. Toxic concentrations of ROS cause severe damage to protein structures, inhibit the activity of multiple enzymes of important metabolic pathways, and result in oxidation of macromolecules including lipids and DNA. All these adverse events compromise cellular integrity and may lead to cell death [100, 101]. Normal cellular metabolic activity also results in ROS generation under regular growth conditions. Thus, cells sense uncontrolled elevation of ROS and use them as a signaling mechanism to activate protective responses [102]. In this context plants have

developed efficient mechanisms for removal of toxic concentrations of ROS. The antioxidant system is composed of protective enzymes (*e.g.*, superoxide dismutase, catalase, peroxidase, reductase, and redoxin) and radical scavenger metabolites (mainly GSH and ascorbate). GSH is an essential component of the antioxidant system that donates an electron to unstable molecules such as ROS to make them less reactive and also can acts as a redox buffer in the recycling of ascorbic acid from its oxidized form to its reduced form by the enzyme dehydroascorbate reductase [103]. Organized remodeling of metabolic networks is a crucial response that gives the cells the best chance of surviving the oxidative challenge.

In *A. thaliana*, oxidative treatment with methyl viologen causes the down-regulation of photosynthesis-related genes and concomitant cessation of starch and sucrose synthesis pathways, meanwhile catabolic pathways are activated. These metabolic adjustments avoid the waste of energy used in non-defensive processes and mobilize carbon reserves towards actions of emergency relief such as the accumulation of maltose, a protein structure-stabilizer molecule [104]. A GC-MS metabolomic study, together with an analysis of key metabolic fluxes of cell cultures and roots of *A. thaliana* treated with the oxidative stressor menadione, revealed the similarities and divergences in the metabolic adjustments triggered in both culture systems. Inhibition of the tricarboxylic acid cycle (TCA) by accumulation of pyruvate and citrate is accompanied by a decrement of malate, succinate, and fumarate pools. This early (0.5 h) response was observed in both systems. Inhibition of TCA cycle concomitantly causes a decrement in the pools of glutamate and aspartate due to the inhibition of the synthesis of TCA-linked precursors 2-oxoglutarate and oxaloacetate, respectively. Another mutual early metabolic redistribution is the redirection of the carbon flux from glycolysis to the oxidative pentose phosphate (OPP) pathway. This is also reflected by the decrement in the glycolytic pools of glucose-6 phosphate and fructose 6-P, and the increment in the OPP pathway intermediates ribulose 5-phosphate and ribose 5-phosphate. Increased carbon flux through the OPP pathway might supply reducing power (via nicotinamide adenine dinucleotide phosphate, NADPH) for antioxidant activity, since oxidative stress decreases the levels of the reductants GSH, ascorbate, and NADPH. After 2 and 6 h of stress progression, metabolic adjustments in response to oxidative stress are different in roots than in cell suspension cultures. In roots, pools of TCA cycle intermediates and amino acids are recovered. In contrast, in cell cultures, the concentrations of these metabolites remains depressed throughout the time course, indicating higher basal levels of oxidative stress in cell cultures. At the end of the treatment time (6 h), 39 metabolites, including GABA, aromatic amino acids (tryptophan, phenylalanine, and tyrosine), proline, and other amino acids, were significantly altered in roots. These results showed the broad spectrum of metabolic modifications elicited in response to oxidative stress and the influence of the biological system analyzed [105].

Redirection of carbon flux from glycolysis through the OPP pathway and subsequent increase in the levels of NADPH was also reported in rice cell cultures treated with menadione. CE-MS analysis of these rice cultures showed the depletion of most sugar phosphates resulting from glycolysis (pyruvate, 3-phosphoglyceric acid, dihydroxyacetone phosphate, fructose-6-phosphate, glucose-1-phosphate (G1P), G6P, G3P, phosphoenolpyruvate) and TCA-organic acids (2-oxoglutarate, aconitate, citrate, fumarate, isocitrate, malate, succinate) and increases

in the levels of OPP pathway intermediates (6-phosphogluconate, ribose 5-phosphate, ribulose 5-phosphate). Incremental increases in the biosynthesis of GSH and intermediates (*O*-acetyl-L-serine, cysteine, and γ-glutamyl-L-cysteine) are also observed in the menadione-treated rice cell cultures [106].

9. Perspectives

Metabolome analysis has become an invaluable tool in the study of plant metabolic changes that occur in response to abiotic stresses. Despite progress achieved, metabolomics is a developing methodology with room for improvement. From a technical perspective, further developments are required to improve sensitivity for identification of previously uncharacterized molecules and for quantification of cellular metabolites and their fluxes at much higher resolution. This will allow the identification of novel metabolites and pathways and will allow linkage to responses to specific stresses, and, therefore, increase our level of knowledge of the elegant regulation and precise adjustments of plant metabolic networks in response to stress.

Another challenging task is the integration of metabolic data with data from experiments profiling the transcriptome, proteome, and genetic variations obtained from the same tissue, cell type, or plant species in response to a determined environmental condition. Integrated information can be used to map the loci underlying various metabolites and to link these loci to crop phenotypes, to understand the mechanisms underlying the inheritance of important traits, and to understand biochemical pathways and global relationships among metabolic systems. Elucidation of the regulatory networks involved in the activation/repression of key genes related to metabolic phenotypes in response to determined abiotic stress is becoming possible. Transcription factors (TFs) are central player in the signal transduction network, connecting the processes of stress signal sensing and expression of stress-responsive genes. Thus engineered TFs have emerged as powerful tools to manipulate complex metabolic pathways in plants and generate more robust metabolic phenotypes [107, 108].

Metabolic networks are highly dynamic, and changes with time are influenced by stress severity, plant developmental stage, and cellular compartmentalization. Since metabolic profiling only reveals the steady-state level of metabolites, detailed kinetics and flux analyses will support a better understanding of metabolic fluctuations in response to stress [109]. Genome-scale models (GSM) are *in silico* metabolic flux models derived from genome annotation that contain stoichiometry of all known metabolic reactions of an organism of interest. Construction of detailed GSMs applied to plant metabolism will provide information about distribution of metabolic fluxes at a specific genotype, a determined developmental stage, or a particular environmental condition. This detailed knowledge of the metabolic and physiological status of the cell can be used to design rational metabolic engineering strategies and to predict required genetic modifications to obtain a desired metabolic phenotype such as optimized biomass production, increased accumulation of a valuable metabolite, accumulation of a metabolite of response towards abiotic stress, or modification of metabolic flux through a specific pathway of significance [110]. Recently advances have been made in this

field. For example, in rice, by using four complementary analytical platforms based on high-coverage metabolomics, molecular backgrounds of quality traits and metabolite profiles were correlated with overall population structure and genetic diversity, demonstrating that quality traits could be predicted from the metabolome composition, and that traits can be linked with metabolomics data. Results like these are opening the doors to modern plant breeding programs [111].

Once a metabotype (metabolic phenotype) is confirmed to strengthen the tolerance to a particular abiotic stressor, the next challenge will be the transfer of this metabolic trait to a non-adapted plant species of interest. Engineering of more tolerant plants will then require the efficient integration and expression of one to several transgenes in order to modify an existent metabolic pathway or reconstruct a new complete one. Development and optimization of protocols for robust transformation of nucleus, mitochondria, and chloroplasts must be made available for higher plants including economically important crops; this will open new opportunities for plant metabolic engineering [112]. Future research progress on these topics will lead to novel strategies for plant breeding and elevating the health and performance of crops under adverse growth conditions to keep up with the ever-increasing needs for food and feed worldwide.

10. Conclusions

Metabolomics is the comprehensive and quantitative analysis of the entirety of small molecules present in an organism that can be regarded as the ultimate expression of its genotype in response to environmental changes, often characterized by several simultaneous abiotic and biotic stresses. Results obtained from a number of metabolomic studies in plants in response to different abiotic stresses have shown detailed relevant information about chemical composition, including specific osmoprotectants, directly related to physiological and biochemical changes, and have shed light on how these changes reflect the plant phenotype. Metabolomic studies are impacting both basic and applied research. Metabolomic studies will generate knowledge regarding how plant metabolism is differentially adjusted in relation to a specific stress and whether metabolic adjustments are stress specific or common to different types of stress. These studies will also reveal how metabolic pathways coordinate their fluxes and enzymes activities in order to strength their cellular energy requirements under stressing conditions. In an applied context, metabolomic approaches are providing a broader, deeper, and an integral perspective of metabolic profiles in the acclimation plant response to stressing environments. This information will reveal metabotypes with potential to be transferred to sensitive, economically important crops and will allow design of strategies to improve the adaptation of plants towards adverse conditions. Ultimately, design strategies will consider plant metabolism as a whole set of interconnected biochemical networks and not as sections of reactions that lead to the accumulation of a final metabolite. The task is challenging as it must take into account that reactions to stress course through a complex metabolic response, including different systematic mechanisms, time-course changes, and stress-dose dependences. Moreover, there are differences among plant tissues, and, as expected, marked differences

between plants at the genus and species levels, exposing intimate correlation with genetic backgrounds. Nevertheless, the application of more advanced metabolomics tools will lead to new knowledge that will accelerate the design and the improvement of plant breeding projects, that surely will lead to the next generation of crops for specific applications in particular circumstances to cope with abiotic and biotic stress on agricultural crops worldwide.

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Chapter 3

Abiotic Stress Responses in Plants: Unraveling the Complexity of Genes and Networks to Survive

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Additional information is available at the end of the chapter

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1. Introduction

Plants are often subjected to unfavorable environmental conditions – abiotic factors, causing abiotic stresses - that play a major role in determining productivity of crop yields [1] but also the differential distribution of the plants species across different types of environment [2]. Some examples of abiotic stresses that a plant may face include decreased water availability, extreme temperatures (heating or freezing), decreased availability of soil nutrients and/or excess of toxic ions, excess of light and increased hardness of drying soil that hamper roots growth [3]. The ability of plants to adapt and/or acclimate to different environments is directly or indirectly related with the plasticity and resilience of photosynthesis, in combination with other processes, determining plant growth and development, namely reproduction [4]. A remarkable feature of plant adaptation to abiotic stresses is the activation of multiple responses involving complex gene interactions and crosstalk with many molecular pathways [5, 6].

Abiotic stresses elicit complex cellular responses that have been elucidated by progresses made in exploring and understanding plant abiotic responses at the whole-plant, physiological, biochemical, cellular and molecular levels [7]. One of the biggest challenges to modern sustainable agriculture development is to obtain new knowledge that should allow breeding and engineering plants with new and desired agronomical traits [8]. The creation of stress-tolerant crop either by genetic engineering or through conventional breeding covered almost all aspects of plant science, and is pursued by both public and private sector researchers [9].

During the last decade, our research groups have focused their research on elucidating the different components and molecular players underlying abiotic stress responses of a broad range of species both model and crops plant. Several attempts to engineer those species with improved abiotic stress traits (drought and salinity) were made and the response of genetically engineered plants was deeply studied after establishment of adequate physiological methods. Now, we are moving efforts to expand our knowledge on plants response to abiotic stresses using holistic System Biology approaches, taking advantage of available high throughput tools such as transcriptomics, proteomics and metabolomics.

The aim of this chapter is to provide a general overview of the main studies made and how the different expertises of our team were pooled to improve our understanding of the biology of abiotic stress responses in plants. We present some details about the main results and perspectives regarding other possible approaches to develop plants better adapted to face the environmental constraints.

2. Physiological mechanisms underlying abiotic stress responses

Stress is a concept imported from physics. It was introduced in the theory of elasticity as the amount of force for a given unit area [10]. In a biological context, stress is usually defined as an external factor that exerts a disadvantageous influence on the plant [11]. Alternatively, stress could be defined as a significant deviation of the optimal condition of life [12].

2.1. Physiological responses to early abiotic stress: Functional decline in the alarm phase — The stress reaction

Three main phases may be considered on plant stress events and responses: i) the phase of alarm; ii) the phase of resistance; and iii) the phase of exhaustion [12]. Lichtenthaler [13] added a fourth phase, the regeneration phase, which occurs only when the stressor is removed before damage being too severe, allowing partial or full regeneration of the physiological functions. The alarm phase starts with the so-called stress reaction, characterized by functional declines due to the stressor factor, offset by restitution counter reactions, in the transition to the phase of resistance. Stressors rarely act separately and individually on a plant. Generally, several stress factors act simultaneously, such as the frequently combined, at sunny, warm and dry summer periods, heat, water and high-light stress [14].

Sensing is the very first event experienced by a plant when one or more environmental factors (biotic or abiotic) depart from their optimum. Stress sensing is a complex issue and there is not a single sensing mechanism common to all stresses. For instance, some stresses directly affect the underground parts of plant bodies (e.g. drought, flooding) whereas other stresses (e.g., photoinbition) affect directly the aboveground structures of plant bodies. It is, thereby, expected that different sensing mechanisms will be involved. The most common model of sensing external stimuli is that of a chemical ligand binding to a specific receptor [15]. This model, however, is suitable only for chemical stresses (e.g., heavy metal stress, nutrient depletion stress), not for physical stresses: primary sensing of temperature stress (heat stress

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or chilling / freezing) do not involve any chemical ligand. The same applies to radiation stress, although in this case an analogy between "ligand – receptor" and "photon – receptor" could be made. Even when molecules are involved, the universal character of the ligand - receptor model is debatable. In fact, in what concerns the rooting system, it is unclear if cells can sense the water concentration in the soil [16]. In contrast, experimental evidences point to the possibility of sensing cell water homeostasis. The isolation of a transmembrane hybride-type histidine kinase from *Arabidopsis thaliana* provides experimental evidence for osmosensors in higher plants [17]. Also sugars generated by photosynthesis and carbon metabolism in source and sink tissues play an important role in sensing and signaling, modulating growth, development, and stress responses [18].

Following sensing, one or more signaling and signaling transduction cascades are activated, preparing restitution counter reactions which will lead to the phase of resistance to stress. Meanwhile, functional declines are generally observed, including the photosynthetic performance, transport or accumulation of metabolites and/or uptake and translocation of ions, as described later in section 2.3. If these declines are not counteracted, acute damage and death may occur. The importance of restitution counter reactions is highlighted in experiments where different rates of stress imposition are compared: a more pronounced decline of physiological functions (photosynthesis, photosynthetic capacity and electron transport rate) was observed when higher plants were rapidly dehydrated than when the rate of water loss was slower [19]. In desiccation resistant bryophytes there is a threshold of water loss rate behind which no physiological restoration is observed [20]. Increased damage with more rapidly imposed stress is due, at least in part, to increased production of active oxygen species (AOS) [21]. Significant differences in the physiological behavior between the phase of alarm and the phase of resistance were highlighted by Marques da Silva and Arrabaça in [22], who found in the C4 grass *Setaria sphacelata* a decrease on the activity of the enzyme phosphoenolpyruvate carboxylase after several days of water stress, in sharp contrast with the several-fold increase of its activity observed after a short period of acute stress.

2.2. Common and distinctive features of salinity, cold and drought stress

Salinity, cold and drought stress are all osmotic stresses: they cause a primary loss of cell water, and, therefore, a decrease of cell osmotic potential. However, the elicitor of cell water loss differs between stresses: i) salinity stress decreases cell water content due to the decrease of external water potential, caused by the increased ion concentration (mainly Na^+ and Cl^-), turning more difficult water uptake by roots and water translocation to metabolically active cells; ii) cold stress decreases cell water content due to the so-called physiological drought, i.e., the inability to transport the water available at the soil to the living cells, mainly the ones of the leaf mesophyll; iii) the decrease of the cell water content under drought stress is due to water shortage in soil or/and in the atmosphere. Anyway, dehydration triggers the biosynthesis of the phytohormone abscisic acid (ABA) and it has been known for a long time that a significant set of genes, induced by drought, salt, and cold stresses, are also activated by ABA [23].

As a consequence of water loss and decreased cell volume, cell sap solute concentrations increase and thereby cell osmotic potential decreases. As cell turgor also decreases, an early effect common to these stresses is a sharp decrease in leaf expansion rate and overall plant growth rate. Furthermore, an additional active decrease of the cell sap osmotic potential is observed, as an attempt to keep cell hydration. In fact, at the metabolic level, a common feature to these three stresses is the osmotic adjustment by synthesis of low-molecular weight osmolytes (carbohydrates [24], betain [25] and proline [26]) that can counteract cellular dehydration and turgor loss [27]. On the other hand, differences between these stresses do also exist. While drought stress is mainly osmotic, ion toxicity, namely Na^+, is a distinctive feature of salinity stress. Cold stress, behinds physiological drought, has an impact on the rate of most biochemical reactions, including photosynthetic carbon metabolism reactions, as enzyme activities are extremely temperature-dependent. Also water stress and salinity stress decrease photosynthesis, which create conditions to increased photoinhibition, particularly under high irradiances.

2.3. Plant bioenergetics as a core to stress sensor

Despite the different physiological responses to early abiotic stress discussed previously, a common point observed is the changes in the plant bioenergetic status. Such changes may involve a decrease in the energy production and/or an increase in energy demand to overcome the stress. The bioenergetics status is often considered as the chemical energy provided by adenylate energy charge (AEC), as defined in [28], for which plants are mainly dependent on photosynthesis.

The effect of abiotic stresses on photosynthesis can be perceptible: i) within the photochemical reactions in the tylakoid membrane; ii) in the carbon reduction cycle in the stroma; iii) in the carbohydrate use in the cytosol and; iv) on the CO_2 supply to the chloroplast dependent of stomata, mesophyll and chloroplast conductance (reviewed by [29,30]). ATP and NADPH resulting from photochemical reactions are used in all others processes except CO_2 supply to the chloroplast in C3 plants, so any limitation in photosynthesis such as those imposed by drought, can alter the plant bioenergetics status [31].

When the ATP and NADPH production by photochemical processes exceed the capacity for utilization in CO_2 fixation, plants can use several processes to dissipate energy and avoid or minimised photoinhibition (see 2.4). These processes include alternative electron sinks dependent of O_2 such as the oxygenase reaction catalised by ribulose-1,5-bisphosphate carboxylase/oxigenase (Rubisco, E. C. 4.1.1.39) which initiates photorespiration [32]. The light-dependent O_2 uptake by photorespiration not only use ATP and reducing power from photosynthetic electron transport system but also cause a loss of the CO_2 fixed by Calvin cycle. Even in plants under no photoinhibitory conditions, photorespiration occur due to the capacity of Rubisco to catalise the carboxylation and oxygenation of ribulose-1,5-bisphosphate, depending on the CO_2/ O_2 ratio. At 25 ºC, photorespiration increases the cost of carbon (C) fixation to 4.75 ATP and 3.5 NADPH per C fixed under atmospheric CO_2 and O_2 Concentrations, which compares to 3 ATP and 2 NADPH per C fixed under no photorespiration conditions, e. g. only 2% O_2 instead of atmospheric 21% O_2 [33]. In plants submitted to drought, a reduction

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of photosynthesis and photorespiration is observed as a result of the lower CO_2 and O_2 availability in the chloroplast. However, in this situation, the photorespiratory pathway is less decreased than photosynthesis, as firstly suggested Lawlor and co-workers [34, 35]. In fact, despite the much higher affinity of Rubisco for CO_2 than O_2, the CO_2 concentration is almost at the sub saturating level in C3 plants. Thus any decrease in stomatal conductance or in the gases solubility limits the carboxylase activity while the oxigenase activity is unaffected or less affected [36, 37]. In C4 plants, the higher CO2 concentration at the Rubisco level allows a lower decrease in the photosynthesis / photorespiration ratio under water deficit [38] than the one observed in C3 plants, despite the C4 pathway having per se specific energy costs. The less efficient light use for CO_2 fixation caused by photorespiration lowers the quantum yields of photosynthesis in C3 plants under drought [39] or high temperature but this was not observed in C4 plants [40]. Since photorespiration is the major cause of a lower bioenergetic balance in photosynthetic tissues of C3 plants, increasing plant growth by overcoming the limitation of photosynthesis imposed by Rubisco is still an important target of research and plant improvement [41-46].

In C3 and C4 plants under water deficit, the photosynthetic rate decreases with the leaf relative water content and water potential [47-52]. This decrease is frequently correlated to the impairment of photochemical processes in C3 plants [53, 54], including inhibition of ATP synthesis [55-56]. It is still unclear if photosynthesis is primarily limited by water deficit through the restriction of CO_2 supply to metabolism (stomatal limitation) [47] or by the impairment of other processes which decrease the potential rate of photosynthesis (non-stomatal limitation). Nevertheless research efforts on these subjects are relevant to improve plants responses to stress [56].

Biochemical modeling of leaf photosynthesis in C3 and C4 plants [57-61] can provide useful insights into the evaluation of stomatal and non-stomatal limitations of photosynthesis, as previously shown in drought stressed *Medicago truncatula* plants [52] and *Paspalum dilatatum* plants under water deficit [38], elevated CO2 [62] and dark chilling [63]. Photosynthesis light curves allow the determination of the relative contribution of respiration, photosynthesis and photorespiration to the light energy dissipation [64]. Additionally, they are an expeditious method to screen plants with improved resistance to water deficit, as also shown with *M.truncatula* transgenic lines [39].

The role of plant mitochondria in the bioenergetic balance is complex and involves cytocrome *c* oxidase but also several other processes such as alternative dehydrogenases and alternative oxidase that are independent of the adenylate control [65]. An increase in leaf respiratory energy demand to overcome the drought stress *via* respiration was referred in leaves in few studies [66-69]. More often, in drought plants, no change or a decrease in respiration is observed in leaves but the variations were always minor comparing to photosynthesis, despite the interdependence of the two processes through photorespiration [70]. However, at the whole-plant level, the contribution of respiration to the plant bioenergetics status is relevant because respiration can account for a release of 30-70% of the C fixed daily in well-watered plants, whereas in drought plants the proportion of C lost increases, mainly due to the decrease observed on photosynthesis [69-73].

2.4. Stress interaction: Photoinhibition as a case study

Photoinhibition, the decrease of photosynthesis and/or photosynthetic capacity due to exposure to excess photosynthetically active radiation, is dependent not only on the radiation level but also on the level of metabolic activity.

Thereby, all stresses that decreased energy demand increased photoinhibition. In fact, photoinhibition occurs when the demand from the carbon reduction cycle for ATP and, mainly, reductive power is decreased and, thereby, not enough $NADP^+$ is available to act as the terminal electron acceptor of the linear photosynthetic electron transport chain. In these circumstances, the photosynthetic electron transport chain becomes over-reduced and AOS such as hydroxyl radicals, the superoxide anion and hydrogen peroxide are formed [74], causing oxidative damage to the components of the photochemical apparatus. It is well established that the main target of oxidative damage is the D1 protein and that photoinhibition occurs when the accumulation of photooxidized D1 surpasses its *de novo* synthesis [75]. Plants developed several mechanisms to cope with high irradiance and avoid photoinhibition. These range from the anatomical to the molecular level. Paraheliotopic leaf movements [76] or leaf nastic growth [77], allowing the vertical orientation of leaves, optimizes the leaf to irradiation angle in order to decrease energy load and prevent photoinhibition. Leaf chloroplast movements, to minimize exposition to high irradiation [78] or to fulfill auto-shading, represents another example of strategies to avoid or minimize photoinhibition.

At the molecular level, non-photochemical quenching of chlorophyll fluorescence regulates energy dissipation at the primary photosynthetic reactions and therefore constitutes the first protection line against photodamage. This dissipative pathway is controlled by the thylakoid lumen pH and the xanthophyll cycle [79] which increases the dissipation of excitation energy by inducing an enzymatic conversion of the carotenoid violaxanthin into antheraxanthin and zeaxanthin. Additionally, a second line of defense is provided by alternative electron cycling such as photorespiration. When photooxidation cannot be avoided, damage in the photosynthetic apparatus occurs, especially in PSII, where the reaction center D1/D2 heterodimer is the main site to be affected, mainly D1 while D2 is affected in a lesser extent [80]. The repair of damaged components is then activated, as D1 has a high turnover rate. However, if the rate of repair fails to keep pace with the rate of damage, photosynthesis is decreased and photoinhibition occurs [75]. Nuclear-encoded early-light inducible proteins (ELIPs) may play a relevant role in the protection mechanism discussed above [81] and it will be addressed in a subsequent 4.3 section of this chapter.

2.5. Stress and plant life-cycle: The case of drought stress

It is well known that drought stress at the early stages of plant life, shortly after germination, may have devastating impacts as both the root system is not yet fully established, in one hand, and stomatal control is not yet fine tuned. However, drought stress at this early life stage did not attract much research attention, because it is easily overcome by farmers through an accurate choice of seedling dates. Drought stress at later phenological stages received most attention, particularly the comparison between drought effects on the vegetative phases and in the reproductive phases over grain production. It is now well established that the effects of

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stress may vary significantly with the phenological stage of plants. Reproductive stages are generally more sensible to stress than vegetative ones, but differences can also be made between different phases of the reproductive stage. Mouhouche *et al.* [82] found in *Phaseolus vulgaris* that periods of flowering were more sensitive than pod elongation and grain filling phases. Casanovas *et al.* [83] reported a decrease of both leaf physiology and grain yield in maize subjected to drought during flowering. Boonjung and Fukai [84] reported that when drought occurred during vegetative stages, it had only a small effect on subsequent development and grain yield. The effect of water stress on yield was most severe when drought occurred during panicle development.

Grapevine provides an interesting example of the complexity of the relationships between drought stress and plant phenology. Traditionally, grapevine is a non-irrigated crop that occupies extensive areas in dry lands and semi-arid regions [85]. Recently, in the Mediterranean region, irrigation was introduced to increase the low land yield. However, wine quality is strongly dependent on the organoleptic characteristics of grapes which, in turn, particularly in what concerns soluble sugar contents, are dependent on moderate drought stress during berry expansion (i.e. in the phases from fruit set to *veraison*). The irrigation strategy must therefore maximize the vineyard production without decreasing berry quality, an objective suitable for deficit irrigation programs (DRI).

Furthermore, a deep understanding of plant carbon assimilation and partitioning mechanisms under different water regimes will be required in the frame of precision agriculture, as, in fact, these mechanisms play a key role in the fine tuning of the balance between berry yield and quality. Hopefully, this will lead to the adoption of criteria for irrigation scheduling based on vine physiology [85].

3. Gene expression and regulation under abiotic stress

3.1. Complexity of gene expression and regulation

Plants have evolved intricate mechanisms at multiple levels that increase tolerance in order to adapt to adverse conditions and to an ever sessile living. Plant growth and productivity are affected to a great extent by environmental stresses such as drought, high salinity, and low temperature. Expression of a variety of genes is induced by these stresses in various plants. The products of these genes impact not only stress tolerance but also in stress response.

Genes induced during stress conditions function not only in protecting cells from stress by producing important metabolic proteins, but also in regulating genes for signal transduction in the stress response. The first group includes proteins that probably function in stress tolerance, such as chaperones or late embryogenesis abundant (LEA) proteins. The second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response [86]. In some cases networks and cascades of expression are activated in response to a stress condition. The regulation of the expression of these networks is being studied during the last decades.

The use of microarray approaches, and more recently, of the Next Generation Sequencing (NGS) methodologies have unveiled new regulatory mechanisms that complicate the understanding and most of all the possibilities to modulate and control these processes in view of improving plant responses and productivity.

The regulation of plant genes can be observed at three levels: transcriptional; post-transcriptional and post-translational. In each level, actions depend on specific molecular elements as well as molecular networks and cascades.

The transcriptional regulation involves the interplay of three major elements: chromatin and its modification and remodeling; cis-regulatory elements which are often binding sites, such as enhancers and promoters, located upstream and downstream the coding region; and trans-regulatory elements, usually transcription factors. Chromatin modification and remodeling involved in plant abiotic stress response have been observed in numerous situations [87]. The sensitization of stress responsiveness is called priming [88, 89]. Priming boosts the plant's defensive capacity and brings it into an alarmed state of defense. Recently, priming was correlated with chromatin modification of promoter region of WRKY transcription factors [90]. The involvement of epigenetic mechanisms in the response to environmental cues and to different types of abiotic stresses has been documented [91,92]. Recent reports have shown that different environmental stresses lead to altered methylation status of DNA as well as modifications of nucleosomal histones.

Promoters are regulatory regions of DNA located upstream of genes that bind transcription factor IID (TFIID) and allow the subsequent coordination of components of the transcription initiation complex, facilitating recruitment of RNA polymerase II and initiation of transcription [93].

Members of dehydration-responsive element-binding (DREB) or C-repeat binding factor (CBF), MYB, basic-leucine zipper (bZIP), and zinc-finger families have been well characterized with roles in the regulation of plant defense and stress responses. Most of these transcription factors (TFs) regulate their target gene expression through binding to the cognate cis-elements in the promoters of the stress-related genes [94]. More recently the WRKY transcription factors are becoming one of the best-characterized classes of plant transcription factors [95]. Several WRKY proteins were shown to be involved in plant drought and salinity stress responses [96]. For example, overexpression of the *Oryza sativa* WRKY11 under the control of Heat Shock Protein 101 (HSP101) promoter led to enhanced drought tolerance [97]. Similarly, the altered salt and drought tolerance of 35S:OsWRK45 and 35S:OsWRK72 Arabidopsis plants may be attributed to induction of ABA/stress-related genes [98,99].

NAC (N-acetylcysteine) proteins are plant-specific TFs which have been shown to function in relation to plant development and also for abiotic and/or biotic stress responses. The cDNA encoding a NAC protein was first reported as the RESPONSIVE TO DEHYDRATION 26 (*RD*26) gene in Arabidopsis [100]. For example OsNAC6 expression is induced by cold, drought, high salinity, and ABA [101]. OsNAC6 showed high sequence similarity to the Arabidopsis stress-responsive NAC proteins ANAC019, ANAC055, and ANAC072 (RD26). It seems that abiotic stress-responsive NAC-type transcription factors, especially the SNAC

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group genes, have important roles for the control of tolerance against environmental stresses such as drought [102].

Post-transcriptional regulation is a second level of gene expression modulation which is represented by four groups of processes: pre-messenger (mRNA) processing (capping, splicing, and polyadenylation), mRNA nucleocytoplasmic trafficking, mRNA turn-over and stability, and mRNA translation [103].

Alternative splicing is widely known to regulate gene expression in plants subjected to low and high temperatures [104]. For example, it was shown that *STABILIZED1* (*STA1*), a gene coding for a nuclear pre-mRNA splicing factor is important under cold stress conditions in *A. thaliana* [105]. Alternative splicing has been reported upon water deficit as well [106].

Since the early 2000's, several reports have associated small RNAs to abiotic stress responses, showing that post-transcriptional regulation of gene expression plays an important role in these phenomena [107]. Small RNAs (20 to 25 nt) are processed from non-coding double-stranded RNA precursors by RNAses of the DICER-LIKE (DCL) family and mediate a series of gene silencing mechanisms. One of these mechanisms cleaves mRNAs or prevents their translation through the mediation of 21 nt microRNAs. The discovery that stress can regulate microRNA (miRNA) levels, coupled with the identification of stress-associated genes as miRNA targets provided clues about the role of miRNAs in stress responses. Functional analyses have demonstrated that several plant miRNAs play vital roles in plant resistance to abiotic stresses [108-110]. Their role in abiotic stress responses will be further addressed in section 3.2.

Messenger RNA translation is dependent on mRNA cytoplasmic cycling [111] namely compartmentalization in P bodies and association to ribosomes. The amount of mRNAs in polysomes is generally reduced during exposure to dehydration or anoxia, while stress-induced mRNAs significantly increase in polysome association [112]. In chloroplasts, RNA binding proteins and several nucleases have been described to adjust the relative half-life of their mRNAs in response to environmental cues, particularly light conditions [113].

At the post-translational level phosphorylation, sumoylation and ubiquitination of proteins are processes that play major roles in the modulation of plant response to abiotic stress. Phosphorylation and de-phosphorylation play major roles in the responses to abiotic stress. Several signal transduction cascades formed by mitogen-activated protein kinases (MAPKs) and SNF-1-related protein kinases (SnRKs) are activated upon water deprivation and osmotic stress through the phosphorylation of specific residues [114]. Among these, SnRK2 proteins have been shown to be involved in ABA-dependent responses to water deficit, like stomata closure [115].

The up-regulation of the *XERICO* gene, encoding a H2-type zinc-finger E3 ubiquitin ligase, results in increased drought tolerance due to an enhanced ABA induced stomatal closure [116]. *XERICO* controls the level of ABA by enhancing the transcription of the key ABA biosynthetic gene *AtNCED*3. The findings indicate that the protein degradation mediated by the ubiquitin/proteasome pathway plays a fundamental role in ABA homeostasis and response [112].

Sumoylation was also reported to participate in responses to phosphate starvation, and to the tolerance to low and high temperatures [117]. An increase in the levels of SUMO-protein conjugates was also detected in water-deprived plants [118].

The concerted actions of the transcriptional, post-transcriptional and post-translational mechanisms ensures temporally and spatially appropriate patterns of downstream gene expression and ultimately the shaping of transcriptome and proteome of stress-exposed plants to switch on adaptive response. The complete understanding of the interplay of these three regulatory systems is crucial for the understanding of the molecular mechanisms governing plant adaptation to environment as well as for plant improvement for stress tolerance.

3.2. miRNAs in plant responses to abiotic stress — An additional post-transcriptional regulation layer may apply

Plant responses to abiotic stress such as water deficit involve an intricate regulation of gene expression at the transcriptional and post-transcriptional levels. MicroRNAs (miRNAs) are a class of small non-coding RNAs molecules (21-24 nt) involved in post-transcriptional regulation of gene expression. miRNAs were shown to be involved in plant development [119-124], biotic [125, 126] and abiotic stress responses [108, 110, 127-130].

In plants, microRNAs repress gene expression by directing mRNA degradation or translational arrest: miRNAs guide Argonaute (AGO) proteins to bind to matching target mRNAs in a RNA-induced silencing complex (RISC), promoting cleavage of mRNAs with near perfect base complementarity and/or inhibiting translation of those with lower complementarity [131-133].

The first reports assigning miRNAs to have a role in shaping plant responses to abiotic stresses were based on small RNA cloning and sequencing [134], complemented with analyses of miRNA expression profiles and miRNA target prediction [108]. Since then, the application of high-throughput sequencing technology and genomic approaches like microarray analyses to evaluate the profile of miRNA expression in various tissues and conditions, associated to improved bioinformatic tools to identify miRNAs and their targets, have allowed an extensive recognition of stress-responsive small RNAs and their targets in various plant species (reviewed in [107]).

Sequencing of miRNAs in Legumes was first reported in *Medicago truncatula* [135] and *Glycine max* [136] but there are references to small RNAs in other Legumes back to 2004, with a size population of small RNA molecules being identified in the phloem sap of *Lupinus albus* [137]. These findings were the basis of a systemic signalling mechanism in which small RNAs movement is facilitated by chaperone proteins to exert their action at a distance.

One of the most extensively studied miRNAs in the context of abiotic stresses have been the miRNAs involved in nutrient deprivation miR395, miR398 and mir399, all identified in the phloem sap of nutrient deprived plants. In fact, studies in Arabidopsis have established that miR395 (sulphate), miR399 (phosphate) and miR398 (copper) regulate these nutrients homeostasis by moving along the phloem to inform the roots of the nutrient status of the shoot [138-139].

The miRNA395 gene-family targets genes involved in sulphate translocation (the low-affinity transporter *SULTR2;1*) and assimilation (the ATP sulphurylases, *APS*) [134, 140,141]. Importantly, miR395 itself is regulated by a transcription factor, the SULFUR LIMITATION 1 (SLIM1) [141]. The miR395/APS-SULTR2;1/SLIM1 regulatory module is involved in root-to-shoot sulphate translocation as a strategy to improve sulphate assimilation in the leaves during sulphate starvation [142].

The miR399 gene-family is strongly and specifically induced by inorganic phosphate limitation in the shoot and targets PHO2, an E2 ubiquitin-conjugating enzyme that represses Pi uptake [109, 140,143-144]. As for miR395, also the expression of miR399 is regulated by a transcription factor, the MYB TF PHOSPHATE STARVATION RESPONSIVE1 (PHR1; [109]). The miR399/PHO2/PHR1 regulatory module operates under Pi deprivation: miR399 is induced by PHR1 in the leaves, travels along the phloem to repress PHO2 expression in the roots thereby releasing several protein targets from ubiquitinylation-dependent degradation, including transporters involved in Pi allocation inside the plants and increasing Pi content in the shoot. A worth mentioning aspect of the miR399 regulatory module is the extra layer of miR399 activity regulation exerted by IPS1 (induced by phosphate starvation1) [145]. IPS1 is a non-protein coding transcript with sequence complementarity to miR399 that sequesters miR399 thus inhibiting its repressing activity over its target. This mechanism designated as target mimicry was first described in plants [145] and more recently discovered in animals [146] and expands the regulatory post-transcriptional gene expression network in which miRNAs are involved.

The miR398 (and miR408) are induced by copper limitation and target genes encondig copper proteins like Copper/Zinc superoxide dismutases, cytochrome *c* oxidase and plantacyanin [147, 148]. Similar to miR395 and miR399, also miR398 and miR408 are regulated by a transcription factor, the SQUAMOSA promoter binding protein–like7 (SPL7) that regulates the expression of several copper-responsive genes [149]. Copper in contrast to sulphate and phosphate is a micronutrient but still the regulation of this nutrient homeostasis is basically similar, as it involves sistemic signalling, a well established regulatory module involving a transcription factor, the miRNA and its target.

The miR395, miR399 miR398 and miR408 were identified in *M. truncatula* by sequencing libraries of small RNAs from the aerial part [135]. Homologs of known miRNA target genes were identified, such as low affinity sulphur transporter for miR395, COX5b (subunit 5b of mitochondrial cytochrome c oxidase) for miR398, PHO2 for miR399 or plantacyanin for miR408. However, our computational prediction identified many hypothetical genes for miRNA targeting ([135] - Additional File 1), rendering experimental confirmation a laborious and unsuccessful task (Trindade, unpublished data).

Some miR398 and miR408 predicted targets were validated by 5'RACE and miR398 and miR408 expression was further investigated in different plant parts and in specific water deficit conditions, showing up-regulation in water deprivation and concomitant down-regulation of their validated targets [129]. These targets were further confirmed by deep sequencing of cleaved miRNA targets (Parallel Analysis of RNA Ends - PARE) [150-151] in *M. truncatula* in

collaboration with the Tamas Dalmay laboratory (School of Biological Sciences, UEA, Norwich, UK) (unpublished data).

Still, the bioinformatic prediction of many hypothetical genes for miRNA targeting raises the question whether we are dealing with true or instead pseudo targets and can have a strong implication on our assumptions about the mechanisms of miRNA functioning as they impose an additional layer of post-transcriptional regulation.

Seitz [152] proposed that many computational identified miRNA targets are indeed pseudo-targets that prevent miRNAs from binding their true targets by sequestering them. They would have the basic features of miRNA targets identified by the target prediction algorithms: complementarity to miRNAs and phylogenetic conservation but are instead modulators of miRNA expression.

These pseudotargets occur naturally in plants [145] and animals [146] and were firstly associated to miRNA regulation of nutrient deprivation but their involvement in other abiotic stress conditions like water deprivation may also be envisaged.

A 5-year EU FP7 project designated "ABStress - Improving the resistance of legume crops to combined abiotic and biotic stress" was recently started [153]. This project will study the small RNAs and epigenetic regulation involved in abiotic and biotic stresses in Legumes using *Medicago truncatula* as a model and it is certainly expected to bring new information about the complex network of regulatory circuitries in which miRNAs participate.

4. Transgenic approaches to improve abiotic stress resistance

The advance in genetic engineering offers new ways to understand the genetic mechanisms of stress-related genes and their contribution to the plant performance under stress [154]. However, while a great degree of success has been obtained in the production of herbicide-, virus- and fungal-resistant plants and plants with fortified nutritional values using transgenic tools, the same has not been the case in production of abiotic stress-tolerant crops [155]. This is largely due to the complex genetic mechanisms that govern abiotic stress tolerance. Additionally, as previously referred, in natural conditions, crops can suffer from different stress combinations, at different development stages and during different time periods.

Recently, several reviews were published concerning genetic engineering for abiotic stress tolerance, most focused in model but also in crop plants (e.g. [156 -161]). Possible targets for genetic engineering towards abiotic stress in plants are genes belonging to structural and regulatory categories. They can be modified (for example truncated) and fused to other genetic components such as signal peptides that direct their expression to specific organelles and/or reporter genes for early detection in transgenic plants. After the proper cloning of the desired genes, they are engineered for their expression to be regulated in a time and space context, using specific promoters. The approach can take into account if it is desirable to have the gene expression upregulated, by sense overexpression of the transgene, or downregulated, by the antisense or RNA interference (RNAi) techniques.

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Presently, numerous genes associated to plant responses to abiotic stress have been identified and characterized in laboratory studies (reviewed in [157, 162-163]). Engineered overexpression of biosynthetic enzymes for osmoprotectants such as glycine betaine [164,165]; stress induced proteins such as LEA proteins [166-167]; scavengers of reactive oxygen species [168,169]; transcription factors [170, 171] or signal transduction components [172-173] were reported. Since stress resistance is a complex trait regulated by several genes acting in a concerted way during the process, it is not surprising that transgenic approaches using a single stress-related gene will only lead to marginal stress improvement [174]. One of the major challenges is the introduction of multiple genes by pyramiding strategies or co-transformation [175-176].

It is also expected that several areas, such as post-transcriptional regulation involving protein modification, protein degradation and RNA metabolism will emerge [163]. An example is the application of miRNAs in the improvement of stress resistance. The discovery of miRNAs involved in the regulation of stress responses and discovering the potential use of these miRNAs to modulate or even increase stress resistance in plants is an open field of research as previously discussed in section 3.2 of this chapter. As an example, Sunkar and co-workers [110] have generated transgenic *Arabidopsis thaliana* plants overexpressing a miR398-resistant form of a plastidic Cu/Zn Super Oxide Dismutase (Cu/Zn-SOD;CSD2) and confirmed that transgenic plants accumulate more CSD2 mRNA than plants overexpressing a regular CSD2 and are consequently much more tolerant to high light, heavy metals, and other oxidative stresses. These results suggest that understanding posttranscriptional gene regulation is important to widen our ability to manipulate stress tolerance in plants and offer an improved strategy to engineer crop plants with enhanced stress tolerance.

The process of generating transgenic lines requires success in the transformation method and proper incorporation of stress resistance genes into plants. The most used method to transfer foreign genes into plant cells and the subsequent regeneration of transgenic plants is based on the natural system, the *Agrobacterium*-mediated plant transformation [177]. Particle bombardment has also been exploited extensively for plant transformation especially in species recalcitrant to *Agrobacterium* infection such as maize. The development of new plant transformation vectors namely using new-plant associated bacteria (such as from the *Rhizobiacea* family) has also proved to be an effective approach to generate transgenic plants from explants/genotypes unsuitable for *Agrobacterium*-mediated transformation methodology [178].

The promoters that have been most commonly employed in the production of abiotic stress-tolerant plants include the cauliflower mosaic virus (CaMV) 35S promoter (mostly used for dicot crops) and the actin 1 promoter (Act-1) (used for expression of transgenes in monocot crops) [155]. As these promoters are constitutive, the downstream transgenes are expressed in all organs and at all stages which is unnecessary as well as demanding on the energy reserves of the cell [170]. In some cases, constitutive expression of a gene normally only induced by stress can have negative effects on growth and development when stress is not present (pleiotropic effects). The use of inducible promoters that allow the expression of a transgene only when it is required could therefore be the ideal solution [179, 180]. There is a strong need to obtain an increased array of inducible promoters, which are expressed only when exposed

to stress situations, and to pair such promoters with the stress tolerance-related genes in the adequate cloning vectors [181]. Additional tests need to be performed to guarantee that obtained stress-inducible promoters work in heterologous plant systems.

Concerning the improvement of stress resistance, the past decade has witnessed the utilization of transgenic approaches for experimental purposes, mainly in model plant systems but not in important agricultural species or crops. Nevertheless, the creation of stress-tolerant crops either by genetic engineering or through conventional breeding has covered almost all aspects of plant science, and is pursued by both public and private sector researchers [161]. One of the major goals of transgenic technology is to produce plants not only able to survive stress, but also capable to grow under adverse conditions with substantial biomass production, thus overcoming the negative correlation between drought resistance traits and productivity, which was often present in past breeding programs [155, 182]. In the case of crop plants, it is ultimately the yield of genetically altered plants under specific field conditions that will determine whether or not a specific gene, or metabolic or signaling pathway, is of technologic importance [3]. One successful case in releasing tolerant plants to abiotic stresses is the transgenic maize line resistant to drought developed by the Monsanto company. This maize line (MON87460) was recently approved in the USA and is able to growth in soils with reduced water content due to the presence of a cold shock protein –CSPB- from *Bacillus subtilus* [183].

During the last decade, our group has engineer model species like tobacco and *Medicago truncatula* with improved abiotic stress traits (drought and salinity), using different stress related genes.

4.1. Engineering trehalose accumulation

Trehalose is a disaccharide, containing two glucose molecules. Trehalose was first discovered in 1832 from the Ergot of rye [184-186] and since then isolated from numerous organisms, including algae, fungi, bacteria, insects and crustaceans. Trehalose is nevertheless considered non-occurring in measurable amounts in plants, with the exception of a few species [184], notably the so called "resurrection plants", able of surviving the loss of most of their water content until a quiescent stage is achieved and upon watering rapidly revive and restored to their former state [187].

Trehalose can be synthesized by three different pathways [188] and the most frequent in nature involves the enzyme trehalose-6-phosphate synthase (TPS; EC 2.4.1.15) that catalyzes the transfer of glucose from UDP-glucose to glucose-6-phosphate to produce trehalose-6-phosphate plus UDP. Another enzyme, trehalose-6-phosphate phosphatase (TPP; EC 3.1.3.12) converts trehalose-6-phosphate to free trehalose [184, 186, 189, 190]. Genes codifying both enzymes have been isolated in several species including *Sacharomyces cerevisiae* and *Escherichia coli* and several plant species such as Arabidopsis and rice [191]. Trehalose may be degraded by the enzyme trehalase (EC 3.2.1.28) [186, 191].

In living organisms, several functional properties have been proposed for trehalose: energy and carbon reserve, protection from dehydration, protection against heat, protection from damage by oxygen radicals and protection from cold [186]. As trehalose, sucrose is one of the

few free disaccharides in nature. Both are non-reducing sugars and synthesized by similar pathways. Contrary to trehalose, sucrose synthesis is mainly limited to photosynthetic organisms [192], where it holds a central position as the major product of photosynthesis and as a transport molecule involved in growth, development, storage, signal transduction and acclimation to environmental stress. Sucrose transport is finally energetically superior to trehalose transport making it more "preferred" to plants metabolism. It is hence often suggested that trehalose is evolutionary more ancient than sucrose [192].

As trehalose is present in so low or in undetectable amounts in most plants, it is unlikely that under natural conditions and with the exception of desiccation tolerant plants, this sugar might play a role in stress protection in plants [193]. Nevertheless, other roles have been proposed for trehalose and trehalose-6-phosphate synthase: regulation of plant growth and development; broad spectrum agent preventing symbiosis between susceptible plants and trehalose producing microorganisms [193-194]; the regulation of carbohydrate metabolism or the perception of carbohydrate availability [190,194-197]; the regulation of embryo maturation [197-199]; implication on vegetative growth and transition to flowering [200]; implication on seedling development [201-202]; and regulation of glucose, abscisic acid and stress signaling [203-205]. According to [190], trehalose plays several roles in carbohydrate metabolism, with a number of processes and pathways being affected.

For all that was stated above, trehalose is one of the most studied osmoprotectants and in recent years there has been a growing interest in trehalose metabolism as a means of engineering stress tolerance in crop plants [191]. Several experiments have been conducted to obtain transgenic plants over-expressing genes codifying enzymes of the trehalose biosynthetic pathway of *E. coli* and *S. cerevisiae*, using both model plants like tobacco (*Nicotiana tabacum*) and crop plants such as potato (*Solanum tuberosum*), rice (*Oryza sativa*) and more recently tomato (*Lycopersum esculentum*). Additional, attempts have been made using an alternative approach: the inhibition of the expression of trehalase gene. Those experiments and their main results are summarized in Table 1.

The previously mentioned genetic engineering obtained a variable degree of success. Generally speaking, transgenic plants were found to have higher tolerance than controls to some form of water stress imposed, following in most cases, confirmed trehalose accumulation. Albeit such fact, trehalose engineered plants frequently had altered phenotypes, particularly dwarfism and leaf abnormalities. Such fact was particularly true for the first transformation events in which genes of microbial origin were used. Later events, in which endogenous or plant origin genes were used seem to counter that tendency [217, 218]. Genetic engineering of plants with trehalose biosynthesis genes seems therefore to be of extreme pertinence to the increase of abiotic stress tolerance in plants, particularly plants of agricultural importance such as cereals and legumes.

4.2. Engineering polyamine accumulation

Polyamines (PAs) are small (low-molecular-weight), positively charged, aliphatic amines that are found in all living organisms. The major forms of PAs are putrescine (Put), spermidine (Spd) and spermine (Spm), although plants also synthesized a variety of other related com-

pounds. Arginine (Arg) and ornithine (Orn) are the precursors of plant PAs. Ornithine decarboxylase (ODC; EC 4.1.1.17) converts Orn directly into Put. The other biosynthetic route to Put, via arginine decarboxylase (ADC; EC 4.1.1.19), involves the production of the intermediate agmatine (Agm) followed by two successive steps catalysed by agmatine iminohydrolase (AIH, EC 3.5.3.12) and *N*-carbamoylputrescine amidohydrolase (CPA, EC 3.5.1.53). In animals and fungi Put is synthesized primarily through the activity of ODC while in plants and bacteria the main pathway involves ADC. Aminopropyl groups, donated by decarboxylated S-adenosyl methionine (dcSAM), must be added to convert Put into Spd and Spm in a reaction catalysed by spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthase (SPMS; EC 2.5.1.22), respectively (reviewed in [220]). Polyamines levels in plants increase under a number of environmental stress conditions, including drought and salinity [221-223]. Several biological roles were proposed for polyamines action in stress situations; PAs could act as osmoprotectants, as scavengers of active oxygen species (AOS) or by stabilizing cellular structures, such as thylakoid membranes [222, 224, 225]. The first reports of transgenic approaches using genes responsible for PA biosynthesis were conducted in two species, tobacco and rice [226-230]. Recently, new insights into the role and regulatory function of polyamines in plant abiotic stress tolerance have been achieved, with several abiotic (salt, drought, freezing, heat) stress tolerant transgenic plants overproducing polyamines being described in the following reviews [220, 231-233].

Among abiotic stresses drought is the main abiotic factor as it affects 26% of arable area [229]. Plants respond to changes in water status by accumulating low molecular-weight osmolytes including PAs. Polyamines may have a primary role of turgor maintenance but they may also be involved in stabilizing proteins and cell structures. The polycationic nature of PAs at physiological pH is believed to mediate their biological activity, since they are able to bind to several negatively charged molecules, such as DNA, membrane phospholipids, pectic polysaccharides and proteins [225].

In respect to the antioxidant activity of PAs, the research data is contradictory; on the one hand, PAs have been suggested to protect cells against AOS and on the other hand, their catabolism generates AOS [232]. PA catabolism produces H_2O_2, a signaling molecule that can act promoting activation of antioxidative defense response upon stress, but can also act as a peroxidation agent. In a recent study, the effect of increased putrescine (Put) accumulation was found to negatively impact the oxidative state of poplar cells in culture due to the enhanced turnover of Put [233]. Gill and Tuteja [234] stated that, while increase Put accumulation may have a protective role against AOS in plants, enhanced Put turnover can actually make them more vulnerable to increased oxidative damage. The higher polyamines, Spd and Spm are believed to be most efficient antioxidants and are considered scavengers of oxyradicals [235].

As plants with elevated putrescine contents are able to tolerate drought stress because Put has a direct protective role in preventing the symptoms of dehydration, higher PAs (Spd and Spm) appear to play an important in role in stress recovery [236]. Recently, transgenic rice plants overexpressing *samdc* (S-Adenosyl methionine decaboxylase gene), with increased Spd and Spm levels, were considered to be non drought tolerant, but showed a more robust recovery

Gene/Promoter	Origin	Plant	Main Effects	Ref.
tps; Rsu- rubisco small unit promoter	Yeast	Tobacco	Increased trehalose levels; Transgenic plants showed less water loss upon leaf detaching.	[206]
*ots*A; *ots*B; CaMV 35S	*E. coli*	Tobacco	Low levels of trehalose in leaves.	[207]
*ots*A; *ots*B; CaMV 35S	*E. coli*	Potato	Absence of trehalose detection.	[207]
tps1; CaMV 35S	Yeast	Tobacco	Higher levels of trehalose; Phenotypic alterations (stunted growth; lancet shaped leaves); Improved drought tolerance.	[208]
*ots*A; *ots*B; CaMV 35S	*E. coli*	Tobacco	Phenotypic alterations (larger leaves and altered stem growth); Higher growth under drought stress.	[209]
*ots*A; CaMV 35S	*E. coli*	Tobacco	Altered phenotypes; Transgenic plants showed less water loss upon leaf detaching.	[210]
*ots*A; *ots*B; Rsu and ABA-inducible promoter	*E. coli*	Rice	Higher trehalose levels; Sustained plant growth; Less photo-oxidative damage Favorable mineral balance under abiotic stress; Stress tolerance.	[211]
*ots*A; *ots*B; Ubi-1 promoter	*E. coli*	Rice	Increased trehalose levels; Absence of phenotypic alterations and altered growth. Tolerance to drought, salt and cold.	[212]
*ots*A; *ots*B; CaMV 35S	*E. coli*	Tobacco	Altered photosynthesis in transgenic plants.	[213]
tps1; CaMV 35S	Yeast	Tomato	Higher trehalose content; Altered phenotypes; Tolerance to drought, salt and oxidative stress.	[214]
tp; CaMV 35S	Pletorus sajor-caju	Tobacco	Higher trehalose content; Unaltered phenotypes; Tolerance to water deficit.	[215]
tre (Antisense); CaMV 35S; Rd29A- osmotic stress inducible	*Medicago sativa*	Tobacco	Reduced trehalase activity in transgenic plants.	[216]
tps; CaMV 35S	*A. thaliana*	Tobacco	Transgenic plants with higher tolerance to several osmotic stresses.	[217]
		M. truncatula	Transgenic lines with higher tolerance to moderate water deficit or ability to recovery from severe water deficit.	[218]
tps; Act-1 promoter	*O. sativa*	Rice	Improved the tolerance of rice seedling to cold, high salinity and drought.	[219]

Table 1. Genetic Engineering of plants towards trehalose accumulation

from drought compared to wild type [236]. The *de novo* synthesis of Spd and Spm in transgenic plants under drought stress, at the expenses of Put, was responsible for the stress tolerance observed in these plants.

The covalent linkage of PAs to proteins appeared to be of extreme importance in plant light-induced stabilization of the photosynthetic complexes and Rubisco therefore exerting a positive effect on photosynthesis and photo-protection. Also in the cytosol, they are involved, mediated by transglutaminase (TGase) activity, in the modification of cytoskeletal proteins and in the cell wall construction/organization [237]. In a recent study, the characterization at the proteomic level of the TGase interaction with thylakoid proteins, demonstrated its association with photosystem II (PSII) protein complexes using maize thylakoid protein extracts [238]. Binding of Put to thylakoid membranes has been proposed to be a photoadaptation response under controlled stress conditions. Campos and collaborators [238] results reinforce the importance of the TGase in photo-protection by polyamine conjugation to light-harvesting complex II (LHCII) proteins.

Recently, PAs were proposed to be components of signaling pathways and fulfill the role of second messengers [220, 231]. Studies with ABA-deficient and ABA-insensitive Arabidopsis mutants with differential abiotic stress adaptations [239] support the conclusion that the up-regulation of PA biosynthetic genes and Put accumulation under water stress are mainly ABA-dependent responses. To reinforce the fact that PAs biosynthesis may be regulated by ABA, several stress-responsive elements, like drought responsive (DRE), low temperature-responsive (LTR) and ABA-responsive elements (ABRE and/or ABRE-related motifs) are present in the promoters of the polyamine biosynthetic genes [239]. Liu *et al.* [240] also found that inward potassium channels were targets for PA regulation of stomatal movements. Since ABA signaling pathway in stomata regulation involves many different components including signaling molecules like AOS, IP_3, Ca^{2+} and nitric oxide (NO), evidences point to an interplay between ABA, polyamines, H_2O_2 and NO in stomata regulation [220].

In our experiments, we transformed the model legume *Medicago truncatula* cv. Jemalong with the arginine decarboxylase gene (*adc*) from *Avena sativa* to overexpress the heterologous ADC enzyme aiming to increase the levels of polyamines in transgenic plants [241, 242]. Several transgenic lines overexpressing This oat adc construct were obtained. The oat *adc* cDNA under the control of a CaMV 35S constitutive promoter was previously transferred into rice plants [228] and those authors found increased Put levels in regenerated plants and observed minimized chlorophyll loss during drought stress. However, constitutive over-expression of this gene severely affected developmental patterns of those plants. Afterwards, the same group used the monocot maize's ubiquitin-1 (Ubi-1) promoter to overexpress the Datura *adc* gene and found that transgenic plants, with increased Put levels, were tolerant to drought stress [230]. The Ubi-1 promoter is known to contain a number of stress-responsive elements that enhance transgene expression under drought stress [230] and hence function as a stress-inducible promoter. Roy and Wu [229] also found that the expression of the *adc* transgene under the control of an ABA-inducible promoter led to stress-induced upregulation of ADC activity and polyamine accumulation in transgenic rice plants. Second-generation transgenic rice plants showed an increase in biomass under salinity–stress conditions.

In our *M. truncatula* system, no altered external morphology was observed in *adc* transgenic plants, that were successfully developed without phenotypic visible alterations and produced seeds (T_2 generation) [241, 242]. One specific transgenic line (L108) expressing the heterologous *adc* transgene had a very high accumulation of Agmatine (22-fold) (the direct product of the ADC enzyme and intermediate in the Put biosynthesis) and moderately related increase of Put (1.7-fold) and Spd (1.9-fold) levels, compared to control plants [242]. These results are consistent with several reports that suggest PAs levels are under strict homeostatic regulation [227, 243].

Nevertheless, several recent studies have concluded on the feasibility of PA biosynthesis engineered for the production of stress-tolerant plants. Accumulating experiments and their main results are summarized in Table 2. The constitutive expression of homologous *adc1* and *adc2* in Arabidopsis resulted in freezing and drought tolerance, respectively [244-245]; with a patent application for "Plant resistance to low-temperature stress and method of production thereof" by [244]. In another work, transgenic tomato lines transformed with the yeast *samdc* fused with a ripening-specific promoter E8, over-accumulate Spd and Spm and, interestingly, showed phenotypes of agronomical importance such as enhanced phytonutrient content and fruit quality [246-247]. Polyamine-accumulating transgenic eggplants exhibited increased tolerance to multiple abiotic stresses (salinity, drought, low and high temperature and heavy-metal) and also biotic resistance against fungal disease caused by *Fusarium oxysporium*. These authors used a construct similar to ours, with the *adc* gene from oat under the control of the constitutive CaMV 35S promoter and found that some transgenic eggplants lines showed an enhanced level of Put, Spd and in some cases also Spd. These lines also showed increase in ADC and also on the activity of the PA catabolic enzyme, diamine oxidase (DAO) [248].

There are several reports in which the plant response to diverse abiotic stress is associated to the stimulation of polyamine oxidation [249]. However, the precise role of polyamine catabolism in the plant response to environmental stress remains elusive [249-250]. Considering these results, further research concerning the PAs changes and the global response of our *M. truncatula* diverse germplasm with altered PA content to multiple stresses should be developed in the near future.

4.3. Engineering accumulation of photo-protective proteins — ELIPs

To cope with environmental stresses, plants activate a large set of genes, which lead to the accumulation of specific stress-associated proteins (reviewed in [253]).The stomatal limitation on photosynthesis imposed by the earlier stages of water deficit (WD) result in a decrease of primary electron acceptors available for photochemistry [47]. If protection mechanisms are not activated, the excess of absorbed energy may induce photo-oxidative damage in chloroplast structures. The nuclear-encoded early-light inducible proteins (ELIPs) may play a relevant role in the protection mechanisms discussed above.

ELIPs and ELIP-like proteins are pigment-binding components of the thylakoid membrane widely distributed among plant species and belong to the chlorophyll a/b-binding protein (cab) family (reviewed in [254, 255]). ELIPs are widely present among different plant species like pea [256], barley [257], *Craterostigma plantagineum* [258], *Dunaliella bardawil* [259], *Sporobolus*

Gene/Promoter	Origin	Plant	Main Effects	Ref.
odc; CaMV 35S	*S. cerevisae*	Tobacco	Increased ODC activity; Increased Put and Nicotine	[251]
samdc; CaMV 35S	Human	Tobacco	Increased SAMDC activity; Spd and Spm levels. Lower Put levels. Thick leaves, stems and stunting.	[252]
adc; Tet- inducible promoter	Oat	Tobacco	Increased ADC activity; Phenotypic alterations pp to Put levels (thin stems and leaves, leaf necrosis, chlorosis, short internodes and growth inhibition)	[226]
adc; CaMV 35S	Oat	Tobacco	Increased ADC activity; ODC and SAMDC normal; Increased Agm; Put, Spd and Spm normal.	[227]
adc; CaMV 35S	Oat	Rice	Increased Put and less chlorophyll loss during drought. Severe altered phenotypes.	[228]
adc; ABA- inducible promoter	Oat	Rice	Increased Put, ADC activity and biomass under salt stress.	[229]
samdc; E8 promoter	Yeast	Tomato	Increased Spd and Spm. Enhanced phytonutrient content and fruit quality	[246, 247]
adc; Ubi-1 promoter	*D. stramonium*	Rice	Higher Put, Spd and Spm levels and drought tolerance	[230]
adc; CaMV 35S	Oat	*M. truncatula*	Increased Agm, Put and Spd levels. Absence of phenotypic alterations and altered growth (second generation homozygous plants).	[241, 242]
adc; CaMV 35S	Oat	Eggplant	Increased Put, Spd and Spm levels; multiple abiotic stress resistance and fungal resistance.	[248]
adc1; *adc2*; CaMV 35S	Arabidopsis	Arabidopsis	Increased Put; freezing and drought tolerance.	[244, 245]

Table 2. Genetic Engineering of plants towards polyamine accumulation

stapfianus [260], *Arabidopsis thaliana* [261], *Tortura ruralis* [262], *Nicotiana tabacum* [263] and recently found in *Coffea canephora* [264].

Contrary to the other members of the *cab* family that are expressed constitutively, ELIPs accumulate transiently during the greening of etiolated plants [265] and in developing plastid membranes [266]. In mature plants, ELIPs also accumulate in response to various stress conditions including ABA or desiccation [258], nutrient starvation [259], high light [267, 268], UV-B [269], cold [270], methyl jasmonate [271], salinity [262] and senescence [263]. ELIPs and

ELIP-like proteins are thought to protect the chloroplast apparatus from photooxidation by: a) acting as transient pigment-binding proteins during biogenesis or turnover of chlorophyll binding proteins [262, 266, 268, 272]; b) binding or stabilising carotenoids like zeaxantin and lutein [266, 268, 273, 274]; c) stabilising the pigment-protein complexes and/or favouring their appropriate assembly [268, 272, 274, 275]; d) dissipating the excessive absorved light energy at the reaction center of the PSII, in the form of heat or fluorescence [276].

We decided to express the *dsp22* gene from *Craterostigma plantagineum* [258] in *M. truncatula*, aiming to investigate the protective role of this ELIP-like protein in the photosynthetic apparatus, during the dehydration and rehydration [81, 241]. We assessed the photochemical performance of in *dsp22* transgenic (A.27) and wild type (M9-10a) plants together with leaf pigment contents and biomass accumulation during dehydration and subsequent recovery. Transgenic *M. truncatula* plants overexpressing the ELIP-like DSP22 protein display higher amount of chlorophyll (Chl), lower Chl *a*/Chl *b* ratio and higher actual efficiency of energy conversion in PSII after dehydration and rehydration, also suggesting a role in pigments stabilization during WD stress [81]. Our results are in agreement with the transient photosynthetic pigment binding function postulated for ELIPs and ELIP-like proteins under disturbing environmental conditions [266, 268]. Additionally, the results indicate that DSP22 may contribute to reduce the impact of photooxidative damage on the PSII complex of *M. truncatula* resulting from WD and recovery treatments. Despite of this assumption, the mechanisms by which DSP22 leads to enhanced photooxidative protection in this model legume are yet not clear and further studies are necessary to support these hypothesis. Nevertheless, the results supports that the expression of photoprotective proteins, such as ELIPs, can be considered a valuable approach to improve abiotic stress resistance in crops.

5. Omics and system biology approaches to understand abiotic stress responses

During the last decade, the "reductionistic" molecular biology and functional biology approaches are being progressively replaced by the "holistic" approach of systems biology. However, molecular biology and systems biology are actually interdependent and complementary ways in which to study and make sense of complex phenomena [277]. Presently, the use and development of post-genome methodologies, such as global analysis of transcriptomes, proteomes and metabolomes integrated in solid bioinformatics platforms, has noticeably changed our knowledge and holistic understanding various plants function, including the response to abiotic stresses [278]. System-based analysis can involve multiple levels of complexity, ranging from single organelles or cells, tissues, organs to whole organisms. These variables can be still combined with multiple developmental stages and environmental interactions suggesting an infinite number of permutations to this complexity [279].

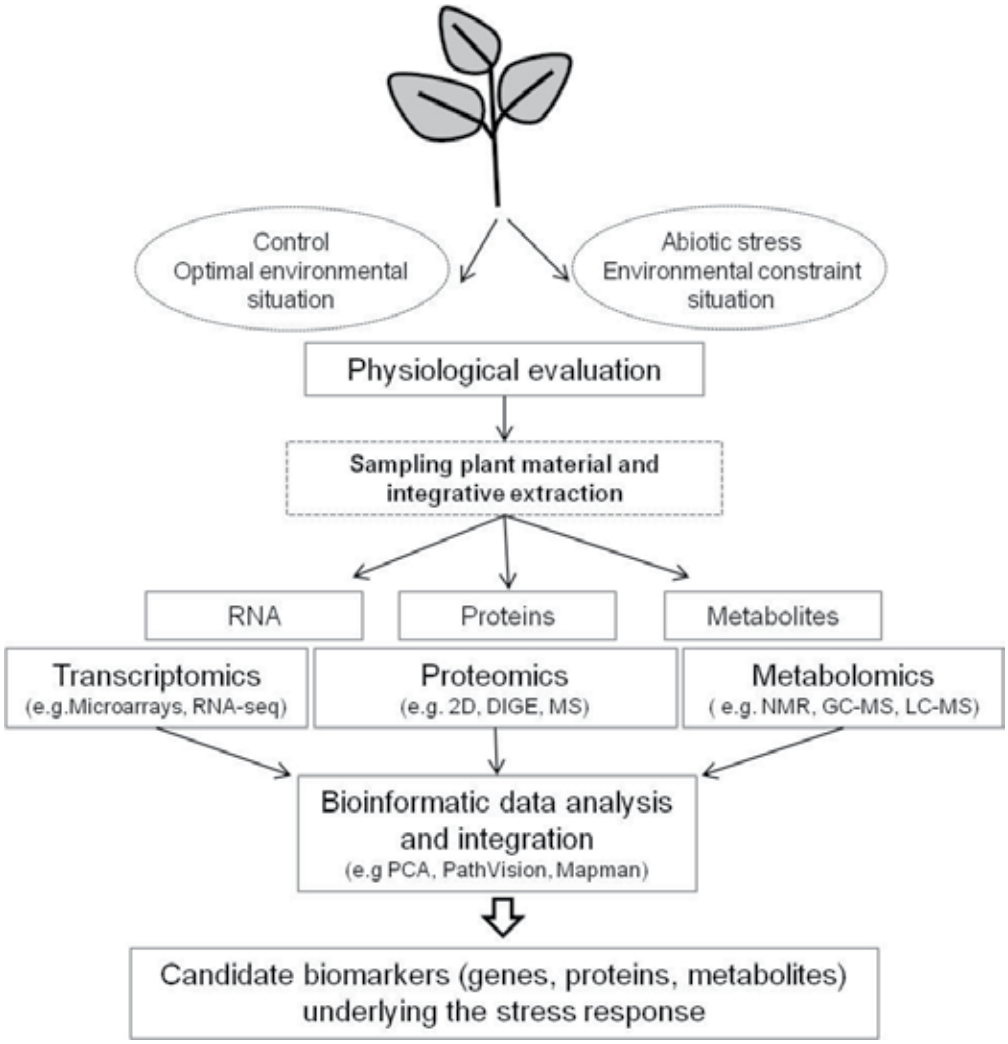

Figure 1. Schematic overview of a common System Biology approach to study abiotic stress responses in plants.

The breakthrough in Omics technologies has led to designing better experiments which provide deep insight into the function of genes and also their effects on phenotypic change in a specific biological context [280]. System biology approaches can circumvent some barriers that had previously blocked the translation of knowledge gained from model plants, like *Arabidopsis thaliana* and *Medicago truncatula*, to other economically important plant species in light of current progress in generating new crop genome sequences and functional resources [279, 281]. It is anticipated that this trend will continue into the next decade in light of current developments in crop functional resources [281] and in view of the exponential number of papers published on abiotic stress studies in plants using a systems biology approaches during the last decade [279].

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Most of the plant system biology approaches relied on three main axes: transcriptomics, proteomics and metabolomics (see Figure 1).

In addition to these previous studies, interaction between DNA-proteins and Proteins-proteins – interactomes - are being also used with success to identify regulatory proteins involved in complex whole plant responses [282]. Bioinformatics has been crucial in every aspect of Omics-based research to manage various types of genome-scale data sets effectively and extract valuable information and facilitate knowledge exchange with other model organisms [278, 283]. A comprehensive list of the analytical bioinformatics platforms available constituting an essential infrastructure for systems analysis can be found in [278].

5.1. Transcriptomics

Transcriptomics, also referred as expression profiling, captures spatial and temporal gene expression within plant tissues or cell populations on a specific biological context (e.g. genotype, growth or environmental condition). In many instances transcriptomic analysis is used to screen for candidate genes for abiotic stress improvement programs [280] or to predict the tentative gene function by the association of differently expressed or co-expressed genes with the plant phenotype alteration [284]. Transcriptomic approaches should incorporate highly specific, sensitive and quantitative measurements over a large dynamic range with a flexibility to identify unanticipated novelties in transcript structures and sequences [285].

Determination of large scale transcript profiles or identification of differentially regulated genes in plants can be performed by various techniques, such as DNA microarrays, serial analysis of gene expression (SAGE) or more recently Digital Gene Expression (DGE) profiling taking advantage of next-generation sequencing (NGS) based tools such as RNA sequencing (RNA-seq) [279, 280, 285]. The hybridization-based method, such as that used in microarray analyses, together with the availability of completed genomes sequences and increasing public repositories of available microarray data and data analysis tools have opened new avenues to genome-wide analysis of plant stress responses [278, 280].

Cassava (*Manihot esculenta* Crantz) is an important tropical root crop adapted to a wide range of environmental stimuli, such as drought and acid soils, but it is an extremely cold-sensitive species [286]. A transcriptome profiling of cassava apical shoots, that were submitted to a progressive cold stress, was conducted using a dedicated 60-mer oligonucleotide microarray representing 20,840 cassava genes has identified a total of 508 transcripts [287]. Those differentially expressed transcripts were identified as early cold-responsive genes in which 319 sequences had functional descriptions when aligned with Arabidopsis proteins. Various stress-associated genes with a wide range of biological functions were found, such as signal transduction components (e.g., MAP kinase 4), transcription factors (TFs, e.g., RAP2.11 and AP2-EREBP), and active oxygen species scavenging enzymes (e.g., catalase 2), as well as photosynthesis-related genes (e.g., PsaL). This work provided useful candidate genes for genetic improvement in this species and suggested that the dynamic expression changes observed reflect the integrative controlling and transcriptome regulation of the networks in the cold stress response of this important tropical root crop.

Drought is the major constraint to increase yield in chickpea (*Cicer arietinum*) [288]. SuperSAGE, an improved version of the serial analysis of gene expression (SAGE) technique, has been employed in the analysis of gene expression in chickpea roots in response to drought [289]. To achieve this goal 80,238 26 bp tags were sequenced representing 17,493 unique transcripts (UniTags) from drought-stressed and non-stressed control roots. A total of 7,532 (43%) UniTags were more than 2.7-fold differentially expressed, and 880 (5.0%) were regulated more than 8-fold upon stress. Their large size enabled the unambiguous annotation of 3,858 (22%) UniTags when searched against public databases. This comprehensive study demonstrated that signal transduction, transcription regulation, osmolyte accumulation, and AOS scavenging undergo a strong transcriptional remodeling in chickpea roots in early drought stress responses, suggesting potential targets for breeding for drought tolerance.

High-throughput transcriptome sequencing and digital gene expression (DGE) profiling are cost-efficient platforms that are predicted to change transcriptomic analysis, eliminating the need for restriction enzyme digestion of DNA samples, PCR-based genomic amplification and ligation of sequence tags; they are additionally a suitable choice for characterizing non-model organisms without a reference genome [290-291]. Furthermore, RNA-seq can produce a complete coverage of transcripts, providing information about the sequence, structure and genomic origins of the entire transcript [285]. The dynamic transcriptome expression profiles of poplar (*Populus simonii* × *Populus nigra*) under salt stress were investigated using Solexa/Illumina digital gene expression technique [292]. A total of 5453, 2372, and 1770 genes were shown to be differentially expressed after exposure to NaCl for 3 days, 6 days and 9 days, respectively. Differential expression patterns throughout salt stress identified 572 genes, most of them mapped to the Gene Ontology term "receptor activity", "transporter activity" and "response to stress". Importantly this study showed that the greatest upregulation was observed for the POPTR_0018s02240.1 transcript encoding a serine/threonine protein kinase. Serine/threonine protein kinases have been reported to confer enhanced multi-stress tolerance in many plants [293], suggesting that this gene can be a suitable target for biotechnological manipulation with the aim of improving poplar salt tolerance.

The recent rapid accumulation of dataset containing large-scale gene expression profiles has supported the development of dedicated web databases acting as large public repositories, where data and underlying experimental conditions are widely described. A very complete and comprehensive list of searching database may be found in [294]. With the completion of the genome sequencing of several model and crop plants, these repositories can constitute important functional resources to be explored to decipher the molecular mechanisms underlying abiotic stress responses.

5.2. Proteomics

Proteomics may be defined as the science that studies the proteome, i.e. the number of proteins expressed in a given cell, tissue, organ, organism or populations. Proteomics is normally associated to two types of studies: 1) the characterization of a proteome in which all the proteins expressed in a given cell, tissue, organ, organism or populations are identified; and 2) differential proteomics in which a proteome of for instance a plant under control conditions is

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compared to the proteome of the same plant under study conditions such as the exposure to a heavy metal or water deficit, or in another example the comparison of protein expression profiles between different varieties of wheat.

Proteomics is heavily dependent on two laboratory techniques, protein electrophoresis (particularly two-dimensional electrophoresis and DIGE – Difference In Gel Electrophoresis) and protein identification using mass spectrometry. For further information on these approaches, kindly refer to the reviews by Minden [295] and Soares *et al.* [296] on respectively DIGE and mass spectrometry based protein identification strategies. Proteomics, particularly differential proteomics, has been widely applied to the study of the effects of several abiotic stresses on plant organs and tissues. The subject has been the object of a recent and extensive review [297]. For this reason, in this section we will provide examples on the use of proteomics to study the effects of abiotic stress in plants.

Evers *et al.* [298] have used both transcriptomics and proteomics to study the effects of cold and salt stresses on the leaf transcriptome and proteome of potato (*Solanum tuberosum*). Results pointed out to a number differentially regulated genes and proteins at the level of both stresses. Interestingly, salt exposure results displayed a strong down-regulation of genes implicated in primary metabolism, detoxication apparatus and signal transduction, whereas upon cold exposure, up and down-regulated genes were similar in number. On the contrary, proteome analysis seems to point out to an increase in protein expression of almost every protein with the exception of those with a role in photosynthesis. The results from this study highlight not only the differences between transcriptome and proteome expression as a consequence of cold and salt stresses but it particularly shows how the proteome analysis tends to be much more thorough and complete than transcriptome analysis.

In another example, DIGE has been used to study the effects of high level of UV radiation on the leaf proteome of artichoke, particularly targeting the levels of inducible antioxidants present in this species [299]. Authors observed a total of 145 spots showing differential expression and were able to identify 111 of them. Most of the proteins differentially modulated were chloroplast located, involved in photosynthesis, sugar metabolisms, protein folding and stress responsive, shedding a new understanding on the physiological and metabolic alternations induced by UV radiation exposure.

The embryo proteome of six rice varieties subjected to water deficit stress has been compared in order to further understand the mechanisms leading to water-stress tolerance in this crop [300]. A total of 28 proteins were identified involved in stress tolerance (LEA proteins), nutrient reservoir activity, among other proteins implicated in diverse cellular processes potentially related to the stress response (e.g., mitochondrial import translocase) in this cereal. Authors were also able to identify several differences and the post-translational level, particularly in the late embryogenesis abundant Rab21 that was more strongly phosphorylated in the embryos of the sensitive varieties than in the embryos of the tolerant ones. Similarly to the example by Evers previously mentioned, this study clearly demonstrates the broadness and completeness of proteome studies, particularly at the level of Post Translational Modifications (PTMs).

These three simple examples illustrate the advantages of the use of (differential) proteomics to study the effects of different abiotic stresses such as water deficit, temperature or UV exposure. Results show a large number of proteins being affected by abiotic stresses and the metabolic pathways that are subsequently affected and at what levels they are affected. The advantages of proteomics are further highlighted by the possibility to study PTMs of key importance in plant's physiological and biochemical responses to stress.

5.3. Metabolomics

Higher plants have the remarkable ability to synthesize a vast array of compounds that differ in chemical complexity and biological activity, playing indispensable roles in chemical defenses against biotic and abiotic stresses [301, 302]. In such context, it is obvious that Metabolomics (i.e. the study of the metabolome, or the set of metabolites found in a given plant tissue or organ) plays a significant role in bridging the phenotype-genotype gap [303]. The increasing number of publications in this subject also supports that metabolomics is not just a new Omics but a valuable tool to study phenotypes and changes in phenotypes induced by biotic and abiotic stresses (reviewed in [303]).

Metabolomics experiments start with the acquisition of metabolic fingerprints or metabolite profiles using various analytical instruments and separation technologies based in the physic-chemical properties of each metabolite [280]. Since there is no single technology currently available (or likely in the near future) to detect all compounds found in plants or any other organism, a combination of multiple analytical techniques, such as gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis (CE) coupled to Mass Spectrometry (MS), and Nuclear Magnetic Resonance (NMR) are generally performed following established protocols (reviewed in [280, 301]).

Metabolomic profiling of plants under stress is an important approach to study stress induced change in metabolites pools. In most of these studies, metabolite profiles are analyzed in combination with transcriptomic analysis: a strong correlation between metabolite levels is often correlated to a specific gene underlying a specific response or phenotype observed [280, 304]. In the recent past, the majority of the metabolic works have occurred in model species such as Arabidopsis [305] but nowadays, such metabolomic technologies are being used with success in forages [306], cereals [307] and other food crops [308].

Common bean (*Phaseolus vulgaris* L.) is one of the most important legume crops for human consumption but its productivity is often limited by low Phosphorus (P) levels in the soil [309]. Coupled to a transcriptomic approach, a non-biased metabolite profiling of bean roots using GC-MS was done to assess the degree to which changes in gene expression in P-deficient roots affect overall metabolism [308]. A total of 81 metabolites were detected and 42 were differentially expressed between −P to +P response ratios. Stress related metabolites identified such as polyols accumulated in P-deficient roots as well as sugars, providing additional support for the role of these compounds for P stress. The metabolomic data supported the identification of candidate genes involved in common bean root adaptation to P deficiency to be used in improvement programs.

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A recent study in maize was conducted to understand the combined effects of enhanced atmospheric CO_2 and drought on the stress responses by monitoring foliar metabolites (LC and GC-MS) and transcripts [307]. The concentrations of 28 out 33 leaf metabolites were altered by drought. Soluble carbohydrates, aconitate, shikimate, serine, glycine, proline and eight other amino acids increased, and leaf starch, malate, fumarate, 2-oxoglutarate and seven amino acids decreased with drought. Overall analysis of both transcriptomic and metabolomic data supported that water stress inhibited C4 photosynthesis and induced photorespiration in this species.

In plants, isoprene is a dual purpose metabolite that can act as thermo-protective agent proposed to prevent degradation of photosynthetic enzymes/membrane structures [310] and/or as reactive molecule reducing abiotic oxidative stress [311]. Gene expression and metabolite profiles of isoprene emitting wild type plants and RNAi-mediated non-isoprene emitting grey poplars (*Populus x canescens*) were compared by using poplar Affymetrix microarrays and non-targeted FT-ICR-MS (Fourier Transform Ion Cyclotron Resonance Mass Spectrometry) [312]. A transcriptional down-regulation of genes encoding enzymes of phenylpropanoid biosynthetic and regulatory pathways, as well as distinct metabolic down-regulation of condensed tannins and anthocyanins, in non-isoprene emitting genotypes was seen, when high temperature and light intensities possibly caused a transient drought stress. The results suggested that non-isoprene emitting poplars are more susceptible to environmental stress and provided new evidences about the physiological and ecological roles of isoprene in the protection of plants from environmental stresses.

6. Conclusions and final remarks

The Intergovernmental Panel on Climate Change 2012 (IPCC, 2012) indicated that temperature rising, drought, floods, desertification and deterioration of arable land and weather extremes will severely affect agriculture, especially in drought-prone regions of the developing world [313]. Regarding food security, this threatening scenario highlights the need for a globally concerted research approach to address crop improvement to mitigate crop failure under marginal environments. One of the major goals of plant improvement is to develop crops fit to cope with environmental injuries but still capable to achieve substantial yield under abiotic stress.

Data from traditional breeding, plant molecular breeding based in the development of molecular markers, candidate gene identification or gene expression profiles and from the use of transgenic approaches are becoming more and more frequent. Resulting plants are being evaluated in controlled conditions (greenhouse and growth chambers) but also, importantly, in the field to confirm the generation of improved cultivars. Despite the difficulty to establish reliable methods to assess new breed or engineered plant phenotypes as result of those approaches, some efforts are anticipated to fulfill the gap between plant molecular biology and plant physiology.

Several stress-resistant genes encoding for functional proteins were identified and introduced via genetic engineering into model species such as *Medicago truncatula*, *Nicotiana tabacum* or *Arabidopsis thaliana*, producing plants with improved abiotic stress tolerance. These results support the future use of this technology into economically important plants species namely crops and trees. As a consequence of the novel findings on the mechanisms underlying the regulation of gene expression under abiotic stress, we could speculate that future genetic engineering approaches might be targeted to these regulatory pathways. Emerging reports where the expression of regulatory molecules such as transcription factors (e.g. NAC proteins) or components of the small RNA pathway (e.g. miR398) are described to successfully produce abiotic stress resistant plants, supporting our hypothesis. Nevertheless, it should be kept in mind that the success of this approach relies on the development of efficient regeneration and transformation methods adequate to the target species or genotype. Future research efforts should be directed to overcome this significant limitation. Although the use of a constitutive promoter (e.g. CaMV 35S) ensured the expression of the target coding sequence, it presents some disadvantages as discussed previously. The use of inducible promoters (e.g. rd29A) that allow the expression of a transgene only when it is required could therefore be the ideal solution.

As stated previously across this manuscript, the nature and complexity of abiotic stress responses supports the use of global, integrative and multidisplinary approaches to understand the different levels of regulation of stress responses. The emerging holistic System Biology approaches still enclose a myriad of unexploited resources for Plant and Agricultural Sciences. Given the increasing development of high throughput genomic tools and concomitant release and progress on plants genome sequencing, it is now possible to gain information in a global scale, providing an overall comprehensive and quantitative overview on the gene-to-metabolite network associated to a particular plant response. The use of such cutting-edge methodologies to a specific plant species requires a previous study of the availability of reference genomes (e.g. Phytozome [314]), metabolite (e.g. Plant Metabolic Network [315]) or proteomic databases (e.g. UniProtKB [316]). Additionally, it requires appropriate laboratory, equipment and bioinformatics facilities and know-how that can be accessed using own institutional infrastructures or taking advantage of established collaborations with renowned research institutional research platforms and /or commercial service providers.

Presently, we are exploring the molecular mechanisms underlying *Medicago truncatula* and *Phaseolus vulgaris* adaptation to water deprivation using a System Biology approach that combines whole plant physiology data with transcriptomics, proteomics and metabolomics. We aim to identify candidate genes to be used in legume improvement programs and also fundamental knowledge on points of transcriptional, post-transcriptional and post-translational regulation of the gene expression under stress in these species. This highlights the efforts that we are currently doing to transfer the developed tools and information gained with the model *Medicago truncatula* to an important grain legume crop. A robust identification of the molecular targets to be used in biotechnological applications will be elucidated. Additionally, some clues about the signaling, regulation and interaction between the different cellular players involved are also expected.

In due time, it is expected that Omics and System Biology approaches provides a comprehensive knowledge of the plant responses to abiotic stresses making a significant progress in developing crops and trees with desirable traits as increasing yield and quality under abiotic stress and contribute to sustainable agriculture development.

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Chapter 4

The Molecular Basis of ABA-Mediated Plant Response to Drought

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Additional information is available at the end of the chapter

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1. Introduction

'Drought stress is as complicated and difficult to plant biology as cancer is to mammalian biology' said Jian-Kang Zhu, a molecular geneticist at the University of California, Riverside. The capacity of a plant to turn on or turn off a series of genes that further alter plant physiology and morphology allows a plant to tolerate, escape or avoid drought stress. Many countries around the world experience drought stress in different ways but it always leads to a decreased annual yield of crops. Deciphering the basis of the molecular response to stress and the mechanism for the adaptation and acquisition of tolerance can facilitate the creation of cultivars with increased drought tolerance. Drought response is a complex mechanism that has been investigated using a broad spectrum of 'omics' techniques, such as molecular genetics, functional genomics, transcriptomics, proteomics and metabolomics combined with advanced phenotyping techniques. The response of plants to dehydration stress has been extensively studied in a wide range of species with particular emphasis on model plants such as Arabidopsis. Taking advantage of the knowledge already obtained from Arabidopsis and other model species, it is possible to gain insight into the stress response in crops such as barley or wheat.

The best known trigger of the cascade of drought signaling is abscisic acid (ABA). Knowledge about the complexity of ABA signaling in regards to stress response is still full of gaps but the recent identification of ABA receptors and the key factors of the first step of ABA signal transduction in Arabidopsis provided an important insight into this mechanism ([1-4]. The actions of the other ABA signaling components, such as phosphatases, kinases, transcription factors and their roles in abiotic stress response during different developmental stages is also documented in crops [5]. Under drought conditions, ABA induces the expression of many genes whose products are involved in the response

to drought, among which are positive and negative regulators of ABA signaling, transcription factors and genes encode enzymes that are involved in the synthesis of osmoprotectants. It is important to mention that ABA is not the only phytohormone involved in stress response. There is much evidence of cross-talk between ABA and other phytohormones, such as jasmonates and ethylene [6].

Recent advances in functional genomics have revealed the importance of posttranscriptional regulation of gene expression performed by microRNA. Deep sequencing methods have enabled the identification of the miRNA involved in drought response in barley and rice. Further analysis also showed their potential roles in stress signaling by identifying their targets [7-8].

The molecular basis of drought response and the interaction between genes and proteins involved in this mechanism can be studied using of advanced molecular techniques only when a good drought assay that mimics natural drought conditions can be applied in the laboratory. Many protocols for drought assays have been developed that can be implemented in the study of different species ranging from Arabidopsis to crops. Another important issue is the method of phenotyping and the spectrum of physiological parameters that are measured [9]. The techniques used most often are: chlorophyll fluorescence, stomatal conductance and relative water content (RWC) [10-12]. Combining these molecular techniques with advanced methods of phenotyping would enable drought tolerant forms to be produced. This would contribute to beginning the Blue Revolution advocated by Kofi Annan in his April 2000 Millennium Address: "We need a Blue Revolution in agriculture that focuses on increasing productivity per unit of water – more crop per drop". This chapter reviews the newest aspects of the molecular and physiological mechanisms of drought stress response in crops.

2. Abscisic acid – The best known stress messenger

Since its isolation from cotton in the 1960s [13], the role of abscisic acid (ABA) in plant development and in the response of plants to environmental signals has been extensively studied. Analysis of Arabidopsis under salt and drought stress has revealed the important role ABA plays in response to these stresses [14-16]. Endogenous ABA concentrations increase under drought stress due to induction of ABA biosynthesis genes [14]. The increase in ABA reprograms the gene expression pattern to regulate water relations through adjustment of cellular osmotic pressure, the closure of stomata, a reduced leaf canopy, deeper root growth and changes in root system architecture [17-19].

Biosynthesis of ABA has been relatively well characterized in Arabidopsis and some data is available for other species, such as maize, tomato, potato and barley [20-24]. Knowledge about ABA biosynthesis derived from studies in Arabidopsis is highly applicable to other plant species, because the pathway and the respective genes are conserved in angiosperms. ABA is synthesized through the cleavage of a C40 carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via ABA-aldehyde [25-27]. The path-

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way begins with isopentyl pyrophosphate (IPP) which is the biological isoprene unit and the precursor of all terpenoids, as well as many plant hormones. The next step is the epoxidation of zeaxanthin and antheraxanthin to violaxanthin which is catalyzed by zeaxanthin epoxidase (ZEP), which was first identified in tobacco [28]. After a series of violaxanthin modifications which are controlled by the enzyme ABA4, violaxanthin is converted into 9-cis-epoxycarotenoid [29]. Oxidative cleavage of the major epoxycarotenoid 9-cis-neoxanthin by the 9-cis-epoxycarotenoid dioxygenase (NCED) yields a C15 intermediate - xanthoxin [30]. This step is the last one that occurs in the plastid. Xanthoxin is exported to the cytoplasm where two-step reaction via ABA-aldehyde takes place. The first step is catalyzed by a short-chain alcohol dehydrogenase/reductase (SDR) that is encoded by the *AtABA2* (*ABA deficient 2*) gene [31-33] and generates ABA aldehyde. Then the ABA aldehyde oxidase (AAO) with the molybdenum cofactor (MoCo) catalyzes the last step in the biosynthesis pathway - the conversion of ABA-aldehyde into ABA [34].

Drought stress has been shown to up-regulate *NCED3* expression in Arabidopsis [14], maize [21], tomato [35], bean [15] and avocado [36]. A significant increase in NCED transcript levels can be detected within 15 to 30 min after leaf detachment or dehydration treatment [15; 37], indicating activation of NCED genes can be fairly quick. Cheng et al. [32] reported that the *AtNCED3* gene (and *AtZEP* (*Zeaxanthin Epoxidase*) and *AtAAO3* (*ABA aldehyde oxidase*)) could be induced in the Landsberg erecta background by ABA and studies in rice showed that *OsNCED3* expression was induced by dehydration [38]. Immunohistochemical analysis, using antibodies raised against AtNCED3, revealed that the protein is accumulated in the leaf vascular parenchyma cells in response to drought stress. it was not detected under non-stressed conditions. These data indicate that the drought induction of ABA biosynthesis occurs primarily in vascular tissues and that vascular-derived ABA might trigger stomatal closure via transport to guard cells [39]. *AtNCED3* expression is up-regulated by drought conditions across observed species and decreases after rehydration. At the same time, the expression level of *AtCYP707A1*, *2*, *3* and *4* (*CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 1*, *2*, *3*, *4*) were induced by rehydration [40-41]. These genes, which encode the hydroxylases that are responsible mostly for ABA catabolism, were identified in Arabidopsis, rice [42], barley [43], wheat [44] and soybean [45]. *OsABA8ox1* (*ABA-8-hydroxylase 1*) expression is induced dramatically by rehydration, which can lead to a decrease in the ABA content in rice leaves [42].

The balance between active and inactive ABA is very important for plant stress response and is achieved not only by biosynthesis and catabolism reactions, but also by conjugation and deconjugation. ABA can be inactivated at the C-1 hydroxyl group by different chemical compounds that form various conjugates and accumulate in vacuoles or in the apoplastic space [46]. The most widespread conjugate is ABA glucosyl ester (ABA-GE) which is catalyzed by ABA glucosyltransferase [47-48]. Lee et al [49] identified the AtBG1 (BETA-1,3-GLUCANASE 1) protein which is responsible for the release of ABA from ABA-GE. Their findings showed that ABA de-conjugation plays a significant role in providing an ABA pool for plants that allows them to adjust to changing physiological and environmental conditions.

The ability of ABA to move long distances allows it to serve as a critical stress messenger. ABA transport was long assumed to be a diffusive process, mainly due to the ability of ABA to diffuse passively across biological membranes when it is in a protonated state [50]. The last step of ABA biosynthesis occurs in the cytosol where pH is estimated to be 7.2-7.4. In the apoplastic space, where ABA is meant to be transported before reaching the target cell, the pH is estimated to be around 5.0-6.0. Although ABA can be passively transported from a low pH to a higher one with a pH gradient, there is a need for the transporter to allow ABA to get into the target cell and to be exported from the cell to the apoplast. During stress response, the strong alkalization of apoplastic pH would slow ABA diffusive transport from the apoplastic space to the target cells. Because of the predominance of a non-protonated ABA state, there is a need for the existence of ABA transporters. The identification of ABA transporters in target cell membranes, such as the cell membranes of guard cells, has resolved the problem of how ABA gets into the cells when passive transport is decreased under stress conditions. One of the identified ABA importers is ABCG40 (ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G40) described by Kang et al [51]. The expression of *ABCG40* is not tissue specific and its product localizes in cell membranes [51]. Kuromori et al [52] identified another ABA importer - ABCG22 (ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G22). The gene encoding this transporter is mainly expressed in guard cells. Also, the expulsion of ABA into the intercellular space is mediated by transporters such as ABCG25 (ARABIDOPSIS THALIANA ATP-BINDING CASSETTE G25). *ABCG25* is expressed mainly in vacuolar tissue, where ABA is synthesized [53].

A breakthrough in understanding ABA signaling occurred recently when several groups identified key ABA receptors. Chemical genetics emerged as the solution for the problem of the identification of receptor. Pyrabactin (4-bromo-N-[pyridine-2-yl methyl]naphthalene-1-sulfonamide) is a synthetic compound that partially mimics the inhibitory effect of ABA during seed germination and seedling development. Using a series of pyrabactin-resistant mutants and the map-based cloning approach, several genes encoding ABA-binding proteins, among them PYR1 (PYRABACTIN-RESISTANCE 1) have been identified [3]. PYR1 is one of the 14 homologs (PYL – PYRABACTIN RESISTANCE LIKE) present in the Arabidopsis genome [1-4]. After receiving ABA from ABC transporters, the PYR/PYL/RCAR-ABA (PYRABACTIN-RESISTANCE 1/ PYRABACTIN RESISTANCE LIKE/ REGULATORY COMPONENT OF ABA RECEPTOR) complex perceives ABA intracellularly and forms ternary complexes inhibiting clade A of PP2Cs (PROTEIN PHOSPHATASE 2C), the negative regulators of ABA signaling, such as ABI1 (ABA INSENSITIVE 1), ABI2 (ABA INSENSITIVE 2), HAB1 (HYPERSENSITIVE TO ABA1) [1-2; Table 1].

This allows the activation of down-stream targets of PP2Cs – the Sucrose nonfermenting 1-related subfamily 2 protein kinases (SnRK2), such as SnRK2.2/D, SnRK2.3/E and SnRK2.6/ OST1/E which are the key players in the regulation of ABA signaling [54-57; Figure 1].

The last enzyme, OST1 (OPEN STOMATA1), displays dominant kinase activity during drought stress response when the ABA signal is relayed to the guard cells. Mutants in *OST1* showed a wilty phenotype under water deficit conditions [58]. Mutants for the other two ABA-activated kinases, *SnRK2.2* and *SnRK2.3*, did not show a drought-sensitive phenotype

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[59]. The triple mutant *snrk2.2/d snrk2.3/I snrk2.6/e* displayed an extremely sensitive phenotype under water deficit conditions. Transcriptomic studies of the triple mutant showed a down-regulation of genes encoding PP2Cs, which suggested a feedback loop in the transcription regulation of PP2Cs by SnRKs [54].

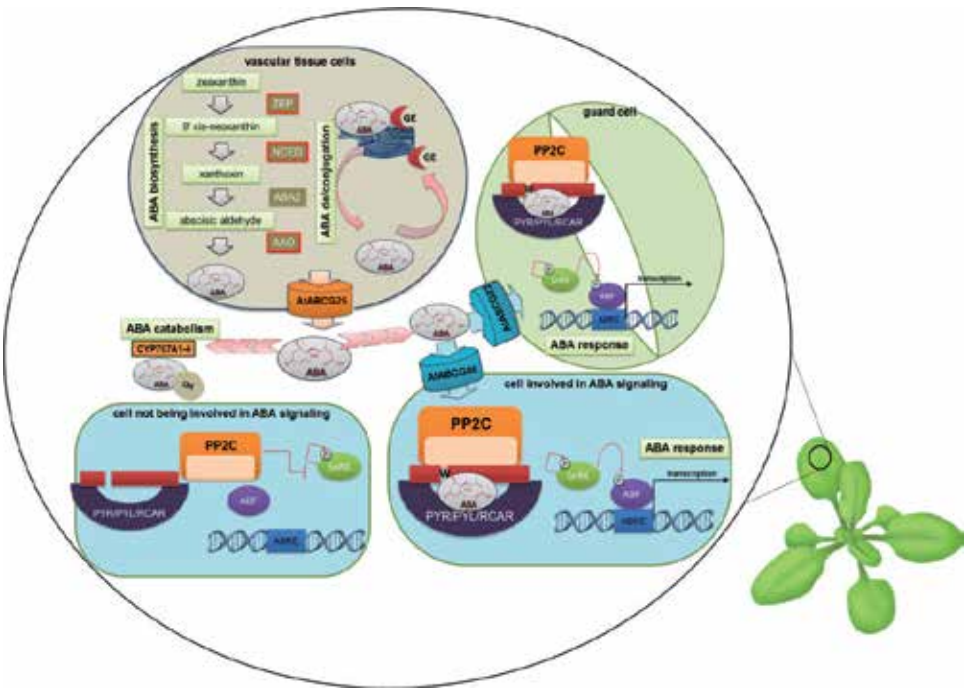

Figure 1. ABA synthesis, catabolism, conjugation and response in a scheme.

One of the earliest plant responses to water deficit condition, and one regulated mainly in an ABA-dependent manner, is the closure of stomata. The closing or opening of the pore is a result of the osmotic shrinking or swelling, of the two surrounding stoma guard cells. ABA acts directly on the guard cells and induces stomata closure via an efflux of potassium and anions from the guard cells [60]. ABA regulation of the membrane ion channels is mediated by increased cytosolic Ca^{2+} resulting from the release of Ca^{2+} from intracellular stores and a Ca^{2+} influx from the extracellular space. It is worth noting that a number of mutations that affect ABA signaling in regards to stomatal action during drought have been characterized. Dominant mutations have been described in genes that encode type-2C phosphatases - ABI1 (ABA INSENSITIVE 1) and ABI2 (ABA INSENSITIVE 2) [61-62], whereas recessive mutations that lead to supersensitivity to ABA in regards to stomata closure are found in genes that encode farnesyltransferase β-subunit - ERA1 (ENHANCED RESPONSIVE TO ABA1) [63-64], a larger subunit of cap binding complex CBP80 (CAP BINDING PROTEIN 80) [65] and the Sm-like snRNP protein SAD1 (SUPERSENSITIVE TO ABA AND DROUGHT 1) [66].

RCAR	PYR/PYL	PP2C interactors
RCAR1	PYL9	ABI1[1],[4], ABI2[1], HAB1[1]
RCAR2	PYL7	ABI1[4]
RCAR3	PYL8	HAB1[3], ABI1[4]
RCAR4	PYL10	ABI1[4]
RCAR5	PYL11	HAB1[3], ABI1[4]
RCAR6	PYL12	PP2CA/AHG3[2]
RCAR7	PYL13	
RCAR8	PYL5	HAB1[3], ABI1[4]
RCAR9	PYL6	ABI1[1],[4], ABI2[1], HAB1[1]
RCAR10	PYL4	HAB1[2], ABI1[4]
RCAR11	PYR1	HAB1[2], ABI1[4]
RCAR12	PYL1	HAB1[2], ABI1[4]
RCAR13	PYL3	HAB1[2]
RCAR14	PYL2	HAB1[2]

Table 1. The nomenclature of the different soluble receptors and their PP2Cs interactors

3. Abscisic acid is not the only phytohormone in stress response

The effectiveness of ABA is regulated not only by the length of a drought or the previous stress history of a given plant, but also by other phytohormones such as jasmonates, cytokinins and ethylene. The role of jasmonic acid (JA) has been well established in regards to plant development and defense responses [67]. Recently, it was also shown that jasmonic acid (JA) and methyl jasmonate (MeJA) are involved in the regulation of drought response. When JA or MeJA are applied exogenously to plants they are converted into a biologically active form (+)-7-iso-Jasmonoyl-L-isoleucine (JA-Ile). JA-Ile is then bound by the receptor SCF^{COI} complex that contains the CORONATINE INSENSITIVE1 (COI1) F-box protein [68-69]. This interaction leads to the degradation of the repressor protein – JAZ (Jasmonate ZIM-domain) by the 26S proteasome, it allows MYC2 (MYC DOMAIN TRANSCRIPTION FACTOR 2) activation of a distinct JA response genes [70-72]. In the absence of JA, JAZ inhibits MYC2 in order to activate the transcription of JA-inducible genes. It was showed that *MYC2* is up-regulated not only by JA, but also by ABA and drought. The described interaction between the protein specific to jasmonates - JAZ and both jasmonates and also ABA and drought-inducible MYC2 suggest the important regulatory role of JA in an ABA-dependent response to drought. A similar mechanism has been described in rice [73]. It was shown that, in addition to ABA, jasmonates also trigger stomatal closure in response to drought in

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various species, including Arabidopsis and barley [74-76]. Low endogenous ABA content in the ABA-deficient mutant *aba2* impairs MeJA (methyl-jasmonate)-stimulated Ca^{2+} elevation, which is, in turn, important metal closure. Furthermore, MeJA stimulates the expression of the ABA biosynthetic gene, *NCED3*. MeJA signaling in guard cells requires the presence of endogenous ABA [77]. Another example of cross talk between ABA and jasmonates during stress response is the up-regulation by JA of *AtPYL4* (*PYRABACTINE LIKE 4*), *AtPYL5* (*PYRABACTINE LIKE 5*) and *AtPYL6* (*PYRABACTINE LIKE 6*), which are members of the *PYR/PYL/RCAR* ABA receptor family [78]. These studies showed the importance and conservation across the species of the role of JA in ABA-dependent response to drought.

Cytokinins (CKs) are another group of hormones involved in stress responses [79-80]. Cytokinins regulate cell proliferation and differentiation [81]. Abiotic stresses, such as drought, decrease the biosynthesis and transport of CKs from roots to shoots [82]. An increased concentration of CKs in xylem has been shown to decrease stomatal sensitivity to ABA [83]. The same effect was observed when exogenous CKs were applied [84-85]. When a plant encounters mild drought conditions, it is not necessary to close the stomata and further limit its photosynthetic rate. Since the decline in CK content increases the stomatal sensitivity to ABA, avoidance of this phenomenon might help in obtaining a better yield from plants that experience mild drought. CK up-regulation can be achieved by reduced expression of a gene that encodes cytokinin oxidase, an enzyme that degrades CKs. In addition to maintaining a better photosynthetic rate, increased levels of CKs lead to enhanced activity of the cell-cycle genes, and the consequent, increase in cell number may result in improved grain filling [86]. The process of grain filling is actually an increase in cell number and cell filling in the endosperm [87]. There is a generally positive relationship between endosperm cell number and grain weight in wheat [88], barley [89], maize [90] and rice [91]. Thus, endosperm cell number is one important factor determining grain weight [87]. Taking into account that endosperm cell number in cereal crops is established during an early phase of development, it is assumed that this step can be regulated by cytokinins [87]. Another manipulation of the CK level in plant tissues was achieved by seed inoculation with CK-producing bacteria, gradually releasing CKs within the physiological concentration range [92]. Wheat plants in which seeds were treated with such bacteria and grown under mild drought condition gave a 30-60% higher yield than non-treated controls. Since a high level of CKs improves grain quality and photosynthesis rate, and a high level of ABA increases root extension rate, osmoprotectant activity, and solute biosynthesis, another aim of breeders is to obtain a high content of both ABA and CKs under mild drought conditions Wilkinson et al. [6].

Ethylene, a gaseous plant hormone that inhibits root growth and development, is involved in stress-induced leaf senescence and can contribute to reducing the rate of photosynthesis [93-95]. ABA can modulate the influence of ethylene on stomatal conductance. Contradictory results have been published regarding the role of ethylene in stomatal action. Desikan et al. [96] showed that ethylene induces stomatal closure, whereas Tanaka et al. [97] and Wilkinson and Davies [98] proved that ethylene can antagonize ABA action in the stomata. This

is probably due to the fact that the concentration of neither hormone is important for the final effect but rather the ratio of ABA to ethylene [99; 18].

4. With a little help from arabidopsis – Transferring knowledge from weeds to crops

A small genome, short life cycle, small stature, prolific seed production, ease of transformation, a completely sequenced genome, a near saturation insertion mutant collection, a genome array that contains the entire transcriptome – these are the major advantages of using the model plant Arabidopsis in studies on the molecular basis of responses to environmental stresses including drought. The identification of stress-related genes, their functions and the pathways they are involved in, has been facilitated by an increasing number of molecular tools, genetic resources and the large number of web-based databases available for Arabidopsis (Table 2).

Genomic resources and results obtained of Arabidopsis provide a resource for exploitation in crops. Using sequence homology, EST (Expressed Sequence Tag) libraries, and the full-length cDNA repositories available for crop species, there is a possibility of a simple transfer of data revealed in Arabidopsis to identify a gene of interest in a crop species (Figure 2).

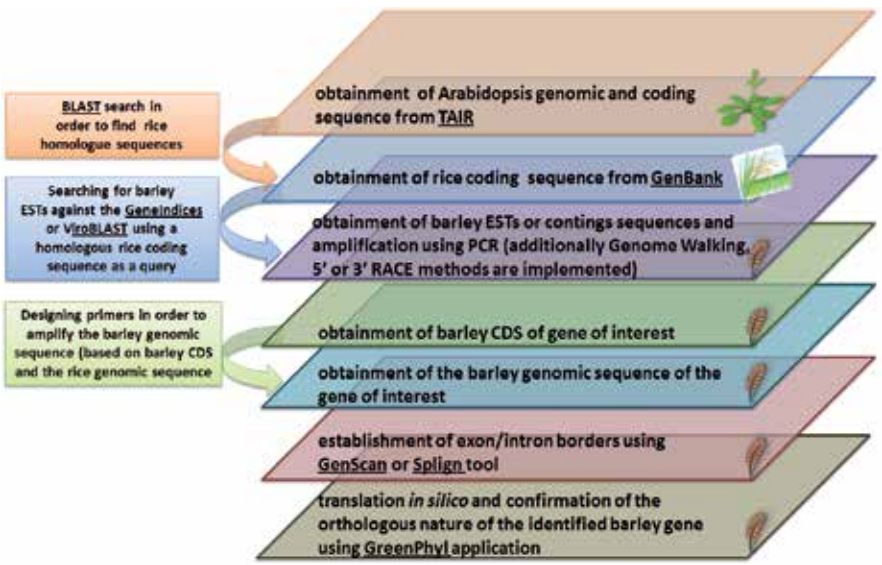

Figure 2. The pipeline of identification of barley homologous gene based on Arabidopsis and rice information. GenBank: http://www.ncbi.nlm.nih.gov/genbank/; TAIR: www.arabidopsis.org; BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi; GeneIndices: http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=barley; GenScan: http://genes.mit.edu/GENSCAN.html; Splign: http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi; GreenPhyl: http://greenphyl.cirad.fr/v2/cgi-bin/index.cgi.

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Type	Resource/ database	URL
Integrative databases	TAIR	*http://www.arabidopsis.org/*
	MIPS	*http://mips.helholtz-muenchen.de*
	PlantGDB	*http://www.plantgdb.org/*
	EnsEMBLPlants	*http://plants.ensembl.org/index.html*
	Brachypodiumdb	*http://db.brachypodium.org/*
	EBI	*http://www.ebi.ac.uk/embl/*
	GenBank	*http://www.ncbi.nlm.nih.gov/genbank/*
	Gramene	*http://www.gramene.org/*
	Oryzabase	*http://www.shigen.nig.ac.jp/rice/oryzabase/*
	GrainGenes	*http://wheat.pw.usda.gov/*
Large-scale collections of full-length cDNA clones	flcDNA *A. thaliana*	*http://rarge.psc.riken.jp/*
	flcDNA *O. sativa*	http://cdna01.dna.affrc.go.jp/cDNA/ http://www.ncgr.ac.cn/ricd
	flcDNA *H. vulgare*	*http://www.shigen.nig.ac.jp/barley/*
	flcDNA *T. aestivum*	*http://trifldb.psc.riken.jp/index.pl*
	flcDNA *Z. mays*	*http://www.maizecdna.org/*
	ViroBLAST	*http://indra.mullins.microbiol.washington.edu/viroblast/viroblast.php*
TF databases	AGRIS	*http://arabidopsis.med.ohio-state.edu/*
	PlantTFDB	*http://planttfdb.cbi.edu.cn/*
	GRASSIUS	*http://grassius.org/*
Microarray bulk data retrieval	NASCarrays	*http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl*
	ArrayExpress	*http://www.ebi.ac.uk/arrayexpress/*
	AtGenExpress	*http://www.weigelworld.org/resources/microarray/AtGenExpress/*
	GEO	*http://www.ncbi.nlm.nih.gov/geo/*
	PlexDB	*http://www.plexdb.org/*
Gene expression analysis resources	Genevestigator	*https://www.genevestigator.com/gv/*
	BAR	*http://esc4037-shemp.csb.utoronto.ca/welcome.htm*
	eFP Browser	*http://esc4037-shemp.csb.utoronto.ca/efp/cgi-bin/efpWeb.cgi*
Functional information	GeneMANIA	*http://www.genemania.org/*
	IntACT	*http://www.ebi.ac.uk/intact/*
	BioGRID	*http://thebiogrid.org/*

Table 2. Web-based resources for gene expression analysis for Arabidopsis and other species, including crops.

In many cases, not only structural proteins, such as ion channels are conserved between Arabidopsis and other plant species, but also regulatory proteins, such as transcription factors. In addition, it is worth adding that entire transcriptional regulons can also be conserved, as in case of the ABA signalosome PYR/PYL/RCAR-PP2Cs-SNRKs. 'Only after we understand how plants respond to stress — in many cases first in Arabidopsis and then applying the Arabidopsis model to crop plants — will we be able to begin engineering stress tolerance' [100].

During the last decade, microarrays have become a routine tool for the analysis of transcripts, not only in model Arabidopsis but also in crops, such as barley and rice. Interestingly, interspecies comparisons between distantly related species, Arabidopsis and rice or barley revealed conserved patterns of expression in the case of many orthologs genes [101-103]. Comparative analyses showed that orthologous of specific genes in rice or barley are also responsive to stress similar to Arabidoposis [103; 102]. Mochida et al. [104] used publicly available transcriptome data to investigate regulatory networks of the genes involved in various developmental aspects including drought in barley. On the basis of a comparative analysis between barley and model species, such as Arabidopsis or Brachypodium, modules of genes putatively involved in drought response have been identified. In addition to these computational approaches, Moumeni et al. [105] have undertaken a comparative analysis of the rice root transcriptome under drought stress. They used two pairs each of drought-tolerant and susceptible rice NILs (Near Isogenic Lines). Global gene expression analysis revealed that about 55% of the genes differentially expressed were in rice roots under drought stress. The drought-tolerant lines showed an up-regulation of the genes involved in secondary metabolism, amino acid metabolism, response to stimulus, defense response, transcription and signal transduction. Proteomic analysis of drought-sensitive and drought-tolerant barley lines performed by Kausar et al. [106] revealed an increased level of metabolism, photosynthesis and amino acid synthesis-related proteins in tolerant genotypes, whereas a decreased level was observed in sensitive forms. The data confirmed the results described previously in other species and should that similar processes play a significant role in barley's adaptation to stress conditions.

5. The huge role of tiny molecules (microRNA) in drought response

Small non-coding RNAs – miRNAs, which were first reported in the nematode *Ceanorhabditis elegans* in 1993 [107] and which are responsible for the phenomenon of RNA interference, have become recognized as very important regulatory components of the cell signaling. miRNAs have been shown to be highly conserved gene expression regulators across species [108-109]. The first plant miRNA was isolated from Arabidopsis [110]. To date, approximately 5000 plant miRNAs have been identified and deposited in miRbase (19.0 release) including 299 miRNA from Arabidopsis, 135 from Brachypodium, 206 from sorghum, 42 from wheat, 591 from rice, 172 from maize and 67 from barley [111]. miRNAs are small regulatory RNAs of a 20-22 nucleotide length that are encoded by endogenous *MIR* genes. Their primary transcripts are partially double-stranded stem-loop structures. Pri-miRNAs in plants

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are processed by DCL1 (DICER-LIKE 1) HYL1 (HYPONASTIC LEAVES 1), SE (SERRATED) proteins into pre-miRNA hairpin precursors which are finally converted into short duplexes – mature miRNAs. The duplexes are then methylated at the 3′ terminus and exported to the cytoplasm. In the cytoplasm, single-stranded miRNAs are incorporated in the AGO (ARGONAUTE) protein, the catalytic compound of the RISC (RNA-INDUCED SILENCING COMPLEX) complex, and guide the RISC to the target mRNAs by sequence complementarity to negatively regulate their expression [112].

Plant microRNAs are involved in various developmental processes including flowering, and leaf, stem and root development [113-115]. Jones-Rhoades and Bartel [116] drew the attention of plant biologists to the miRNA engagement in stress response for the first time. To gain an insight into the role of miRNAs in the regulation of transcripts in response to drought, several projects on the identification of the miRNAs related to stress response in crops were undertaken. Using deep sequencing techniques, Zhou et al [117] identified nineteen new miRNAs that are induced by drought in rice, among them eleven down-regulated and eight up-regulated miRNAs. In addition, they identified nine miRNAs that showed an opposite expression to that observed in drought-stressed Arabidopsis (Table 3). A similar approach was used by Kulcheski et al. [118] in soybean, which revealed 11 miRNAs that are related to drought stress (Table 3). Based on bioinformatic prediction and then verification of the obtained results using RT-qPCR, Xu et al. [119] identified 21 miRNAs differently expressed during water stress in maize (Table 3). A similar approach using bioinformatic prediction of miRNAs on dehydration stress was undertaken by Kantar et al. [7], who found four miRNAs that are related to drought stress in barley (Table 3). Deep sequencing of a small RNA library in the case of barley was performed by Lv et al. [8]. They showed that six miRNAs specific for stress response. hvu-MIRn026a, hvu-MIRn029, hvu-MIR035, hvu-MIR156d exhibited higher expression in response to salt and drought stress, whereas hvu-MIR396d and hvu-MIR399b showed a higher expression only in drought-stressed plants. Additionally, the authors observed that hvu-mir029 was highly expressed after drought treatment and at a very low level under non-stressed conditions, which suggests the important role of this molecule in water deficit response (Table 3).

To understand the function of newly identified miRNAs, the putative target transcripts have to be predicted. In order to identify microRNAs target transcripts, Kantar et al [7] performed computational studies and a modified 5′ RLM-RACE (RNA ligase-mediated 5′ rapid amplification of cDNA ends) in barley. Seven cleaved miRNA transcripts were retrieved from drought-stressed leaf samples as targets for hvu-MIR165, hvu-MIR166, hvu-MIR156, hvu-MIR2055, hvu-MIR171, hvu-MIR172, hvu-MIR397 and hvu-MIR159. The identified targets are mainly transcription factors that play a role in plant development, morphology and determination of the flowering time. *SCRL6* (*SCARECROW LIKE 6*) encodes a transcription factor that is involved in diverse plant developmental processes such as leaf or root growth and is the target of hvu-MIR171, *ARF10* (*AUXIN RESPONSIVE FACTOR 10*) encodes a transcription factor that negatively regulates auxin signaling and is the target of hvu-MIR160, *SBP* (*SQUAMOSA PROMOTER BINDING PROTEIN*) is a transcription factor that is mainly important for leaf development and is the target of hvu-MIR156a, and *MYB33* (*MYB DOMAIN*

PROTEIN 33) is a transcription factor that is involved in ABA and GA signaling and is the target of hvu-MIR159a [7].

Species	Identified miRNA related to drought	References
rice	osa-MIR170, osa-MIR172, osa-MIR397, osa-MIR408, osa-MIR529, osa-MIR896, osa-MIR1030, osa-MIR1035, osa-MIR1050, osa-MIR1088, osa-MIR1126, osa-MIR395, osa-MIR474, osa-MIR845, osa-MIR851, osa-MIR854, osa-MIR901, osa-MIR903 and osa-MIR1125, osa-MIR156, osa-MIR168, osa-MIR170, osa-MIR171, osa-MIR172, osa-MIR319, osa-MIR396, osa-MIR397, osa-MIR408	[117]
soybean	gma-MIR166-5p, gma-MIR169f-3p, gma-MIR1513c, gma-MIR397ab, gma-MIR-Seq13, gma-MIR-Seq11, gma-MIRSeq15, gma-MIR166f, gma-MIR-482bd-3p, gma-MIR4415b, gma-MIR-Seq07	[118]
maize	zma-MIR161, zma-MIR397, zma-MIR446, zma-MIR479, zma-MIR530, zma-MIR776, zma-MIR782, zma-MIR815a, zma-MIR818a, zma-MIR820, zma-MIR828, zma-MIR834, zmaMIR1, zma-MIR2, zma-MIR3, zma-MIR4, zma-MIR5, zma-MIR6, zma-MIR7, zma-MIR8, zma-MIR9	[119]
barley	hvu-MIR156, hvu-MIR166, hvu-MIR171, hvu-MIR408	[7]
	hvu-MIRn026a, hvu-MIRn029, hvu-MIR035, hvu-MIR156d, hvu-MIR396d, hvu-MIR399b	[8]

* red indicates down-regulation by drought, green indicates up-regulation by drought, blue indicates regulation opposite to that observed in Arabidopsis, black indicates no information about regulation by drought

Table 3. miRNA related to drought in different crop species.

6. From the cell to the organism level – Phenotyping of drought-treated crops

In order to understand gene-to-phenotype relationships in the plant response to drought stress, it is vital to decipher the physiological and genetic bases of this process. Recent advances in crop physiology, genomics and plant phenotyping have provided a broader knowledge and better tools for crop improvement under stress conditions [120]. Maintaining a high yield under drought conditions has become a priority for breeders. However, the physiological basis of yield maintenance under drought is not yet fully understood, of the complexity of the mechanisms that plants can use to maintain growth in conditions due to water deficit [120]. Quantitative trait loci (QTL) for genes conferring a yield benefit under drought conditions first need to be identified in phenotypic screens and then incorporated into crops using marker-assisted selection [121]. Direct selection for yield in drought-prone environments, however, has proven to be difficult. Drought stress is a dynamic process and

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can occur at different periods of the crop cycle and with different intensities. Consequently, plants have developed various strategies in response to drought: tolerance, escape and avoidance. Ludlow [122] defined three strategies plants use to cope with drought stress: drought tolerance is the ability of a plant to cope with water deficit through low tissue water potential, drought escape is defined as completion of the life cycle just before a severe drought starts, and drought avoidance is plant maintenance of high tissue water potential by minimizing water loss or maximizing water uptake. The final mechanism conveys the ability to survive and recover rapidly after a severe stress through protective mechanisms, such as cell wall folding, membrane protection, and the accumulation of antioxidants [123-124].

In order to incorporate traits that confer drought tolerance into molecular breeding programs, phenotyping protocols are extremely important [125]. With the wide availability of genetic resources, such as mutant populations (TILLING) or mapping populations, high-throughput phenotyping will become an essential asset in closing the gap between plant physiology and genetics [126- 127]. It is worth noting that a complex set of both abiotic and biotic stresses shapes the natural environment during plant development drought stress is just one of many factors. It is hard to exclude one of the stress pathways and to analyze it in isolation from others because the cascade of stress response is a complicated web of overlapping pathways. When studying drought tolerance in plants, it is very difficult to control and monitor the level and onset of water deficit, since it is a dynamic process and a combination of the available water in the soil and the plant water status. Continuous measurements are needed in order to link the level of drought experienced by the plant with the physiological changes occurring in response to it [125]. Under greenhouse conditions, water use can be monitored by weighing the pots or using TDR (Time Domain Reflectometry) soil moisture meters [128]. The water supply can be regulated at high-throughput automated screening facilities by using the classical water withdrawal approach [14] and maintaining a constant soil water status [129].

Another difficult issue is how to describe plant response to drought at the physiological level using properly chosen physiological, but also morphological, traits. In breeding programs for improved drought tolerance, crop traits associated with the conceptual framework for yield drought adaptation have been proposed by Passioura [130]. This framework has three important drivers: (1) water uptake (WU), (2) water-use efficiency (WUE) and (3) harvest index (HI). Several traits are highly associated with these three aspects of Passioura model. With regard to WU, the best method would be direct selection for variation in root architecture but since this is hard to perform, stomatal conductance, mainly the canopy temperature, is measured. This provides indirect indicators of water uptake by roots [131]. To estimate WUE, carbon isotope discrimination is used. A high affinity of Rubisco for the more common ^{12}C isotope over the ^{13}C indicates a lower WUE, whereas a lower discrimination value indicates a higher WUE [131]. In the case of HI, the extreme sensitivity of reproductive processes to drought may result in reproductive failure, which is associated with a low HI value [132].

Water stress reduces photosynthesis in the leaves of higher plants. It is linked with a decreased diffusion of CO_2 from the atmosphere to the site of carboxylation [133-134]. Underlying this process is the stomatal closure during short-term drought and photoinhibition damage, and the inactivation of RuBisCO under long-term stress [135].

Stomatal closure is one of the first responses to drought conditions which might result in cell dehydration or runaway xylem cavitation [136]. A good illustration of this process is stomatal behavior in the midday, when either stomatal closure or decreased stomatal conductance can be observed. Both responses are mediated by ABA synthesized in response to dehydration conditions [18]. When decreased stomatal conductance is combined with sustained high irradiance, leaves are subjected to excess energy relative to the available CO_2 and the rate of reducing power can overcome the rate of its use in the Calvin cycle. These processes lead to the down-regulation of photosynthetic and even photoinhibition. Plants have evolved mechanisms of defense to protect photosynthesis. Such protection can be achieved by the regulated thermal dissipation that occurs in the light-harvesting complexes [137].

Processes associated with the photosynthetic apparatus can be measured using chlorophyll fluorescence. Experiments with chlorophyll fluorescence were first carried out by Kautsky and Hirsch [138]. Since then, this technique has progressed quickly and chlorophyll fluorescence can be easily measured using commercially available chlorophyll fluorimeters which enable the measurements of the photochemical and non-photochemical processes involved in the fluorescence quenching that occurs in the presence of light [139]. The Fv/Fm ratio representing the maximum quantum yield of the primary photochemical reaction of photosystem II (PSII) is the most often used parameter. Environmental stresses that affect PSII efficiency lead to the characteristic decrease in the value of this parameter [140]. Fluorescence kinetics of chlorophyll a, the 'OJIP/JIP-test' named after the basic steps of the transient by which parameters quantifying PSII behavior are calculated (O is the fluorescence intensity F0 (at 50 μs); J is the fluorescence intensities FJ (at 2 ms); I is FI (at 30 ms) and P is the maximal fluorescence intensity, FP = FM) is an informative tool for studying the effects of different environmental stresses on photosynthesis [141-142;10;143]. This analysis offers simple equations to express the equilibrium between the inflow and outflow of the entire energy flux within PSII; it also provides information about the fate of absorbed energy. Some of the parameters calculated using the JIP-test are related to energy fluxes for light absorption (ABS), the trapping of excitation energy (TR) and electron transport (ETR) per reaction center (RC) or per sample area called cross-section (CS). Their estimates are based on the analysis of several groups of measured and calculated parameters. Analyses performed using these parameters are quick and the measurements are non-invasive [10].

In addition to the photosynthesis process, it was observed that the alteration of leaf angle caused by dehydration, towards smaller angles, would diminish intercepted radiation and carbon assimilation, and also have an important protective role against excess solar energy [144]. There is also a correlation between the rate of photosynthesis and the age of the leaf. Younger leaves tend to be more resistant to drought than older ones. When a severe reduction in the size of the leaf canopy occurs, as a result of shedding older

leaves, it allows a plant to recover faster following rehydration [145]. Photosynthetic recovery following rehydration plays a pivotal role in drought-tolerance mechanisms and prevents a dramatic decline in crop yields [146]. It was shown that recovery from a severe stress is a two-step process. The first phase occurs during the first hours or days after rewatering and corresponds to an improvement of leaf water status and the reopening of stomata [147]. The second stage lasts a few days and requires the *de novo* synthesis of photosynthetic proteins [148-149].

It is also worth noting that other phenotype analyses should be performed in order to obtain a complete picture of the stress response of a given plant. Relative Water Content (RWC), which was proposed by Sinclair and Ludlow [12], is the most often used assay to assess plant response to a water deficit. This simple test allows the establishment of relative water content in a leaf of control and drought-treated plants. Detached leaves are weighed and saturated with water for 24 h, then again weighed and dried for 48 h and weighed again. RWC is calculated from the following formula: RWC (%) = [(FM - DM)/(TM - DM)] * 100, where, FM, DM, and TM are the fresh, dry and turgid masses of the tissue weighted, respectively.

The degree of cell membrane stability (CMS) is considered to be one of the best physiological indicators of drought-stress tolerance. It can be evaluated using measurements of solute leakage from plant tissue [150-151].

In response to drought stress, plants are able to adjust osmotic pressure by synthesizing osmoprotectants such as proline, the water soluble carbohydrates that behave like a molecular weapon against dehydration within the cell. There are several methods used in order to estimate the accumulation of endogenous proline or sugars in drought-treated plants [152].

Several morphological traits that have an impact on drought tolerance have been observed. Growth inhibition resulting from drought-induced ABA biosynthesis was observed in plants exposed to stress [153]. A number of studies have shown that wax deposition on the leaf surface increased in response to drought and an associated improvement in drought tolerance was observed in oat, rice, sorghum, wheat and barley plants that had an increased wax layer [154 -157]. Enhanced drought tolerance was also gained by plants having a reduced number of stomata, which was probably dependent on the accumulation of waxes [158]. Yang et al [158] performed analysis on an ox-*win1/shn1* (overexpressor *wax inducer 1/shine 1*) mutant. *WIN1/SHN1* encodes a transcription factor that regulates the expression of genes that control the accumulation of cuticular wax. Analyses performed by Yang et al [158] showed that induction of *WIN1/SHN1* expression by drought is correlated with an increased expression of the genes involved in wax accumulation, and on the other hand, a decreased expression of the genes involved in stomatal development. These results suggest that the drought-tolerant phenotype of analyzed by Yang et al [158] forms caused by induction of *WIN1/SHN1* may be due to a reduced number of stomata as well as wax accumulation.

There are now several high-throughput phenotyping techniques available for the measurement of some of the traits described above. One of these is thermal infrared imaging, or infrared thermography (IRT), which is used to measure the leaf or canopy temperature.

Evaporation is a main determinant of leaf temperature. There is a direct relationship between leaf temperature, transpiration rate and stomatal conductance [159-161]. Drought-tolerant genotypes can maintain a higher stomatal conductance and also a higher rate of photosynthesis, as was mentioned above, thus these genotypes could be identified as having a lower canopy temperature than the sensitive genotypes [162-163].

7. GM crops – are they a solution?

Genetic modification of crops is a controversial issue. Some aspects of genetic modification that have potential to improve drought tolerance in crops are presented here. Biotechnological approaches may involve the overexpression of genes related to osmotic adjustment, chaperones and antioxidants [reviewed in 164-165]. Also, ectopic expression or suppression of regulatory genes, such as genes that encode transcription factors, is widely used [166]. Recent studies on rice led to the identification of genes involved in three pathways that can be manipulated in order to improve drought tolerance in crops: the gene that encodes β-carotene hydroxylase, which confers drought resistance by increasing xanthophylls and ABA synthesis [167], the *DST1* (*DROUGHT AND SALT TOLERANT 1*) gene that regulates stomatal closure and density under drought stress [168] and the *TLD1/ OsGH3.13* (*INCREASED NUMBER OF TILLERS, ENLARGED LEAF ANGLES, AND DWARFISM*) gene whose down-regulation enhanced drought tolerance in rice [169]. Although several genes that can improve the drought tolerance of crops have already been identified, progress in the commercialization of the traits controlled by these genes has been slow [165]. One of the genes that has been successfully introduced into a crop plant and that gave improved drought tolerance in field trials was the gene encoding Cold Shock Protein B (CspB) RNA chaperone from *Bacillus subtilis*. The *CspB* gene is important in the ability of bacteria to adapt to cold, and its overexpression in plants was shown to provide drought tolerance in Arabidopsis, rice and maize [170]. Results from field experiments showed that a maize line expressing the *CspB* gene had a higher yield under water deficit conditions than the control and expressed a yield equivalent to the control under non-stressed conditions. Tests are in progress in 2012 on commercial farms, [171; http://www.monsanto.com/products/Pages/corn-pipeline.aspx#firstgendroughttolerantcorn]. The value of a biotechnological approach to improving crop yields under drought stress conditions is becoming evident with the first demonstrations of improved drought tolerance in crops in the field (reviewed in [171]).

8. Conclusions and perspectives

In order to achieve a full understanding of drought-response mechanisms in plants and to make use of this understanding to produce crops with improved drought tolerance, there is a need to combine the data derived from different studies. Detailed analyses of the networks of protein interactions, the co-expression of genes, metabolic factors, etc. should provide insights into the key regulators of drought response [172-173]. Biotechnological approaches

can also be promising in improving drought tolerance in crops based on previously obtained and integrated knowledge [171].

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Chapter 5

Root Development and Abiotic Stress Adaptation

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Additional information is available at the end of the chapter

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1. Introduction

As soon as plants became independent from homogeneous aquatic environments, root-like organs were developed. The interface between land and water bodies was probably the medium for the earliest land plants. Taking into account that those ancestral root-like organs did not face problems of water and nutrient acquisition, they were probably rather simple. As the earliest plants colonized this medium, the sandy substrate was replaced by heterogeneous soil, promoting more sophisticated vegetation and expanding the limits of land plant colonization. Therefore, to increase the efficiency of exploration of heterogeneous soil, during plant evolution the ancestral root-like organ was replaced by a complex root system (RS) as the one we now know [1-3]. Land plants nowadays present a wide diversity of root system architectures (RSA; spatial configuration of the root system) among species, from non-branched to highly complex branching patterns, achieving the most effective performance regarding anchorage and the acquisition of water and nutrients. Each kind of RSA is guided by a genetically controlled post-embrionary root developmental program (PERDP). This program is not rigid, and actually permits high phenotypic plasticity in response to stressing environmental conditions. PERDP is essentially driven by two cellular processes, cell division in the apical root meristem and new lateral meristems formed from the pericycle, and cell expansion performed in the root elongation area. This particular characteristic permits plants, which are sessile organisms, to change their root architecture to adapt to abiotic stress [4-6]. Soils provide plants with water and nutrients; however, nutrients and water are distributed in a heterogeneous or patchy manner. In order to enhance nutrient capture, plant roots have modified their root architecture to explore those nutrient-rich zones. In the last two decades, progress has been made understanding the physiological, molecular and biochemical basis of how the PERDP could be modified by abiotic environmental cues [5, 7]. The aim of this chapter is to provide a review of how abiotic stress modulates post-embryonic plant root development. We will begin with a discussion of origin, anatomy, morphology and kinds of RS. Then, we will review recent advances in the knowl-

edge of molecular, genetic and cellular processes that modulate post-embryonic root development in the model plant *Arabidopsis thaliana* making emphasis in the cell cycle. We will continue to focus on the modulation of PERDP in response to salinity and water. We will describe the changes in the RS induced by nutrients such as nitrogen, potassium and iron. The modulation of RSA by phosphorous will be discussed taking into account molecular, genetic and cellular responses. Finally, we will discuss how abiotic stress modulates apical root meristem activity.

2. Root system

Raven and Edwards (2001) define: "roots are axial multicelular structures of sporophytes of vascular plants which usually occurs underground, have strictly apical elongation growth, and generally have gravitropic responses which range from positive gravitropism to diagravitropism, combined with negative phototropism". The apical meristem of one (lower vascular plants) to many (all seed plants) diving cells produces a root cap acropetally and initials of stele, cortex and epidermis basipetally. The branching of roots involves the endogenous origin of new root apical meristems in the pericycle [2]. The most conserved functions of roots present in extant plants are anchorage to substrate, and uptake of water and mineral nutrients. The evolution of multicellular organs such as roots was necessary to successful colonization of land by early plants [1, 4].

2.1. Origin and evolution

Over 470 million years ago, in the mid-Palaeozoic era, took place one event with far-reaching consequences in the history of the life, the origin and early evolution of embryophytes (land plants). It appears that margins of drying pools were the place where early embryophytes evolved from algal ancestors. The earliest land plants probably presented a system of rhizoid-like filaments that performed the rooting functions (anchorage and uptake water and nutrients) helped by associated fungi. They grow in superficial soil produced for weathering of rock surface similarly to bryophytes (mosses). Their appearance started changes on energy and nutrient fluxes among terrestrial and freshwater ecosystems and consequently for the evolution of animal, bacteria and fungi groups that lives in those habitats. Roots as the ones we know now are present only in vascular plants (tracheophyta), they evolved in the sporophyte of at least two different lineages of tracheophytes, lycophytes (licopods) and euphyllophytes (ferns and seed plants), during the Early and middle Devonian. Roots of early Euphyllophytes started to penetrate deeper into substrate increasing the anchorage and funding the inorganic nutrients produced by rock leaching. In Euphyllophytes a fundamental difference in the anatomy of embryonic roots among seed plants and free-sporing monilophytes, suggesting that roots evolved independently. At this time root developed more branched axes and finer structures involved in the nutrient uptake, root hairs. In Carboniferous (300 millions of years ago) gymnosperms appear and their RS is highly branched and depth penetration, they break up rocks letting exposed mayor rock area exposed to weathering. By late Cretaceous (100-65 millions of years) angiosperms are presents showing similar root system as exant angiosperms [1, 2, 8].

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During the Devonian period (415–360 million years ago) apparition and radiation of embryophytes with roots caused large changes to the global level. The early land plants with rhizoid-like filaments that penetrated the top few centimeters of soil, were replaced by plants with deep RS with complex structures. The apparition of those organs that actively penetrate the rock with the capacity of uptake and transport mineral nutrients permitted the development of structurally complex above-ground structures to photosynthesis, which increased the amounts of carbon fixed on the continent. The increase of primary production of early land plants changed the global carbon cycle and generates new complex soils which increased the border of land inhabited by plants. On one hand, the high rates plant production in this period allowed deposition of carbon on continental area from plant-drive organic matter, organic molecules secreted into the soil; on the other hand, the increase weathering rate of rocks by root penetration and secretion of organic compounds permitted the mining of rock-derived inorganic nutrients. Those changes in habitat turned up to be a part of a stimuli cycle in plant evolution, as themselves allowed the primary production to rise, which produced changes, and so on. The apparition of RS during Devonian allows that most of land surface was covered by plants, since Carboniferous forest (300 million of years ago), through late Cretaceous where basal angiosperms appeared (100-65 million of years ago) until days [1-3].

2.2. Classification and architecture

The RS consists of all roots that a plant has. It can be classified according to branch structure, root activity or development. The classification based on development is the more typical and useful to analyze the RS growth. This approach ontogenetically classified roots into three categories: primary root (PR), lateral root (LR) and adventitious root (AR; Figure 1 A). This classification reflects the differences between monocotyledonous and dicotyledonous RS. During germination PR is the first root to emerge from seed in both monocotyledonous and dicotyledonous, and is derived from embryonic root. In most of dicotyledonous LR are formed post-embryonically from pericycle cells (Figure 1 B-C) generating a branching system called primary root system. Depending on the length of LR relative to the primary axis (PR), the morphology of the RS will vary between tap rooted (Figure 1 A) and diffuse [3, 6, 9, 10]. Many monocotyledonous form PR and LR in a manner alike to dicotyledonous, in addition form nodal roots (AR) to generate a 'fibrous' adventitious roots system [6, 10, 11]. The morphology of the RS itself is very consistent, depends on the species, however, the spatial configuration of the RS (number, position and growth position of PR, LR and AR) called root system architecture (RSA) is highly variable, even among genetically identical plants. RSA is generated during post-embyonic root development and is guided by a plastic genetic program which is modulated by environmental cues [4, 9].

3. Root system development

Root development can be divided in two main stages: a) embryonic development (ED) and b) post-embryonic development (PED). During the ED, through a suite of highly regulated and reproducible stages, the fertilized egg cell rises into an embryo. In the embryo, the primary

meristems, body axes and major tissue layers are established [12-15]. Unlike metazoans, almost all the body of the mature plant is generated during the PED. The PE begins during germination, when the mitotic activity of meristems commences. Primary root meristems occupy one end of the main body axis and originate the RS [9, 14, 16]. During the post-embrionary root development traits such a i) primary meristems activity, ii) cell elongation, where both determine the anatomy, length and trajectory of roots and iii) de novo formation of secondary meristems and organs increase the branching to explore new soil zones [5, 6].

In Arabidopsis, the root consists of a series of concentric cylinders of different tissues (Figure 1 B), and this pattern is formed by sequential and ordered cell divisions during embryogenesis [17]. The outer epidermal layer covers all root tissues, and by itself contains the trichoblasts, a cell lineage that produces root hairs by tip growth, providing the root with additional anchoring and nutrient uptake surface. Cortex layers give mechanical support and protection while the endodermis forms an ion barrier. Inwards the endodermis, the pericycle cells maintain meristematic properties that can give place to root primordia or diverge into vascular tissues or cambium during secondary root growth. This pattern is established during the embryogenesis by a series of asymmetric and formative divisions [18, 19].

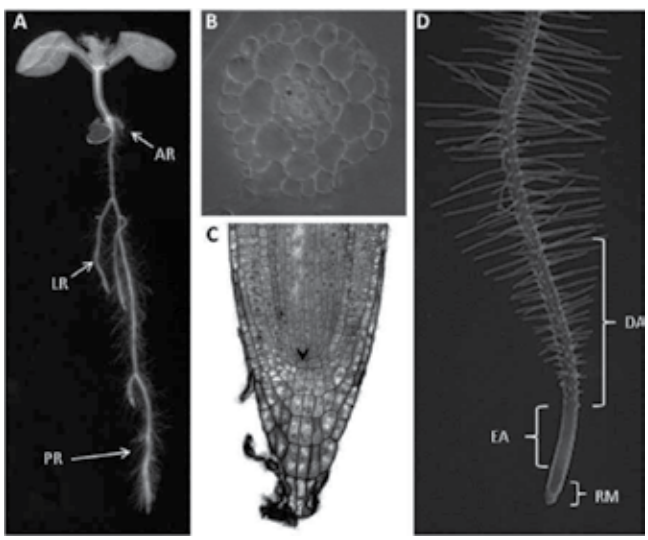

Figure 1. Arabidopsis root system. Typical tap root system of dicots (A). Transversal section of primary root (B). Longitudinal section of primary root meristem (C) and primary root tip. Primary root (PR), lateral roots (LR), adventitious roots (AR), pericycle cell layer (*), QC cells (arrow), root meristem (RM), elongation area (EA) and differentiation area (DA).

3.1. Cellular proliferation, elongation and differentiation

Root growth is produced by the biosynthesis of cell wall combined with cell division. In the root meristem (RM) (Figure 1C-D), the cell layers apart from the epidermal and root cap ones are originated around a region that consist of three or four slowly proliferating cells, the

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quiescent center (QC) (Figure 1 C), which has a role organizing the meristem and is also involved in the stem cell identity maintenance QC removal results in the de novo formation of a new QC with adjacent initial cells and stem cells adjacent to the cortex and endodermal stem cells yield to epidermal initial cells and the lateral root cap [20-22]. Directly upwards from the QC the proximal meristem is located, as the distal meristem is located below, and within the meristems the forward growth is carried on as cells divide and grow there at a steady rate. When reaching certain distance from the meristem, in elongation area (EA)(Figure 1D) division is arrested and the cells start to elongate. Elongated cells are associated with endoreplication, a process of DNA replication without actual cell division which accumulates genome copies in the cell and uses part of the machinery associated with cell cycle, and involves the inactivation of mitotic CYC-CDK (Cyclin- Cyclin Dependent Kinase) complexes [23-25]. Pericycle and cambium cells, distanced from the root tip, maintain the potential to reenter division, forming LRs or transitional cells at the meristem end, depending on localized auxin responses [26] or oscillating gene expression [27].

3.1.1. Cell cycle

The cell cycle is a temporal regulator of proliferative cell division, and it is comprised of mitosis, cytokinesis, post-mitotic interphase (G1), DNA synthetic phase (S) and post-synthetic interphase (G2)[28]. The conjunction of all these is the key force driving organogenesis and growth in plants and other eukaryotes. The mitotic cycle is driven by the periodic activation of a multicomponent system that relies on CDKs as key regulators. CDKs combine with different CYCs to trigger the transition from the G1 to S phase and the G2 to M phase, and a wide variety of components control the activity of these kinases, thus becoming part of a complex molecular network that is still being studied [29-31]. In plants, a number of core cycle regulators have been revealed to exist [32, 33] and what appears to be distinctive in plants is that they appear to have many more CYCs and CDKs in comparison to animals and yeasts [21, 24]. The reason of this abundance of putative function overlapping components can be the one suggested in [34], postulating that that plants have evolved a combinatorial resource pool consisting of around ninety different CDK-CYC complex variants, thus explaining to an extent the plasticity of plant development regulation, as they provide with a strategy to recognize distinct stimuli and environments, and thus promote different phases of the cell cycle. Cell cycle progression and controlling mechanisms include transcriptional regulation, protein-protein interaction, phosphorylation-dephosphorylation and protein degradation [29, 30, 35, 36]. As recently reviewed [36], the evidence obtained from interaction studies suggests that Arabidopsis CDKA;1 primarily binds to CYCDs to promote the G1/S transition and to CYCA3 to drive the S phase progression while CDKA;1 pairs with CYCD3 to drive the M phase progression. In contrast, CDKBs presumably interact preferentially with CYCA2 and CYCBs to promote the G2/M transition and the M phase progression [37-39]. In Arabidopsis, the accumulation of the CYCB1;1 transcript is correlated with meristematic tissues [40], activated from early S phase in synchronized cells with no significantly increase during G2 phase [41]. Together with environmental and hormonal stimuli, the coordination of the different cell cycle control processes lead to a balance between cell division and expansion that ensures the correct embryonic and post-embryonic development. As part of the extensive toolset that plants

possess in order to finely tune the mitotic and endoreplicative cycles, the phase-specific activation of CYC-CDK complexes via temporal transcription is a mechanism that is evidently used but not completely understood in plants. In synchronized Arabidopsis cell cultures many cell cycle genes present highly specific expression windows during the mitotic cell cycle [41, 42]. For example, the expression of several CYCAs is dramatically upregulated at the G1/S transition and S phase, while others are accumulated at G2/M transition, as well as all CYCBs. Most of CYCDs are expressed during G1 and S phases, with the exception of a few ones, like CYCD3:1, expressed during G2-M. In the case of CDKs, CDKA;1 is expressed throughout the cell cycle, with constant transcript levels, the CDKB1s are present from S to M phase, and CDKB2s are detected specifically from late G2 to M phase.

3.1.2. Cell cycle control in root meristem

The expression windows of cell-cycle control genes can be extrapolated to their expression in the actively dividing cells of the root meristems. In these and all dividing cells, The G1/S transition is generally controlled by the E2F-DP-RBR (E2F-Dimerisation Partner-Retinoblastoma Related) pathway. One of the three Arabidopsis-encoded E2F transcription factors forms dimers with one of the two DP proteins to bind to certain promoter sites in the transcriptional target genes to promote the G1/S transition, including those required for DNA replication and repair. In G1, CYCD-CDKA complexes phosphorylate RBR, releasing the E2F-DP dimers to allow them to bind to the transcriptional activation sites [43-46]. In the other hand, E2Fc-DPb dimers act as transcriptional repressors with yet unknown target genes, although their repressing mechanism appear to be independent from the RBR pathway [47]. Meanwhile, genes expressed during G2 and M phases contain M phase-specific activator (MSA) elements in their promoters, recognized by three Myb repeats (MYB3R) transcription factors, discovered for the first time in tobacco [48]. The Arabidopsis genome encodes five MYB3R proteins (MYB3R1-5), from which MYB3R1 and MYB3R4 are the closest homologs of the G2/M specific transcriptional activators NtMYBA1 and NtMYBA2, with the first having a stable transcript level through the cell cycle, and the latter having an expression peak during G2/M transition, suggesting that MYB3R1 is post-translationally regulated. The expression of many G2 to M specific genes possessing MSA elements in their promoters is visibly down-regulated in the *myb3r1 myb3r4* double mutant, but not completely abolished [49] suggesting an alternative mechanism controlling the transcription of G2 and M phase genes. Additionally to E2F and MYBs, there are other transcription factors that control cell cycle phase-specific gene expression, as the DNA-binding with one finger (DOF) transcription factor, OBP1, whose overexpression shortens the cell cycle with elevated expression of many other cell cycle genes, and that normally upregulates the expression of replication-specific transcription factors and CYCD3;3 [50]. Another form of controlling the activity of CYC-CDK complexes is through post-translational mechanisms, and among them, the ubiquitin-mediated degradation of cell cycle proteins is the most determinant for the correct timing in the progression of the cell cycle [51-53]. A number of ubiquitin-dependent degradation pathways have been associated with the mitotic cell cycle, and the E3 ubiquitin ligases participate in all cases, marking target proteins by polyubiquitination and subsequent proteolysis. The Skp-cullin1-F-Box (SCF) E3 ligase regulates primarily the G1/S transition while the Anaphase Promoting Complex/

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Cyclosome (APC/C), a Cullin-RING finger E3 ligase, is most active from the M phase to G1 phase. APC/C complex is composed by at least 11 subunits, and in the Arabidopsis genome, all APC/C components except for APC3/CDC27/HOBBIT are encoded by a single gene [54]. All APC/C subunit mutants studied so far accumulate mitotic CYCs in embryo sacs, suggesting that they're substrates of the APC/C [52, 54]. Apart from its core components, APC/C also pairs with co-activators, known as CDC20/FIZZY and CDC20 HOMOLOG1 (CDH1)/FIZZY-RELATED (FZR), which confer substrate specificity and are activated during distinct phases of the cell cycle with equally distinct activities. The Arabidopsis genome contains five CDC20 and three CDH1 genes, also called CELL CYCLE SWITCH 52 (CCS52), and even if their modification of the APC/C activity during the cell cycle is not fully established, CCS52A1 and CCS52A2 participate in meristem maintenance [55]. Notably, they act through different mechanisms and exhibit different expression patterns as well. The expression of CCS52A1 starts at the elongation zone of Arabidopsis roots and stimulates mitotic exit and an entry into the endoreplication cycle, whereas CCS52A2 is expressed at the distal part of the root meristem and is required to maintain the cell identity in the QC. The *ccs52a2* mutation activates the QC cell division, contrasting with the occasional division behavior normally presented, and when its promoter is switched with the one of CCS52A1, the expression of the latter rescues the phenotype in *ccs52a2*, suggesting homologous function. In vertebrates, negative regulators also modify the activity of APC/C. These regulators, called Early mitotic inhibitor1 (Emi1) and Emi2 directly bind to CCS52 and CDC20, inhibiting the APC/C activity. No direct plant orthologs are identified, but recent studies have shown that GIGAS CELL1 (GIG1)/OMISSION OF SECOND DIVISION 1 (OSD) and UV-INSENSITIVE4 (UVI4)/POLYCHROME (PYM) act as their functional homologs in plants [56, 57] by physically interacting with the APC/C activators CCS52 and CDC20. Their overexpression causes an accumulation of CYCB1;2 and CYCA2;3, respectively, by the inactivation of the APC/C, suggesting that it also could have an effect on root meristem maintenance by the inhibition of the APC/C-CCS52 complex activity. Another important way to control and modulate the CYC-CDK complexes activity involves said complexes binding directly to CDK inhibitors, proteins that interfere with the ability of CYC-CDK to phosphorylate their substrates. Plants have two classes of CDK inhibitors – KIP-RELATED PROTEINs (KRPs) and SIAMESE (SIM)/SIAMESE RELATED (SMR). The Arabidopsis genome encodes 7 KRPs, KRP1-7, and at least 13 SIM/SMRs. Recent analyses have shown that all 7 KRPs purify conjoined with CYCDs and CDKA [36] suggesting that they inhibit the activity of the CYCD-CDKA complexes, as it has been proposed previously [58], but not excluding the possibility of them inhibiting the activity of CYCD-CDKB complexes as well [59]. The seven KRPs display overlapping but distinct expression patterns in the Arabidopsis shoot apex, some of them present strongly in dividing cells, like KRP4 and KRP5, while KRP1 and KRP2 are present in differentiating cells [60]. KRPs have a role driving the endoreplication cycle as well, also by inhibiting CDK activities [61, 62]. The SIM/SMR family of CDK inhibitors is found only in plants, and is required to repress the mitotic cell cycle in trichomes via the interaction of SIM with the CYCD-CDKA complex [63]. Another member of the SIM family, SMR1/LGO, is implicated in the control of endoreplication in sepals [64], maintaining the presence of elongated, endoreplication-undergone giant cells in the sepals, which are lost in the *smr1/lgo* because they progressed through additional cell divisions instead of endore-

plication. Recent studies [34] show that both SIM and SMR/LGO are purified jointly with CDKB1;1 while other SMRs interact with CDKA;1, thus suggesting that CDKB1;1 could be directly inhibited by SIM/SMR1 leading to endoreplication onset.

3.1.3. Cell cycle control in post-embryonic root development

The cell cycle relies not only on its own molecular machinery to determine cellular fate. Post-embryonic plant development needs a highly precise coordination of cell cycle-directed signaling to correctly drive cells to form new tissues or cell types, as is evident in root development. Molecular genetic studies have uncovered several key regulators involved in developmental cell cycle control, and many of them have shown to be transcriptional regulators, but how are they linked to cell cycle control has not been well characterized. SHORTROOT (SHR) and SCARECROW (SCR) are members of the GRAS family of transcription factors required for the asymmetric division of cortex/endodermis initial cells (CEI) in the root apical meristem [65, 66]. This tissue-formative division generates two new cellular kinds- cortex and endodermis, making the CEI cell division control a key requisite for a proper root development. It has been demonstrated that both SHR and SCR directly regulate the expression of CYCD6;1, present at G1 and S phases, by binding to its promoter [67]. CYCD6;1 is expressed specifically in CEI and CEI daughter cells, and the asymmetric division of CEI is significantly decreased in the *cycd6;1* mutants. Additionally, when CYCD6;1 is expressed ectopically in the *shr* mutant background, it partially compensates the division defects presented by the latter, supporting the idea of CYCD6;1 being downstream of the SHR/SCR pathway. Other cell cycle genes, like CDKB2;1 and CDKB2;2, have their expression regulated by SHR and SCR, and when these CDKs are overexpressed in endodermal cells, the formative cell division of the CEI is promoted. However, they do not appear to be direct targets of SHR and SCR, implying that the activation of these CDK genes is linked by another control factor. Cell proliferation needs to be restored in the xylem-pericycle cells for the LR initiation and this process can be induced by auxin in many plant species, like Arabidopsis. LR development starts by the degradation of INDOLE ACETIC ACID 14(IAA14)/ SOLITARY-ROOT (SLR), dependent on auxin, that leads to the de-repression of two related AUXIN RESPONSE FACTORs (ARFs), ARF7 and ARF19 [68]. These ARFs are required for the subsequent expression of LATERAL ORGAN BOUNDARIES 18 (LBD18] and LBD33 transcription factors, which form a LBD18-LBD33 heterodimer that activates the expression of the E2Fa, one of the E2F genes induced at LR initiation, by binding directly to its promoter [69]. E2Fa expression is increased by auxin treatment at the LR initiation site and this auxin-dependent E2Fa expression is lost in the *iaa14/slr-1* mutant background. Expectedly, the number of LR primordial is decreased in the *e2fa* mutants, evidencing a requirement of E2Fa for LR emerging and establishing a link between auxin signaling and cell cycle progression during LR development. Another unrelated pathway that is also involved in the auxin-induced LR formation has KRP2 downregulated by auxin [70]. Under low auxin conditions, the CYCD2;1-CDKA activity is repressed by the presence of KRP2. Upon auxin treatment, both gene expression and protein accumulation of KRP2 is reduced, leading to an increase in the CYCD2;1-CDKA activity and subsequent enhancement of LR induction. A possible hyperphosphorylation of RBR resulting in the activation of E2Fb directly caused by the CYCD2;1-CDKA complex activity has been suggested [69]. A model on the basis of available information on the density and

orientation of auxin transporters, cell shape, and auxin transport parameters predicts a maximum auxin concentration in the QC and a steep auxin gradient in the proximal meristem, which drops according to the cell number from the quiescent center [71, 72]. This agrees with the auxin levels found in protoplasts derived from different apical cell types, as well as with the expression patterns of auxin responsive genes, such as members of the PLETHORA (PLT) family, in the different root tissues [73]. PLT 1 and PLT2 are known to be crucial for interpreting this gradient in the terms of root growth and development. They encode for AP2-domain transcription factors, and losing of their function results in the loss of stem cells, arrest of transit-amplifying divisions and reduction of cell expansion [74]. PLT pathway has other effects over cell cycle control. Histone acetyltransferase, a chromatin modifier and required to maintain the dividing ability in meristem cells, is also required to sustain PLT expression and support both transit-amplifying divisions and the root stem cell status at the root apex [75]. Moreover, the action of SUMO E3 ligase is vital to repress endoreplication in shoot and root meristems, and in the root, this SUMO E3 ligase acts in the PLT pathway [76]. It can be said then that the root tip is characterized by an auxin maximum, and auxin is required to support transit-amplifying divisions [77].

4. Root system development and abiotic stress

Abiotic cues as water and nutrient availability limit plant productivity in almost all ecosystems in the world. Typically, RS has to growth in media where the biotic and abiotic components are distributed heterogeneously. Soils are complex, a broad range of chemical a physical process occurs due to intrinsic soil characteristics and the action of biotic factors. Thus, this complexity presents several challenges to survive. As soon as the root makes contact with the soil must sense and integrate biotic and abiotic cues in order to adjust their genetic program of post-embryonic root development (PERD). This capacity to change their PERD allows them change their architecture to find the supplies of water and nutrients that could be limited and localized [3, 4, 12]. Environmental cues such as water, salinity and nutrient can modulate the ARS.

4.1. Regulation of root system architecture by water availability and salinity stress

Water and salinity can indirectly modulate the RSA because they can produce unfavorable changes in the nutritional composition of the soil, the distribution of said nutrients, the density and compaction of soil, and the type of soil particles [9]. Those interactions complicate the dissection of specific transduction pathways involved in root growth and development [78] The RS is the first to perceive the stress signals for drought and salinity, therefore its development is deeply affected by their availability in soil. In many agriculturally important species, the whole plant growth is inhibited during water starvation, however, RS is more resistant than shoots and continues growing under low water potentials that are completely inhibitors for shoot growth [79]. Notably, while growth of PR is not appreciably affected by water deficit, the number of LRs and its growth are significantly reduced [80]. It has been suggested that the reduction of the LR formation may be caused by the suppression of the activation of the lateral root meristems, not because of the reduction of the initiation in the LR per se, as primordia

generation is unaffected [9, 80-82]. Mutants with alterations in the development of LRs respond differently to drought stress [80, 83]. Suppression of the growth of LR by drought has been widely accepted as an adaptive response to ensure the plant survival under unfavorable growing conditions [83]. Another factor that plays an important role in growing and development of plants to tolerate the drought stress is the hydrotropism [84, 85]. A recent study showed that a gradient of moisture generated by water stress causes an immediate degradation of amyloplasts in the columella cells of plant roots, producing a minor response to gravity and an increase of hydrotropism [86]. However, it is unknown how the gravity signals interact with other environmental signals to modulate the direction of root growth. Less known are the adaptations in root morphology and its relevance to salinity tolerance. Many halophytes have developed morphological adaptations, like the formation of specialized organs to expel salt out of their leaves, which allows them to keep the water and take out the salt in an active manner. Glycophytes have not developed permanent changes on its morphology to deal with salt, but they can adjust the root growth and its architecture in response to salinity, like in the case of Arabidopsis [87]. Also it has been observed that Arabidopsis RS exhibit a reduced gravitropism under salt stress, growing against the gravity vector [88]. Arabidopsis RS exposed to a simultaneous salinity and gravity stimuli responded to salinity with a change in growing direction in a way that apparently represents an adaptive arrangement between gravitropic and saline simulation. Control of the relation between gravitropism and hydrotropism allows plants to direct the root growing for a better water uptake, giving an advantage during development of the radical system under stress conditions. It is known that the salt stress inhibits the growth of the PRs in Arabidopsis seedlings, although it has been reported that salt stress also modulates root gravitropism of PR in young seedlings. In vertical position, five day seedlings germinate normally in MS medium (Murashige and Skoog) containing different concentrations of sodium chloride (NaCl), however the direction of root growth changes according to the increase of NaCl concentrations, and the root curves in stressed plants with 150 mM NaCl in the medium [88].These results suggest that the salt stress and the induction of signal translations by stress modulate the direction of the root, despite of the gravity. Some reports suggest that the gravitropic signal and the answers in root apex are controlled, at least partially by Salt Overly Sensitive (SOS) signaling pathway. Therefore, this pathway might interact with the gravity sensor system in the cells of the columella to direct root growth in a coordinated way[88]. Abscisic acid (ABA) and auxins participate in a complex signal system that plays a very important role in the development of the RSA under drought conditions. These hormonal effects (levels) even though are considered as intrinsic [82] can change in response to environmental cues. Cytokinins, gibberellins and abscisic acid are produced in roots to be transported to other tissues, where they play their roles in development and growth. Although auxins are the major determinants of root growth [89], cytokinin and especially abscisic acid [90-92] have been proposed as potential chemical signals in response to water stress to modulate RSA. The decrease in water potential of roots caused by salinity is the factor that triggers the production of ABA in different species [93]. A condition of mild osmotic stress also inhibits the LR formation in a dependent way of ABA [80, 82, 83, 94]. In Arabidopsis, the reduced water availability dramatically inhibits the formation of LR, but not by the suppressing of initiation of LR at the lateral primordia. This inhibition does not occur

in lateral root mutant 2 (*lrd2*) nor in two ABA deficient [80, 82]. Abscisic acid and a recently identified gen *LRD2* are linked to repression of LR formation in response to osmotic stress. It is very interesting to note that these regulators are also related to the establishment of RSA without apparent effect of osmotic stress. The mutant *lrd2* presents an altered response to exogenous application of ABA, while ABA-deficient mutants and *lrd2* show an altered response to inhibitors of polar auxin transport [95-97] suggesting a joint interaction of the hormonal signaling pathway in the regulation of LR formation. Some authors propose a model where the promotion or suppression of hormonal signaling pathway and regulators as LRD2 determine the type of LR primordium (LRP) and coordinate the RAS in response to environmental stimuli [87]. In contrast, under drought stress conditions or osmotic stress, activation of the LR meristem is suppressed by ABA-mediated signals, producing few small LRs [80, 98]. While auxins seem to be the main initialization hormone, pattern and emergence of LRs; ABA is the main hormone that controls the environmental effect (like drought and salt stress) over the RSA [99].

4.1.1. Cellular responses

4.1.1.1. Epidermis

Root epidermis is the first tissue that makes contact with salt; hence, it is the first to perceive osmotic and ionic changes in cells and the first one that triggers rescue mechanisms. The accumulation of sodium in the cells and the resulting ionic imbalance is the main cause of inhibition of plant growth and yield decrease [100]. Therefore, maintaining low intracellular sodium levels is critical for plant adaptation to water and salinity stress. Plants use different strategies to fight against salinity damage in every organizational level, from cellular, biochemical, molecular to anatomic, morphological and phenological level. At cellular and molecular level, plants cells keep a low cytosolic sodium (Na^+) content by means of compartmentalization and ionic transport regulation [100, 101]. During salinity stress, processes of membrane transport play a very special role. Some transport mechanisms implied in the perception of salt stress are: water output of the cell by osmotic gradient, the decrease of the availability of potassium (K^+) in roots due to the reduced activity of this cation in soil solution, where sodium competes for binding sites for K^+ transporters in PM (plasma membrane) including low and high affinity, also the increased efflux of K^+ by selective and non-selective channels [102] and finally that these ionic events initially evoked in the PM of epidermal root cells are propagated to intracellular organelles (mainly vacuoles) and other plant tissues such as leaves. Considering the entry of Na^+ and K^+ loss, preventing worsening of the K^+/Na^+ cytosolic relation is a key criterion for resistance to salt stress. Once the stress is perceived, the respective signalization triggers and changes in metabolism and genetic expression take place; all these are related with defense mechanisms [102, 103]. For the response to osmotic changes in metabolic compartments, it occurs an immediate osmotic adjustment by synthesizing compatible osmolytes and inorganic ions capture [104], for the toxic component of stress is performed a compartmentalization of harmful ions and ion transport [105]; and it generally occurs a restriction of unidirectional Na^+ entry via non-selective cation channels (NSCC) [105, 106] and high affinity potassium transporters (HKT) [107, 108], the Na^+ efflux from the cytosol

by the Na^+/H^+ exchanger in the PM [100] or its capture by tonoplast [109]; changes of metabolism and signalization by polyamines and Reactive Oxygen Species (ROS) and the antioxidant activity [110, 111].

4.1.1.2. Reactive oxygen species

ROS fluctuations in time and space can be interpreted as signals to regulate growth, development, cell death and stress responses [112, 113]. Understanding the mechanisms that control ROS signaling in cells in response to water stress and salinity could therefore provide a powerful strategy for increasing crop tolerance to these environmental stress conditions [114]. Among the targets of ROS action at the cellular level, there are ion channels that mediate ion exchange in the PM. In the PM of roots and guard cells H_2O_2, stimulates the channels activated by hyperpolarization that mediate the influx of Ca^{2+} and NSCC [112, 115, 116] and inhibit the K+ outward and inward rectifier currents [117]. The stimulation of the influx of Ca^{2+} in guard cells appears to mediate the induction of stomata closure by ABA [116, 118-120]. At the same time it was reported that the OH• activates a Ca^{2+} inward and K+ outward currents in epidermal protoplasts derived from mature and growth zone of Arabidopsis roots [115]. A larger stimulation of the inward current of Ca^{2+} in the growth zone may indicate that ROS are involved in growth regulation via Ca^{2+} signaling. Moreover, the OH• produced by NADPH oxidase in Arabidopsis root hairs activated a Ca^{2+} inward rectifier conductance causing an increase in cytosolic Ca^{2+} allowing the root elongation [112]. Recently it has been reported that under severe water stress autophagy programmed cell death occurs in the region of the root apical meristem [121]. There is evidence that this defense mechanism is promoted by the accumulation of ROS in stressed meristematic cells of root tips. Analysis of the expression of BAX inhibitor-1 (AtBI1, apoptotic inhibitor) and the phenotypic response of the mutant *atbi1-1* under severe water stress indicates that AtBI1 and the pathway of endoplasmic reticulum stress response modulates the induction of PCD by water stress. As a result, thin and short roots induce an increase in their tolerance to stress. These authors also propose that under severe drought stress, plants activate the PCD program in the root apical meristem, removing the apical dominance; so they can remodel the RSA to adapt to stressful environments [122].

A slight drought stress increases the expression of enzymes associated with root morphology (Xyloglucan endotransglucosylase) while other structural proteins (actin and tubulin) are downregulated, these proteins are strongly correlated with root growth since its function is the vesicular carrying in cells with polarized growth (e.g. root hairs) allowing its growth and hence an augmentation in the surface of water uptake. However, when there is a greater stress, these structural proteins increase their expression. It is believed that alterations in the expression of these proteins are positively correlated with the of LR development that partially has an indirect effect on whole plant photosynthetic process [123]. While the decrease of lateral root development is a well-known response to water stress, none of the mutants that are resistant to drought stress have a reduced number of LR [124]. Only a few transcription factors have shown to regulate the formation of roots under drought conditions, among them stands the MYB96 transcription factor since it plays an important role in LR growth under drought

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stress conditions [124], these same authors found that overexpression of MYB96 promotes resistance to drought and reduced lateral root density.

4.2. Regulation of root system architecture by nutrients

In soil nutrients such as phosphorus (P), nitrogen (N), potassium (K) and iron (Fe), are distributed in a heterogenous patching pattern. As soon as the PR emerges from the seed, it has to grow. As growth goes on, *de novo* LR are formed to generate the particular RS morphology and architecture. These nutrients alter root patterning through particular signal transduction pathways. Thus, during their life plants change their PEDP in order to increase exponentially the root-soil interaction area and find the nutrient-rich regions [5, 125-129]. The changes in Arabidopsis root system are specific for each nutrient. P, N and K starvation dramatically alter primary root length (Figure 2).

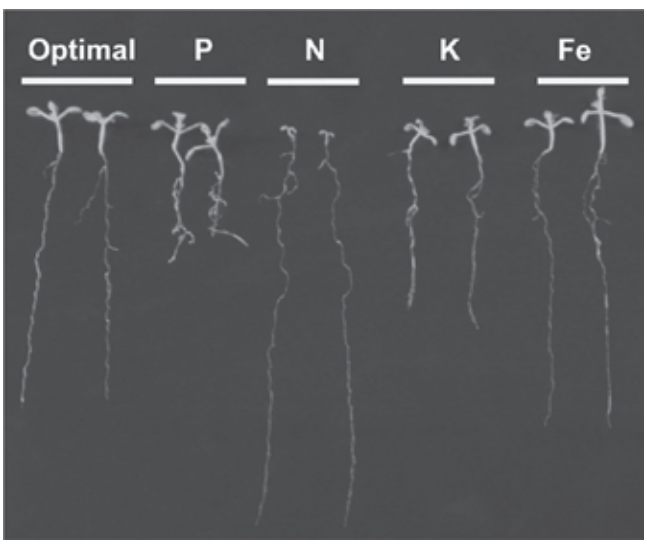

Figure 2. Changes in root system architecture of Arabidopsis seedlings when growth on media depleted of phosphorous (P), nitrogen (N), potassium (P) and iron (Fe).

4.2.1. Phosphate starvation

Root system in boot monocotyledonous and dicotyledonous plants, present a set of developmental modifications that tend to increase the exploratory capacity of the plant [130]. When Arabidopsis growth under limiting P conditions their RSA changes dramatically such as reduction in primary root length, increased formation of LRs and greater formation of root hairs [126, 128]. On optimal P conditions the newly formed root cells are added by the mitotic activity of primary meristem. These cells then get away from the meristem and increase their length, and the elongation process ends when the cells start to differentiate. When plants are P starved, cell division in the primary root meristems gradually reduces and the cells start to

prematurely differentiate until total inhibition of cell elongation and loss of meristematic activity occur (meristem exhaustion). At the end, root tips change their physiological characteristics and the exhausted meristem becomes a structure which takes part in P uptake. In this process, root tips locally detect P deficiency, this response being mediated by at least LPR multicopper oxidase genes [12, 131, 132]. Recently, iron (Fe) has been reported to play a role as well in the control of these PED reprogramming [133]. This change of root architecture is due to the fact that, in both meristematic and elongation areas, the content of ROS is reduced as long as the determined PED goes on [134].

In the past decade the changes in RSA evocated by P availability has been widely studied, several genes that regulates the root architectural changes has been idetified, trascription factor such as WRKY75, ZAT6 (ZINC FINGER 6), Pi-responsive R2R3 MYB (MYB62) and BHLH32 (BASIC HELIX_LOOP_HELIX 32) [135-138] are key regulators in this response. Mutants affected in the RSA changes induced P availability have been isolated: *pdr2* (*phosphate deficiency response 2*), *lpi* (low phosphorus-insensitive) *siz1* [SAP (scaffold attachment factor, acinus, protein inhibitor of activated signal transducer and activator of transcription) and *Miz1* (Msx2-interacting zinc finger), SIZ] [139-141]. It has been reported that ethylene is involved in modulating Pi-starvation-responsive root growth, it may restrict elongation of PR, but promote elongation of LRs [142] HPS4/SABRE (important regulator of cell expansion in Arabidopsis) antagonistically interacts with ethylene signalling to regulate plant responses to Pi starvation. Furthermore, it is shown that Pi-starved *hps4* mutants accumulate more auxin in their root tips than the wild type, which may explain the increased inhibition of their primary root growth when grown under Pi deficiency [143]. Gibberellins and ROS also trigger responses involving DELLAs proteins which control the rate and timing of cell proliferation and they will be dealt with in further sections.

4.2.2. Nitrogen

N is fundamental for biological molecules, such as nucleotides, amino acids, and proteins. Plants need to acquire nitrogen (N) efficiently from the soil for growth and development. In soil, nitrate (NO^{3-}) is one of the major N sources for higher plant and their concentrations vary in both time and space. Plants are able to sensing these variations of NO^{3-}, which is one of the most important environmental signals affecting plant physiology and development [144]. The effects of N supply on plant development have been particularly studied in Arabidopsis. NO^{3-}-free medium drastically reduces shoot biomass production and appears to have little effect on PR length (Figure 2). However, NO^{3-} has a dual role on LRs. On one hand, the uniform exposure of RS to high nitrate (>10 mM) inhibits lateral root growth at a specific developmental step corresponding to the activation of the meristem in LRP after their emergence[145-147]. As a high NO^{3-} supply on only one part of the RS is able to repress lateral root growth on the whole RS, it has been proposed that nitrate accumulation in the aerial tissues is responsible for this LRP arrest, suggesting that long-distance signals to the root are involved. On the other hand, when the entire RS is exposed to low nitrate concentration (10 μM) and only one part of the RS is exposed to a high nitrate, there is local proliferation of LR. NO^{3-} locally promotes LR growth and increased lateral root growth rate due to a higher cell production in the lateral root meristem [145, 146, 148]. The local stimulation of lateral root growth by nitrate-rich patches is a striking example of the

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nutrient-induced plasticity of PERDP. This stimulation could be dependent on NRT1.1 (Nitrate Transporter 1). This is partially due to the fact that NRT1.1 represses LRP emergence and growth of young LRs in the absence of nitrate. NRT1.1 transports nitrate and facilitates auxin transport in a concentration-dependent manner. NRT1.1 represses LR growth at low nitrate availability by promoting basipetal auxin transport out of the LRP, towards the parental root [149]. MADS-box transcription factor NITRATE REGULATED (ANR1) and Auxin signaling F-box protein 3 (AFB3) are key regulators of RSA in response to nitrate availability. The *Chlorate-resistant 1* mutant (*chl1)* is *ANR1* affected, and is less responsive to the localized NO^{3-}-rich patches similarly to transgenic plants in which ANR1 expression is down-regulated. In the tips of LR and LRP, ANR1 is expressed and is localize with NRT1.1 [150]. The *afb3-1* mutant shows altered root development response to nitrate. *AFB3* is an auxin receptor gene induced by nitrate in the primary root tip and pericycle; its mRNA is the target of miR393 that is induced by the products of NO^{3-} assimilation.

4.2.3. Potasium and iron

Contrasting with physiological and molecular responses to low K and Fe, changes in RSA have been scarcely described. Potassium deficiencies arrest LR and PR development in Arabidopsis (Figure 2) [129]. K^+ transporters play a crucial role in SRA changes in response to K^+ availability. Disruption of the root-specific K^+-channel AKT1 in the *akt1-1* Arabidopsis mutant causes reduced ability of plants to grow in low potassium media (100 μM)[151]. In Arabidopsis, changes in the gravitropic behavior of RS were also observed in low potassium media. The genes of the *KUP/HAK/KT* family are homologous to bacterial KUP (TrkD) potassium porters. The *trh1* (tiny root-hair 1) mutant, which is disrupted in *AtKUP4/TRH1* gene shows agravitropic behavior in its roots independently of K^+ concentration in the media when grown on vertical agar plates, and also, *ProTRH1:GUS* expression is limited to the root cap where gravity is sensed. Interestingly, agravitropic responses in *trh1* are complemented by exogenous auxin. This mutation is associated with the loss of auxin pattern in the root apex. Thus, TRH1 is an important part of auxin transport system in Arabidopsis roots [151-153].

Typically, the root architectural changes in response to low availability of Fe include ectopic formation of root hair due to modulation in their position and abundance [154]. Recently, Giehl et al. (2012) analyzed the changes in LR architecture in response to localized Fe supply in wild-type and Fe acquisition and translocation- defective mutant plants. They found that lateral root elongation is highly responsive to local Fe and that the symplastic Fe pool in LR favors local auxin accumulation. They identified the auxin transporter AUX1 as a major Fe-sensitive component in the auxin signaling pathway that mainly directs the rootward auxin stream into LRs that have access to Fe.

4.3. Meristematic activity regulation by abiotic stress

To cope with environmental changes, plants have to adapt their growth timing and pattern by altering rates of cell proliferation and differentiation. The expression of several cell cycle genes is increased or decreased upon external cues (Figure 3) [155] but it is poorly understood the full molecular basis supporting these transcriptional controls, and if the cell cycle control modifications happen to fall into the post-translational category, the current knowledge is also

very limited. However, there have been identified several key players in stress-induced cell cycle modifications that have cast the first light over the understanding the talk between environmental signals and the mitotic or endoreplication cycle. Gibberellins (GAs), plant hormones, promote cell expansion by disrupting growth inhibitory proteins named DELLAs [156] and also promote cell proliferation in Arabidopsis [157]. In the root meristem of GA-deficient mutants, cell division rate is decreased and the phenotype is rescued by GA treatment. DELLA proteins are also involved in this regulation, as non-degradable forms of DELLA inhibit cell proliferation. Low levels of GAs in GA-deficient mutants enhance the expression of certain CDK inhibitor genes – KRP2, SIM, SMR1 and SMR2- with a DELLA-related mechanism, and cell proliferation defects shown by these mutants can be recovered by overexpressing CYCD3;1. These findings tend to indicate that GA signaling drives cell proliferation by modulating the activity of CYC-CDK complexes, at least partially mediated by the DELLA-dependent expression of CDK inhibitors, and thus making DELLA a potential intermediate in the signal transduction channel connecting environmental signals and cell cycle progression. This is proposed to be a consequence of reduced cell expansion and associated division of the endodermis layer in the root apical meristem [158, 159], suggesting a role for the endodermis in controlling the growth rate in the root apical meristem. Another potential link is RICE SALT SENSITIVE 1 (RSS1), controlling the cell cycle progression under various abiotic stress conditions [160]. The *rss1* mutants do not present evident growth defects under normal conditions, but they display hypersensitivity to high salinity, ionic stress and hyperosmotic stress. Under these conditions, in *rss1*, shoot and root meristems are severely affected, showing a reduced population of proliferating cells, leaving RSS1 as a required factor for proliferative cell status in the meristem. RSS1 is expressed during the S phase of the mitotic cycle and its protein is degraded via APC/C during the M/G1 transition. RSS1 interacts with a Type 1 Protein Phosphatase (PP1), known in humans to inactivate Retinoblastoma (Rb) proteins through dephosphorylation, which is inhibitory to the G1/S transition [161]. Sugars can act as signaling molecules in assorted biological processes, and even that sucrose-dependent cyclin expression is known since a decade ago [162], LR formation through sucrose induction is a good example of sugar-dependent reactivation of cell proliferation [163]. This recent study shows that the expression of CYCD4;1 levels in root pericycle cells is dependent on the sucrose availability, and that reduced CYCD4;1 levels in *cyca4;1* mutants or wild-type (wt) roots grown in the absence of sucrose cause LR density to drop. It is not clear how sucrose upregulates CYCD4;1 in specifically in that kind of cells, but these findings suggest that the transcriptional effect has to do with sucrose-dependent regulation of LR density. Notably, auxin does not have an effect over the expression of CYCD4;1 in pericycle cells, and restores the reduced LR density phenotype of cyca4;1 mutants, suggesting that CYCD4;1 has no role in the auxin-mediated LR initiation pathway. CYCD3;1, is also responsive to sucrose availability, but the effects of this over CYCD3;1 activities are not clear [164]. Endoreplication progress is also affected by several environmental signals. E2F3/ DEL1, an atypical E2F present in Arabidopsis, and that functions as a transcriptional repressor, is one of the key regulators that negatively controls the entry into the endoreplicative cycle [165]. It has been suggested that the balance between the transcriptional activator E2Fb and repressor E2Fc controls light-dependent endoreplication through the antagonistic modification of the DEL1 expression [166]. E2Fb and E2Fc compete

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for the same DNA-binding site of the DEL1 promoter and enhances the DEL1 expression, respectively. Under light conditions, E2Fb is the preferred binding partner, enhancing DEL1 expression and consequently repressing the endoreplicative cycle [167]. In the dark E2Fb is degraded, allowing E2Fc to bind to the DEL1 promoter, repressing DEL1 expression. Ultraviolet-B (UVB) radiation damages DNA molecules by forming cyclobutane pyrimidine dimers (CPDs) which prevent DNA transcription and translation. Plants remove CPDs by photolyases, and these enzymes are encoded by a PHOTOLYASE 1 (PHR1) [168, 169]. It has been shown that in addition to CCS52A2, a known target of DEL1, DEL1 represses the transcription of the PHR1 gene and thereby coordinates DNA repair and endocycle triggering [167]. After UVB treatment, DEL1 expression is strongly downregulated, permitting the upregulation of PHR1 and thus leaving the cell able to repair its DNA.

Environmental and nutrient availability condition changes affect root apical meristem organization [170]. ROS and Reactive Nitrogen Species (RNS) have been reported to be rapidly induced by several kinds of environmental stresses in a variety of plant species to regulate the plant response to biotic and abiotic stresses. In particular, oxidative stress caused by drought and salinity, has been proposed that ROS production is an obligatory element of the response to induce an adequate acclimatization process [114]. Therefore, the degree of accumulation of ROS is what determines whether it is a part of the signaling mechanism (low production) or a harmful event (high production) to plants, making the control of production and degradation of ROS the crucial element for plant resistance to stress [114, 171-173]. ROS is never completely eliminated, as it plays an important role in signaling and growth regulation [174]; ROS quenching inhibits the root growth [115], and overexpression in Arabidopsis of a peroxidase localized mainly in the elongation zone stimulates root elongation [175]. This calls for redox control of the cell cycle, which is possibly linked to A-type cyclins, shown to be differentially expressed under oxidative stress in tobacco, resulting in cell cycle arrest [176]. It is also known that low temperatures [177, 178], metals [179] and nutrient deficiency [180] induce the presence of ROS and RNS in specific tissues. These forms of stress affect root morphology by reducing primary root growth and promoting branching, but the mechanisms of the redox generation-sensing are not well understood.

The typical response of the Arabidopsis radical system to low phosphorous (P) availability is an example to illustrate how complex these processes are. A recent study showed that ROSs are involved in the developmental adaptation of the RS to low P availability [181]. Rapidly growing roots of plants within a normal P medium synthesize ROS in the elongation zone and QC on the root, whereas seedlings within low P mediums showed a slow growth of the PR, and the ROS normally found in the QC relocate to cortical and epidermal tissues. In a previous study [131], it has been indicated that Arabidopsis plants under low P conditions show a decreased number of cells in the root apical meristem, and it decreases until it is depleted. In these roots, all root apical meristem cells differentiate and the QC is almost indistinguishable. A possible cause of this response to P starvation could be the cell cycle arrest modulated by ROS and CYCAs, but it is more complicated, as the response is also modulated by auxin [170, 182] and gibberellin-DELLA pathways [183]. Interestingly, DELLAs promote survival by reducing the levels of ROS [184], suggesting a link between the gibberellin-DELLA cell cycle

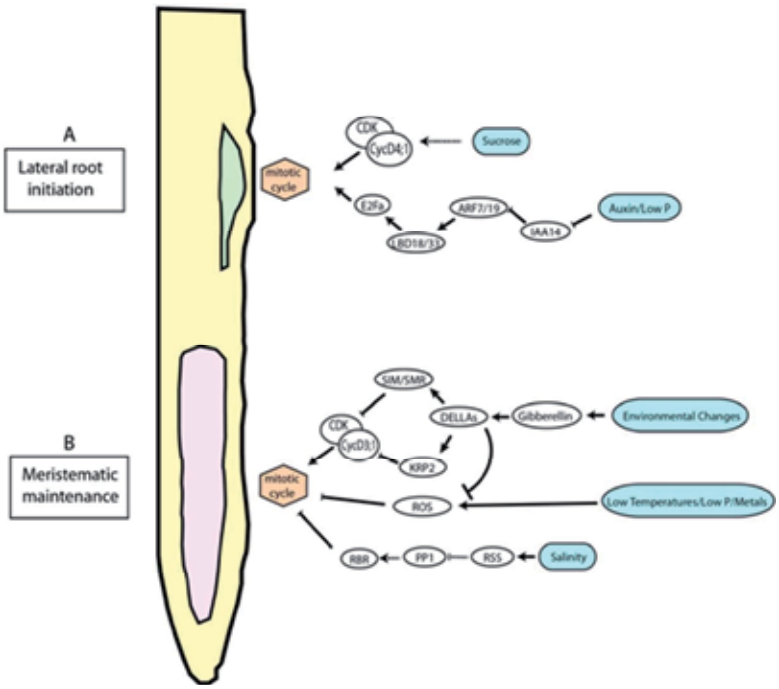

Figure 3. Abiotic Stress affects root mitotic cycle. A) Lateral root formation responds to sucrose availability in medium through an unknown link that enhances CycD4;1 expression in pericycle cells, allowing them to proliferate; it also responds to low P availability through the activation of the auxin pathway. Auxin controls lateral root initiation through the E2F mechanism, promoting the degradation of IAA14 and thus activating ARF7/18 transcription factors, subsequently activating LBD18/33 factors which in turn bind and activate the promoter of the cell cycle-enabling E2F transcription factor. B) Meristematic maintenance also responds to diverse environmental changes. Through the gibberellin pathway, DELLA proteins inhibit cell cycle progression by enhancing the accumulation of CDK inhibitors. DELLAs are influenced by various environmental factors including light and temperature. These factors, as well as metals and nutrient deficiency as in low P, promote the accumulation of ROS, known for inhibiting cell cycle in tobacco cells. Interestingly, DELLAs promote survival by lowering the levels of ROS, indicating a novel pathway to maintain cell cycle in the meristems. Salinity affects it by activating RSS1, required to maintain the mitotic cycle in the meristem. The putative mechanism comprises RSS1 interacting with a type 1 protein phosphatase (PP1), regulating its activity at the G1/S transition.

control pathway and ROS pathway in the developmental adaptation to the RS to low P availability It requires further study to precise the way these signals crosstalk and determine the developmental adaptation of the RS to low P availability by means of cell cycle progression control, as well as additional efforts to reveal the manners by which other regulatory pathways responding to abiotic stress interact with and influence the cell cycle control mechanisms.

5. Conclusion

Sensing and responding to environmental cues by roots enable plants to overcome the challenges posed by their sessile lifestyle [10]. As we mentioned above, RS is important to

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plants due to a wide variety of processes, including nutrient and water uptake from soil, which is a complex medium with high spatial and temporal environmental variability. Thus, it is not surprising that RSA is highly influenced by environmental cues [9, 148]. The importance of RSA in plant productivity stems from the fact that many soil resources are unevenly distributed or are subject to localized depletion, so that the spatial deployment of the RS will largely determine the ability of a plant to exploit those resources [4].The PERDP which regulates the changes in RSA, can be considered as an evolutionary response to medium with high spatial and temporal variability in resource supplies [148]. The genetic controls regarding root deployment (PERDP) are still largely unknown. A great effort has been made to understand the molecular components that regulate the formation, proliferation and maintenance of meristems, either being embryo or pericycle-originated. Nevertheless, the facts behind their regulation by environmental factors still leave many questions to be solved.

Plants are important to humans, as they provide food, fuel, fibres, medicines and materials. As the global population is projected by the UN to rise to over 9 billion by 2050, the improvement of crops is becoming an increasingly pressuring issue. The new challenge arisen is to solve the current and future obstacles to the maintenance of food supply security through higher crop yields [10]. Water and nutrient availability limit the productivity in most agricultural ecosystems. In all environments characterized by low water and nutrient availability, RSA is a fundamental aspect, the acquisition of soil resources by RS systems is therefore a subject of considerable interest in agriculture [4]. RSA and PERDP are important agronomic traits; the right architecture in a given environment allows plants to survive periods of water of nutrient deficit, and compete effectively for resources [9]. Most of drought-resistant rice varieties have a deeper and more highly branched RS than sensitive varieties [9].

Understanding the RSA and the PERDP holds potential for the exploitation and opening of new options for genetic manipulation of the characteristics of the root, in order to both increase food plant yield and optimize agricultural land use. Improved access to deep soil water, inherently reducing the need for irrigation, is one potential benefit that could be achieved by exploitation of RSA. Increase in root branching and root hair in crops may enable plants to make more efficient use of existing soil nutrients and increase stress tolerance, improving yields while decreasing the need for heavy fertilizer application [9, 10]. Understanding which structures and environmental cues that regulate proliferation and elongation of the RS cells will allow us to develop strategies to generate crops that possess greater soil exploration capacities in order of a more efficient usage of nutrients and water present in the soil.

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Chapter 6

Extreme Temperature Responses, Oxidative Stress and Antioxidant Defense in Plants

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Additional information is available at the end of the chapter

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1. Introduction

Temperature stress is becoming the major concern for plant scientists worldwide due to the changing climate. The difficulty of climate change is further added considering its precisely projecting potential agricultural impacts [1, 2]. Temperature stress has devastating effects on plant growth and metabolism, as these processes have optimum temperature limits in every plant species. Global climate change is making high temperature (HT) a critical factor for plant growth and productivity; HT is now considered to be one of the major abiotic stresses for restricting crop production [3]. The US Environmental Protection Agency (EPA) indicates that global temperatures have risen during the last 30 years [4], and it was mentioned that the decade from 2000 to 2009 was the warmest ever recorded.

High temperature stress is defined as the rise in temperature beyond a critical threshold for a period of time sufficient to cause irreversible damage to plant growth and development [5]. The growth and development of plants involves a countless number of biochemical reactions, all of which are sensitive to some degree to temperature [6]. Consequently, plant responses to HT vary with the extent of the temperature increase, its duration, and the plant type. Worldwide, extensive agricultural losses are attributed to heat, often in combination with drought or other stresses [7].

Low temperature (LT) or cold stress is another major environmental factor that often affects plant growth and crop productivity and leads to substantial crop losses [8, 9]. Chilling stress results from temperatures cool enough to produce injury without forming ice crystals in plant tissues, whereas freezing stress results in ice formation within plant tissues. Plants differ in their tolerance to chilling (0-15°C) and freezing (<0°C) temperatures. Both chilling and freezing

stresses are together termed low temperature or cold stress: the damage due to cold stress can range from chilling injury and freezing injury to suffocation and heaving. In general, plants from temperate climatic regions are considered to be chilling tolerant to variable degrees, and their freezing tolerance can be increased by exposing to cold, but non-freezing, temperatures; this process is known as cold acclimation. However, generally the plants of tropical and subtropical origins are sensitive to chilling stress and lack this mechanism of cold acclimation [9]. Low temperature may affect several aspects of crop growth; viz., survival, cell division, photosynthesis, water transport, growth, and finally crop yield.

The cellular changes induced by either HT or LT include responses those lead to the excess accumulation of toxic compounds, especially reactive oxygen species (ROS). The end result of ROS accumulation is oxidative stress [10-12]. In response to HT, the reaction catalyzed by ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) can lead to the production of H_2O_2 as a consequence of increases in its oxygenase reactions [13]. On the other hand, LT conditions can create an imbalance between light absorption and light use by inhibiting the activity of the Calvin–Benson cycle. Enhanced photosynthetic electron flux to O_2 and over-reduction of the respiratory electron transport chain (ETC) can also result in ROS accumulation during chilling which causes oxidative stress [14]. Plants have evolved a variety of responses to extreme temperatures those minimize damages and ensure the maintenance of cellular homeostasis [15]. A considerable amount of works have explored that there is a direct link between ROS scavenging and plant stress tolerance under temperature extremes [12]. Thus, the improvement of temperature stress tolerance is often related to enhanced activities of enzymes involved in antioxidant systems of plants. Plants exposed to extreme temperatures use several non-enzymatic and enzymatic antioxidants to cope with the harmful effects of oxidative stress; higher activities of antioxidant defense enzymes are correlated with higher stress tolerance. Different plant studies have revealed that enhancing antioxidant defense confers stress tolerance to either HT or LT stress [16-19].

In this chapter, we review the recent research findings those revealed variable responses of plants to extreme temperatures. We also focus on the oxidative stress and antioxidant defenses that are invoked by plants for survival under temperature stress conditions.

2. Plant responses to high temperature

2.1. Seed germination and emergence

Seed germination and seedling vigor are important traits for obtaining a good plant stand and subsequent high yields of a crop. Seed germination is highly dependent on temperature as temperature is one of the basic requisites of this process. However, the range of temperature in which seeds perform better germination depends largely on crop species (Table 1). Soil temperature is one of the major environmental factors that influences not only the proportion of germinated seeds, but also the rate of emergence and the subsequent establishment, even under optimum soil and irrigation conditions [20].

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Crop species	Temperature (°C)		
	Minimum	Maximum	Optimum
Rice (*Oryza sativa*)	10	45	20-35
Wheat (*Triticum aestivum*)	20	40	25-30
Maize (*Zea mays*)	10	40	25-30
Soybean (*Glycine max*)	10	35	25-30
Tomato (*Solanum lycopersicum*)	11	30	15-27
Cucumber (*Cucumis sativus*)	18	30	25-30
Egg plant (*Solanum melongena*)	15	33	20-25
Peeper (*Capsicum* spp.)	15	35	20-30
Pumpkin (*Cucurbita moschata*)	15	40	20-25
Water melon (*Cucumis melo*)	15	35	25-30
Lettuce (*Lactuca sativa*)	4	25	15-20
Carrot (*Daucus carota*)	11	30	15-25
Cabbage (*Brassica oleracea* var. *capitata*)	8	35	15-30
Spinach (*Spinacia oleracea*)	5	30	15-20

Table 1. Ranges of temperatures for seed germination of different crops [21, 22]

Roberts [23] documented three distinct physiological processes during seed germination which are affected by temperature: 'first, temperature, together with moisture content, determines the rate of deterioration in all seeds; second, temperature affects the rate of dormancy loss in dry seeds and the pattern of dormancy change in moist seeds; and, third, in non-dormant seeds, temperature determines the rate of germination' (for review see [24]).

The effect of HT on germination was investigated in various crops and serious impacts of HT on seed germination were observed. Increasing temperature between base and optimum temperatures increased the rate of germination and total percentage germination, but temperatures above optimum decrease the germination percentage [Prasad et al. 2006]. Essemine et al. [25] observed that very HT (45°C) did not allow adequate rate of germination due to cell death and embryo damage in *T. aestivum* during the early stage of development (first 6-d of growth), indicating that HT is not favorable to wheat growth and did not permit establishment of new seedlings. In some cases, plants grown under HT also produce low quality seeds which have poor germination and vigor. Recently, Kumar et al. [26] observed that growth of roots and shoots in hydroponically grown *Phaseolus aureus* seedlings was not inhibited at 35/25°C (day/night temperature), but at 40/30 and 45/35°C, 18 and 34% reduction of shoot growth was observed. The root growth at these temperatures was inhibited by 13 and 23%, respectively. When *Vigna mungo* seeds were exposed to 10, 20 and 30 min of heat (50°C), Piramila et al. [27] observed that seed germination as well as vigor index was significantly reduced by high

temperature. Pant et al. [28] observed that when seeds of *Cassia tora* were incubated under normal room temperature they exhibited 92% germination but when exposed to 40, 50 and 60°C continuously for 10 d the germination percentage decreased to 85, 63 and 32%, respectively. Several earlier investigators have suggested that HT may be necessary for adequate release of energy for germination and growth [29-31], but it can also reduce plant emergence. Hall [32] stated that the maximum threshold temperatures for germination and emergence are higher for warm-season than for cool-season annuals. For instance, the threshold maximum seed zone temperature for the emergence of *Vigna unguiculata* is about 37°C, whereas in *Lactuca sativa*, it is 25-33°C.

2.2. Growth and morphology

The most observed effect of heat stress on plants is the retardation of growth. As heat stress often occurs simultaneously with drought stress, the combination of drought and heat stress induce more detrimental effect on growth and productivity of crops than when each stress was applied individually [24]. In higher plants, heat stress significantly alters cell division and cell elongation rates which affect the leaf size and weight. However, it was reported that heat stress resulted in significant increases in leaf numbers, particularly when reproductive development was arrested without any decrease in leaf photosynthetic rates [20, 24]. Exposure of plants to severe heat stress decreased the stem growth resulting in decreased plant height [20]. Rahman [33] reported that plant height of wheat plant ranges from 66.4-97.3 cm and 55.7-82.3 cm in normal and heat stress condition, respectively. While studying with *T. aestivum*, Ahamed et al. [34] observed that sowing time mediated heat stress negatively influenced the plant height and number of tillers of 4 different genotypes. In a recent study, Al-Busaidi et al. [35] observed that high atmospheric temperature cause significant water loss which negatively influenced the growth and biomass production in biofuel plant, *Jatropha curcas*. Parallel to shoot growth heat stress often decreases root growth, number of roots and root diameter [36].

High temperature decreased shoot dry weight, relative growth rate (RGR) and net assimilation rate (NAR) in maize, millet and sugarcane [5, 37]. In their review, Wahid [5] mentioned that HT can cause considerable pre- and post-harvest damages, including scorching of leaves and twigs, sunburns on leaves, branches and stems, leaf senescence and abscission, shoot and root growth inhibition, fruit discoloration and damage. High temperature also alters the internal morphology (anatomy) of plants and these changes are generally similar to those under drought stress. Under HT stress, there is a general tendency towards reduced cell size, closure of stomata and curtailed water loss, increased stomatal density and trichomatous densities, and larger xylem vessels in both roots and shoots [5]. Several lines of study indicate that exposure of plants to HT resultes in the disintegration of ultrastructural characteristics, mainly attributed to a lower stomatal density, larger stomatal chamber with a larger stomatal opening area, thinner leaves, loose arrangement of mesophyll cells, a partially developed vascular bundle and unstable organelle structure. Zhang et al. [38] examined the microscopic and ultrastructural characteristics of mesophyll cells in flag leaves of both HT sensitive and tolerant rice genotypes grown under heat stress (37/30°C) and reported that the membrane permea-

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bility increased in both sensitive and tolerant plants under HT stress. However, under the HT stress, the tolerant plants showed tightly arranged mesophyll cells in flag leaves, fully developed vascular bundles and some closed stomata, whereas the sensitive plants suffered from injury because of the poor structures of these organs [38]. Recently, Johkan et al. [22] observed that the number of tillers in wheat plants decreased in response to HT, especially high night-time temperatures, however shoot elongation was promoted.

2.3. Physiological effects

Physiological processes of plants are largely affected by the alteration of surrounded environmental temperature. The ability of plants to cope with extreme temperature is a complex process and is determined by environmental factors and also by the genetic capability of the plant. In general, stability of life processes in most plants is comparatively wide which ranges from several degrees above zero to around 35°C [6]. The increase of temperature up to a certain level increases plant growth, photosynthesis, respiration and enzyme activity and after that these parameters tend to decline (Fig. 1). Respiration rapidly increases with temperature and drops drastically after an extreme tolerable temperature. Photosynthesis is a comparatively less sensitive than respiration process but its declining pattern is as like as respiration. The average rate of enzymatic reactions increases twofold with every 10°C increase in temperature within the range. The optimal temperature for structural integrity and activity of most enzymes are within the range of 30–45°C; and enzymes are irreversibly denatured and inactivated at temperatures higher than 60°C with the exception of thermophilous organisms. Thus each life process has its own referred critical or lethal temperature after that it can not proceeded and causes permanent damage to cell structures and ultimately the cell, plant death as well [6].

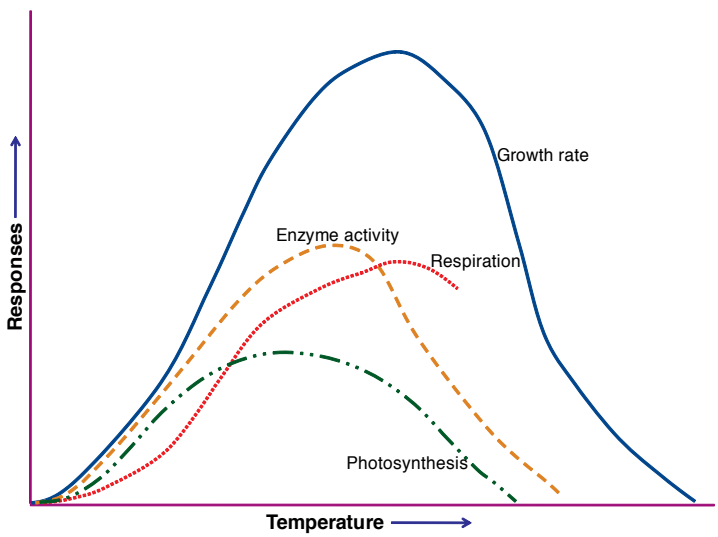

Figure 1. Schematic illustration of the effect of temperature on major physiological processes of plants [6, 39]

2.4. Photosynthesis

Temperature plays one of the most important roles in the rate and ability of a plant to photosynthesize effectively. In general, there is a positive correlation between change in temperature and photosynthesis. But when temperatures exceed the normal growing range (15°C to 45°C) of plants heat injury takes place and HT hurts the enzymes responsible for photosynthesis. Even in the absence of heat stress injury, photosynthesis would be expected to decline as temperature increases because photorespiration increases with temperature faster than does photosynthesis [40].

In tobacco leaves HT stress (43°C for 2 h) decreased the rate of photosynthesis by 38% compared with that of the rate of photosynthesis at optimal temperature (25°C). After 1 d recovery, it reached only about 75% of its control. Under HT condition, the stomatal conductance (g_s) also decreased significantly [41]. Prasad et al. [42] reported that high night temperature (31.9°C/27.8°C) decreased chlorophyll (Chl) content and photosynthetic rate by 8% and 22%, respectively, compared to optimum night temperature. Deactivation of RuBisCO is one of the causes associated with the decline in photosynthesis under HT. Many authors reported that the heat-induced deactivation of RuBisCO is the primary constraint for photosynthesis at moderately HT and showed that Chl fluorescence signals from PSII are not affected by temperatures that cause significant deactivation of RuBisCO [43]. While studying with oak (*Quercus pubescens* L.) leaves, Haldimann and Feller [43] concluded that regardless of whether temperature was increased rapidly or gradually, rate of photosynthesis decreased with increasing leaf temperature and it was reduced more than 90% at 45°C as compared to 25°C. Stomatal conductance is also an important factor that modulate photosynthesis rate in plants. Eamus et al. [44] have shown from the stomatal conductance response of *Eucalyptus haemastoma* leaves to temperature declined with leaf temperatures above about 30–32°C, with a considerable reduction at 40°C. In *Semillon* leaves, Greer and Weston [45] observed that 4-d heat exposure at 40°C caused a sustained reduction in photosynthesis that was 95% attributed to reduced g_s which suggest that stomata of this plant was highly susceptible to heat. Recently, Greer and Weedon [46] observed that average rates of photosynthesis of *Vitis vinifera* leaves decreased by 60% with increasing temperature from 25°C to 45°C. This reduction in photosynthesis was attributed to 15–30% stomatal closure.

It was noted that an increase in temperature of 10°C to 15°C above normal growth temperature leads to alteration of photosynthetic pigments and thus limiting photosynthesis. The reasons for decreasing in photosynthetic pigments under HT may be attributed to the inhibition of biosynthesis, changes in ultrastructure of chloroplast, especially the membrane, and photodeterioration [47, 48]. Tewari and Tripathy [47] observed that heat stress significantly reduces Chl content in *T. aestivum* which was due to inhibition of porphobilinogen deaminase activity and thus reduction in protochlide content in the seedlings upon exposure to short duration of heat stress (42°C). Heat stress has been reported to reduce Chl content, Chl *a*/*b* ratio and Chl:Car ratio in various plant and tree species like *Festuca arundinacea* [49] and *Solanum spp.* [50], *T. aestivum* [48]. Recently, Almeselmani et al. [51] investigated the performance of heat tolerant (C306) and heat susceptible (PBW343) wheat genotypes under HT (35/25°C day/night). They observed that HT significantly reduced leaf Chl content in both genotypes at any stages of

growth. Heat stress reduced leaf Chl content by 23 and 48% in C306 at anthesis and 15 days (d) after anthesis respectively, while in PBW343, 29 and 61% reduction in Chl content at anthesis and 15 d after anthesis, respectively, was observed under HT as compared with normal temperature [51]. As a result, HT significantly reduced the leaf photosynthetic rate in both genotypes at all three stages of plant growth compared to their respective control. In another study Chl *a* in the *L. esculentum* leave was reduced by 10-32%, Chl *b* by 10% and 5% reduction in the ratio of Chl *a* and Chl *b* observed after 2-d of heat treatment [52]. However, the effect of HT on the photosynthesis of the crop also depends on other climatic parameters. In addition it is not always obvious that HT reduces the rate of photosynthesis. For instance, HT had no effect on the photosynthetic temperature response of potato [53] and pea leaves [54]. Furthermore, growth of maize leaves at HT had no effect on their rates of photosynthesis [55].

2.5. Water relations

Plant water status is considered as the most important variable under changing ambient temperatures [56]. Plant water relation is more affected under the combined heat and drought stress, than the condition of heat and sufficient moisture level. High temperatures affect seedlings, first, by increasing evaporative demand and tissue damage. High temperatures-induced increased transpiration and water transportation is another necessary tool for plant survival under extreme temperatures. Death of a large number of *Pinus ponderosa* seedlings were observed at 63°C but among those a few were survived those maintained basal stem temperatures as much as 15°C lower than the surrounding air by keeping higher g_s, transpiration rate and water transportation. Here, water transport through seedling stems may help to cool plant by the heat transferring mechanism. Heat exchange calculations demonstrated that rapid water flow through seedling stems can absorb sufficient energy to reduce the stem temperature by 30°C during peak sunlight hours [57]. *Triticum aestivum* and *Hordeum vulgare* were grown in soil that was well watered or not watered in controlled chambers at 15/10, 25/20, 35/30 and 40/35°C day/night temperatures. After two days soil water content, leaf relative water content, leaf water potential, leaf osmotic potential, leaf turgor potential and osmotic adjustment were nearly constant at all temperatures when soil was well watered but were affected strongly by HT when water was withheld [58]. Morales et al. [59] indicated that HT-induced reduction in leaf water status was caused mainly due to reduction in hydraulic conductance leading to decrease in water absorption or due to reduced g_s. In *Lotus creticus* elevated night temperatures caused a greater reduction in leaf water potential in water-stressed as compared to well-watered plants [60]. In sugarcane, leaf water potential and its components were changed upon exposure to heat stress even though the soil water supply and relative humidity conditions were optimal, implying an effect of heat stress on root hydraulic conductance [61].

2.6. Dry matter partitioning

Dry matter (DM) partitioning varied widely under different temperatures and crops. Stresses like water deficit and heat slower down the assimilation process and the mineral uptake during the grain filling period. Assimilates those are transferred directly to kernels and remobilization

of assimilates stored in vegetative plant parts both together contribute to grain yield [62]. Sometimes under HT, it happens that the sink activity lost due to the earlier panicle senescence where the source activity still exists as the leaf senescence does not occurs [63, 64]. In those cases, grain filling was terminated earlier than complete leaf senescence. Kim et al. [64] reported that consistently HT increased the rates of grain filling, fraction of DM partitioning to panicle and leaf senescence while it reduced the durations of them under the temperature regime of 24.4 and 21.9°C in temperate variety of *O. sativa*. There is a positive contribution of the delayed leaf senescence to grain filling and yield of crop. There are various reasons for loss of sink activity at HT which may result from a decline of translocation ability or loss of activity of starch synthesis-related enzymes. The sucrose synthase activity of rice grain has been observed to be positively correlated with grain sink strength and starch accumulation [65, 66]. Another reason for decreased grain filling duration of rice under HT is reduction of cell size on the dorsal side close to the vascular bundles [67]. In a pot experiments with 4 *Arachis hypogaea* genotypes varying in heat tolerance were grown at either 28/22 or 38/22°C from 21 to 90 d after planting (DAP). High temperature reduced total dry weight by 20 to 35%, seed harvest index by 0 to 65%, and seed dry weight by 23 to 78% [68]. There are several reports regarding HT induced declines in shoot DM, relative growth rate and net assimilation rate in *Z. mays*, *Pennisetum glaucum* and *Saccarum officinarum* [37, 69]. In the medicinal plant *Panax quinquefolius* L., partitioning of DM to roots in the cool greenhouse and in the field was 73%, whereas it was 62.5% in the heated greenhouses [70].

2.7. Reproductive development

It is notable that reproductive development of plants is more sensitive to HT because plant fertility is considerably reduced as temperatures increase [71]. For heat-sensitive plants such as tomato, no fruit set occurs at day/night temperatures of 35/23°C [72]. Studies on common bean [73], and peach [74] showed that elevated temperatures during flower development can markedly reduce the fruit set. The decrease in the fruit set has generally been attributed to low pollen viability and germinability at HT in crop species such as tomato [75] and groundnut [76]. Porch and Jahn [77] reported that *Phaseolus vulgaris* exposed to pre-anthesis heat stress resulted in pollen and anther development abnormalities. In soybean, pollen viability was lower at day/night temperature conditions of 37/27°C than at 27/27°C which resulted in a lower pod setting [78]. High temperatures inhibit flower differentiation and development, and result in smaller ovaries in pistillate and bisexual flowers [22].

The diurnal variation of temperature is also important for reproductive growth and development. Spikelet fertility of rice is sensitive to night temperature, where the degree of sensitivity depends upon the developmental stage of the spikelet [79]. Later, Peng et al. [80] observed a strong negative linear relationship between the number of fertile spikelets and increase in night temperatures. Ledesma et al. [81] examined the effect of two day/night temperature regimes (30/25°C and 23/18°C) on fruit set and fruit growth in two strawberry cultivars (Nyoho and Toyonoka). It was recorded that high day/night temperature of 30/25°C reduced the number of inflorescences, flowers, and fruits in both cultivars compared with control (23/18°C). The percentage of fruit set in Nyoho was not significantly different between the two temperature

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treatments, while in Toyonoka it was much lower at 30/25°C than at 23/18°C. Ripening time was shorter at 30/25°C than at 23/18°C in both cultivars.

2.8. Yield

As HT negatively affected plant establishment, growth, DM partitioning, reproductive growth and photosynthesis, it ultimately poses serious consequence on crop yield. Several lines of study indicated the reduction of crop yield under HT which greatly varies with the degree and duration of temperature as well as genotypes of the crop (Table 2). Mendham and Salsbury [82] reported that HT can reduce crop yield by affecting both source and sink for assimilates. The decrease in grain length and width of cereals was found to be associated with a reduction in the average endosperm cell area observed under high night temperature [67]. In addition, cereals generally respond to HT through an increase in the rate of kernel growth, which lead to a decrease in the duration of DM accumulation [79]. Kernel dry weight reduced from 79 to 95% in field conditions in B-73 inbred line of maize under heat stress [83]. Shah and Paulsen [84] demonstrated that photosynthesis and leaf area, shoot, grain biomass and sugar contents of kernels rapidly decreased under HT. High temperature affected the endosperm development in maize and reduced grain yield during endosperm cell division [85]. Prasad et al. [20] observed that an increase in temperature from 32/22°C to 36/26°C and 40/30°C decreased seed yield of sorghum by 10 and 99%, respectively. Djanaguiraman et al. [86] grew sorghum plants both under normal (32/22°C) and HT (40/30°C) and observed significant differences in yield attributes and yield. After 45 d of treatment, plant height, leaf dry weight, seed weight and total dry weight decreased by 22, 14, 53 and 36%, respectively compared to optimum temperature. Compared to other crops, wheat is the most sensitive to HT as the reproductive growth, especially grain filling is greatly facilitated by LT. Mohammed and Tarpley [87] found almost 90% less grain in the plants grown in high night temperature (32°C) compared to normal temperature (27°C). Plants grown under high night temperature showed 20% decrease in grain weight compared to plants grown under normal temperature. Johkan et al. [22] reported that HT resulted in more immature grains and decreased yields in *T. aestivum* because of dark respiration. In a recent study, Prasad et al. [42] observed that spring wheat plants grown under HT (31/18°C) showed a significant reduction in number of grains spike^{-1} (50%), total dry weight (20%), grain yield (39%) and harvest index (24%) as compared to optimum temperature (24/14°C).

Plant species	Temperature and duration	% reduction	References
Cicer arietinum	35/16°C (day/night), 10 d; during flower and pod development	Pod plant^{-1}: 53% Seed yield: 48%	[88]
Brassica spp.	35/18°C, 10 d; during bud formation, flowering, and pod development	Main stem pods: 75% Seeds pod^{-1}: 25% Seed weight: 22%	[89]
Cicer arietinum	35/16°C (day/night), 10 d during early flowering and pod development	Seed weight: 40% Harvest index: 7%	[90]

Plant species	Temperature and duration	% reduction	References
Sorghum bicolor	Increase in temperature from 32/22 to 36/26 and 40/30°C (day/night); from emergence to maturity	Seed yield: 10 and 99%, respectively	[20]
Triticum aestivum	5°C higher than optimum; from sowing to 60 DAS, 61-80 DAS and 81 DAS to maturity	Grain spike^{-1}: 18% Grain weight: 19% Grain yield plant^{-1}: 46%	[91]
Sorghum bicolor	40/30°C (day/night), 63 d	Seed weight plant^{-1}: 53%	[86]
Oryza sativa	35/30°C (day/night); at heading stage	Panicle weight: 7% Spikelet weight: 16%	[92]
Oryza sativa	32°C (10 h night temperature; stating from 20 DAE until harvest)	Yield per plant^{-1}: 90%	[87]
Oryza sativa	34°C, 7 d; during grain filling stage	Panicles plant^{-1}: 10% Grain yield plant^{-1}: 39% Harvest index: 30%	[93]
Capsicum annum	29/23°C (day/night); from 7 DAT to edible maturity	No. of fruits plant^{-1}: 28% Yield plant^{-1}: 62% Fruit length: 18% Fruit diameter: 20%	[94]
Triticum aestivum	31/18°C (day/night); from heading to harvest maturity	Grain number spike^{-1}: 50% Grain yield plant^{-1}: 39%	[42]
Oryza sativa	27°C during grain filling period	Grain weight: 4%	[95]

DAS – days after sowing, DAT – days after transplanting, DAE – days after emergence

Table 2. Reduction in yield components and yield of different crops as affected by high temperature

3. Plant responses to low temperature

Cold or LT stress comprises of chilling (<20°C) and freezing temperatures (<0°C) those hamper the plant growth and development in many ways. Chilling-sensitive plants exposed to LTs usually show water-stress symptoms due to decreased root hydraulic conductance and leaf water and turgor potentials [96]. Cold stress effects on crop plant have been studied since long time in many economically important crops [97-100] among which some of are chilling sensitive and unable to survive cold temperatures. Low temperature affects the plants in every stage of life starting from germination up to maturity.

3.1. Germination

Chilling injury is a serious problem during germination and early seedling growth in many plant species. For instance, optimum temperature range for germination of rice seed lies

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between 20 and 35°C, and the temperature of 10°C is cited as the minimum critical value below which rice does not germinate [101]. There are many reports on positive correlation between germination at LT and root development at an early stage; and between the germination and seedling establishment [102]. Angadi et al. [103] observed that the number of days to 50% germination in *B. napus* was only 3 d at 8°C which was nearly 13 d at 2°C. This LT effect was more pronounced in *B. rapa*, because at 2°C, emergence was less than 50%, even after 20 d of sowing [103]. Buriro et al. [104] reported that the increase in temperature significantly enhanced germination and related traits in wheat cultivars. All the wheat varieties germinated well (80-97%) sown at 10-30°C. The maximum seed germination, vigor index occurred at 20-30°C and these temperature regimes were identified as optimum for wheat seed germination. The delay in germination percentage and the reduced germination percentages were observed in *Gossypium hirsutum* at LT below 20°C [105]. In *T. aestivum*, the germination is drastically hampered at temperature below 8-10°C [106].

3.2. Growth and morphology

Low temperature stress inhibits various metabolic reactions thus preventing the expression of full genetic potential of plants which is expressed by different phenotypic symptoms [107]. Some of the common LT injury in plants are reduced seedling growth, seedling discoloration, leaf yellowing, leaf whitening, white specks, white bands, withering after transplanting, a reduced rate of tillering, stunting and so on [102]. According to Angadi et al. [103], temperatures below 10°C result in slower and reduced growth and premature stem elongation in *B. napus*, *B. rapa* and *Raphanus sativus*. It is well reported that plants at their seedling stage are very much sensitive to cold stress. At early stage of plant growth, and various phenotypic symptoms in response to chilling stress are surface lesions, chlorosis, necrosis, desiccation, tissue break down and water soaked appearance of tissues, reduced leaf expansion, wilting [108, 109]. Nahar et al. [99] observed varieties of cold injury symptom is rice including stunted growth, yellowing of leaves, abnormal number of tiller, malformed grain, abnormal colors in grain.

Generally, exposure to cold temperature affects crop growth and development in two ways concurrently. First, developmental events in the shoot apex are affected which directly determine the differentiation of the panicle and hence potential yield and spikelet fertility resulting in fewer grains. Second, photosynthesis is impaired which reduces growth and results in indirect yield loss because there is less carbohydrate available for grain production [110]. The cellular structures and components are also damaged due to the cold which have been studied for a long time. The development process and ontogeny of the organelles may be disrupted by chilling stress [111]. Nahar et al. [98, 99] observed cold stress induced morphological symptoms like stunted plant, bushy plants, early maturity, yellowing of leaves. Modifications of cellular components include swelling of mitochondria, plastids and thylakoid lamellae, vesiculation of thylakoid, accumulation of lipid droplets and eventually the disorganization of the entire plastid [112], reduction in ribosomal numbers, dilation of endoplasmic reticulum, vesiculation of cytoplasmic membranes, condensation of nuclear chromatin, invagination of plasmalemma and vacuolation of membranous vesicles [111, 113] were noticed

during chilling stress. Without these in extreme cases, chilling results in accelerated senescence and eventually plant death [114].

3.3. Reproductive development

Reproductive phases of the life cycle of plants are more vulnerable to cold stress [115]. The reproductive phase faced to LT stress is influenced diversely in its different sub-phases. During the development of male gametophyte LT causes disruption of meiosis, tapetal hypertrophy, stunted development of pollen grain, anther protein degradation, pollen sterility, pollen tube deformation. In female gametophyte development its effects are characterized by reduced style and ovary length, disruption of meiosis, reduced stigma receptivity, callose deposition in style, damage to embryo sac components, and arrest of the fertilization process. At flowering LT may cause delayed flowering, bud abscission, sterile or distorted flowers, while at grain filling the source-sink relation is altered, kernel filling rate is reduced and ultimately small sized, unfilled or aborted seeds are produced [109, 116]. Flower buds of *Simmondsia chinensis* can be damaged or killed by temperatures of −2° to −5.5°C [117]. Farrell et al. [118] observed that cold water significantly reduced panicle emergence in rice. Low temperatures affect not only normal heading but also panicle exsertion and prevent the normal elongation of internodes of rice. Cold stress (11°C) caused abnormality in panicle initiation process, delayed heading, incomplete panicle emergence, sterile and malformed spikelet and spikelet degeneration symptom in rice genotypes [98, 99b; Fig. 2, 3]. In canola low, but nonfreezing temperatures prior to flowering slow the rate of plant development, delayed flowering, slower the rate of flower opening and reduces the amount of pollen shed and in severe frost the pod abortion is evident in many species [119]. The effect of LT prevailing at the early stages of plant growth is sometimes associated with the premature flowering in Chinese cabbage [120].

Figure 2. Spikelet degeneration (A) and incomplete of panicle exsertion (B) of rice due to cold stress

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Figure 3. Sterile spikelets of rice due to cold stress

3.4. Cell membrane damage

Extreme temperature injuries caused either by cold or by HT first attack on the cell membrane. There are many studies where cellular membranes have been shown as the primary site of freezing injury in plants [121, 122]. Cell membrane is damaged in two ways viz. disruption of protein lipid structure, protein denaturation and precipitation of solutes that indulges the membrane permeability. Due to LT stress the fatty acids become unsaturated and the lipid protein ratios of the membrane become altered which ultimately affect the membrane fluidity and structure as well [90]. The flexible liquid-crystalline phase is converted in to a solid gel phase, thereby affecting the cellular function in different ways, viz. increased membrane permeability increases ion leakage, allows the entrance of undesirable anions and cataions into the cell, obstructs the exchange of essential ions, hampers the osmosis and diffusion processes, etc. All the phenomena are responsible for disrupting cellular homeostasis [97]. Conversion of cellular water into ice is a major reason for cell rupture in cold stress. At first the ice formation occurs in apoplast having low solute concentration, this creates a vapor pressure between cytoplasm and apoplast and results in the migration of unfrozen cytoplasmic or cytosol water to the apoplast. This water gives a pressure which is the cause of enlargement of existing ice crystals and the pressure towards the cell wall and cell membrane which leads to cell rupture [123, 124].

3.5. Photosynthesis

Low temperatures may disturb the key organs of photosynthesis, including chloroplast and thylakoid membranes, causes swelling of plastids and thylakoid lamellae, vesiculation of thylakoid, accumulation of lipid drops and ultimately disorganization of entire plastid [111,

113]. Low temperature also disrupts the systems including electron transport, carbon cycle metabolism and g_s. Among the photosynthetic apparatus PSII is the primary target of damage under LT stress. Moreover, LT reduces the activities of stromal and carbon assimilation enzymes like Calvin cycle enzyme, ATP synthase, and restricts RuBisCO regeneration and limits the photophosphorylation [125]. Another impact of LT exposure is the decline of carbon export from leaves which results in the accumulation of soluble carbohydrates [126]. Yordanova and Popova [127] stated that exposure of wheat plants to a LT (3°C) for 48 h and 72 h resulted in decreased levels of Chl, CO_2 assimilation and transpiration rates. Photosynthesis is strongly reduced below 18ºC [128], while temperatures around 4ºC dramatically depress photosynthetic performance [129]. The decline of photosynthetic capacity in LT is related to a decrease in the quantum efficiency of PSII and the activities of PS I, the ATP synthase and the stromal enzymes of the carbon reduction cycle [125]. Partelli et al. [130] showed that coffee plant resulted in 30% reduction in Chl *a*, 27% reduction in Chl *b*, 29% reduction of total Chl when the day/night temperature decreasing from 25/20° to 13/8°C. For Car, 86% reduction of α-carotene, 57% reduction in β-carotene, 68% reduction in α/β-carotene ratio, 32% reduction in lutein, but 21% increase in zeaxanthin. In *O. sativa*, the total Chl content was reduced by 50% due to exposure to LT (15/10 ºC) for 2 weeks [131]. In a recent study, Reda and Mandoura [48] reported that even at LT stress of 3°C the enzyme chlorophylase is still activated and led to a decline in Chl in *T. aestivum* plant.

3.6. Water and nutrients movements and uptakes

Cell membrane plays major roles in water and nutrient movements within and outwards the cell. Intra- and extracellular water and nutrient movement are inhibited due to LT due to memebrane damage under LT. There can be of two types of abnormalities during LT stress. Cold damages the membrane that makes the membrane permeable to undesired nutrients and ions and causes ion leakage; another is cell membrane and cell wall can be ruptured by the cold which is also responsible for disrupting cellular homeostasis by destroying both intra and extracellular nutrient and water movements [132, 133]. Severe dehydration may also occur due to freezing of cell constituents, solutes and water [134]. Available literature states that when temperatures drop below 0°C, the ice formation generally begins in the intracellular spaces because the intracellular fluid has a higher freezing point as compared to the other suborganales of cell [134, 135]. Low and freezing temperatures also lead to cellular dehydration, reduce water and nutrient uptake and conduction by the roots in some plants, thus causing osmotic stress [107]. Yadav [134] stated that dehydration during cold occurs mainly due to reduction in water uptake by roots and a hindrance to closure of stomata. The success or failure of a seedling in the field is strongly related to the development of its root system under cold stress [136]. In root of cucumber it was found that chilling caused injury to the cortical cells and further long time exposure increased the density of cytoplasm and damage the endoplasmic reticulum [137]. Chilling-sensitive plants exposed to LT usually show water-stress symptoms due to decreased root hydraulic conductance; and decreased leaf water and turgor potentials [96]. Freezing-induced increase in water viscosity is partly accounted for an initial decrease in root hydraulic conductance [138]. During cold stress another phenomenon is common with the imbalanced water movement. The metabolic functions are altered those include production

of more enzymes, isozymes though they help in maintaining more catalytic activity to cope with the LT stress [139].

3.7. Yield components and yield

Reproductive phase products are the key components of economic yield and hence LT stress during the reproductive phase has significant economic and social consequences. All the adverse effects of cold stress ultimately lower the yield of crop. Low temperature-induced yield reduction is a common phenomenon in many crops [98, 99, 140, 141]. Low temperature often causes flower abortion, pollen and ovule infertility, breakdown of fertilization, poor seed filling, decreases in seed setting which ultimately reduce the grain yield [116]. In *O. sativa*, LTs are responsible for 30–40% yield reduction in temperate growing areas [142]. It was observed that about 16 and 37% yield reduction in the rice variety of BRRI dhan46 and BRRI dhan31 due to late sowing mediated LT stress [98]. In another report, it was observed that LT stress near about 11°C caused yield reduction in maximum genotypes and only 23 genotypes were screened out among the 244 genotypes considering their better yielding ability under LT [99]. The reduction in yield in *C. sativus* L. was from 15 up to over 18% due to low soil temperature [143]. In *C. melo* L., total yields decreased linearly for cold stress (21 and 32 h) which accounted for 10% lower than normal condition [143]. Frost prevailing just after flowering can result in yield reduction and the quality or grade loss. Premature flowering or bolting is occurred due to LT stress which has economic importance for the Chinese cabbage industry because advanced flower stalk development results in an unmarketable head [120]. In *B. napus* and *B. rapa* various abnormal structures were observed like reduced diameter and extensive white patches, white reticulation, red-brown pigmentation, folded seed, extensively shriveled seed, etc. those are the causes for reduced market value of this crop[103].

4. Responses of perennial crops to extreme temperature

Like annual crops, perennial crops are also sensitive to extreme temperature. Fruits and nut trees are important crop plants which often face extreme temperature stress induced damages. Every fruit tree species has a range of optimum temperatures (Table 3) above or below which the growth and yield markedly reduced. The mean temperatures range for optimum growth of most tropical fruits are about 24-30°C [144, 145]. For instance, mango (*Mengifera indica*) tree can tolerate HT up to 48°C only for a certain period of time [146], on the contrary it has only partial tolerance to LT. In another study, Schaffer et al. [147] observed that monoembryonic mango cultivars tend to be more LT tolerant than polyembryonic cultivars [147]. However, several studies have shown that LT promote reproductive morphogenesis in mango. Dinesh and Reddy [145] studied the responses of fruit trees to temperature and observed differential responses to temperature in different fruit species. They concluded that lychee and longan require a warm sub-tropical to tropical climate that is cool but also frost-free or with only very slight winter frosts not below -4°C, and with high summer heat, rainfall, and humidity. In longan, stressful temperatures of <15°C at the young fruit stage reduce fruit growth potential and final size as reported by Young et al. [148]. Stressful LT also induces excessive fruit drop

and severe fruit cracking [148]. In a previous study, Tindal [149] reported that rambutan is adapted to warm tropical climates of approximately 22–30°C and is sensitive to temperatures below 10°C. In mangosteen, LT (below 20°C) markedly slowed down the overall growth of the tree, whereas HT (above 35°C) caused some stresses on the trees [150]. Low temperatures during floral development in fruit trees result in fewer healthy flowers. On the other hand HT during floral development causes dryness and leads to sterility. Larcher [151] showed that banana, papaya, mango, grape and orange are sensitive to LT and lose their quality and productivity. Aslamarz et al. [152] studied several walnut cultivars and genotypes and found the temperature had a great influence on the performance of trees. On the basis of the heat requirements, walnut trees were classified as: low requirement, medium requirement, or high requirement. In most of the cases, HT regimes result in the best quality fruits but extremely HTs for extended periods of time are known to cause damage.

Tree species	Optimum temperature	References
Mangifera indica	24-27°C	[153]
Litchi chinensis	25-35°C	[149]
Psidium guajava	23-28°C	[154]
Artocarpus heterophyllus	16-28°C	[155]
Dimocarpus longan	20-25°C	[154]
Durio zibethinus	24-30°C	[154]
Nephelium lappaceum	25-32°C	[154]
Musa spp.	20-35°C	[156]
Vitis vinifera	10-35°C	[157]
Cocos nucifera	20-32°C	[158]
Anacardium occidentale	20-35°C	[159]

Table 3. Optimum temperature ranges for growth of some tropical fruit trees

5. Oxidative stress under extreme temperature

Oxidative stress has been mentioned as a common metabolic route of different stresses [160], and the regulation of oxidative stress has been mentioned as an indication of abiotic stress tolerance of plants by different studies [3, 161-163]. Several recent reports indicated that under abiotic stress production of free radicles or ROS markedly increased [3, 164-166]. Temperature stress accelerates the generation of ROS including singlet oxygen ($^1O_2$), superoxide radical ($O_2\bullet^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•), thereby induced oxidative stress [10, 167]. In plant cells, ROS are continuously produced as a consequence of aerobic metabolism in all the intracellular organelles, particularly in the chloroplast, mitochondria and

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peroxisomes [160, 168; Fig. 4]. But the chloroplast is considered as the main source of ROS in plants.

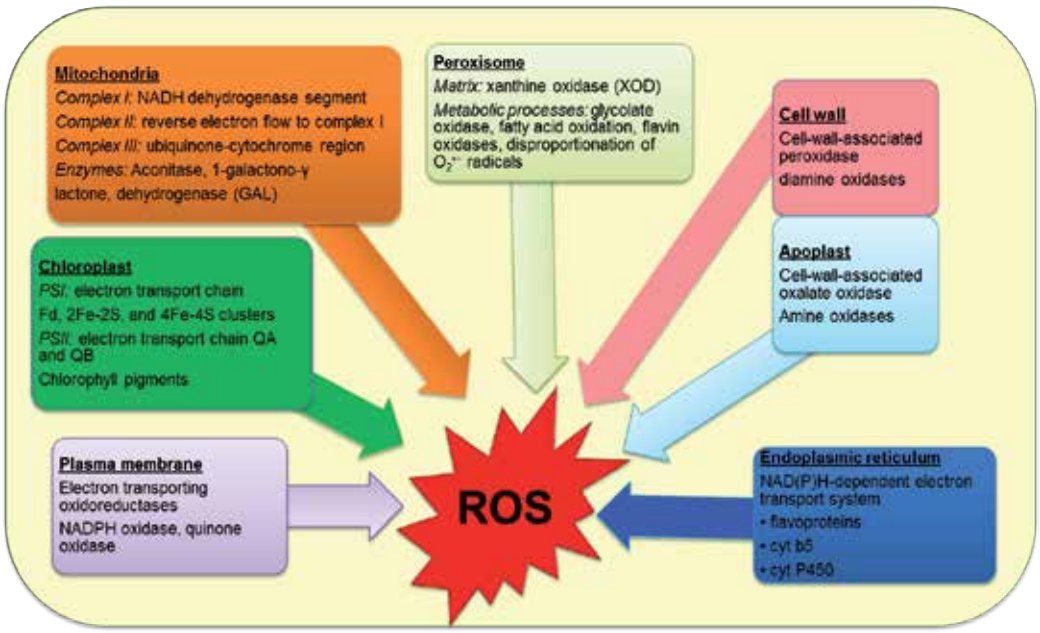

Figure 4. Sites of production of reactive oxygen species (ROS) in plants

Superoxide radical ($O_2^{\bullet-}$) is formed in many photooxidation reactions (flavoprotein, redox cycling), Mehler reaction in chloroplasts, mitochondrial ETCs reactions, glyoxisomal photorespiration, NADPH oxidase in plasma membranes and xanthine oxidase and membrane polypeptides. Hydroxyl radical ($OH^{\bullet}$) is formed due to the reaction of H_2O_2 with $O_2^{\bullet-}$ (Haber-Weiss reaction), reactions of H_2O_2 with Fe^{2+} (Fenton reaction) and decomposition of O_3 in apoplastic space [169, 170]. Hydroxyl radicals ($OH^{\bullet}$) can potentially react with all biomolecules like, pigments, proteins, lipids and DNA, and almost with all constituent of cells. Hydroxyl radical is not considered to have signaling function although the products of its reactions can elicit signaling responses, and cells sequester the catalytic metals to metallochaperones efficiently avoiding $OH^{\bullet}$ [169, 170]. Singlet oxygen ($^1O_2$) is formed during photoinhibition, and PS II electron transfer reactions in chloroplasts. This radical directly oxidizes protein, polyunsaturated fatty acids, and DNA [171, 172].

The main effects of ROS include autocatalytic peroxidation of membrane lipids and pigments, modification of membrane permeability and functions [3, 173]. During the time of temperature stress, ROS level can increase dramatically which can result in significant damage to cell structure [174]. Vallelian-Bindschedler et al. [175] reported that even very short heat pulses can result in oxidative bursts of $O_2^{\bullet-}$ and/or H_2O_2. Heat stress may disturb the homeostatic balance of cell and promote lipid peroxidation, either by increasing the production of reactive

oxygen species or by decreasing the O_2 radical scavenging ability in the cell [176]. The drastic increase in lipid peroxidation due to HT stress was reported by many researchers [162, 177]. Several lines of study indicated that under heat-stress conditions, malondialdehyde (MDA), a product of peroxidation of unsaturated fatty acids, has been used as a good indicator of free radical damage to cell membranes [12, 178, 179]. In wheat seedlings (8-d old) gradual increase in the accumulation of H_2O_2 was observed (0.5, 0.58, 0.78 and 1.1 μmol g^{-1} FW) in response to differential heat shock treatment of 22, 30, 35 and 40°C for 2 h [180]. The effect of a long-term (24 h) HT (42°C) shock on oxidative damages in *T. aestivum* seedlings was investigated by Savicka and Škute [181] in respect of the changes in $O_2^{\bullet-}$ production and MDA content. The effect of HT was analyzed at the early (4-d-old) and late stages (7-d-old) of seedling development. The increase of $O_2^{\bullet-}$ production, which was observed in the first leaf of wheat seedlings at all stages of development, led to an increase of MDA concentration. Parameter changes in the level of $O_2^{\bullet-}$ production were observed in the roots of wheat seedlings grown under HT exposure for 24 h at all stages of development, but MDA concentration in the roots of experimental and control seedlings did not differ significantly at the early and late stages of development. The level of $O_2^{\bullet-}$ production in coleoptile cells increased after a HT exposure at the late stages of seedling development. They concluded that growth inhibition of the root system could be connected with a powerful oxidative stress, evidenced by a significant increase (68%) of $O_2^{\bullet-}$ production in root cells during the early stages of seedling development and an insignificant increase (6%) of $O_2^{\bullet-}$ production 2 d after a HT exposure, as compared to control seedlings. The increase of $O_2^{\bullet-}$ production was also observed in roots after a HT stress during the late stages of development, and this effect was present 2d after a HT exposure (6 and 42%, respectively). Moreover, $O_2^{\bullet-}$ production after 2d at the late stages was more intensive than at the early stages of development (79% and 22%, respectively). In contrast, in the first leaf cells at late seedling development stages a higher level of $O_2^{\bullet-}$ production was observed immediately after exposure (65%) as compared to 2 d after HT exposure (34%). The MDA content increased by 27% in the first leaf in 2 d after exposure at the early stages of seedling development, and this trend also continued during the late stages of development (58%) [181]. Kumar et al. [182] observed that high temperature of 40/35°C (day/night temperature) resulted in 1.8-fold increase of MDA content in rice genotypes and 1.2- to 1.3-fold increase in maize genotypes over the control treatment. At 45/40°C, a further increase of MDA content was observed in both the crops, which were 2.2- to 2.4-fold increase in rice and 1.7-fold in maize genotypes compared to control. Similarly at 40/35°C the H_2O_2 level showed 1.9- to 2.0-fold elevation in rice genotypes and 1.4- to 1.6-fold elevation in maize genotypes relative to their respective controls. Moreover, at 45/40°C, H_2O_2 content increased further in higher rate in maize genotypes.

Low temperature is also responsible for the production of ROS in plant cell [183, 184]. In extreme cold beyond the plants tolerant level or in chilling sensitive plants the activities of antioxidant enzymes are reduced which accelerate the accumulation of ROS in higher amount. Production of ROS severely affects electron transfer and biochemical reactions [12, 108]. Low temperature-induced oxidative stress decreases phospholipid content, increases lipid peroxidation, free and saturated fatty acid content [185-187]. This stress damages lipid, protein, carbohydrate and DNA [Gill SS and Tuteja 2010], thus it alters the enzyme activities, bio-

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chemical reactions and plant physiological processes including photosynthesis, respiration, nutrient movements, transpiration which negatively affect plants survival. In extreme cases ROS induced oxidative stress causes cell death [160].

However, recent studies have shown that ROS could also play a key role in mediating important signal transduction events. The rates of ROS production during temperature stress could play a central role in stress perception and protection [12].

6. Antioxidant defense under temperature stress

Plants have various enzymatic and non-enzymatic defense systems to minimize the deleterious effects of ROS which include the enzymes: catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione *S*-transferase (GST), glutathione peroxidase (GPX), superoxide dismutase (SOD) etc. as well as non-enzymatic compounds such as ascorbate (AsA), glutathione (GSH), carotenoids etc. However, under the extreme condition, ROS production overwhelm the scavenging action of the antioxidant system, which results in extensive cellular damage and death. In such cases external protectants as well as genetic manipulation of defense genes can work in upregulating the defense system which is also true for temperature stress induced oxidative damage. There are numerous plant studies which indicate the tolerance to temperature stress in plants is positively correlated with an increase in antioxidants [3, 17, 18, 188, 189].

Under HT stress, plants are found to accumulate enhanced amount of non-enzymatic antioxidant and upregulate the activities of antioxidant enzymes. However, in most of the cases these enhanced activities are not sufficient for stress tolerance in plants, especially in susceptible genotypes [3, 18]. Almeselmani et al. [18] observed that the activities of SOD, APX, CAT, GR and POX were increased significantly at all stages of growth in heat-tolerant cultivars (C 306) in response to heat stress while the susceptible cultivar (PBW 343) showed a significant reduction in CAT, GR and POX activities. While investigating the induction of antioxidant enzymes (SOD, APX, GR and CAT) in wheat shoot under HT stresses, Badawi et al. [190] observed that three wheat genotypes (Fang, Siete Cerros and Imam) showed differences in their antioxidant enzyme activities. Importantly, Fang, the heat tolerant genotype, showed higher SOD, APX, GR and CAT activities under HT stress compared to the other two genotypes which indicated the role of the antioxidant defense system in conferring heat stress tolerance. Djanaguiraman et al. [86] observed that HT stress decreased antioxidant enzyme activities and increased oxidant production in sorghum. In their study, SOD, CAT and POX activities were decreased in heat stress (22, 15 and 25% lower than control plants) and the greater inhibition of all antioxidant enzymes in heat-stressed plants relative to control plants indicates greater inactivation of all antioxidant enzymes by heat stress. On the other hand, the application of selenium (Se) decreased oxidative damages by enhancing antioxidant defense resulting in higher grain yield. In addition, the increase in antioxidant enzyme activities and decrease in ROS content by Se was greater in HT than in optimum temperature. In *Cicer arietinum* plants,

Kaushal et al. [191] reported that heat stress (45/40°C) induced the activities of enzymatic (SOD, CAT, APX, GR) and levels of non-enzymatic (AsA, GSH) antioxidants. However, the plants growing in the presence of proline reduced the oxidative injury which was coupled with elevated levels of enzymatic and non-enzymatic antioxidants which indicated the upregulation of the antioxidant defense system could imparts partial heat tolerance to chickpea plants. Recently, we investigated the effect of HT stress (38°C for 24 and 48 h) on antioxidant defense system and the protective role of NO in coffering stress tolerance in *T. aestivum* L. cv. Pradip) seedlings [Hasanuzzaman et al. 2012b]. We observed that AsA content markedly decreased upon heat treatment but GSH and glutathione disulfide (GSSG) content increased. Heat treatment resulted in an increase in the activities of antioxidant enzymes - APX, GR, GPX and GST. However, supplementation of heat-treated seedlings with sodium nitroprusside (SNP) significantly increased the content of AsA and GSH as well as the GSH/GSSG ratio [165]. Heat treated seedlings which were supplemented with SNP also upregulated the activities of APX, MDHAR, DHAR, GR, GST and CAT. This study clearly indicated the role of antioxidant defense to develop stress tolerance in plant under HT. Bavita et al. [192] reported that the upregulation of the antioxidant system by NO possibly contributed to better tolerance against HT induced oxidative damage in wheat.

A higher AsA content was found to associate with higher antioxidative capacity and higher cold tolerance in rice [184]. Streb et al. [193] found to increase the contents of AsA and α-tocopherol in chilling-tolerant cereal leaves which helped to maintain better photosynthesis levels as compared to the chilling sensitive varieties. Fortunato et al. [194] stated that the elevated ROS production indicated by H_2O_2 and $OH^{\bullet}$ was reduced by the over production of AsA and α-tocopherol contents under LT stress in *Coffea* sp. The ratio of GSH/GSSG is also important because higher of this ratio is an indication for better tolerance to stress. Under stressful condition including the cold the higher GSH/GSSG ratio is desirable for the sufficient amount of GSH in the AsA-GSH cycle [195]. Takáč et al. [196] showed that the activities of some antioxidant enzymes are partially correlated with the chilling sensitivity of maize cultivars and thus the antioxidant enzymes posses a significant importance in the chilling tolerance of *Z. mays*. In rice, a greater efficiency of antioxidant enzymes was observed in chilling-tolerant cultivars and the activities of those were far higher than chilling-susceptible cultivars [19]. Wang and Li [90] observed that both heat and cold altered the antioxidant defense system in grape plants. However, exogenous salicylic acid (SA) pretreatment enabled the grape leaves to maintain relatively higher activities of APX, GR, MDHAR, and redox ratio in the AsA–GSH pool both under normal temperature and heat or cold stress. They also suggested that Ca^{2+} homeostasis and antioxidant systems are involved in SA-induced heat or cold tolerance. Zhao et al. [189] observed that the chilling tolerance of tomato cultivars could obviously be indicated by higher activities of CAT, APX, POX and SOD. Zhang et al. [2009] found that chilling stress reduced the activities of antioxidant enzymes viz. SOD, POD, CAT and APX in *C. sativus*. However, these changes were significantly restored by exogenous application of putrescine (Put) and spermidine (Spd) which rendered the plants tolerant to chilling. Zhao et al. [189] reported that the chilling tolerance of tomato cultivars could obviously be designated by the higher activities of CAT, APX, POX and SOD enzyme. Chu et al. [197] observed that Se treatments significantly increased the content of anthocyanins,

flavonoids, and phenolic compound of seedlings subjected to LT stress which was mainly due to the ability to scavenge ROS. They showed a significant increase in activities of POD and CAT in Se treated wheat seedlings under LT. Liu et al. [198] found insufficient antioxidant defense in *Cucumis sativus* seedlings under chilling (4°C). But when the seedlings were pretreated with 1.0 mM SNP (NO donor) and exposed to LT they observed that treatment with NO donor stimulated the activities of various enzymes such as SOD, GR, POD and CAT which indicates that exogenous NO enhanced chilling stress tolerance. It was also observed that due to SNP treatment the MDA content was significantly decreased (27%) in chilling-stressed seedlings as compared to stress alone. Yang et al. [199] observed that the enhanced activities of SOD, CAT, APX and POX in *C. sativus* plants reflected better tolerance to chilling. The activities of SOD, APX, GR and POX increased in cold-acclimated *Cicer arietinum* plants at the chilling stress of 2 and 4°C which enhanced their chilling tolerance [200].

7. Conclusion and future perspectives

The extreme temperatures those are consequences of present-day global climate changes are considered as major abiotic stresses for crop plants. Different plant studies clearly show that temperatures exceeding the limits of adaptation substantially influence the metabolism, viability, physiology, and yield of many plants. Plants exposed to extreme temperatures often show a common response in the form of oxidative stress. However, the extent of damage caused by extreme temperatures depends greatly on the duration of the adverse temperature, the genotypes of the exposed plants, and their stage of growth. There is ample need to develop temperature tolerance in crop plants by exploring suitable strategies. Numerous research findings support the notion that induction and regulation of antioxidant defenses are necessary for obtaining substantial tolerance against environmental stress. The development of genetically engineered plants, by the introduction and/or overexpression of selected genes, would to be one feasible strategy. However, plant adaptation to either HT or LT is a multigenic response which is very complex in nature. Thus the task of identifying the traits those correlate with stress tolerance is incredibly difficult for researchers.

At present, number of genes have been identified in different studies but the knowledge of the transcriptional control of extreme temperature responses is limited. In addition, the regulation of these transcriptional responses is far more complex than previously believed. In recent years, a number of exogenous protectants, such as proline, glycinebetaine, nitric oxide, silicon, selenium, salicylic acid, and polyamines have been tested and found to be beneficial in protecting plants against damage from temperature extremes. Therefore, more advanced research should conduct focusing on the development of plants those restrain genes which promote the accumulation/synthesis of these beneficial elements and compounds. Considering these facts, a well organized approach should combine to investigate the molecular, physiological, and metabolic aspects of temperature stress tolerance both at the cellular and the whole plant level.

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Chapter 7

Programmed Cell Death as a Response to High Light, UV and Drought Stress in Plants

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Additional information is available at the end of the chapter

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1. Introduction

Because of their sessile nature, plants are unable to avoid fluctuating environment conditions like high light, ultraviolet radiation, drought, salt stress, heat, cold or flooding. Upon certain threshold of these changes, plant cells can no longer maintain proper metabolic processes and programmed cell death (PCD) is induced.

PCD is an essential cell suicide process in animals, yeast and plants. In multicellular organisms, it plays an important role in the cell homeostasis maintenance, tissue specialization, removing of damaged or infected cells and acclimatory response. In contrast to necrotic death, which proceeds via swelling, lysis and leakage of cell content, PCD is a highly regulated and organized process. This controlled disassembly of cell involves the condensation, shrinkage and fragmentation of both cytoplasm and nucleus and DNA laddering. Furthermore, while PCD can occur during development and is regulated by complex mechanisms, necrosis does not require the activity of proteases nor nucleases and is not associated with signal transduction pathways [1].

There are two main categories of PCD in plants: developmentally- and environmentally-induced PCD. The first one is a genetically encoded process which plays a crucial role in the development of some tissues and organs. It is involved in tracheary elements formulation during xylem differentiation. Tracheary elements are long cells that transport water and mineral salts, and serve as a structural support in vascular plants. Their formation occurs after secondary wall synthesis and begins with a collapse of the central vacuole and release of lytic enzymes, followed by degradation of cellular content [2]. Another example of developmentally-induced PCD is a formation of unisexual flowers in monoecious species (e.g. maize), bearing generative organs of both sexes on the same plant. Sex determination in these species involves the developmental arrest of one of the organ primordia - either the

female or male within a bisexual floral meristem [3]. The production of complex leaf shapes also frequently employs PCD. Such remodeling of leaf blades occurs in *Monstera obliqua*, *Monstera deliciosa* or lace plant [4]. These species tend to induce death pathway in some patches of cells and thus form distinctive perforations within the leaf [5]. PCD is also engaged in such processes as dying of aleurone cells in seeds of monocots, root cap shedding or anther dehiscence. Senescence, which is the final stage of vegetative and generative development, preceding plant organs death, also involves PCD. In deciduous trees, senescence is exhibited in the changes of leaves color developing during autumn. It enables the active turnover of cellular material and its use in other organs. For example, nutrients, such as nitrogen, recycled from leaves are used for the synthesis of proteins that will be stored in stems and will support growth in the following vegetative season. Moreover, PCD during senescence helps to block spreading of diseases to still vital parts of the plant [6].

Developmental PCD is induced by internal factors and occurs at defined time and in particular plant tissue. On the contrary, environmentally-induced PCD is triggered by different stimuli ranging from pathogen infection to environmental factors [7]. During infection of plant leaves by pathogens, a specific gene-for-gene (avr–R) interaction triggers defense responses. Upon such plant-microbe interaction, cell death takes a form of so-called hypersensitive response (HR) and includes a burst of reactive oxygen species (ROS). HR leads to the formation of a lesion which is clearly delimited from surrounding healthy cells and thus prevents the spread of pathogen throughout the plant tissue. Certain mutations in many plant species have been demonstrated to cause spontaneous, HR-resembling lesions, which suggests that this type of cell death is under a genetic control. Such lesion mimic mutants are divided into two groups: related to the initiation of PCD (inappropriate induction of PCD and formation of localized lesion spots) or propagation (inability to stop PCD once it has been initiated). Both these groups of mutants are currently widely investigated since they can provide insight into the general mechanism of PCD in plants [8–12]. The existence of these two classes suggests that genetically distinct processes underlie the lesion formation: the initiation of cell death and its spread to surrounding cells as well as the existence of communication signals between dying and healthy cells in determining the lesion size.

In natural habitats, plants are constantly exposed to a variety of environmental stresses that can lead to the disturbance in cellular homeostasis and consequently limit crop yield. Programmed cell death is a fundamental cellular process associated with the defense responses to abiotic stimuli such as excessive irradiation, ozone, ultraviolet radiation, heat, cold, drought or flooding. One of the example factor triggering PCD is hypoxia, a condition in which plant is deprived of oxygen supply. In response to waterlogging and lower O_2 concentration in the ground, cortex of the root can form aerating tissue called aerenchyma [13]. The internal air spaces are generated through PCD and facilitate gas diffusion from aerial organs to waterlogged roots [14]. Although it is unfavorable for biomass production, the selective death of cells and tissues under abiotic stresses eventually provides survival advantages for the whole organism. At the organismal level, PCD helps to maintain tissue and organ homeostasis, enables developmental adaptation and nutrient resorption from dying cells thus increases the probability of survival. It also leads to the signals transduction from

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cells undergoing PCD to healthy, not-affected cells and triggers stress tolerance and acclimation to adverse conditions [6,15].

2. Hallmarks and the regulation of programmed cell death

While the cascade of events and molecules regulating PCD have been already well described in animal cells, mechanisms underlying plant PCD remain still inexplicable. Therefore, numerous studies in plants rely on the comparison of PCD mechanisms to animals. Apoptosis (well-studied form of animal PCD) features in cell shrinkage, chromatin condensation, cleavage of DNA (called DNA laddering) and nuclear fragmentation. The mechanism of PCD depends on a family of cysteine proteases called caspases that cleave their target proteins after aspartic acid residues. Caspases are synthesized in the cell as inactive precursors (procaspases). Once activated, caspases cleave and activate other procaspases which results in a self-amplifying cascade. They can also cleave other proteins such as nuclear lamins or proteins that hold DNA-degrading enzymes in inactive form, releasing DNases to cut DNA. The destructive protease cascade is irreversible, therefore caspase activity needs to be tightly controlled. Procaspase activation is induced by the release of electron carrier protein - cytochrome c from mitochondria to the cytosol. The family of Bcl-2 proteins regulates the activation of programmed cell death. Some members of this family (e.g. Bcl-2) block the release of cytochrome c, inhibiting apoptosis. Others (e.g. Bax and Bak) act as PCD inducers, promoting cytochrome c leakage. IAP (inhibitor of apoptosis) proteins are another family involved in apoptosis regulation as they bind to some procaspases, preventing their activation or to caspases, inhibiting their activity. Proteins that block IAPs are released together with cytochrome c which increases the efficiency of cell death process [16].

Many hallmarks of plant PCD seem to be similar to animals such as cytoplasm shrinkage, chromatin condensation and DNA cleavage, mitochondrial swelling, disruption of organelles and plasma membrane collapse [17]. The major difference in executing PCD between animals and plants lies in the process of removing the cell content after its death. While in animal cells, removal action is undertaken by other cells to avoid the activation of inflammatory response, in plants there is a leakage of the cell content into the apoplast and remains are not engulfed by surrounding cells [10]. Moreover, plants exhibit some distinctive features of PCD that result from the presence of chloroplasts and the significance of vacuoles [18,19]. Plant vacuoles represent important storage organelles that are the repository of hydrolytic enzymes such as proteases, lipases and nucleases. Vacuoles are therefore postulated to play a role in the turnover of organelles and cytoplasm during autophagy as a part of clean-up system for dying cells. The component of this system is a caspase-like protease - the vacuolar processing enzyme (VPE) which plays a crucial role in such PCD pathways as senescence, lateral root formation and hypersensitive response. Upon receiving pro-apoptotic signals, VPE activates hydrolases that execute the degradation of vacuolar membrane resulting in the release of hydrolytic enzymes and subsequent degradation of cell content [19].

Chloroplasts are strongly suggested to be key players during cell death responses as they constitute an important source of defense signaling molecules such as ROS, reactive nitrogen species (RNS) and defense hormones like salicylic acid (SA) and jasmonic acid (JA). The oxidative burst is one of the earliest and most common plant response to abiotic and biotic stimuli [20]. The application of chloroplast-targeted, ROS-generating herbicides such as methyl viologen (paraquat) induces cell death with the typical apoptotic traits [21].

Some of key proteins controlling animal cell death such as the Bcl-2 family and caspases have been proven to be not conserved in plants. It suggests that plants have developed some unique mechanisms of PCD [15]. Although orthologs of caspases have not been found in plants based on the sequence similarity, several studies using caspase-specific peptide inhibitors suggested the presence of caspase-like proteases (metacaspases) [22]. These caspase inhibitors have been demonstrated to prevent chemically-, UV- or HR-induced PCD [23–25] indicating that caspase-like proteins are indeed involved in the regulation of PCD in plants. Metacaspases (MC) differ from animal caspases in their substrate specificity as they cleave proteins after arginine or a lysine residues. Nine predicted metacaspase-encoding genes have been found in *Arabidopsis thaliana* and divided into two classes, depending on the presence (type I) or absence (type II) of the N-terminal zinc-finger domain that has the homology to the LESION SIMULATING DISEASE 1 (LSD1) protein (see later) [26,27]. This domain is known to participate in protein–protein interactions and could indicate that oligomerization is important for MCs type I activation. The catalytic activities of AtMC4, AtMC5 and AtMC8 have been found to be Ca^{2+}-dependent while AtMC9 is active under mildly acidic conditions. Thus, alterations in cellular Ca^{2+} concentration and pH, that are common during various stresses, may help to control MCs activation. The sequence of AtMC4 has also revealed potential self-cleavage sites that may facilitate additional regulation of protease activity to achieve sensitive control of PCD [28]. Additionally, metacaspase ATMC4 (AtMCP2) has been proven to play a positive regulatory role in biotic and abiotic stress-induced PCD [29]. AtMC1 and AtMC2, belonging to type I metacaspases, have opposite roles in the cell death control. There is a genetic evidence that AtMC2 acts as a negative regulator of AtMC1-induced PCD. Therefore, it is hypothesized that proteins belonging to MC family execute either anti- or pro-apoptotic functions and compete with each other in making the cell life-death decisions [30].

Although no orthologues of Bcl-2 family genes (*Bcl-2* or *Bax*) have been found in plants, there are some studies demonstrating that the expression of these genes in plants can regulate programmed cell death pathway [31,32]. Transgenic plants overexpressing animal antiapoptosis genes such as *Bcl-2* have been proven to exhibit enhanced tolerance to both biotic and abiotic stress conditions [33,34]. Moreover, the homologue for animal Bax Inhibitor (BI) has been identified in *Arabidopsis* [35] and proven to inhibit cell death in plants expressing mammalian Bax [36]. *Arabidopsis* BI-1 (AtBI-1) has been reported to localize in the endoplasmic reticulum (ER) and to contain predicted transmembrane α-helices in the sequence, that are conserved in two other AtBI-1-related proteins: BI-2 and BI-3. These proteins are hypothesized to function in a similar fashion to the Bcl-2 family - as regulators of pro-death or survival pathways [10]. In plants, mRNA level of AtBI-1 increases during leaf senescence

and under different abiotic stresses. The loss of function in AtBI1 results in the mutant hypersensitivity to environmental stimuli, whereas its overexpression in retarded PCD [37].

Numerous signals are constantly integrated by the cell to decide whether to enter or not the cell death pathway. Different plant hormones are involved in the regulation of cell death under unfavorable conditions. One of the most important is SA which is intensively produced in cells after pathogen infection or various abiotic stresses [38]. Many lesion mimic mutants have constitutively elevated levels of SA [39]. At high concentrations, SA functions as a cell death inducer in cooperation with other signals. It can be also transported beyond the site of synthesis, acting as a signaling molecule and mediating systemic acquired resistance (SAR) - a whole-plant resistance response that prepares plant for another infection [40]. The existence of SA-dependent generation of ROS and the feedback control of SA synthesis by ROS have been also demonstrated [41]. SA and ROS have been proposed to work in a potentiation feedback loop which acts to amplify signals leading to cell death. Another cell death signaling molecule - nitric oxide (NO) has been also demonstrated to regulate key steps in SA biosynthesis during pathogen infection [42]. Additionally, NO has been proven to cooperate with ROS and SA in inducing cell death [43]. Other phytohormones regulating cell death under stress conditions are JA, gibberellic acid (GA), abscisic acid (ABA) and ethylene (ET). The latter is involved in the regulation of PCD during different developmental processes and responses to environmental stimuli. ET has been proven to participate in the formation of aerenchyma in roots under hypoxia [14]. Antisense inactivation of the ET biosynthetic enzyme - ACC oxidase delays leaf senescence and cell death in tomato [44]. Ethylene is also required for the continuation of ROS accumulation - external supply of ET during cell death increases ROS production and causes accelerated spreading of cell death [45]. JA is a plant signaling molecule best known for its role in the wound response but it is also produced during wide range of biotic and abiotic stresses. It is involved in the inhibition of ROS- and ET-dependent lesion propagation [46]. JA derivatives such as methyl jasmonate (MeJA) are also engaged in the regulation of plant immune responses [47]. Upon exposure to stress, MeJA is produced and causes the activation of PCD through the induction of ROS generation, alterations in mitochondrial dynamics and photosynthetic collapse [48]. Another phytohormone - GA has been proven to promote cell death in cooperation with ROS, whereas ABA delays GA-induced PCD. Such counteracting role of these hormones relates to their influence on the ROS-scavenging enzymes expression [49]. ABA has been also shown to delay ET- and GA-induced cell death in rice epidermal cells [50]. All these interactions between phytohormones and ROS indicate the complexity of PCD regulation. Overmyer and colleagues [39] suggested the following series of events during oxidative stress-triggered PCD. At the site of lesion initiation, the action of ROS is amplified. Increased ROS accumulation together with SA induces the cell death. During the initial process, JA signaling is hindered by SA and ET. Meanwhile, the burst of ET from the initial site disperses to surrounding cells, amplifies ROS production that promotes the lesion spread. This is the signal to induce competitive reactions to PCD. Cell death results in the production of JA which acts as a negative regulator of the oxidative cell death cycle. JA, through the suppression of SA biosynthesis/signaling and the attenuation of ET sensitivity, inhibits the lesion propagation.

During early events of hypersensitive response, ion fluxes are induced. Ca^{2+} influx caused by external hydrogen peroxide application has been demonstrated to be sufficient in triggering HR in soybean cells [51]. Moreover, several cell death signaling proteins in plants exhibit a function associated with lipids. Two *Arabidopsis* mutants, *eds1* and *pad4* have been proven to be defective in HR signaling. *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY 1*) and *PAD4* (*PHYTOALEXIN DEFICIENT 4*) genes encode proteins with triacylglycerol lipase function [52,53] which provides a genetic evidence that phospholipid signaling is involved in the induction of PCD. The level of phosphatidic acid (PA), produced by the phospholipase D (PLD) increases during defense response [54]. One of PLD isoforms in *Arabidopsis* has been shown to impair ROS-mediated PCD in response to biotic and abiotic stimuli [55], indicating the role of PA as a negative signal of cell death propagation. It is also hypothesized that the perturbation in sphingosine transport may cause cell death in plants since the mutation in *ACD11* gene encoding a sphingosine-transport protein, results in a lesion-mimic phenotype that is dependent on EDS1, PAD4, SA and light [56]. EDS1 and PAD4 are extensively studied regulators of PCD. They constitute a regulatory hub that transduces redox signals in response to biotic and abiotic stresses. Both EDS1 and PAD4 are also important activators of SA signaling and mediate antagonism between JA and ET pathways during defense responses [57]. Furthermore, they are responsible for the biotic and abiotic stress-induced PCD in the *LESION SIMULATING DISEASE 1* (*LSD1*) mutant [58,59]. The *lsd1* mutant fails to limit the spread of PCD under long photoperiod or after the infection with avirulent pathogens. It is one of the best characterized mutants in terms of programmed cell death. The *lsd1* mutant exhibits a runaway cell death (RCD) phenotype (Figure 1B) manifested in the inability to restrict the progression of cell death once it has been initiated [9,11], which provides a genetic evidence for LSD1 as a PCD repressor.

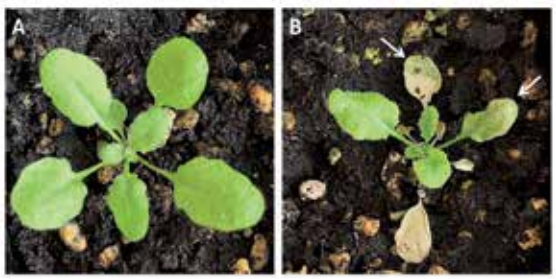

Figure 1. Runaway cell death (RCD) phenotype in the *lsd1* mutant. 3-week-old *Arabidopsis thaliana* rosettes grown in long photoperiod (>11 h); A – wild type; B – *lsd1* mutant. Arrows indicate leaves undergoing runaway cell death.

The *lsd1*/*cao* double mutant which has reduced photosystem II (PSII) antenna size and thus reduced light absorption capacity due to the *cao* mutation in chloroplast signal recognition particle (cpSRP43), exhibits higher non-photochemical quenching (see later) and inhibited RCD after excess light exposure in comparison to the *lsd1* mutant. Therefore, the RCD in the *lsd1* mutant has been linked to the amount of light energy absorbed in excess by the PSII light harvesting complex [11]. It has been shown that ET and ROS production in the *lsd1*

mutant plants is elevated after plastoquinone reduction. The RCD in the *lsd1* mutant plants has been also proven to be inhibited by the mutation in *EIN2*, which encodes an ethylene receptor. Additionally, the artificial impeding of foliar gas exchange in *lsd1* has been shown to induce RCD, while high CO_2 level has prevented cell death in this mutant. Importantly, *lsd1* phenotype depends on EDS1 and PAD4, since in double mutants *eds1*/*lsd1* and *pad4*/*lsd1* PCD is inhibited [11,14,60]. The formation of ROS by plasma-membrane-bound NADPH oxidase has been proposed to play a major role in RCD in the *lsd1* mutant during the shift from short to long photoperiod, since the inhibition of this enzyme diminishes the formation of lesions [61]. All these results suggest that LSD1 acts as a ROS rheostat and is necessary for acclimation to conditions that promote oxidative stress. While LSD1 has been proven to negatively regulate the cell death, a highly similar protein - LOL1 (LSD1 like 1) is suggested to be a positive PCD-regulator [62]. It has been proposed that LSD1 and LOL1 might function in an antagonistic fashion to regulate the cell death propagation. Both LSD1 and LOL1 are putative transcription factors (TF) or scaffold proteins since they possess zinc-finger domains responsible for DNA/protein binding. Such Zn-finger motif of the C2C2 class has been found in plants, algae and protozoa, but not in animals. Apart from LSD1 and LOL1, only five other *Arabidopsis* proteins contain one or more LSD1-like Zn-finger domains: LOL2, LOL6 and already mentioned metacaspases: AtMC1, AtMC2 and AtMC3 [12]. The second and third Zn-finger domains of LSD1 are responsible for interacting with metacaspase AtMC1, which is a positive regulator of PCD. The *atmc1* mutation is able to suppress cell death in *lsd1.* Furthermore, the interaction of LSD1 with AtbZIP10 transcription factor prevents its translocation to the nucleus. AtbZIP10 has been proven to be a positive mediator of RCD observed in the *lsd1* mutant [63].

3. Reactive oxygen species in plants

The signaling during PCD proceeds mainly through the regulation of reactive oxygen species [60,64,65]. ROS are produced continuously as by-products of various pathways localized in chloroplasts, mitochondria and peroxisomes. They can occur as free radicals: superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), perhydroxyl radical ($HO_2^{\bullet}$), alkoxy radical ($RO^{\bullet}$) or in non-radical forms: singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Most abiotic stresses evoke the overproduction of ROS in plant tissues. Because of their high reactivity, ROS can cause damage of proteins, lipids, carbohydrates and nucleic acids, ultimately leading to cell death (Figure 2).

The single reduction of O_2 results in the formation of $O_2^{\bullet -}$. From $O_2^{\bullet -}$ other more reactive ROS like $^{\bullet}OH$ or $HO_2^{\bullet}$ can be formed. The Haber-Weiss reaction generates hydroxyl radical from hydrogen peroxide and superoxide. In this reaction $O_2^{\bullet -}$ donates an electron to Fe^{3+}, reducing ferric ion to ferrous:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$

The second step of •OH formation is the Fenton reaction in which reduced form of iron (Fe^{2+}) transfers electrons to H_2O_2:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

$O_2^{\bullet-}$ can be also protonated to form $HO_2^{\bullet}$. Furthermore, $O_2^{\bullet-}$ can react with another free radical species as $NO^{\bullet}$ to generate peroxynitrite ($OONO^-$) [66]. Another form of ROS – singlet oxygen is the first excited electron state of O_2 that originates when an electron is elevated to a higher energy orbital (Figure 3).

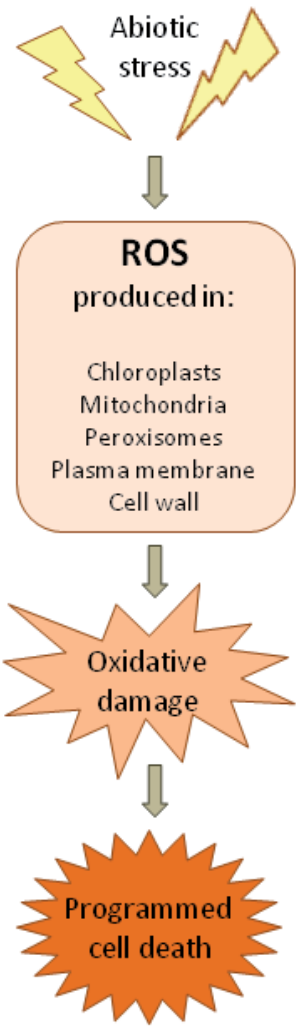

Figure 2. ROS production and programmed cell death induced by abiotic stress (according to Gill and Tuteja, 2010) [66].

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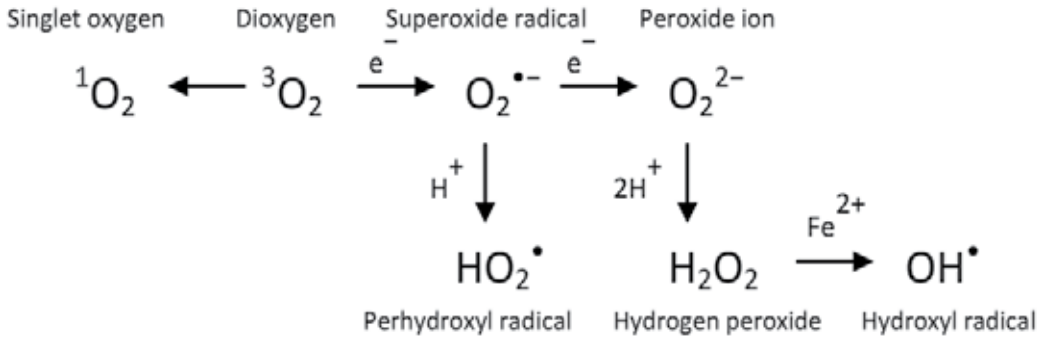

Figure 3. Formation of different ROS (according to Gill and Tuteja, 2010) [66].

Very reactive $^1O_2$ can be formed by the reaction of O_2 with photo-excited chlorophyll. Inadequate dissipation of excess energy during photosynthesis can lead to the generation of chlorophyll triplet state. The Chl triplet state can react with $^3O_2$ to yield $^1O_2$ [67]. The formation of $^1O_2$ has extremely damaging effect on PSI and PSII and other components of photosynthetic machinery. The $^1O_2$ lifetime has been measured to be approximately 3μs [68]. During this time, some part of $^1O_2$ fraction is able to diffuse over several hundred nm, reacting with proteins, pigments, nucleic acids and lipids [69]. In *Arabidopsis* mutants favoring $^1O_2$ production, photooxidative stress has been demonstrated to cause dramatic increase in lipid peroxidation (LPO) that precedes cell death [70]. Plant chloroplasts avoid the accumulation of $^1O_2$ by employing β-carotene, tocopherol and plastoquinone to remove it. If the elimination is not sufficient, $^1O_2$ can lead to the upregulation of genes involved in defense responses against photooxdative stress [69]. These genes have proven to be different from those induced by $O_2^{\bullet-}$ and H_2O_2 and it has been suggested that $^1O_2$ acts as a signal molecule that activates several stress-responsive pathways [71]. The reaction of superoxide radical reduction produces hydrogen peroxide. H_2O_2 is moderately reactive but has relatively long lifetime (1ms) in comparison to other ROS. H_2O_2 has been shown to be required in a broad range of physiological processes such as stomata movements [72], cell cycle [73] as well as growth and development [74]. It plays a dual role. At low, physiological concentration, H_2O_2 acts as a signal molecule involved in defense and acclimatory responses to various biotic and abiotic stimuli. At high concentrations, it reacts with cell constituents, e.g. inactivates enzymes by oxidizing their thiol groups and triggers PCD [75]. The highly reactive ROS - hydroxyl radical, produced in Haber-Weiss reaction can potentially react with many organic compounds including DNA, proteins and lipids. Due to the absence of enzymatic mechanism for $^{\bullet}OH$ elimination, its excess production leads to cell death [76].

It has been estimated that 1 - 2% of O_2 consumed by plants is sidetracked for ROS production in different subcellular compartments [66]. Organelles such as chloroplasts, mitochondria or peroxisomes are major sources of ROS in plant cells since they exhibit an intense rate of electron flow and highly oxidizing metabolic activity. In chloroplasts, PSI and PSII are major sites where the singlet oxygen and superoxide radicals are generated. Under non-stress conditions, the electron from excited photosystem is transferred to $NADP^+$, reducing it

to NADPH. However, under various abiotic stresses, the electron transport chain (ETC) tends to be overloaded and a part of the electron flow is diverted from ferredoxin to O_2, reducing it to $O_2^{\bullet-}$. The photoreduction of O_2 at PSI proceeds via Mehler reaction and produces $O_2^{\bullet-}$, which is disproportionated to H_2O_2 and O_2 with the use of superoxide dismutase. H_2O_2 is rapidly detoxified to H_2O by the ascorbate peroxidase pathway (Figure 4A). Because of the electron flow from water in PSII to water in PSI that occurs in this process, it has been termed the water–water cycle [77]. This cycle does not only scavenge $O_2^{\bullet-}$ and H_2O_2, but also generates a pH gradient across thylakoid membranes which enhances non-radiative dissipation of light energy by non-photochemical quenching (see later). Therefore, the water–water cycle is considered to function as a dissipatory mechanism of the excess energy [77,78].

H_2O_2 is also produced during a process that proceeds concurrently to the photosynthesis – photorespiration. During photosynthetic carbon assimilation, ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme (Rubisco) uses CO_2 to carboxylate ribulose-1,5-bisphosphate (RuBP). CO_2 uptake results in the formation of two molecules of 3-phosphoglycerate (3-PGA) that are utilized for biosynthetic reactions and the recycling of the RuBP acceptor molecule. However, Rubisco can also use O_2 to oxygenate RuBP, forming one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG). The latter cannot be used for biosynthetic reactions and is considered as an inhibitor of the chloroplast function. Photorespiration functions to convert 2-PG back to 3-PGA and thus to recover carbon. It constitutes a series of reactions taking place in chloroplasts, peroxisomes, and mitochondria. 2-PG is dephosphorylated to glycolate in the chloroplast and transported to the peroxisome where it is oxidized to glyoxylate. O_2 is the electron donor in this reaction, which results in H_2O_2 generation. Glyoxylate is transaminated to glycine which is transported to the mitochondrion, where two molecules of glycine are converted to serine and the remaining carbon and nitrogen are released as CO_2 and NH_3, respectively. The amine group is used to form a new glycine from glyoxylate and the resulting hydroxypyruvate is reduced to glycerate. Finally, glycerate is phosphorylated in the chloroplast to form 3-PGA, which can be fed back to the Calvin cycle [79,80].

The production of ROS is also an unavoidable consequence of the aerobic respiration. It occurs under normal respiratory conditions but can be enhanced in response to biotic and abiotic stress. ROS produced in mitochondria are regarded to be essential in PCD regulation [81]. In mitochondria, $O_2^{\bullet-}$ is mainly produced in complex I, ubiquinone and complex III of ETC [82]. This $O_2^{\bullet-}$ can be further converted into highly toxic $^{\bullet}OH$ which may penetrate membranes and leave the mitochondrion [83]. Hydroxyl radical can also initiate the peroxidation of mitochondrial membrane polyunsaturated fatty acids (PUFA) that leads to the formation of cytotoxic lipid aldehydes, alkenals and hydroxyalkenals, such as malonyldialdehyde (MDA). Lipid peroxidation products may cause cellular damage by reacting with other lipids, proteins and nucleic acids. The mitochondrial ETC produces significant amount of ROS but the mitochondrial enzyme - alternative oxidase (AOX) can prevent ROS overproduction [84]. Some studies, performed on tobacco plants, have demonstrated that the lack of AOX induces PCD while the AOX overexpression decreases the lesion size during HR [85,86].

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Another source of ROS - peroxisomes are small, spherical organelles with an oxidative type of metabolism. There are two sites of $O_2^{\bullet-}$ generation in peroxisomes: in the organelle matrix, where xanthine oxidase (XOD) catalyzes the oxidation of xanthine and hypoxanthine to uric acid and in the membrane, by components of peroxisomal ETC. The main metabolic processes responsible for H_2O_2 generation in peroxisomes are photorespiratory glycolate oxidase reaction, fatty acid β-oxidation, enzymatic reaction of flavin oxidases and disproportionation of superoxide radicals [87].

ROS are also generated in the apoplast by NADPH oxidases residing in the plasma membrane and generating superoxide radicals. The extracellular $O_2^{\bullet-}$ is quickly mutated into H_2O_2 or converted to $^{\bullet}OH$. The latter initiates a series of reactions that cause a plasma membrane damage, finally leading to cell death. Two *Arabidopsis* respiratory burst oxidase genes, *RBOHD* and *RBOHF*, that encode NADPH oxidases have been proven to be responsible for ROS production during the HR. Enzymes such as cell wall peroxidases, germin-like oxalate oxidases and amine oxidases have been proposed as a source of hydrogen peroxide in the apoplast. The alkalization of apoplast upon elicitor recognition precedes the production of H_2O_2 by pH-dependent cell wall peroxidases [88].

The peroxidation of lipids is considered as one of the most damaging processes occurring in the cell. The damage of membrane is often considered as a parameter determining the level of cell destruction under various stresses. Upon ROS overproduction, polyunsaturated precursors undergo lipid peroxidation, forming small hydrocarbon fragments such as ketones or aldehydes. LPO in both cellular and organellar membranes affects proper cellular functions and aggravates oxidative stress by the production of lipid-derived radicals [89]. This process often affects PUFA, since they contain multiple double bonds in between which lie methylene (-CH2-) groups with reactive hydrogens. Hydroxyl or perhydroxyl radicals combining with a hydrogen atom produce water and a fatty acid radical. The fatty acid radicals are unstable and react rapidly with molecular oxygen, creating a peroxyl-fatty acid radical ($ROO^{\bullet}$). Once initiated, $ROO^{\bullet}$ can further propagate the peroxidation chain reaction by abstracting a hydrogen atom from PUFA side chains. The resulting lipid hydroperoxide easily decomposes into several reactive species including: lipid alkoxyl radicals, MDA, alkanes and lipid epoxides. Thus, LPO generates multiple peroxide molecules and results in the membrane fluidity decrease, its leakiness to substances that do not normally cross it, the damage of membrane proteins and ion channels. It has been found that such PUFAs as linoleic and linolenic acids are particularly susceptible to ROS attack [90]. Increased level of LPO has been demonstrated in many abiotic stress studies, for instance under salt stress in *Oryza sativa* [91].

Apart from lipid peroxidation, the accumulation of ROS leads to protein oxidation. Only few types of these covalent modifications are reversible, most of them are irreversible [92]. A widely used marker for protein oxidation is their carbonylation level. The oxidation of amino acids such as arginine, histidine, lysine, proline, threonine and tryptophan causes the formation of free carbonyl groups, that may inhibit or alter the protein activity and increase the susceptibility towards proteolytic attack [90]. Proteins with sulfur-containing amino acids and thiol groups are often the target for ROS. Cysteine and methionine are especially reac-

tive with $^1O_2$ and $^{\bullet}OH$. Activated oxygen radical can abstract the hydrogen atom from cysteine residue to form a thiyl radical that cross-links to a second thiyl radical and leads to the formation of disulphide bridges. Oxygen can also be added onto the methionine residue to form a methionine sulphoxide. The best characterized response to the oxidation of peptide residues is the induction of proteases that break down the oxidized proteins [93].

DNA damage, triggered by ROS is particularly dangerous for the cell since it causes replication errors and genomic instability. From all ROS, $^{\bullet}OH$ has the most damaging effect to DNA as it can modify all components of the nucleic acid molecule: purines, pyrimidines and the deoxyribose backbone [94]. The major types of DNA damage resulted from oxidative stress are the formation of dimers between adjacent pyrimidines, cross-links, base deletion, strand breaks and base modifications such as alkylation and oxidation. To counteract the DNA damage, plant cells evolved mechanisms for the DNA repair in both nucleus and mitochondria. These include the direct inversion of modifications or the replacement of the whole nucleotide [95].

To protect themselves against toxic oxygen intermediates, plant cells possess a vast antioxidant system. Stress-induced ROS accumulation is counteracted by both enzymatic and non-enzymatic antioxidants. Enzymatic ROS scavengers include superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione reductases (GR), monodehydroascorbate reductases (MDHAR), dehydroascorbate reductases (DHAR), glutathione peroxidases (GPX) and glutathione-S- transferases (GST). Low-molecular, non-enzymatic antioxidants include ascorbic acid (AsA), glutathione (GSH), proline, α-tocopherol, carotenoids and flavonoids [73].

Metalloenzyme SOD is the most effective enzymatic antioxidant which is ubiquitous in all subcellular compartments. SODs remove $O_2^{\bullet-}$ by catalyzing its dismutation (Figure 4A):

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow 2H_2O_2 + O_2$$

This reaction eliminates $O_2^{\bullet-}$ and hence decreases the risk of $^{\bullet}OH$ formation. SODs are classified into three types, depending on their metal cofactor: copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD) and iron (Fe-SOD). Different types of SODs are located in different cellular compartments [96]. *Arabidopsis thaliana* genome encodes three Fe-SOD (FSD1, FSD2 and FSD3), three Cu/Zn-SOD (CSD1, CSD2 and CSD3) and one Mn-SOD (MSD1) [97]. Mn-SOD has been found in mitochondria and peroxisomes of plant cells [98]. Cu/Zn-SOD isoenzymes have been found in the cytosol and in chloroplasts of higher plants. Fe-SODs are usually associated with chloroplasts [99]. The upregulation of SODs during biotic or abiotic stress-triggered oxidative stress has a critical role in the overcoming of adverse conditions and in the plant survival. Many reports indicate that the overexpression of different SODs leads to the generation of abiotic stress-tolerant plants, e.g. Mn-SOD overexpressing *Arabidopsis* has shown increased salt tolerance [100] and Cu/Zn-SOD overexpressing transgenic tobacco has demonstrated multiple stress tolerance [101]. Interestingly, FSD2 and FSD3 play also an essential role in the chloroplast development, protecting chloroplast nucleoids from ROS [102].

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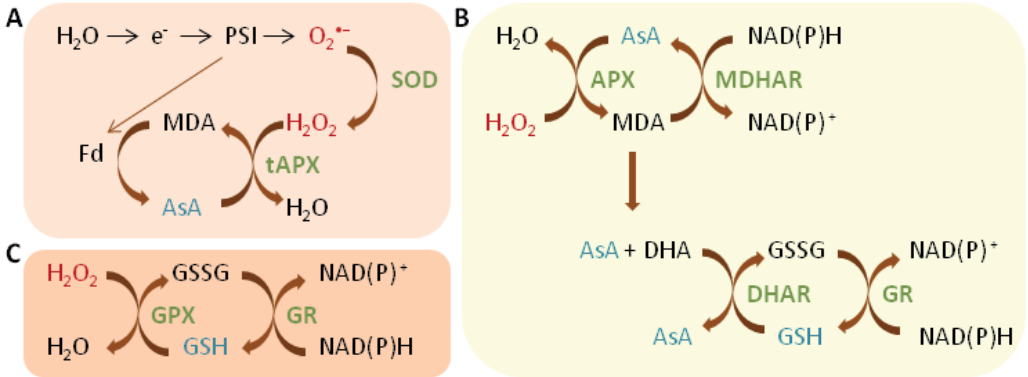

Figure 4. Different pathways for ROS scavenging in plants: A - the water–water cycle (Mehler reaction); B - the ascorbate–glutathione cycle; C – the glutathione peroxidase cycle. Superoxide dismutase (SOD) acts as the first line of defense converting $O_2^{\bullet-}$ into H_2O_2, then ascorbate peroxidases (APX), glutathione peroxidases (GPX) and catalases (CAT – not shown) eliminate H_2O_2. In contrast to CAT, both APX and GPX require ascorbate (AsA) or glutathione (GSH) regenerating cycles that use electrons from the photosynthesis (A) or NAD(P)H (B, C) as reducing power. ROS are indicated in red, ROS-scavenging enzymes in green and low-molecular antioxidants in blue. Abbreviations: DHA - dehydroascorbate; DHAR - DHA reductase; Fd - ferredoxin; GR - glutathione reductase; GSSG – oxidized glutathione; MDA - monodehydroascorbate; MDAR - MDA reductase; PSI - photosystem I; tAPX - thylakoid-bound APX (according to Mittler et al., 2004) [73].

Catalases are tetrameric enzymes containing heme with the potential to dismutate H_2O_2 into H_2O and O_2.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

CAT1 and CAT2 are localized in peroxisomes and cytosol, whereas CAT3 is targeted to mitochondria. Increased CAT activity has been reported in various abiotic stress studies in different species, e.g. under drought stress in wheat [103]. Moreover, a vast number of research indicate that CAT overexpression leads to the abiotic stress tolerance, e.g. wheat catalase expressed in transgenic rice has been demonstrated to improve the tolerance against low temperatures [104].

Another group of antioxidising enzymes - ascorbate peroxidases are involved in H_2O_2 scavenging in water-water and glutathione-ascorbate cycles and use ascorbic acid as the electron donor (Figure 4A and B). The reaction catalysed by APXs is the transfer of electrons from ascorbate to hydrogen peroxide, producing dehydroascorbate and water

$$H_2O_2 + C_6H_8O_6 \rightarrow 2H_2O + C_6H_6O_6$$

In *Arabidopsis thaliana*, the presence of eight APX isoenzymes has been confirmed: soluble cytosolic (APX1, APX2, APX6), bounded to the microsome membrane (APX3, APX4, APX5), chloroplast stromal (sAPX) and thylakoid (tAPX). Higher expression levels of APXs have been demonstrated during different stress conditions and their overexpression has been pro-

ven to enhance the plant resistance, e.g. tobacco plants with higher chloroplast APX expression are more tolerant to the salt stress and water deficit [101].

Glutathione reductases are oxidoreductases participating in the glutathione-ascorbate cycle (Figure 4B). They play an essential role in the defense against ROS by sustaining reduced status of glutathione (GSH), a tripeptide molecule involved in many regulatory and antioxidative processes in plants. They are localized predominantly in chloroplasts, but small amounts have been also found in mitochondria and cytosol [105]. GRs catalyze the NADPH-dependent reduction of the oxidized form of glutathione (GSSG) (Figure 4B and C) thus are important for maintaining the GSH pool. Increased GR activity has been demonstrated in various abiotic stress studies, e.g. in drought stressed rice seedlings [106]. Transgenic plants with lower GR activity have shown enhanced sensitivity to oxidative stress while these with higher GR have been proved to be abiotic stress tolerant. Elevated chloroplastic GR activity has been demonstrated to decrease chilling-induced photoinhibition in transgenic cotton [107].

Monodehydroascorbate reductase is an enzymatic component of the glutathione-ascorbate cycle (Figure 4B). MDHARs are present in chloroplasts, mitochondria, peroxisomes and cytosol, where they participate in H_2O_2 scavanging [108]. They exhibit high specificity for monodehydroasorbate as the electron acceptor and use NADH as the electron donor (Figure 4B):

$$NADH + C_6H_7O_6 \rightarrow NAD^+ + C_6H_8O_6$$

Overexpression of MDHAR in the transgenic tobacco has been demonstrated to increase the tolerance against ozone, salt and osmotic stress [109].

Dehydroascorbate reductases function in the regeneration of ascorbic acid from the oxidized form (Figure 4B) and therefore regulate cellular AsA redox state. DHAR overexpression has been demonstrated to enhance salt tolerance in *Arabidopsis* [110] as well as drought and ozone stress resistance in tobacco [111].

Glutathione S-transferases are a large and diverse group of enzymes with 54 members reported in *Arabidopsis* [112]. They catalyze the conjugation of electrophilic substrates with glutathione. Plant GSTs are known to participate in herbicides detoxification, hormone homeostasis maintenance, sequestration of anthocyanin and regulation of PCD in response to biotic and abiotic stimuli. They are mostly located in the cytoplasm but chloroplastic, nuclear and apoplastic isoforms have also been reported [113].

Glutathione peroxidases are another group of isoenzymes that use GSH to reduce H_2O_2, organic and lipid hydroperoxides (Figure 4C). A family of seven related proteins (AtGPX1-AtGPX7) residing in cytosol, chloroplasts, mitochondria and endoplasmic reticulum has been identified in *Arabidopsis* [114]. Overexpression of GPX in transgenic tobacco has been demonstrated to confer tolerance towards chilling and salt stress [115].

Apart from enzymes participating in redox homeostasis maintenance, plants possess a vast number of non-enzymatic compounds acting as antioxidants. Ascorbic acid (vitamin C) is present in all plant tissues but especially high levels occur in photosynthetically active or-

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gans, meristems and some fruits. Mitochondria play a central role in the metabolism of AsA in plants. Ascorbic acid reacts with oxidants such as $O_2^{\bullet-}$ and $^{\bullet}OH$, transfering a single electron and forming its own radical ion in the following reaction:

$$RO^{\bullet} + C_6H_7O_6^{-} \rightarrow ROH + C_6H_6O_6^{\bullet-}$$

The oxidized form of ascorbate - dehydroascorbate (DHA) is relatively unreactive and do not cause cellular damage. However, it has a short lifetime and needs to be regenerated back into AsA. Ascorbic acid antioxidative capacity provides a protection to membranes by direct scavenging ROS and by regenerating α-tocopherol from tocopheroxyl radical. In chloroplasts, AsA acts also as a violaxantin de-epoxidase cofactor, sustaining dissipation of excess excitation energy [116].

Another powerful antioxidant – glutathione is a tripeptide (γ-glu-cys-gly). In plant tissues it occurs in a reduced form (GSH) and plays a central role in several physiological processes, including detoxification of xenobiotics, signal transduction, conjugation of different metabolites, differentiation, senescence and cell death regulation [117]. By serving as an electron donor, GSH is converted into oxidized form - two glutathione molecules linked by a disulfide bond (glutathione disulfide, GSSG). Once oxidized, glutathione can be reduced by glutathione reductases that use NADPH as an electron donor (Figure 4C). The GSH/GSSG ratio is often used as a measure of cellular redox state. GSH is necessary to maintain reduced state of cell, counteracting inhibitory effects of ROS. It plays a key role in the antioxidative defense system by regenerating other antioxidants like AsA via the glutathione-ascorbate cycle (Figure 4B). GSH is particularly important in chloroplasts since it helps to protect the photosynthetic apparatus from oxidative damage [118].

Proline is considered as another important antioxidant and potential inhibitor of PCD. It has been well established that it acts as an osmoprotectant and protein-stabilizing agent. However, it has been also proven to be the $O_2^{\bullet-}$ and $^{\bullet}OH$ scavenger and inhibitor of LPO [119]. Increased concentration of proline has been correlated with enhanced tolerance to various abiotic stresses, e.g. transgenic tobacco cells with silenced proline dehydrogenase, accumulating more proline than wild-type cells, have shown improved osmotolerance [120]. Overexpression of proline biosynthetic pathway genes has been also found to increase the drought stress tolerance in transgenic soybean [121].

Out of four tocopherol isomers (α, β, γ, δ) found in plants, α–tocopherol (vitamin E) has the highest antioxidative activity because of the presence of three methyl groups [122]. α-tocopherol, a lipid soluble antioxidant molecule is considered as a potential scavenger of ROS and lipid radicals in membranes. It has been shown to prevent the chain propagation step in the lipid autooxidation reaction [123]. It has been demonstrated that oxidative stress activates the expression of tocopherols synthesis pathway genes. Higher tocopherol level has also been reported during water stress [124].

Another group of plant compounds with antioxidant abilities are lipid soluble carotenoids. They play various functions in the plant metabolism such as absorption of light at wave-

length between 400 and 550 nm (light-harvesting role), assembly and stabilization of light harvesting complex proteins (structural role), and protection of the photosynthetic apparatus from free radicals (antioxidant role) [66].

Apart from the antioxidant function, flavonoids are responsible for flowers, fruits and seeds pigmentation, protection against UV, defense against pathogens and signal transduction during stress. Mutant plants, deficient in chalcone synthase and chalcone isomerase that are unable to accumulate flavonoids have been demonstrated to be more sensitive to UV light [125]. Many genes encoding flavonoid biosynthesis components are induced under stress conditions. Considerable increase in flavonoid level has been demonstrated in response to abiotic stresses such as wounding, drought and nutrient deprivation [126].

Under steady state conditions, ROS are eliminated by antioxidative mechanisms described above (78). Different abiotic and biotic stresses such as drought, high salinity, heavy metals, high light, UV radiation, high/low temperature or pathogen attack may disturb the balance between the ROS production and scavenging. The equilibrium between ROS production and scavenging influences their mode of action as protective, signaling or damaging factors. The increase in cellular ROS level can cause significant damage to cell structures, cell death and in consequence loss in crop production [127]. The vast role of ROS in the response to environmental conditions and cell-death signaling are well documented [65,128]. There are results suggesting that H_2O_2 antagonizes the $^1O_2$-mediated signaling and that the cross-talk between signaling pathways, transferred by different ROS, may contribute to the overall response of plant exposed to adverse environmental conditions [129]. Moreover, ROS interact with several other signaling pathways including NO and hormones like SA, JA and ET. Such interactions and the ROS/hormonal balance determine whether the cell will stay alive or enter the PCD pathway [14,39,60]. Finally, the role of ROS as messenger molecules cannot be underestimated, since it has been demonstrated that they trigger the transduction of stress signals and systemic acclimation to adverse environmental conditions [130,131].

4. High and excess light stress

Light is an essential factor in the regulation of plant growth, development and stress responses but it is also responsible for the production of reactive oxygen species leading to PCD. The cell death phenotype of many lesion mimic mutants of *Arabidopsis thaliana* and *Zea mays* is dependent on light [132–134]. Plant cells have been equipped with sophisticated light-perception mechanisms and signaling pathways that are very important for the plant defense. Three families of photoreceptors collecting different light qualities exist in plant cells: phytochromes (PHY), cryptochromes (CRY) and phototropins (PHOT). They localize in the plasma membrane, cytoplasm or nucleus. While photoreceptors play mainly a regulatory role, providing information about diurnal and seasonal light-quality changes, the light-quantity sensing system is located in chloroplasts. The absorption of photons by photosynthetic apparatus is possible owing to chlorophylls located in light-harvesting complexes (LHCs) of photosystem II (PSII) and photosystem I (PSI) in the thylakoid membrane

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of chloroplasts. PSII is enriched in chlorophyll b molecules which results in a maximum absorption at the orange/red light spectrum (650–680 nm), whereas, PSI is enriched in chlorophyll a molecules and absorbs in the far red (700nm). The reaction centers of PSII and PSI are coupled by a chain of electron carriers. A spectral imbalance of light may result in an unequal excitation of two photosystems, leading to increased or decreased ROS production [130]. Therefore, the distribution of light-absorbing antenna complexes between PSII and PSI is under control and can be regulated through a short-term adaptation (e.g. state transition) or long-term acclimation processes. State transition is a reversible phosphorylation of the main LHCII protein and its migration between PSII and PSI [135,136]. Thylakoid-associated kinase 1 (TAK1) is essential for this process since it is responsible for the phosphorylation of thylakoid proteins [137]. In contrast, long-term responses employ modifications of the photosynthetic complexes structure through the adjustment of LHCII and PSII size or PSI/PSII ratio [138,139]. Both short-and long-term processes are triggered by the perception of imbalanced photosystem excitation via redox signals that come from the photosynthetic electron transport (PET) chain, especially from one of electron carriers - plastoquinone (PQ) [130,140].

In natural environment, plants are often exposed to high light (HL) intensities that lead to the absorption of more light energy than can be used for carbon dioxide fixation [77]. The amount of absorbed light energy that is excessive and cannot be used for photosynthetic metabolism is termed excess excitation energy (EEE) [77,130]. In response to EEE, there is an immediate increase in the electron transport rate and in consequence redox changes of PET components. Alterations in the redox status of PET, especially the reduction of PQ pool leads to the expression deregulation of nuclear and chloroplastic genes that encode photosynthesis components such as LHC proteins [141–143] and antioxidants like APX [144,145]. The response to EEE involves not only the alteration in photosynthetic flux but it is also accompanied by changes in the water status and temperature of the leaf, and in consequence it is associated with elevated ABA levels, changes in the redox state of glutathione pool and increased activity of heat shock transcription factors [146,147]. If the accumulation of ROS exceeds the ability of removing them by antioxidant systems, it may cause a photooxidative damage of the photosynthetic apparatus which may lead to cell death, manifested by bleaching, chlorosis or bronzing of leaves [148]. Therefore, the avoidance of EEE, its dissipation and HL tolerance are fundamental for the plant survival. EEE-mediated PCD can be considered as a beneficial process, as it triggers signal transduction to systemic cells and their acclimation to high light [130,131].

Avoidance strategies include such processes as: movements of chloroplasts, decrease in the number of photosynthetic reaction centers, curling of leaves and increase in the thickness of cuticular wax [149]. During HL treatment, chloroplasts have been demonstrated to move to the anticlinal wall (Figure 5) and this response has been proven to be mediated by blue/UVA receptors [150].

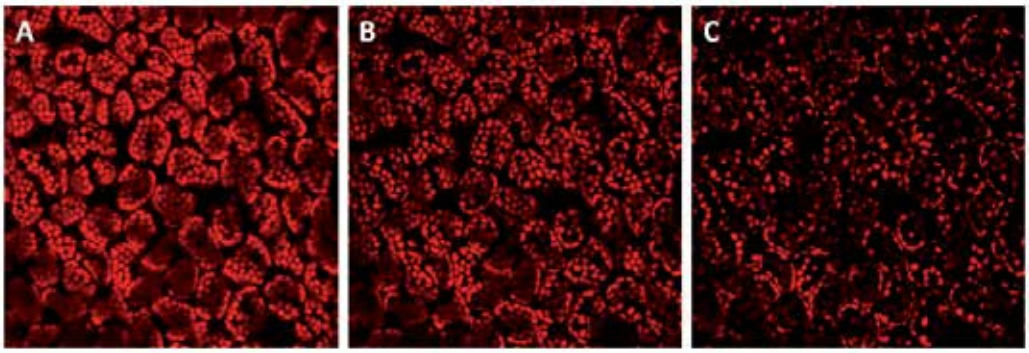

Figure 5. Chloroplast high light avoidance movements. Dark-acclimated *Arabidopsisthaliana* leaf strips have been exposed to a laser beam and the autofluorescence of chloroplasts has been recorded directly after switching on the laser (A), 7 min (B) and 30 min (C) after laser exposure.

Plants have also developed several mechanisms for removing the excess of energy. Dissipation of EEE can be achieved by the combination of photochemical (qP) and non-photochemical quenching (NPQ) processes. Photochemical quenching increases the utilization of photosynthetic electrons by metabolic pathways such as the water-water cycle or photorespiration. The consumption of electrons through water-water cycle is achieved by combined action of the O_2 reduction at PSI to O_2^- and the chloroplast antioxidant system. The reduction of O_2 is much lower than the disproportionation of O_2^-, catalysed by SOD and the following H_2O_2 processing to H_2O, catalysed by APX. Therefore, the water-water cycle shortens the lifetime of O_2^- and H_2O_2, suppresses the production of $^{\bullet}OH$, and prevents photoinhibition [77]. Another energy sink preventing photoinhibition of photosynthetic apparatus by EEE is photorespiration. During this process, photo-produced ATP and reducing equivalents are consumed, preventing the overreduction of PET. However, the photorespiratory cycle leads to the production of H_2O_2 that has to be elimainated by antioxidant systems [151].

Non-photochemical quenching processes relay on the transfer of excitation energy to carotenoids that are able to dissipate it as heat during the xanthophyll cycle (VAZ cycle). The xanthophyll cycle involves the conversion of violaxanthin to de-epoxidised zeaxanthin, via the intermediate antheraxanthin. This enzymatic cycle is performed by violaxanthin de-epoxidase and plays a key role in the stimulation of energy dissipation within light-harvesting antenna [152]. In *Arabidopsis*, the chlorophyll binding protein - PSII subunit S, encoded by *PsbS* gene has been proven to be required for NPQ [153].

Excess energy is sensed by the photosynthetic apparatus not only as a result of HL, but also other environmental factors such as UV radiation, limitations in nutrient availability, drought, salinity or high/low temperatures. All these abiotic stimuli are accompanied by oxidative stress, manifested in the overproduction of reactive oxygen species. If the level of ROS is too high for antioxidant system to eliminate them, cellular macromolecules and structures can be damaged, which triggers PCD. Several studies clearly demonstrate that

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programmed cell death is affected by light. The spread of wound-induced PCD in maize tissue has been shown to be transmitted by chloroplast-produced ROS [134]. It has also been found that the HR-mediated cell death is accelerated by the loss of chloroplast function [154]. Moreover, a study using light- and dark-grown plant cell culture has proven that they respond differently to PCD-inducing stimuli, resulting in various levels of DNA fragmentation and cell-content condensation [65]. Direct induction of programmed cell death by exposure of *Arabidopsis* rosettes to excess light (EL) (2000 μmol of photons m^{-2} s^{-1}) has also been demonstrated [130].

Although ROS produced during the progress of PCD are damaging for the cell, they are also needed as messenger molecules preparing other cells for a struggle with stress conditions. H_2O_2 is thought to freely diffuse across biological membranes, thus it has been proposed to directly influence the function of extra-chloroplastic signaling components. The possibility that H_2O_2 acts as an intracellular messenger molecule has been suggested since it triggeres systemic response to EL [130]. When low-light-grown *Arabidopsis* rosettes have been partially exposed to EL, unexposed leaves have become acclimated to EEE and to photooxidative stress. This phenomenon, termed systemic acquired acclimation (SAA) is attributed to chloroplasts, as it is associated with specific changes in redox status of the photosynthetic electron carrier chain. Such redox changes lead to the alternation in transcription profiles, triggering SAA. By the use of photosynthesis inhibitors: 3,4-dichlorophenyl-1,1-dimethylurea (DCMU), which blocks the reduction of PQ and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), that blocks PQ oxidation, it has been demonstrated that the PQ pool redox status is predominantly responsible for SAA [60,130]. DCMU has been also proven to inhibit effects of EEE, such as higher NPQ, production of ET, H_2O_2 and decrease in stomatal conductance. On the other hand, DBMIB has been shown to trigger the production of ET, H_2O_2 and stomatal closure even under low light. These results indicate that the redox status of PQ pool and NPQ play a critical role in the initiation of response to EEE, in ET and H_2O_2 signaling and consequently in the PCD regulation [11,60,130,155,156]. Activation of the SA-dependent pathway in response to EEE has been also observed after an initial induction of ROS/ET signaling [60]. Other hormones such as JA and ABA, synthesized at least partially within the chloroplast, also participate in response to EEE [149]. A large number of transcripts encoding different antioxidant defense enzymes is induced in local and systemic leaves after EL treatment. Apart from APX1 and APX2 [130], higher expression level has been proven for GPX2, GSTs, APX3 and CSD1 [60,157]. Furthermore, a recent study has suggested that local and systemic responses to EL are associated with changes in the plasma membrane electrical potential (photoelectrophysiological signaling - PEPS). During the EL incident, PEPS has been shown to be initiated by quantum redox changes in PSII, transduced by bundle sheath cells and its systemic propagation has been proven to be dependent on APX2 function. Therefore, PEPS is suggested as a new component of signaling cascades that regulate light acclimatory responses. Furthermore, it has been proposed that leaves are able to memorize different EL episodes and use this information for improving their acclimation and survival under prolonged periods of unfavorable light conditions [131].

In the last decade a significant progress has been made in improving light-induced oxidative stress tolerance in plants. Various components of antioxidative system involved in ROS scavenging have been up- or down-regulated to develop transgenic lines with altered antioxidants levels. Overexpression of enzymes involved in AsA biosynthesis has been shown to confer oxidative stress tolerance in tomato plants [158]. Increased AsA content has been also demonstrated to enhance high light stress tolerance in *Arabidopsis* [159]. Higher concentration of GSH has proven to protect potato plants against oxidative damage [160]. Moreover, reduced level of light-mediated cellular damage has been observed in transgenic tobacco plants overexpressing chloroplast-localized Cu/Zn-SOD (161) and thylakoid membrane-bound APX [162]. MDHAR overexpression in *Arabidopsis* has been demonstrated to enhance the tolerance towards photooxidative stresses [163]. Moreover, some studies have reported that combined expression of antioxidant enzymes in transgenic plants acts synergistically on stress tolerance, e.g. simultaneous overexpression of Cu/Zn-SOD and APX in tobacco chloroplasts enhances the resistance to the photooxidative stress in comparison to their single overexpression [164].

5. UV radiation stress

Being exposed to sunlight, plants need to deal with the damaging effect of ultraviolet (UV) radiation which reduces the genome stability, impeding their growth and productivity. These effects result from damage to cell components including not only nucleic acids, but also proteins and membrane lipids. Upon UV exposure, strongly mutagenic cross-linked forms of DNA can be produced [165]. In order to minimize effects of UV radiation, plants accumulate UV-absorbing secondary metabolites, perform the monomerization of UV-induced pyrimidine dimers (DNA repair) and neutralize generated ROS [166,167].

UV radiation consists of UV-C (below 280 nm), UV-B (280-320 nm) and UV-A (320-390). Although UV-C is not physiologically relevant to plants since it is efficiently blocked by the stratosphere, the UV-C-triggered cell damage is comparable to induced with UV-B radiation, which reaches Earth's surface [168]. Therefore, UV-C radiation has been widely used to study DNA damage and repair mechanisms upon UV stress [169].

UV has been demonstrated to trigger apoptosis in animals [170] and apoptosis-like changes in *Arabidopsis*, including DNA laddering, changes in nucleus morphology (crescent shape) and its fragmentation [168]. It has been also proven to induce oxidative burst in plant cells [171], considered as the main cause of cell death, which aims at the limitation of damage spreading. Light is necessary for UV-C-triggered cell death and caspase-like proteases participate in this process since caspase-inhibitors are able to block the onset of DNA fragmentation [25,172]. Recent study performed on *Arabidopsis* protoplasts has shown that during the early stage of UV stress, a burst of ROS in chloroplasts and adjacent mitochondria is detected. Mitochondria dysfunction has been also observed, manifested by changes in their distribution, mobility and the loss in mitochondrial transmembrane potential. Moreover, the pre-incubation with antioxidant molecule - ascorbic acid or inhibitor of photosynthetic elec-

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tron transport - DCMU decreases the ROS production and retards PCD. These results prove that mitochondria and ROS act as mediators in the UV-C-induced cell death [173] and that AsA can be considered as an important antioxidant during this process [174], which is consistent with what has been reported in various types of PCD. It has been also shown that *Arabidopsis* proteins AtDAD1 and AtDAD2 (defender against apoptotic death), localized in the endoplasmic reticulum membrane can suppress DNA fragmentation, indicating an involvement of the ER in UV-C-triggered PCD pathway (25). The microarray approach has identified numerous genes responsible for ROS scavenging, signaling, transcription regulation and involved in DNA replication or conformation changes that have been deregulated after exposure to UV-C radiation [175]. Metacaspase-8 (AtMC8) has been proven to be strongly up-regulated by UV-C. Overexpression of AtMC8 in *Arabidopsis* has resulted in more severe cell death, while knocking-out AtMC8 has reduced the UV-C-triggered PCD, which suggests that metacaspase-8 is a part of PCD pathway activated by UV radiation [176]. The activation of PCD program upon UV helps plants in eliminating damaged cells to control cell quality and quantity after the trauma.

6. Drought stress - soil water deficit

Drought is one of the most unfavorable environmental factors that affects growth and development of plants and consequently limits plant productivity. Plants have developed specific acclimation and adaptation mechanisms to survive the soil water deficit. In response to drought, plants can exhibit either escape (ability to complete the life cycle before severe stress) or resistance mechanisms. Resistance mechanisms include drought avoidance and drought tolerance. The latter depends on the cell turgor maintenance by accumulating osmolytes and soluble sugars [177]. There are several examples of molecules that help to maintain an osmotic balance under dehydration conditions: sugars, polyols and proline [178]. Proline is accumulated in the cytoplasm and chloroplast stroma while other solutes (sugars, organic acids, potassium) are cumulated in the vacuole. When the cellular water content decreases, they stabilize cellular structures through hydrophilic interactions and hydrogen bonding [179]. A similar role is fulfilled by late embryogenesis abundant (LEA) proteins - a family of unstructured proteins. LEA proteins accumulate in response to dehydration and ABA treatment. Because of their high hydrophilicity and solubility in water, it has been proposed that they play a role in protecting cytoplasmic structures during dehydration [180]. The avoidance mechanism is possible by the maintenance of high water potential in plant tissue despite soil water deficit. It can be achieved by: improved water uptake under stress, the ability to hold water as well as by the reduction of its loss through smaller leaf area and lesser stomatal and cuticular conductance. One of the first acclimation responses to drought is the decrease in leaf growth, which helps to maintain the cell turgor and reduces the transpiration area. In *Arabidopsis*, the size of leaf is regulated by both cell division and cell expansion. Under drought stress, *Arabidopsis* leaves have been demonstrated to compensate for the low rate of expansion by the extension of expansion duration [181]. The cell expansion is a process of cell wall loosening performed by enzymatic and non-enzymatic compo-

nents, from which expansins are key proteins engaged in this process. The onset of cell expansion involves pumping of protons into the cell wall, which makes the surrounding more acidic. As a result, expansins become activated. They loosen connections between cellulose microfibrils, which leads to the cell wall relaxation, water uptake and consequently the cell expansion [182]. It has been demonstrated that the mild osmotic stress causes the induction of expansin genes [183].

Photosynthesis is one of major processes affected by water deficit since stomata closure causes reduced CO_2 diffusion to the chloroplast. As a result of the inhibition of photosynthesis and the predominance of photorespiration, ROS are generated [184]. It has been demonstrated that in drought-stressed plants, the ABA-controlled stomata closure is mediated by H_2O_2 [185]. Under severe drought stress, some antioxidant enzymes have been shown to be highly induced [186]. However, studies on many drought-stressed crop species showed an inconsistency in their expression since in some cases they have been induced, but in other repressed, suggesting that different ROS balance may be required during different response phases [187].

During water deficit, ROS are responsible for the induction of leaf senescence, which is executed through the programmed cell death and plays an important role in the plant survival. It contributes to the nutrients remobilisation during stress and allows the rest of plant to benefit from them and stay alive. Drought-induced PCD enables also the abscission of some leaves and thus the avoidance of further water loss through the transpiration. It occurs gradually and is manifested by specific biochemical and molecular changes such as chromatin condensation, thylakoid swelling, lipid peroxidation, degradation of chlorophyll (leaf yellowing) and proteins. Apart from ROS, cytokinins and ABA have been shown to be involved in the regulation of water-deficit-triggered senescence [123]. Recent studies have shown that the water deficit triggers PCD not only in green tissues but also in plant root tips. Apical meristem cells of primary roots undergoing PCD, demonstrate increased size of vacuole, degradation of organelles and the collapse of plasma membrane [188].

Early events in the perception of drought stress signals include the activation of transcription factors belonging to such classes as DREB/CBF (e.g. DREB1a, DREB2a), ABF (e.g. ABF2, ABF4), MYB (e.g. MYB2), MYC, NAC and WRKY. Many of them possess stress responsive cis-regulatory elements in their promoter sequences like abscisic acid-responsive elements (ABRE) and drought-responsive elements (DRE) [189–191]. The plasma membrane-associated NTL4 (NAC transcription factor) after drought or ABA treatment has been shown to be proteolytically activated and transported to the nucleus where it induces expression of NADPH oxidase involved in ROS generation [192]. Moreover, the dehydration stimulates expression of BAX inhibitor-1 (AtBI-1). The *atbi1* mutant has been shown to display more severe cell death, indicating that ER-located AtBI-1 modulates the water-deficit-induced PCD [188]. Drought stress has been also proven to regulate the expression and activity of aquaporins - a family of channel proteins that facilitate the transport of water along transmembrane water potential gradients [193].

Upon soil water deficit, the accumulation of ABA and the induction of ABA-associated signaling genes occur. ABA induces various second messengers such as cytosolic Ca^{2+}, ROS

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and NO in guard cells. These signals evoke ion efflux through plasma membrane ion channels, resulting in the reduction of guard cell turgor pressure and stomata closure to reduce water loss through the transpiration [194]. Mutants with the perturbation of ABA synthesis or signaling display drought hypersensitivity, manifested in significant growth reduction which suggests that ABA is needed for the proper response to drought [177]. In *Arabidopsis*, stomata closure has been shown to be regulated by *ABI1* and *ABI3* (*ABA-insensitive 1* and *3*) belonging to a group of genes identified through mutant screens and being associated with ABA-mediated metabolic responses to stress. Under drought, *ABI1* transcription is up-regulated while *ABI3* is usually down-regulated. Recently, *ABI3* has been hypothesized to be essential for the successful drought recovery [195]. The cell-surface ABA receptors have not been recognized yet. However, a recent study has proposed the flowering time control protein A (FCA) and the chloroplastic magnesium protoporphyrin-IX chelatase H subunit (CHLH) as candidates for ABA receptors, both of which have been shown to bind ABA *in vitro* [196]. Two genes have been found to play a crucial role in the prevention of stomata opening - *GPA1* (*G PROTEIN ALPHA SUBUNIT 1*) and *PLDα1* (*PHOSPHOLIPASE D ALPHA 1*) [197]. PLD-produced phosphatidic acid has been also shown to play an important role in the plant response to drought stress [198].

Similarly to ABA, JA also triggers stomata closure and such response is conserved among various plant species [199]. At the early stage of moderate drought, plants accumulate high concentrations of ABA and induce ABA-responsive genes. At this stage, no significant differences in JA-responsive genes are observed. At later stage of drought stress, ABA level returns to normal, while JA synthesis and JA signaling genes are significantly down-regulated. This suggests the negative correlation between ABA and JA pathways [177]. The high concentration of JA is probably undesirable during drought stress, as it inhibits the cell expansion and results in stunted growth [200]. Therefore, plants down-regulate JA synthesis and signaling pathways to minimize the inhibitory effect of JA on growth, establishing a new hormone homeostasis.

Downstream of early stress perception events, signaling molecules are activated. Such secondary messengers include Ca^{2+} ions and ROS. They induce further genes that are needed to establish a new cellular homeostasis leading to drought resistance and tolerance [180]. Recent studies have strongly proven that drought response progresses through mitogen-activated protein kinase (MAPK) pathways [201]. In yeast and animals, MAPK-regulated pathways take part in the production of osmolytes and antioxidants. These MAPK pathways are activated by receptors/sensors such as protein tyrosine kinases, G-protein–coupled receptors and histidine kinases. Among these, G-protein–associated receptors have been proposed to serve as one kind of membrane-bounded receptors for ABA. A family of histidine kinases (HK) have been also identified in plants [199]. An *Arabidopsis* AtHK1 has been suggested to be involved in the osmotic stress signal transduction [202]. Other members of this family, ATHB7 and ATHB12 have been proposed to maintain the reduction of plant growth under drought, which is an acclimation response to survive prolonged drought stress [177].

The elucidation of mechanisms controlling drought stress responses has enabled to engineer plants by the expression of specific stress-related genes. Although it was believed that the modulation of osmoregulatory genes would be the best strategy, attempts failed to result in any significant drought-stress tolerance improvement [203]. However, constitutive expression of some LEA proteins has conferred tolerance to soil water deficit in transgenic rice [204] and wheat [205]. Moreover, tomato plants overexpressing *Arabidopsis* CBF1 (DREB1B) have exhibited improved drought tolerance [206]. Similar results have been obtained for transgenic *Arabidopsis* and rice plants overexpressing stress-responsive NAC genes [207].

7. Conclusions

Plants have evolved various strategies to acclimate to different environmental stresses. The most fundamental strategy is the development of high plasticity of plant tissues. It has been demonstrated that programmed cell death plays an important role in this plasticity and subsequent adaptation to unfavorable conditions. There is a growing evidence that PCD is a crucial process in both morphogenetic changes execution and the following adaptation. Although each decade brings a vast number of research, our understanding of plant PCD and its underlying mechanisms is still in its early stage. Further insight into details of the PCD molecular machinery in plants is important, since it is an attractive target for improving stress tolerance and plant yield under adverse conditions. Essentially, it could lead to the generation of pathogen-resistant and stress-tolerant crops as well as fruit varieties with an extended shelf life.

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Abbreviations

ABA - abscisic acid; AOX - alternative oxidase; APX - ascorbate peroxidase; AsA - ascorbic acid; BI - Bax-Inhibitor; CAT – catalase; Chl – chlorophyll; DBMIB - 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU - 3,4-dichlorophenyl-1,1-dimethylurea; DHA – dehydroascorbate; DHAR - dehydroascorbate reductase; EEE - excess excitation energy; EL - excess light; ER - endoplasmic reticulum; ET – ethylene; ETC - electron transport chain; FAD - flavin adenine dinucleotide; GA - gibberellic acid; GPX - glutathione peroxidase; GR - glutathione reductase; GSH - reduced glutathione; GSSG – oxidized glutathione; GST - glutathione-S- transferase; HL - high light; IAP - inhibitor of apoptosis; JA - jasmonic acid; HR -

hypersensitive response; LEA - late embryogenesis abundant; LHC - light-harvesting complex; LPO - lipid peroxidation; LSD1 - LESION SIMULATING DISEASE 1; MC – metacaspase; MDA – malonyldialdehyde; MDHAR - monodehydroascorbate reductase; MeJA - methyl-jasmonic acid; MAPK - mitogen-activated protein kinase; NADPH - nicotinamide adenine dinucleotide phosphate; NO - nitric oxide; NPQ - non-photochemical quenching; PA - phosphatidic acid; PCD - programmed cell death; PEPS - photoelectrophysiological signaling; PET - photosynthetic electron transport; PG – phosphoglycolate; PGA – phosphoglycerate; PLD - phospholipase D; PSI and PSII - photosystem I and II; PQ – plastoquinone; PUFA - polyunsaturated fatty acids; qP - photochemical quenching; RCD - runaway cell death; RNS - reactive nitrogen species; ROS - reactive oxygen species; Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP - ribulose-1,5-bisphosphate; SA - salicylic acid; SAA - systemic acquired acclimation; SAR - systemic acquired resistance; SOD - superoxide dismutase; TF - transcription factor; UV - ultraviolet radiation; VPE - vacuolar processing enzyme; XOD - xanthine oxidase.

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Section 2

Applications in Agriculture

Chapter 8

Water Use and Drought Response in Cultivated and Wild Apples

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Additional information is available at the end of the chapter

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1. Introduction

Water availability is the single most important factor determining plant survival. Many environments experience water-limited periods of various degrees and duration. The issue of water availability for agricultural crops where maintenance of high yields over variable growing seasons is desirable is particular critical. For plants, the strategy choices for survival can be summarized as dehydration avoidance (e.g., deep rooting), dehydration tolerance (e.g., accumulation of osmoprotectants) and drought escape (e.g., reproductive completion before the dry season). Drought adaptation in most plants is controlled by complicated interactions between anatomy, physiology and biochemistry, all of which are directly or indirectly under genetic control [1-3].

2. Water use efficiency and drought resistance

All living organisms have evolved mechanisms for adapting to changes in their environment, whether biotic such as pest related, or abiotic such as physically effected. For plants this is especially challenging, since they are unable to relocate to avoid adverse conditions. As a result, numerous strategies employed by plants leading to successful acclimation have been identified. Broadly speaking, these adaptive mechanisms can be divided into two major categories, namely morphological modifications and physiological adjustments. Through combinations of these basic strategies, plants can respond quickly to environmental cues, often maintaining the response for relatively long periods [4,5].

The term drought resistance is sometimes confused with water use efficiency (WUE). Drought resistance is determined primarily by 'drought avoidance' (high plant water status maintained

under water deficit) and/or 'drought tolerance' (capacity to sustain plant function in a dehydrated state) [6,7]. Drought resistance in a genetic/physiological context refers to the ability of one genotype to yield 'better' than another during severe drought stress. On the other hand WUE is defined as the ratio between diffusion of CO_2 into the leaf (photosynthesis) and loss of H_2O through transpiration, indicated as WUE = A/E, where A is carbon assimilation and E is transpiration. It is positively correlated with carbon isotope discrimination ($\Delta^{13}C$) based on the stable carbon isotope ratio, $\delta^{13}C$ ($^{12}C/^{13}C$ relative to a standard, i.e. PeeDee Belemnite), in the plant tissue relative to the atmospheric ratio and is calculated as: $\Delta^{13}C = \delta^{13}C$ in air - $\delta^{13}C$ of the plant/1- $\delta^{13}C$ of the plant. Since most gas exchange occurs via the stomata, it is expected that guard cell function would be closely associated with WUE. Indeed, the size and density of stomates correlates well with water use efficiency [8-10]. For drought resistance, yield is not necessarily adversely affected by resistance, whereas for WUE reduced transpiration through stomatal closure is often accompanied by reduced yield potential through reduced carbon assimilation, particularly in herbaceous C_3 plants (however, see below). Other parameters, such as root depth, leaf size, and trichome size and density have also been linked to water use efficiency [2], but they have also been linked to drought resistance as well [6].

Different methods have been used to measure drought resistance and WUE [11]. These methods measure the energy status of water in plant tissues and the trans-port processes into and out of the soil-plant-atmosphere continuum. In general, these methods isolate specific plant tissues at instantaneous moments in time, whereas $\Delta^{13}C$ represents a time-integrated value of the ratio of C_i (intercellular CO_2 concentration) to ambient CO_2 which, as previously indicated, reflects the plant's capacity for gas exchange via stomata [12] and discrimination of rubisco against ^{13}C. The use of carbon isotope discrimination to select in-dividuals with higher WUE has been applied successfully to cereal breeding programs (13). The extent of $\delta^{13}C$ varies substantially among wheat genotypes, and heritability is high because genotype X environment interactions are relatively low [14, 15]. Rebetzke et al. [16] reported on the selection of plants with greater biomass, harvest index and kernel weight using results from contrasting high and low $\Delta^{13}C$ groups in combination with a backcrossing program. There were significant correlations between $\Delta^{13}C$ and yield and between $\Delta^{13}C$ and biomass. The resulting high yielding strain, 'Drysdale', produces around 10% more grain under drought conditions than other dry-area wheat varieties.

3. Adaptation and the relationship of $\delta^{13}c$ to yield

Adaptive changes in populations growing in different environments have been amply demonstrated in a variety of plants [17]. Divergence among populations associated with different environments provides the raw material for speciation and differentiation among closely related species. Higher fitness of genotypes in their native environment compared to genotypes transplanted from contrasting environments provides evidence of local adaptation [9]. For example, when two populations of *Boechera holboellii* growing in xeric and wet environments were grown in reciprocal transplant experiments, significantly higher survival was observed with plants growing in their native habitat [18]. Furthermore, genes identified

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by cDNA-AFLP analysis showed genotype-specific expression patterns related to the indigenous environment (population). In another study populations of *Encelia farinosa* growing over a broad rainfall gradient in the deserts of southwestern North America, were evaluated for leaf characteristics. Leaf pubescence declined as mean water availability increased [19]. Variations among populations for both pubescence and carbon isotope discrimination persisted when the plants were grown in common environments differing in water availability, indicating a genetic basis for variation in these traits [20].

WUE in trees adapted to different environments has been documented in several forest species [21-24]. For example, a study of four birch (*Betula pendula* Roth) clones from environments with different rainfall amounts indicated strong clonal differences in a number of water use and photosynthetic traits, including leaf $\delta^{13}C$ values [21]. A follow-up study correlated differences in leaf and root morphological parameters and carbon partitioning with clones from the drier environments [25]. Although individual leaf areas were smaller in drought-treated clones, regardless of their region of origin, total leaf and specific leaf areas (leaf area/leaf weight) were actually higher for the drought-treated clones from the drier environment. This is in contrast to the often observed reduction in leaf surface area seen in plants exposed to water deficit (WD) [26].

Poplar species are differentially adapted to a variety of environments, and because poplar is a rapidly growing tree with heavy water use, there is growing interest in developing lines that are drought resistant. Links between productivity and $\Delta^{13}C$ varied in a study comparing different poplar genotypes, suggesting that genotypes displaying simultaneous high productivity and improved water use efficiency could be selected [27]. To obtain more practical information regarding productivity and WUE, a field study was conducted on the same genotypes analyzed in the previous study. Significant clonal diversity was observed for several traits related to productivity and for Δ which showed high heritability ($H2 = 0.71$) [28]. A lack of correlation between above ground biomass and Δ was reflected in several clones where high productivity was combined with improved WUE. This observation supports previous studies with cereals indicating that WUE and yield can be inherited as separate traits.

Yield of deciduous tree fruit crops is not measured as total biomass yield in commercial production as are agronomic crops such as corn, wheat and rice or forest trees. In commercial orchards it is common practice to reduce yield potential of fruit trees by as much as 50% to insure large fruit size and high fruit quality [29]. Consequently, the paradigm that increased WUE is tied to reduced yield potential is not necessarily valid for tree fruit production. For example, Glenn et al. [30] have demonstrated the practicality of identifying peach cultivars with high WUE without compromising productivity. This study, taken together with those cited previously, demonstrates the feasibility of selecting for improved WUE without loss in productivity.

4. Adaptation, WUE and $\delta^{18}O$

Transpiration rate (E) affects water loss to the atmosphere and is negatively correlated with WUE. Despite the fact that atmospheric ^{18}O is low (ca. 0.2% of total oxygen), plants tend to accumulate ^{18}O and ^{2}H in leaf water due to the difference in vapor pressure between heavy

water and 'normal' water and to differences in diffusivity with air. However, the relationship between E and isotopic enrichment is complex. For example, variation in E can be caused by changes in evaporative demand and/or changes in stomatal conductance, gs [31]. If the source of variation is evaporative demand, then as E increases, ^{18}O enrichment increases. On the other hand, if stomates are the source of variation, then as E decreases (stomates close), leaf water enrichment increases. How does this relate to ^{18}O enrichment of plant organic matter? Plants accumulate ^{18}O in their tissues as a result of the exchange of oxygen between water and carbonyl oxygens in triose phosphates. An enrichment of about 27 parts per thousand (ppt) has been observed in the organic material of several different plants relative to leaf water [32]. This suggests that differences in ^{18}O enrichment can be used to distinguish genotypes with favorable yields and stomatal function. In fact Barbour et al. [33] recently demonstrated a reliably negative correlation between yield and $\delta^{18}O$ in wheat which was used to identify water use efficient varieties for breeding.

5. Specific genes associated with WUE and/or drought responses in apple and other plants

The recent advent of global gene expression methodology has spawned a number of studies of abiotic stress responses, including drought, in several plant species [34-38]. In Arabidopsis, a compilation study of microarray analyses on plants subjected to a variety of stress treatments highlighted overlap among genes up-regulated in the early stages of all the stress responses [39]. Studies on WD stress in cereals and dicots have cataloged a large number of genes up-regulated during treatment [35-37, 40, 43]. Comparisons among these studies reveal that a number of genes are reproducibly up-regulated in response to WD regardless of how the stress was imposed or what plant system was involved, including apple (Table 1) [44].

6. Genes associated with wue

Recent reports of genes associated with regulation of transpiration demonstrate the complexity of water use and transport, as well as the overlap in gene response to other stresses. *ESKIMO1*, which was originally associated with cold response, has recently been shown to affect both drought and salt responses in Arabidopsis. Insertional mutation lines inactivating *ESK1* had reduced transpiration rates and were only slightly less drought tolerant than wild type [45]. Furthermore, there was a reduction in biomass when mutant plants were grown without stress, suggesting that alterations in WUE were reducing both transpiration and CO_2 exchange; biomass differences between WT and *esk1* lines were negligible under stress. Another example of pleotrophic gene effects on plant physiology was reported in a study by Masle et al. [46]. A leucine-rich repeat receptor kinase (*ERECTA*) previously associated with inflorescence development was also shown to regulate transpiration in different races of Arabidopsis. The gene is implicated in epidermal cell expansion, cell-cell contact and mesophyll cell proliferation, but its relationship to reduced transpiration may be linked to differences in stomatal

Genes Up-regulated in 'Royal Gala' Roots	Other Plants	Tissue	Citation
HMW HSP	*Arabidopsis*	various	[40][2]
	Poplar proteome	white roots	[41]
aquaporin	*Arabidopsis*	various	[40]
	barley	leaves and roots	[35]
	maize	leaves and roots	[43]
	Poplar proteome	white roots	[41]
protease inhibitor	*Arabidopsis*	various	[40,42]
	chickpea	whole seedlings	
Histone H2	*Arabidopsis*	various	[40]
	chickpea	whole seedlings	[42]
	maize	leaves and roots	[43]

[1] Apple ('Royal Gala') genes are from two SSH root libraries of water deficit-treated plants (manuscript submitted)

[2] This citation is a review of several different studies of water deficit-response in *Arabidopsis*.

Table 1. Genes up-regulated in water deficit-treated apple roots vs other plants responding to water deficit[1]

density between lines. Interestingly, there was no compensation in biomass for the reduction in E, indicating that as with WUE and $\Delta^{13}C$, there are workable strategies for increasing WUE without sacrificing carbon assimilation under normal growth conditions.

Using a suppression subtractive hybridization (SSH) approach to drought-responsive gene isolation in a commercial apple, 'Royal Gala', we identified numerous genes commonly found to be WD responsive in other plants. We also identified several up-regulated genes unique to apple roots (Table 2; manuscript submitted). Some of these genes may reflect the role of roots in nutrient transport during stress, including a copper chaperone and a high affinity nitrate transporter that is a putative Arabidopsis NRT2.4 homolog.

Genes Up-regulated in 'Royal Gala'		
Roots	**Bark**	**Leaves**
BYPASS1	Anthocyanin reductase	Auxin/Aluminum-induced protein
Serine acetyltransferase	GAST1-like gene	Proteasome maturation factor
High affinity nitrate transporter	Asparagine synthetase	Asparagine synthetase
NPR1	Chloroplast membrane protein Tic40	Glyoxylate aminotransferase

[1] These genes have not yet been reported as up-regulated in response to WD in these tissues of other plants.

Table 2. Genes uniquely up-regulated in WD-treated apple tissues[1]

7. Genes associated with drought avoidance and escape

An example of the rapid evolution of a drought escape mechanism (early flowering) was demonstrated in a population of *Brassica napa* subjected to a multiyear drought [47]. Comparison of seeds collected from individual plants before the drought with those obtained from the drought-affected population indicated a significant earlier onset of flowering in the latter population. This observation was further expanded in a study of quantitave trait loci (QTLs) in maize associated with flowering time (*Vgt1*) where a *cis*-acting region upstream of a transcription factor was shown to be the link between a QTL and the early flowering trait [48]. The authors speculate that natural genetic variations in flowering time enabled the selection of maize lines adapted to a range of latitudes and growing seasons, including drought tolerance. Another study of natural variation in ecotypes of Arabidopsis [49] found a strong positive genetic correlation (rG = 0.98) between $\delta^{13}C$ (drought avoidance) and flowering time (drought escape). They also observed compelling evidence for pleiotropy in lines varying in FLOWERING LOCUS C, suggesting that correlated evolution of $\delta^{13}C$ and flowering time could be partly explained by coordinated allele fixation altering both traits.

In alfalfa a gene encoding a zinc-finger motif is expressed in roots [50, 51]. The protein encoded by *Alfin1* binds DNA at a specific *cis*-elemtent and is proposed to be a root growth regulator, as transgenic lines overexpressing the gene show enhanced root growth under both normal and high salt conditions [52]. These same transgenic lines were significantly more salt tolerant, and presumably more drought tolerant (high salt concentrations in the media decrease water uptake), althought drought tolerance per se was not measured.

8. Genes associated with drought tolerance and resistance

Studies of specific genes associated with dehydration responses have been conducted in a number of plants, and roles for many of these genes have been correlated with specific morphological or physiological traits known to be involved in drought resistance. Abscisic acid (ABA) signaling and stomatal function are correlated with WUE and drought resistance, so it is not surprising that several genes involved in ABA perception and stomata opening/closing respond to severe dehydration. Two calcium-dependent protein kinases from Arabidopsis have been implicated in slow-type anion channel activation [53]. In the double mutants, ABA and Ca^{2+}-induced stomatal closing were impaired, but not completely. These genes may contribute to a rapid Ca^{2+}-reactive response resulting in stomatal closure, as opposed to the slower Ca^{2+}-programmed response which maintains long-term stomatal closure. Similar studies have also implicated a G protein-coupled receptor, GCR1, in ABA signaling perception in guard cells and during seed germination [54]. To examine stomatal function in more detail, Klein et al. [55] used a T-DNA insertion disrupting At*MRP5*, an ABC transporter in Arabidopsis. The mutants had reduced transpiration rates and showed increased water use efficiency. In a similar study using T-DNA insertion disruption of At*MRP4* (another type of ABC transporter), Klein et al. [56] found the mutant lines to be more drought susceptible. In a

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recent study of the effects of overexpressing or silencing early response to dehydration (*ERD15*) in Arabidopsis, Kariola et al. [57] observed decreased drought tolerance in the overexpressing lines, whereas the silenced lines were hypersensitive to ABA and showed enhanced tolerance to drought. This study also suggests a negative role for *ERD15* in mediating stress-related ABA signaling.

The cuticle is an important barrier to moisture loss in plants, therefore genes associated with cuticle synthesis and turnover are expected to contribute to plant water status. A recent study in alfalfa demonstrated that increased wax production activated by a putative TF (transcription factor) [*WXP1*] also enhanced drought tolerance in transgenic plants [58]. Similar studies with other transcription associated factors have also demonstrated a correlation between increased wax synthesis and drought tolerance [59, 60].

Constitutive expression of a barley Group III LEA (late embryogenesis abundant) protein placed in wheat under control of the maize *ubi1* promoter resulted in improved water use efficiency and higher total dry mass in the majority of transgenic lines [61]. Overexpressing a small molecular weight heat shock protein (*HSP17.6*) conferred drought tolerance in Arabidopsis transgenic lines [62]. The authors also demonstrated that *HSP17.6* had chaperone-like activity and could protect citrate synthase from chemical denaturation. Taken together these studies emphasize the different mechanisms so far discovered that affect water use and drought resistance in plants and indicate that different genes in the same pathway may be used by plants to control WD responses.

9. Regulation of pathways/signaling networks associated with dehydration

Most studies of dehydration responsive signaling pathways implicate ABA directly in altering specific gene expression [63]. In fact genes that respond to ABA usually have multiple copies of an ABA response element (ABRE) or a combination of an ABRE with other motifs such as Myb, Myc or coupling elements [for example, 64]. A second pathway involves drought response element binding (DREB) proteins, particularly *DREB2*-encoding genes, and may also involve ABA indirectly [65]. A recent report on the isolation of a fourth CBF (*CBF4*) from Arabidopsis suggests that this TF only responds to drought, in contrast to observations reported for CBFs1-3 which respond to both cold and drought stress [66].

Compelling evidence indicates that the ABA pathway likely involves Ca^{2+} signal transduction as an early step and important relay system for dehydration responses. ABA can also increase reactive oxygen species through higher levels of H_2O_2 [67, reviewed in 68]. Other studies have suggested stress-responsive pathways that operate through osmotic sensing independently of ABA [69]. An osmotic sensor similar to bacterial two-component receptors has been identified in Arabidopsis [70]. The gene was able to complement several mutations in yeast osmosensors and activated the *HOG1* response pathway through a mitogen-activated protein kinase. No doubt other signaling components, both ABA-dependent and independent, will be identified in the near future.

Numerous studies support the existence of extensive cross-talk between plant hormone signaling pathways [71-74]. It is therefore expected that both the salicylic acid and Jasmonic acid (JA)/ethylene pathways indirectly influence the expression of genes that respond to drought. Along these lines an Arabidopsis mutant (*rcd1*) belonging to the ADP-(ribosyl)transferase domain-containing subfamily of the WWE family exhibits reduced sensitivity to ABA, ethylene and Me-JA, suggesting that it acts at an integrative node in hormonal signaling regulating different stress-responsive genes [75]. *rcd1* is just one example of many where one gene participates in multiple pathways.

10. Studies in *Malus sieversii*

Genetic polymorphisms from twenty populations of *M. sieversii* in Xinjiang, China were analyzed with RAPD markers to assess genetic diversity [76]. Based on the bands generated with 42 randomly chosen primers, variation within a population (83.1%) was higher than among populations (16.9%). The authors conclude that *M. sieversii* is a rich source of genetic diversity.

Evaluation of six *Malus* species using a variety of morphological and physiological traits led to the conclusion that *M. toringoides* and *M. sieversii* were the most drought tolerant of those analyszed [77]. Measurement of root parameters indicated that *M. sieversii* root surface area decreased in response to drought to ~25% of the well-watered control in contrast to *M. toringoides* roots which decreased to ~ 43% of the control. On the other hand, root surface activity (absorption) declined the least in *M. toringoides* (17%) compared to *seiversii* (33%). When data from all the measurements were taken into consideration, *M. toringoides* and *M. seiversii* were the top two most drought resistant species.

A study of the contribution of rootstock source to drought resistance was conducted using 'Gale Gala' apple scions grafted onto *Malus sieversii* or *Malus hupehensis* roots [78]. Differential responses of the grafted material to drought stress were determined by a number of physiological and morphological traits. Typical reductions in biomass, growth rate and leaf area are observed under drought conditions, but *M. sieversii* showed smaller reductions in these traits during drought treatment than *M. hupehensis*. Furthermore, a larger increase in whole plant WUE was measured in grafts on *M. sieversii* rootstocks compared to *M. hupehensis*.

Problem Statement: Many of our agronomically important fruit trees are derived from a rather narrow genetic base. To provide methods for enhancing apple germplasm resistance to drought and other dehydrative abiotic stresses it is imperative that we identify those genes that contribute to drought survival. Once these genes are identified and characterized they can be used in marker assisted selection strategies or altered by genetic engineering.

Application Area: The origin of the domesticated apple is believed to be in Central Asia via the silk route through Kazakhstan [79]. The predominant species contributing to the domestication of the modern apple is believed to be *Malus sieversii* which is thought to be the progenitor of *M.* × *domestica* and a possible source of resistance alleles lost during domestication

[80]. A significant secondary contributor to the genetics of current apple varieties is the European crabapple, *Malus sylvestris* [79] which may also possess important resistant genes lost in the modern varieties.

Several studies of *M. sieversii* material collected from geographically and climatologically different sites in Kazakhstan have concluded that significant genetic diversity can be captured in small-sized sub-populations of these site collections [81, 82]. In a subsequent study of the Kazakhstan collection, Richards et al. [83] concluded that differentiation in genetic diversity was greater among individual families than among sites and that gene diversity and allelic richness varied significantly among collection sites. The use of this material to study drought responses in apple at the morphological and genetic levels without the complication of grafted rootstocks provides the cornerstone of our approach to identifying novel drought resistant mechanisms or factors contributing to drought susceptibility.

Figure 1. *Malus sieversii* collection sites in Kazakhstan [after 80].

Research Course: In order to isolate and characterize genes responding to drought from a commercially important cultivar as a standard for comparison, we used SSH on cDNA prepared from 'Royal Gala' subjected to a moderate-severe drought. Genes identified by this method were further characterized for their expression in various tissues under drought treatment or in fully watered controls.

To begin analyzing *M. sieversii* lines for drought responsiveness, we first surveyed core populations of individuals collected from xeric site 6 and later, xeric site 9 for WUE using stable carbon isotope composition ($\delta\ ^{13}C$) to identify individuals with better WUE. Morphological features, e.g. leaf area, leaf length, stomatal density and number of leaves per current year's branch length were evaluated [84]. Individuals showing $\delta\ ^{13}C$ extreme values were then

Individual Seedling ID	Site[a]	Rainfall[b]	$\delta^{13}C$[c]	Fire Blight	Scab[d]	Juiciness	Surface Russet
GMAL3975.k	6	250 mm	-27.09 ppt	R	R	dry	0
GMAL3685.e	6	250 mm	-29.30 ppt	R	S	dry	10%
GMAL3623.f	9	450 mm	-26.33 ppt	S	S	moderate	1%
GMAL4455	4	800 mm	-26.30 ppt	R	R	medium	20%

[a] Geographical location of populations in Kazakhstan.

[b] Annual rainfall; source Forsline et al. (2003)

[c] ppt = parts per thousand; differences of 0.5 ppt are significant.

[d] R = resistant, S = susceptible

Table 3. Example of phenotypic diversity of select lines from *M. sieversii* Kazakhstan populations

clonally propagated for simulated drought experiments where photosynthesis and stomatal conductance were determined and roots, bark and leaves were collected for gene expression analysis. We duplicated these studies using 'Royal Gala', a relatively drought tolerant variety for comparison [85].

Methods Used: We used standard methods for the morphological and physiological measurements. For the drought experiments, young trees (~1 m tall) of 'Royal Gala' propagated by shoot proliferation were grown for several weeks in a controlled environment with standard light and temperature conditions [44]. A simulated moderate-severe drought were imposed by withholding water until the pots reached 40% of full saturation and maintained for 2 weeks at this level after which the trees were sampled. A parallel control group was grown under the same conditions, but watered to full capacity every other day. Samples from roots, bark and leaves (fully expanded) were taken and quickly immersed in liquid N_2. Roots were washed for 5 min in room temperature tap water, blotted dry and placed in liquid N_2. Bark was removed by scraping the outer layers (down to the xylem) directly into liquid nitrogen. All samples were stored at -80°C until use. Bark was lyophilized prior to storage at -80°C.

Total RNA was isolated, cDNA prepared and SSH performed using the protocol reported by Bassett et al. [86] for peach. For gene analysis, we designed primers for several genes shown to be associated with dehydration responsiveness in apple [44; manuscript submitted]. Each primer pair was quality tested and used to prime RT-qPCR reactions in order to quantitate gene expression in different tissues. The qPCR reactions were conducted using a kit containing all reagents (Life Technologies, Applied Biosystems, Grand Island, NY) and the reaction parameters were as follows: 95°C 5 min, followed by 35 cycles of 95°C 1 min, 60-65°C 1 min, 72°C 1 min and a final extension of 72°C for 10 min. Primers for a translation elongation factor (TEF2) was used as an internal control for the qPCR experiments [87]. The relative standard curve method was used to analyze the data.

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10.1. Status and results

Analysis of 'Royal Gala' response to a simulated moderate-severe drought: Clonally replicated individuals growing on their own roots were generated for the suppression subtractive hybridization experiments. We identified several hundred different genes up-regulated or down-regulated in roots, bark and leaves when DNA from drought-treated tissues served as 'tester' and DNA from well watered controls acted as 'driver' (10-fold higher amount). Some genes unique to our experiments that increased in response to drought are shown in Table 2. A number of drought-responsive genes were common to genes isolated from other plants systems, both dicot and monocot. Figures 2-4 show the relative expression of some of the common and unique genes in apple roots, bark and leaves.

Most of the genes examined regardless of tissue origin showed around a two-fold difference between watered and water-deficit treaments. A few genes were substanitally up-regulated in response to drought. including the auxin-induced gene from leaves (8-fold increase) and asparagine synthase from bark (4-fold increase). A few genes in roots were also significantly up-regulated in response to drought treatment, one of which was NPR1 (3-fold increase; manuscript submitted). From this information, including the expression of genes not shown here, we have generated a list of potential up-regulated genes responding to a relatively long term drought that can be used to determine if there is any correlation of expression in *M. sieversii* lines with high and low WUE values.

10.2. Promoter comparison of NRT2.4 from apple, peach and Arabidopsis

A high affinity nitrate transporter gene from the 'Royal Gala' root SSH library (see Table 2) was shown by RT-qPCR to be elevated in roots and bark in response to drought treatment (manuscript submitted). Approximately 700 bp upstream of the ATG start codon was obtained from the genomic sequence of 'Golden Delicious' (Genome Database for Rosaceae; http://www.rosaceae.org/). Several *cis*-elements associated with tissue specificity or stress response were identified. To identify elements preserved during evolution, promoter regions from peach NRT2.4 and an Arabidopsis AtNRT2.4 gene were analyzed for comparison (Figure 5). All three genes contained consensus, well defined TATA boxes within 80-100 bp of the translation start. A number of MYB and MYC binding sites were identified in similar positions in all three promoters. The peach promoter was missing a root-specific element seen in both MdNRT2.4 and AtNRT2.4. Interestingly, both the apple and peach promoters contained a drought responsive element on the reverse strand. Overall the elements identified in the apple NRT2.4 promoter are consistent with the expression analysis results.

Screening the *M. sieversii* population at site 6 in Kazakhstan: A core diversity population of *M. sieversii* trees (34 individuals representing 14 sibling groups) collected from Kazakhstan site 6 and maintained as seedlings at the Geneva, NY ARS Plant Genetic Resources Unit, was sampled for stable carbon isotope discrimination to select individuals with extreme values compared to 'Royal Gala'. The results are shown in Figure 6. Two individuals from each end of the WUE spectrum were chosen for further characterization.

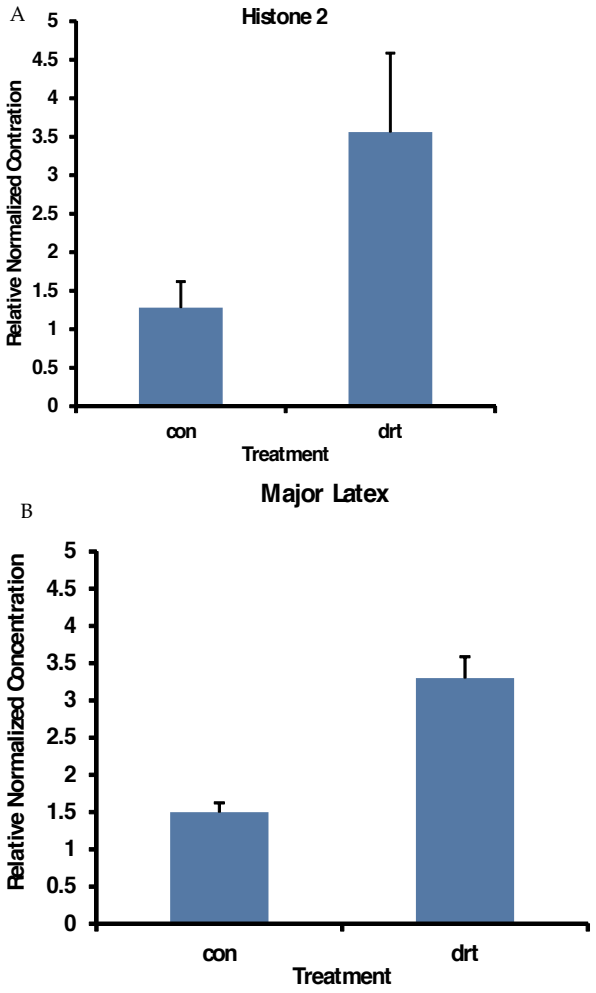

Figure 2. Relative expression of root genes up-regulated by drought treatment. Experiments were conducted as described in Methods. Test transcripts were normalized against TEF2. con: well watered controls; drt: treated to 40% saturation for two weeks. A: relative expression of Histone H2b gene; B: relative expression of the Major Latex protein gene.

M. sieversii lines GMAL4002.e and GMAL3975.k were propagated by shoot proliferation techniques to obtain a number of clonal individuals on their own roots. At the same time, 'Royal Gala' was propagated as a standard for comparison. Individuals from each line (4-6 trees per treatment) were acclimated under controlled conditions of light, water, fertilizer and temperature. Water was withheld from half of each group, while the other half received sufficient water to saturate the pot. The water-deficit trees reached 40% of pot saturation in about 7-10 days, at which time the individual plant and a comparable control were tagged. At the end of two weeks of treatment, the plants were individually sampled. Out of six

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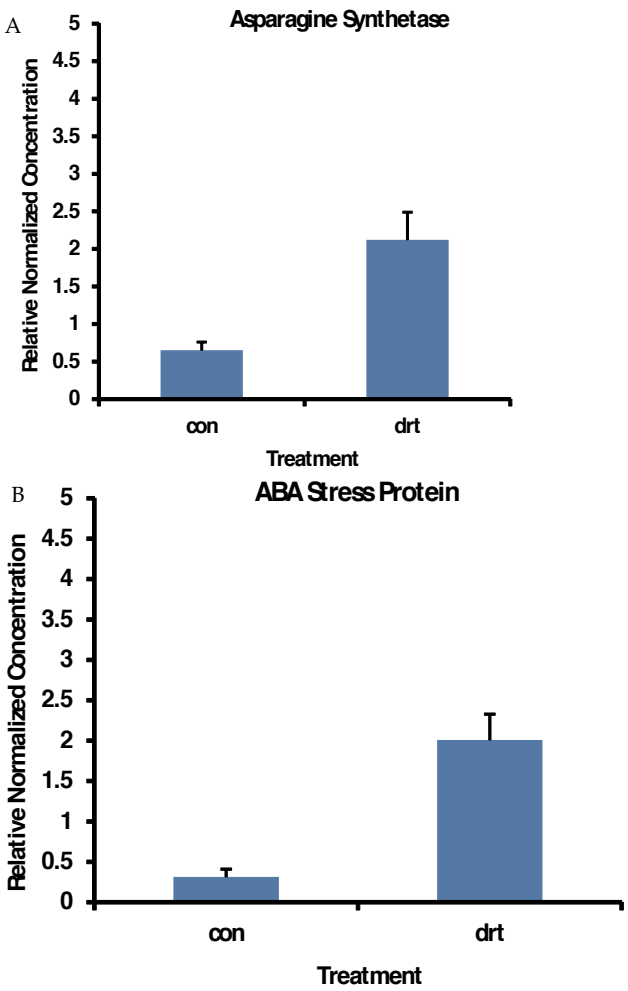

Figure 3. Relative expression of bark genes up-regulated by drought treatment. Experiments were conducted as described in Methods. Test transcripts were normalized against Actin. con: well watered controls; drt: treated to 40% saturation for two weeks. A: relative expression of asparagine synthetase gene; B: relative expression of an ABA stress protein gene.

GMAL4002.e plants, four began to show signs of severe wilting within a few days after reaching 40% as illustrated in Figure 7. This is consistent with WUE measurements which indicated that this particular line was not adept at maintaining healthy water status under the water deficit regime. Well watered GMAL4002.e controls showed no signs of wilting throughout the experiment. In contrast, GMAL3975.k with a WUE close to that of 'Royal Gala' showed no signs of wilting under the well-watered regime or water deficit stress (Figure 6). These results indicate that WUE can be used to identify apple lines that are drought sensitive, as well as drought tolerant.

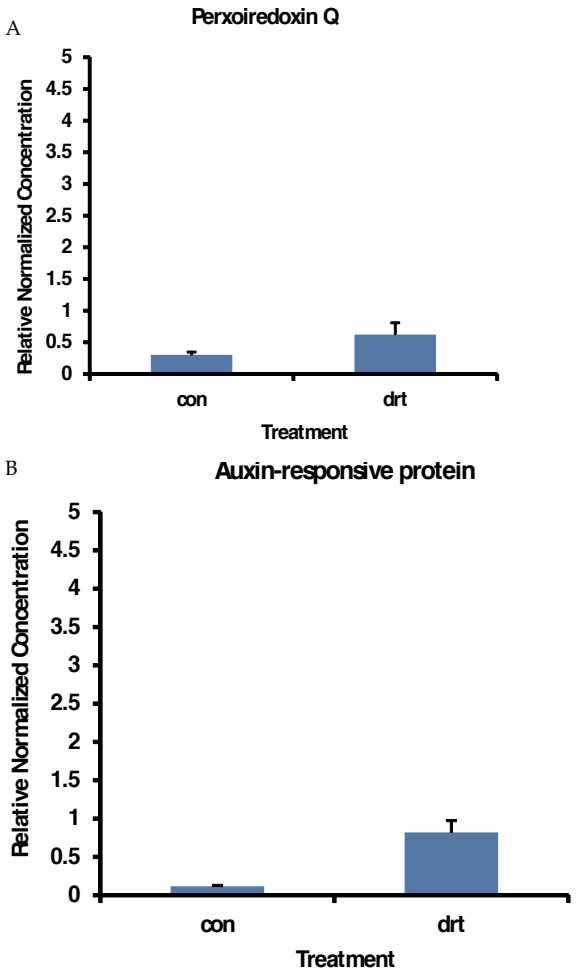

Figure 4. Relative expression of leaf genes up-regulated by drought treatment. Experiments were conducted as described in Methods. Test transcripts were normalized against Actin. con: well watered controls; drt: treated to 40% saturation for two weeks. A: relative expression of a peroxiredoxin gene; B: relative expression of an auxin-responsive/ripening protein gene.

Leaf size and number have been shown to respond negatively to drought, resulting in longer intervals between newly initiated leaves and smaller sizes, all features designed to reduce transpiration to conserve water. Leaf morphological features and stomatal characteristics were examined in the site 6 subpopulation [84]. GMAL3683.o had the smallest leaves by all traits measured, whereas GMAL3687.d and GMAL3989.f had the largest leaves by area. Within the GMAL3683 sibling group, GMAL3683.o leaf area (8.3 cm^2) clearly segregated from the other three members (average = 20.3 cm^2).

Stomatal density has also been linked to drought tolerance and sensitivity. Therefore, we also examined stomata size and density in the site 6 *M. sieversii* population. GMAL3691.m had the

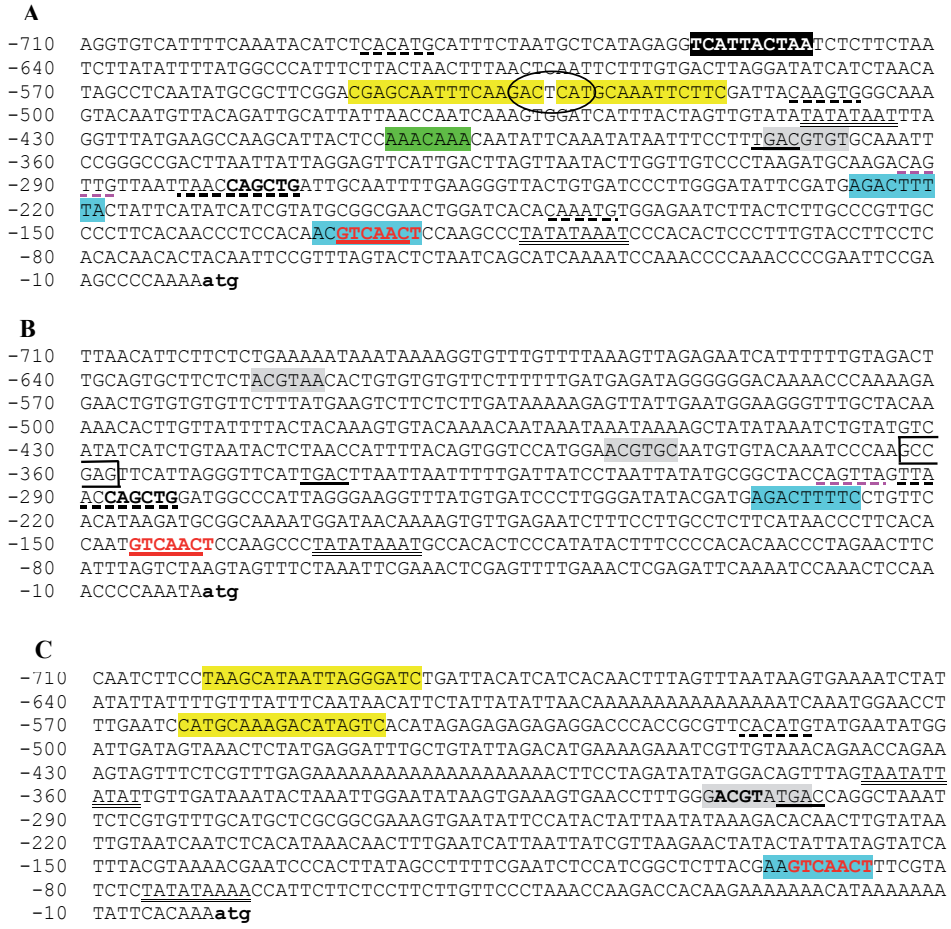

Figure 5. Figure 5. Comparison of NRT2.4 promoter regions from apple and Arabidopsis. Only the first 700 bases upstream of the translation start (**atg**) are shown. *Cis*-elements were identified by PLACE [88] and PLANTCare [89]. A: Promoter region from apple MdNRT2.4; B: Promoter region from peach genome; C: Promoter region from Arabidopsis AtNRT2.4. **AAACAAA:** anaerobic induction [90]; TGACG: WRKY stress responsive binding element [91]; **A^{A}/$_{C}$GTCA** and^{G}/$_{A}$**GACTTTTC:** bZIP and NF-B binding sites, respectively [92]; **CAAGCATGCTTCTTGC:** consensus root-spscific element [93]; **TATA box:** PolII binding; **TCATTACTAA:** wound-inducible element [94]; ACGTG/AT: ABRE core element [95]. An element of unknown function in the Arabidopsis NRT2.4 gene promoter (**GTCAACT**) is also present in the MdNRT2.4 and peach promoters. A *cis*-element for hypoosmolarity-responsiveness [96] is identified by an oval. Dashed underlines indicate a MYB (WAACCA) binding site [97] and MYC core sequences (CANNTG); fuschia-underlined MYC element on the negative strand in A and B (CAACTG) is associated with drought response [73]. A CBF/DREB element [98, 99] is boxed in A and B.

largest stomates, whereas GMAL3684.a had the smallest. GMAL3689.n had the highest density of stomates (58 per 0.09 mm^2); GMAL3685.e had the lowest density (23 per 0.09 mm^2). There was no correlation between leaf and stomate features in the *M. sieversii* site 6 population, nor was there a correlation between the leaf and stomate extreme values and the ^{13}C extreme values.

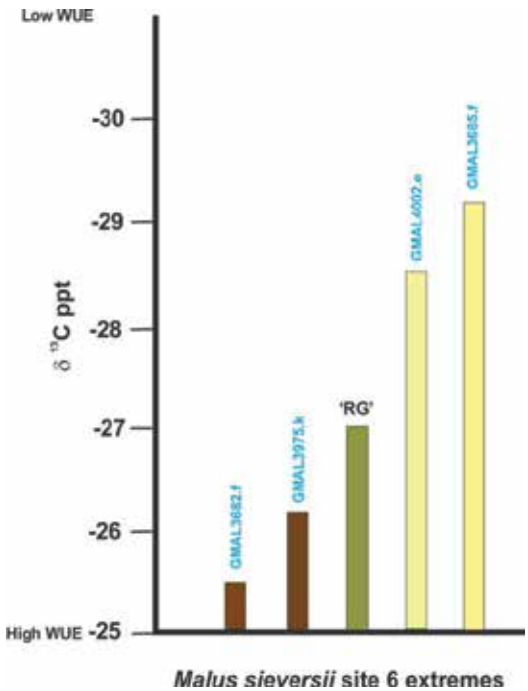

Figure 6. Stable carbon isotope analysis of select *M. sieversii* individuals from the site 6 population Measurements were made from branches representing current year's growth. Collection of samples was made from dormant trees for three years and averaged. RG: 'Royal Gala' standard.

In other words, although leaf and stomate physical characteristics might contribute to the drought response of *M. sieversii* individuals from site 6, another mechanism(s) appears to better explain the WUE data.

Further Research: The overall goal of this project is to link drought responsiveness and/or WUE to specific apple genes. To this end we are interested in candidate genes that are either up-regulated in response to drought (drought 'defensive' genes) or down-regulated (drought survival 'assisting' genes). The latter can be used to identify genetic alterations that could hamper the physiological state attained by up-regulated genes and therefore to be avoided in breeding programs. Up-regulated genes are of interest because of their obvious association with drought resistance. We have developed primers for many of the drought up-regulated genes identified in 'Royal Gala' and have tested them against individual lines of *M. sieversii*. Experiments to quantify their expression in the roots, bark and leaves of the site 6 lines representing WUE extremes are currently underway. We are also replicating additional *M. sieversii* lines on their own roots for simulated drought experiments like the ones shown in Figure 7 to provide physiological, morphological and molecular biological data to detect associations to drought resistance or susceptibility. Since the *M. sieversii* site 6 and 9 populations have undergone rapid adaptation to the xerophytic environments they currently occupy, it seems likely that alterations in patterns of expression could account for their survival. To

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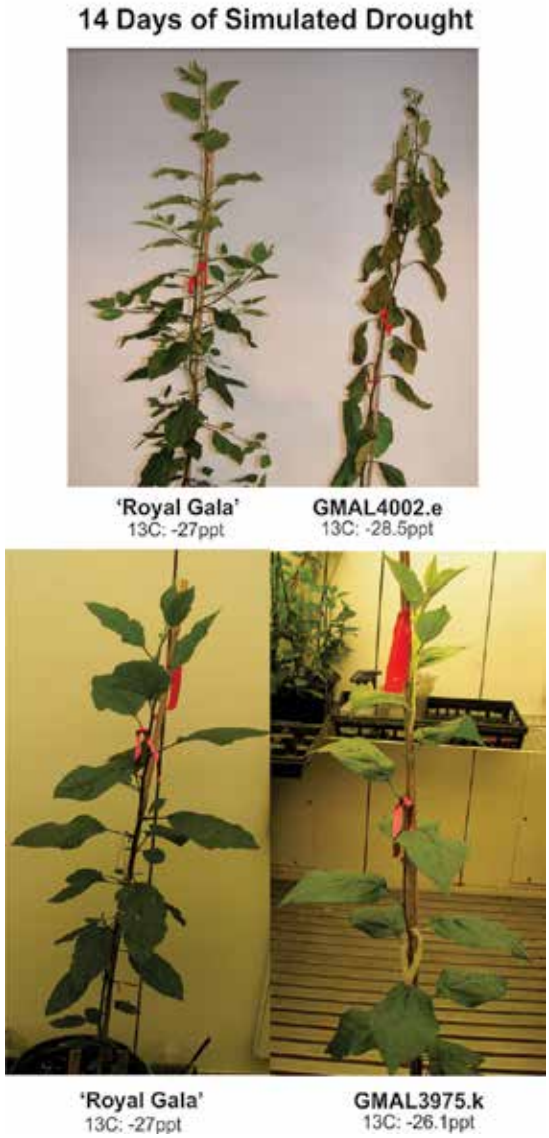

Figure 7. 'Royal Gala' and different *M. sieversii* genotypes under severe water deficit conditions. Delta ^{13}C values are included under the cultivar/genotype name. A difference of 0.50 ppt is considered significant. Note the wilting observed with GMAL4002.e compared to 'Royal Gala' under identical SD conditions. Also note GMAL3975.k and 'Royal Gala' respond similarly to the SD. 13C = $\delta^{13}C$

this end we are planning experiments to determine the kinetics of expression of candidate genes over a broader time period in select *M. sieversii* lines. Finally, we plan to examine promoter elements for single nucleotide polymorphisms or other alterations that might influence expression and to identify changes in methylation that might contribute to differences in expression between the more drought resistant lines and those that are more sensitive.

The information generated from these experiments can be used in breeding programs to select drought resistant progeny using marker assisted selection. In addition the use of genetic engineering of select genes is another potentially successful approach to obtaining new varieites with improved drought resistance or enhanced WUE. With increasing competition between agriculture and urban populations for fresh water and with climate change which predicts increasing episodes of intense drought periods worldwide, there is a critical need for the development of crop varieties with more efficient use of water and the ability to survive longer drought periods. Since apples are a good source of nutrition and can be conveniently stored and shipped, they are a logical target for improvement.

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Chapter 9

Tolerance to Lime - Induced Chlorosis and Drought in Grapevine Rootstocks

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Additional information is available at the end of the chapter

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1. Introduction

Many woody crop plants such as grapevine are traditionally grown with scion varieties grafted onto rootstocks. The selection of an appropriate rootstock provides a powerful tool to manage the growth and fruiting of the scion (Jones, 2012). In the grapevine propagation, the use of rootstocks is not a new matter. The evidence of the use of rootstocks can be found out even in works written by the Roman author Columella who occupied himself with agriculture and viticulture. However, the use of rootstocks obtained a new dimension after the phylloxera calamity, which destroyed European vineyards in the second half of the 19th century.

Rootstocks were introduced to Europe after the phylloxera invasion, a pest which rapidly spread through vineyards and destroyed large areas of sensitive cultivars. At present, grafting European varieties on pathogen-resistant rootstock is a normal procedure and many rootstock varieties have been developed by plant breeders (Arrigo & Arnold, 2007).

When choosing a suitable rootstock it is important to select one with a good tolerance to phylloxera and well as being to the specific climatic conditions and soil characteristics of individual vineyard sites. The rootstock connects the grafted plants with soil and influences mutual relationships. The root system of the rootstock enables the uptake of water and nutrients from soil. The rootstock also shows a marked effect on the growth intensity of grafted plants. When selecting a suitable rootstock, it is important to consider characteristics and parameters of the site. The most important of them are the following: depth of the soil horizon, water-holding capacity of soil, slope and exposure of the site, and climatic conditions. The architecture of the root system of the plant is also very important for its resistance/tolerance to drought. In the case of grapevine, selection and use of a suitable rootstock may help to solve problems of plant protection and of overcoming extreme soil conditions. Adaptability of plants

to environmental conditions, e.g. their tolerance to lime, low soil pH, soil humidity, salts etc., is very important.

Nowadays, grapevine plants are more and more influenced by various kinds of environmental stress. The most important kinds of abiotic stress are the following: extreme temperatures or too high (or too low) irradiation, water logging, drought, lack of minerals in soil (their deficiency) and too high salinity of soil (Koyro et al., 2012). Stress can be defined as an environmental factor that shows a negative effect on the living organism (Levitt, 1980).

This review tries to summarise data about the adaptation of rootstocks to soil (pedological) conditions, viz. their resistance to lime-induced chlorosis and drought. Regarding the global warming, these properties of rootstocks are very important also under conditions of the Central European viticulture.

2. Root system of grapevine plants

The most important functions of the grapevine root system involve anchoring of plants in soil, storage of reserve substances, uptake and conduction of water and dissolved nutrients within the plant and synthesis of growth hormones. The root system consisted not only of older, lignified roots but also of a great number of new ones that are used above all for the uptake of nutrients. Regarding their diameter, roots can be divided into two groups. Thick roots (with the diameter above 2 mm) represent a great proportion of root biomass. They create the "architecture" of the root system, enable the transport of water and nutrients and fulfil the function of a reserve organ. Fine roots (with the diameter below 2 mm) enable above all the uptake of water and nutrients. These are above all root hairs that develop on thicker roots. Root tips consist of root caps and apical meristem. This apical meristem assures growth and development of roots. Root growth takes place in the elongation zone which is approximately two milimeter long. Root tips are also the place of synthesis of plant hormones (gibberelins and cytokinines). These growth hormones are transported via conductive tissues into the aboveground parts of the plant and participate in processes controlling the balance between roots and tops, initiation of flowering, and growth and development of berries. Cell division (and, thus, the growth of roots) is controlled by auxins that are transported from tops of annual shoots into roots via phloem. The elongation growth is influenced by gibberellins that are synthesised in roots. Root hairs are localised behind the elongation zone of roots and assure the uptake of water and nutrients. It is capable to release organic compounds into the soil and to participate in propagation and development of microflora existing in the root zone (Pavloušek, 2011a).

The distribution of roots in soil is influenced by soil and environmental factors, e.g. temperature, degree of aeration, texture, availability of water and nutrients, pH value and frequency and depth of tillage operations (Richards, 1983, Morlat & Jaquet, 1993).

The root system of grapevine plants is mostly created by the root system of rootstocks. The root system of grapevine rootstocks enables the uptake of water and nutrients from greater

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soil depths. Distribution of root systems of individual rootstocks enables to identify their uptake capacity for nutrients. This means that different rootstocks have a different capacity to uptake individual nutrients from soil (Somkuwar et al., 2012).

Within the framework of their response to edaphoclimatic conditions individual rootstocks also show differences in growth capacity of their roots, water uptake, transport of water into annual shoots, metabolic activity and storage of carbon.

Water uptake and its transport represent one of the most important functions of roots. The distribution of roots in soil and the root turnover are the key parameters of water uptake; they are also important for the hydraulic redistribution (Bauerle et al., 2008a).

Root architecture refers to the spatial configuration of the root system, specifically focusing on the geometric properties of root axes and laterals, mostly concerned with the entire root system characteristics (Lynch, 1995). Typically, root distribution studies include root biomass or root length as a function of soil depth, distance from the plant stem, and position between neighbouring plants (Bassoi et al., 2003).

The available soil volume is probably the most important factor dictating the size and the distribution of root system (Saayman, 1982). The spatial root distribution is predominantly a function of the soil environment, while root density is a function of rootstock (Southey & Archer, 1988).

From the viewpoint of tolerance to abiotic factors associated with climatic and soil conditions it is important to study and understand both vertical and horizontal distribution of roots in soil. For example the Dog Ridge and Salt Creek rootstocks, which belong to *Vitis champinii*, put forth prolific root systems of thickness of < 2 mm and 2-5 mm in the top depths of 0-30 cm up to 60 cm away from the trunk, and later thicker roots of > 5 mm beyond 60 cm from the trunk. However, St. George, which belongs to *Vitis rupestris*, has less root length in all categories at all blocks horizontally up to a 150 cm distance from the trunk. In the vertical direction, Salt Creek showed the greatest root length in the category of < 2 mm at a depth of 0-30 cm, while Dog Ridge and St. George were at par for root length. However, at depths of 31-60 cm and 61-90 cm, Dog Ridge produced the greatest root length, followed by Salt Creek and St. George (Simkuwar et al., 2012).

The rootstock shows a significant effect not only on the distribution of roots in soil but also on the architecture of the root system. In *Vitis rupestris,* major roots create vertically a narrow angle and can penetrate deep into the bottom soil layers. On the other hand, roots of *Vitis riparia* are distributed in a wide angle and most of them are situated in a shallow top layer of soil (Perold, 1927). Swanepoel & Southey (1989) analysed the distribution of roots within a wider spectrum of rootstocks growing under conditions of South Africa (Table 1).

Also abiotic factors may influence growth and development of the root system. In this case, above all the vertical distribution of roots within the soil horizon is important.

Rootstock	Number of roots per m^2	Rooting index	Number of roots per diameter (mm) class				
			<0.5	0.5-2	2-5	5-10	>10
Berlandieri 13/5	2069	39.6	1792	226	38	10	3
101-14 Mgt	1604	27.1	1210	337	43	11	3
775 P	1006	44.7	839	145	16	4	2
1103 P	2660	41.9	2199	399	53	6	3
99 R	1138	28.9	833	267	30	8	0
110 R	1468	29.9	1103	319	37	9	0
140 Ru	635	20.2	483	122	21	6	3

Table 1. Distribution of roots of different rootstocks cultivated under conditions of South Africa (Swanepoel & Southey, 1989). (Rooting index = number of roots <2 mm/ number of roots ≥2 mm).

To assure a good tolerance of plants to drought and lime is necessary assure a proper development of the root system (Figure 1). Deep loosening of soil is also very important (Figure 2).

Figure 1. Root system of a five-year-old vine in clay loam soil.

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Figure 2. Deep lossening of soil in clay loam soil.

An adequate care about the root system and its proper development is very important and should be started immediately after the establishment of a new vineyard. This means that it is necessary to create and maintain a required ratio between thick and thin roots. The root system can be modelled by means of a cut off of roots so that they produce more branches, the total volume of root mass increases and the sorption area for the uptake of nutrients grows up. Usually and most frequently, this cut off takes place during the process of deep soil loosening and/or deep supplementary fertilisation. After these operations, the total volume of roots is partly reduced but the recovery is very quick. If, however, this intervention is too drastic, it may show a negative effect on the overall growth process of grapevine plants. The cutoff and shortening of roots should be performed at best at the beginning of the growing season, i.e. in the period of exogene dormancy, and should not be done every year because the optimum development of roots would be disturbed and plants could be under a permanent stress. It is recommended to use a deep additional fertilisation in three-year cycles.

A good understanding of effects of a limited availability of water on the growth of the root system and its functions has a principal influence on the selection of a suitable method of vineyard management (Schultz, 2010) and also on the selection of a suitable rootstock. In both temperate and Mediterranean regions, the growth of grapevine root system takes place above all within the period starting on flowering and finishing at the beginning of softening of berries (Comas et al., 2010). Drought-resistant rootstocks (e.g. 1103P) show a capability to create new

roots also during the periods of summer drought (Alsina et al., 2010). In these periods, the majority of roots is formed in depths below 60 cm, where the groundwater is available (Bauerle et al., 2008b).

To understand well to principles and processes of growth and development of the root system is therefore very important for understanding to stress situations caused by abiotic factors, especially by drought. The architecture of the root system is a genetic characteristic of rootstocks and soil, climatic and growing conditions modify it only very slightly. In the European „cool climate viticulture" rootstocks with *Vitis rupestris* in their pedigree are relatively rare in spite of the fact that their roots penetrate deep into the soil horizon. Practically, the only representative of these rootstocks is the Moravian rootstock Schwarzmann that was selected in Bzenec (Czech Republic) to the end of the 19th century and was very popular in former Czechoslovakia. Unfortunately, the results of introduction of other rootstocks with *Vitis rupestris* in their pedigree into the European „cool climate viticulture" have not been very successful yet.

3. Tolerance of grape rootstocks to lime-induced chlorosis

Lime-induced iron chlorosis, i.e. the condition of a reduced availability of soluble iron to the grapevine plants due high concentrations of bicarbonate ions in calcareous soils, can seriously impair the health condition of vines. The lime-induced chlorosis affects yield and quality of grapevines growing in lots of calcareous areas world-wide (Bavaresco et al., 1994).

A high content of active and total lime in soil can induce symptoms of chlorosis in grapevine plants and, thus, negatively influence the growth and yielding capacity of grapevine plants as well as the quality of harvested grapes. On the other side, however, lime present in soil participates very significantly to the manifestation of sensory properties of wine. From the geological point of view, it is a very important component of „terroir", which directly influences the character of produced wine. Wine-growing regions situated on calcareous subsoils can be considered for localities that are very suitable for production of quality wine. As typical examples it is possible to mention French Champagne wine region or Czech limestone Pálava Hills.

Selection of suitable, lime-tolerant rootstocks represents one of possible ways how to react to an increased content of calcium in soil and, at the same time, to preserve quality of plants and harvested grapes. Although the genetic improvement of tolerance of rootstocks to chlorosis represents a very long process, it still represents the best method of fight with iron chlorosis (Nikolic et al., 2000, Pestana et al., 2003). Differences in the uptake and distribution of nutrients within the plant may be influenced by the variety of rootstock because it may show a rather different absorption capacity as far as individual minerals are concerned (Rizk-Alla, et al., 2001). And just these differences in the absorption capacity may be associated also with the occurrence of the lime-induced chlorosis.

Rootstocks 333 EM and 41B (originating from crossing *Vitis berlandieri x Vitis vinifera)* belonged to the first rootstocks selected for a high degree of tolerance to calcium so that they could be

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used in vineyards established on calcareous soils. A. Ruggeri, an Italian breeder and selectionist, was the author of the rootstock 140 Ru (*Vitis berlandieri x Vitis rupestris*), which also showed tolerance to lime chlorosis (Fregoni & Bavaresco, 1986). The rootstock Fercal, selected in France by Pouget and Ottenwaeter (1978) is one of the most modern rootstocks showing a very high degree of tolerance to calcium.

3.1. Deficiency symptoms of lime-induced chlorosis

A correct identification of symptoms of lime-induced chlorosis is one of the first presumptions of a successful fight against this deficiency. Iron deficiency chlorosis is one of the major problems affecting a variety of crop species grown in calcareous soils (Gruben & Kosegarten, 2002). Iron deficiency causes various morphological and physiological changes in plants (Bertamini & Nedunchezhian, 2005).

Although the symptoms of lime-induced chlorosis are visible on the whole grapevine plant, the most important ones can be probably observed on leaves.

Iron-deficient plants are characterized by the development of a pronounced intervenial chlorosis similar to that caused by magnesium (Mg) deficiency but occurring first on the youngest leaves. Intervenial chlorosis is sometimes followed by chlorosis of the veins, causing the whole leaf to become yellow. In severe cases, the leaves become white with necrotic lesions (Abadia, 1992).

Typical symptoms of lime-induced chlorosis are the inter-vein yellowing of leaves and a decrease in plant biomass because, under conditions of iron (Fe) deficiency, a decreased photosynthetic performance of plants is induced by a lower content of chlorophyll in leaves (Bavaresco & Poni, 2003). Chlorosis is a disease manifesting itself by yellowing of young leaves, whereas more mature leaves are frequently green. Plant growth is often considerably depressed, independent of whether young leaves are chlorotic or green (Mengel, Bübl, Scherer, 1984). The impaired formation of new leaves and restricted leaf growth is a typical and more sensitive symptom of Fe-deficiency than is leaf chlorosis (Kosegarten *et al*.,1998)

Chlorotic symptoms also vary from year to year as a result of environmental variables, like yields, temperature, rains. In soils where shallow layers are less rich in $CaCO_3$ than deeper layers, it is likely that vines develops chlorosis only when the age and roots explore layers with poor conditions for Fe uptake (Tagliavini & Rombola, 2001).

As the lime-induced chlorosis is the result of the relationship existing between soil conditions and grapevine root system, it is manifested also in growth characteristics of grapevines. The lime-induced chlorosis of grapevine was characterized by a dramatic reduction of shoot growth, grape production and leaf Fe content, and a distribution of dry matter towards roots more than to the clusters (Bavaresco, Giachino, Pezzutto, 2003).

Vines growing on high-bicarbonate soil significantly reduced the dry matter production of individual organs and the total plant weight. Lime stress conditions increased the percent distribution of dry matter in the stem and roots and decreased that one in the fruit (berries and cluster stems) (Bavaresco & Poni, 2003).

A high content of lime mostly causes a low availability of iron, which is a result of its non-solubility occurring in soils showing higher values of pH. Under such conditions, iron cannot be uptaken by roots of plants (Hell & Stephan, 2003).

This lime-induced iron deficiency shows a strong effect not only on grapevine plants but also on some other economically important fruit species cultivated on calcareous soils. It is quite common also in peach, pear, quince-tree, kiwi, and citrus fruit plantations (Tagliavini & Rombola, 2001).

Lime-induced stress conditions show a strong effect on production of grapes and reduce the yield of grapes per vine. When growing grapevine on calcareous soils, a lower number of grapes per annual shoot depends on stress conditions existing in the preceding growing season (when the flower buds were differentiated) while a small size of grapes and berries is a consequence of iron deficiency in the current year (Bavaresco, Presutto, Civardi, 2005).

Because the lime-induced chlorosis affects above all the growth of grapevine plants, influences the total leaf area capable of photosynthetic activities and thus also yield and quality of fruit and for that reason it is possible to say that iron deficiency is caused mainly by higher levels of calcium carbonate and the resulting high contents of bicarbonates in soil. These high levels of bicarbonate ion are typical just for these calcareous soils (Pestana, Faria, De Varennes, 2004; Mengel, Breininget, Bübl, 1984). Under such conditions, the occurrence of chlorosis symptoms is quite common and for that reason this type of chlorosis may be defined as a lime-induced iron chlorosis or, abbreviated, lime-induced chlorosis (Pestana *et al*., 2004).

The identification of chlorosis symptoms in vineyards is very important because it enables to perform protection of plants against mechanisms that induce this plant disease. The identification can be performed by means of leaf analysis that enables to estimate contents of individual macro and microelements in leaf blades and/or leaf petioles.

The leaf analysis enables to identify all factors that can influence the availability of nutrients in soil and their uptake by plants; it also can provide information about the nutrient balance of plants in the moment of sampling (Pestana et al., 2003).

3.2. Causes of the occurrence of lime-induced chlorosis

Although the reasons of the occurrence of this type of chlorosis seem to be relatively definite, the mechanism of its occurrence is still not explicitly defined. It seems that different forms of iron present in soil and their availability for plants contribute a lot to the occurrence of this type of chlorosis. Even in very small amounts, iron represents one of those minerals, which are utilised by plants to assure their sound growth. Iron is used by plants in two forms, viz. as Fe^{2+} and Fe^{3+}.

Iron chlorosis affects susceptible plants growing on calcareous soils. Different kinds of carbonates induce different degree of chlorosis. Chlorosis is high for magnesite, hydromagnesite and calcite and low for aragonite and nil for dolomite (Fregoni, 1980). There are several different views concerning iron concentration in soil and its relationship to the occurrence of chlorosis. In some cases the lime-induced chlorosis can occur under conditions of a low content

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of iron in soil and leaves (Bavaresco et al., 1992) while in other its symptoms may be observed at very high levels of iron in leaves (Mengel et al., 1984b). Iron also plays an important role in activities of the enzymatic system of plants: it actively participates in photosynthetic reduction-oxidation reactions, respiration, biosynthesis of proteins and chlorophyll, biological binding of atmospheric oxygen, and in reduction of nitrates and nitrites (Tagliavini & Rombola, 2001).

Cultivated plants differ in their susceptibility to Fe deficiency in calcareous soil; some are not much affected while others show severe leaf symptoms of chlorosis (Tagliavini & Rombola, 2001). In cultivars grown under conditions of a high content of carbonates in soil the content of chlorophyll can decrease dramatically with the increasing age of plants (Shaaban et al., 2007).

The total content of lime in soil is not very useful for predicting the development of the occurrence of this type of chlorosis. Active carbonates (active lime) is more reactive and, therefore, able to build and maintain high levels of HCO^{-}_{3}; for that reason it is a more reliable indicator (Tagliavini & Rombola, 2001). In viticulture, the evaluation of conditions suitable for the induction of chlorosis the following parameters are usually taken into account: total carbonates (%), active lime (%) and CPI (chlorotic power index). Evaluated rootstocks are then classified on the base of these analytic parameters. This concept resulted in the so-called "chlorotic power index" (CPI) (Juste & Pouget, 1972. In: Huglin & Schneider, 1998). This means that the amount of active lime is related to the amount of Fe extracted by ammonium oxalate. Table 2 shows degrees of chlorosis intensity in relation to different values of CPI (Lupascu et al., 2009).

CPI value	Intensity of chlorosis
0	None
≤ 5	Small
6 - 15	Medium
16 -35	High
≥ 36	Very high

Table 2. Degrees of chlorosis intensity in relation to different values of CPI (LUPASCU et al., 2009).

The content of active lime in soil is a parameter, which is frequently used when selecting rootstocks for cultivation of grapevine plants in calcareous soils (Champagnol, 1984).

The susceptibility to chlorosis is the most important selection criterion for rootstocks in many European wine-growing regions where such a condition is prevalent due to occurrence of highly calcareous soils.

Two basic strategies how to classify grapevine plants according to their capability to adapt themselves to conditions, under which the lime-induced chlorosis can occur (Bavaresco, 1990):

- Strategy I involves four types of response in the roots as follows: a) enhancement of H-ions release, b) formation of rhizodermal or hypodermal transfer cells, c) enhancement of ferric

iron reduction to ferrous iron, d) enhancement of release of reducing/chelating compounds e.g. phenols.

- Strategy II is characterized by an enhancement of release of non-proteinogenic amino acids and by a high affinity uptake system.

Bavaresco (1990) formulated the following hypothesis: the response mechanism of tolerant grapevine rootstocks corresponds probably with Strategy I (Bavaresco et al., 1989) however, the vines are normally grafted and the behaviour of the whole plant towards lime-induced chlorosis is governed by the following two properties: (i) by the ability of roots to satisfy iron requirement of leaves; (ii) by the iron requirement of leaves to secure a normal iron nutrition of the plant (Pouget & Ottenwalter, 1973).

The reason that Fe deficiency results in a rapid inhibition of chlorophyll formation is not fully understood, even though this problem has been studied for many years (Bertamini & Nedunchezhian, 2005). The reduction of plant biomass of susceptible plants is related to a reduced root growth due to soil bicarbonate and to a lower photosynthesis rate which also depends by a decrease of leaf chlorophyll, under Fe stress conditions (Bavaresco, Giachino, Pezzutto, 2003). According to the growth rate of sink tissues and such organs as the roots, shoot apex, fruits and storage organs can be limited by supply of photosynthates from the source leaves or by a limited capacity of the sink to utilize the photosynthates (Marschner, 1995). In some cases, lime-induced chlorosis is related to a low Fe uptake and its translocation to leaves (Bavaresco et al., 1992), in others to a high content of Fe in leaves, which has to be somehow inactivated (Mengel, Breininget, Bübl, 1984; Bavaresco et al., 1993).

Screening tests of tolerance to chlorosis are performed on plants grown under conditions of a high content of bicarbonates in soil. This evaluation can be performed also *in vitro* on a medium containing a high level of bicarbonates (Bavaresco et al., 1993). The identification of real causes of the occurrence of the lime-induced chlorosis under conditions of a given vineyard is very important for the improvement and/or elimination of these biotic stress situations. The selection of a suitable rootstock is very important above all in situations when the uptake of iron is blocked due to a high content of lime in soil and also due to unsuitably chosen rotstocks.

3.3. Tolerance to lime-induced chlorosis in wild species and rootstocks varieties

Rootstocks represent a very important part of the concept how to prevent the occurrence of lime-induced chlorosis in vineyards. A perfect knowledge of soil conditions existing in a given locality and also of the resistance of individual rootstocks to lime enables to optimise the management of selection of rootstocks on the base of soil conditions.

Use of genotypes tolerant to chlorosis induced by iron blocking is a reliable tool how to solve problems of chlorosis occurrence (Jimenez *et al*., 2008).

Chlorosis resistance or susceptibility of grapevine varieties and rootstocks is related not only to the root ability to supply adequate iron to the leaves, but also to their iron requirements, which can differ between genotypes. On the basis of this concept, the grapevine varieties were ranked according to their chlorosis resistance or tolerance (Branas, 1974). Breeding also greatly

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contributes to the selection of lime-resistant rootstocks. Breeding efforts to get proper genotypes included successfully crossing between wild grape species, and some chlorosis-resistant rootstocks are now available for the grapevine growers of the many calcareous areas worldwide (Fregoni, 1980; Pouget, 1980; Bavareso, Fraschini, Perino, 1993). Lime-tolerant grapevine rootstocks have some specific physiological mechanism to overcome chlorosis when grown on calcareous soils, including and improvement of root Fe uptake and reducing capacity (Varanini & Magioni, 1982; Bavaresco, Fregoni, Fraschini, 1991).

Vitis riparia and *Vitis rupestris* are very important species in the history of the rootstock breeding activities. These two species are not very tolerant to calcareous soils. *Vitis berlandieri* is recognized for adaptation to calcareous soils. *Vitis vinifera* is species tolerant to calcareous soils (Cousins, 2005). Knowing the characteristics of the important parental species and rootstock varieties used in rootstock development helps us to understand the viticultural attributes of individual rootstocks families.

Data about the tolerance of rootstocks to lime-induced chlorosis, as mentioned by Cousins (2005) and Chauvet & Reynier (1979) are presented in Table 3.

Rootstocks	Tolerance to chlorosis	Reference
SO 4	Medium	COUSINS (2005)
Börner	Low	
420 A	Good	CHAUVET & REYNIER (1979)
Kober 5BB SO4	Medium	
140 Ruggeri	Very Good	
1103 Paulsen 110 Richter	Medium	
Fercal	Very Good	

Table 3. Tolerance of rootstocks to chlorosis (after Cousins, 2005, Chauvet & Reyiner, 1979).

From the viewpoint of the resistance to chlorosis, the rootstocks registered in the State Variety Book of the Czech Republic can be ranked from the most resistant to the most sensitive as follows: *Craciunel 2 – SO 4 – Kober 125 AA – Kober 5 BB – Teleki 5 C – Amos – LE-K-1*. These results are very important from the viewpoint of the use of rootstock varieties for propagation and growing of grapevine in the Czech Republic (Pavloušek, 2008).

In table 4, the classification of rootstock variety, content of active lime and values of CPI are described (Juste & Pouget, 1972 In: Huglin & Schneider (1998).

Rootstock	Content of active lime (%)	CPI value
Vialla	-	2
Riparia Gloire	6	5
196-17	6	-
101-14	9	10
216-3	9	-
44-53	10	-
3309	11	10
1616	11	-
Rupestris du Lot	14	20
99R,110R,1103P,SO4	17	30
5BB,420A, 34 EM	20	40
161-49	25	50
140 Ru	25	90
41B	40	60
333 EM	40	70
Fercal	-	120

Table 4. Classification of rootstocks on the base of the content of active lime and CPI (Juste & Pouget, 1972. In: Huglin & Schneider, 1998).

Recently, the species *Vitis cinerea* is very often used when selecting new rootstock types. In the Czech Republic, rootstock breeders used the German rootstock Börner and the Czech hybrid Bruci [(*Vitis berlandieri x Vitis rupestris) x Vitis cinerea*] as donors of resistance to phyloxera. Hybrids with a very high tolerance to chlorosis originated from parent combinations [Binova x (Binova x Teleki 5C) x Börner] and (Teleki 5C x Börner). These hybrids originated from combinations of *Vitis berlandieri, Vitis riparia* and *Vitis cinerea*. Hybrid combinations with *Vitis rupestris* and *Vitis amurensis* showed mostly only a medium tolerance to chlorosis. A simple hybrid (Binova x Börner) showed also a medium tolerance to lime-induced chlorosis (Pavloušek, 2009).

4. Tolerance of grape rootstocks to drought

Drought stress is one of the most important abiotic stress factors which are generally accompanied by heat stress (Zulini *et al*., 2007).

In recent years, climatic changes can be observed worldwide. Warm years are more frequent and periods of drought are longer. This means that modern viticulture must look for methods how to react to this increasing frequency of periods of drought.

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The grapevine (*Vitis vinifera*) has different physiological and morphological mechanisms enabling it to maintain growth and production also under conditons of water deficiency (Kondouras et al., 2008).

In Europe, varieties of *Vitis vinifera* are traditionally cultivated in non-irrigated regions. Yield of grapes as well as the quality of berries is therefore dependent on the adaptability of grapevine plants to drought. A good understanding and control of the water regime of plants as well as influencing their tolerance to drought stress on the base of application our knowledge of plant physiology and molecular biology may significantly increase not only productivity of plants but also quality of environmental conditions.

In grapevine, water supply of plants plays an important role in processes of plant growth and formation of berries. A limited supply of water reduces not only the growth of annual shoots but also the weight of berries and the final yield of grapes. A marked lack of water may result in reduced yields and an impaired quality of grapes. This means that in the course of the growing season the occurrence of stress induced by water deficit shows a significant effect on physiological functions of grapevine plants. Although the grapevine (*Vitis vinifera*) is a species showing a very good tolerance to drought, a severe stress may sometimes markedly influence qualitative properties and parameters of grapes. When using plant material adapted to drought conditions, it is possible to avoid losses caused by a severe water stress (Van Leeuwen *et al*., 2009).

Selection and breeding of grapevine rootstocks and varieties with a higher water-use efficiency represents a possibility how to adapt viticultural production to current climatic changes (Vandeleur et al., 2009, Flexas et al., 2010).

4.1. Properties influencing the tolerance of grapevine plants to drought

In the course of phylogenesis the grapevine (*Vitis vinifera* L.) plants have developed various physiological and morphological mechanisms, which enable them to maintain their growth and fertility even under conditions of a limited availability of water.

Although grapevine (*Vitis vinifera* L.) is considered to be a species adapted to drought stress, the combined effect of high irradiation, high temperatures and low atmospheric water pressure tension would presumably act as major constraint for the leaf photosynthesis, particularly under conditions of severe soil water deficits usually encountered by this crop (Flexas *et al*., 1998).

Physiological responses of plants to water deficit are linked to a condition of recognition of stress by the root system, turgor changes and water potential and consequently stomatal conductance, internal CO_2 concentration and photosynthetic activity decrease. From a molecular perspective, several genes expressed under stress conditions are activated, such as genes linked to the biosynthesis of abscisic acid and synthesis of specific proteins (Chavaria & Pessoa Dos Santos, 2012). This means that in plants the water stress is manifested by many different mechanisms.

A limitation of growth of annual shoots and leaves represents one of the first symptoms of water deficiency (Stevens et al., 1995). The sensitivity of roots is usually lower than that of annual shoots (Dry *et al.*, 2000). The growth intensity of annual shoots can be used as one of very sensitive indicators of grapevine water status (Patil et al., 1995, Pellegrino et al., 2006, Lebon et al., 2006, Pavloušek, 2011b).

In summer, water available to the plant can often be insufficient because of a lack of precipitation or a low level of its reserves in soil. This can lead to a reduction in the vigour of the plant, its productivity, and quality of the crop. The growth of above-ground parts of grapevine plants is associated also with the growth of roots and this is directly dependent on the availability of water in soil. In periods of drought, roots of some grapevine rootstock varieties can penetrate deep into the soil horizon and thus produce new and new roots.

The growth of roots is also dependent on the relationship, which exists between the rootstock variety and soil conditions (Morlat & Jacquet, 2003). Rootstock genotype has a major influence on root density (Southey & Archer, 1988; Williams & Smith, 1991) even though the distribution of grapevine roots is significantly dependent on both edaphic conditions (Smart *et al*., 2006) and vine (Archer & Strauss, 1985). In extremely drought soils, however, the growth of roots of some botanical species may be significantly reduced (Comas et al., 2005).

The tolerance of grapevine to drought is also dependent on the quality of the root system, its architecture, the distribution of individual types of roots within the soil and the density of the root system in the place of water and nutrients uptake. On the other hand, however, the architecture of the root system can be influenced also by spacing of planarity and method of vineyard tillage.

Roots are usually the first point where the stress is perceived by plants and where they respond to the existing stress conditions. The grapevine tolerance to drought is *de facto* the capability of plants to produce selectively new roots in those places where the groundwater is available. The water stress has a dominant effect on the growth of the grapevine and affects both the growth and the development of grapevines.

The growth inhibition of annual shoots decreases transpiration of plants and reduces the total volume of conductive tissues (Lovisolo et al., 2010). The transport of water from soil to roots and (via conductive tissues) also to annual shoots and other above-ground parts of grapevine plants is dependent on activities of aquaporins. Aquaporins are members of the major membrane intrinsic protein family, where can act as water channels and can regulate cell-to-cell water transport (Maurel et al., 2008). Aquaporins play an important role in the process of water absorption. The availability of aquaporins on the surface of roots is changing during the day and depends on the photoperiodicity (Chavaria & Pessoa Dos Santos, 2012).

The physiological mechanisms related to drought tolerance vary from genotype to genotype. It is necessary to screen genotypes for drought tolerance and take into consideration all important aspects, e.g. photosynthesis rate, transpiration rate, stomatal conductance and relative water content occurring at different level of water stress (Satisha *et al*., 2006). Grapevine varieties adapt themselves to water deficits by means of various mechanisms, e.g. by changes

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in the leaf area (Gómez Del Campo *et al*., 2003), xylem vessel size, and/or conductivity (Lovisolo & Schubert, 1998).

Stomata enable a control of water regime in plants because they balance and stabilise values of water potential existing between their leaves and the atmosphere. Stomatal closure is one of the first responses to soil drying, and a parallel decline in photosynthesis and stomatal conductance under progressive water stress has already been reported (Medrano *et al*., 1997). Within the framework of stomatal activities there are relationships among metabolism of abscisic acid (ABA), hydraulic signals, regulation of activities of aquaporins and electric signals that are manifested when measuring the water potential of leaves (Lovisolo et al., 2010). This means that the reaction of stomata is mediated by ABA, which is produced within the framework of a response to the stress induced by drought in roots; this newly synthesised ABA is then transported into other parts of the plant (Loveys et al., 1984).

Plants respond to the lack of water by a quick closing of stomatal opening so that a further loss of water via transpiration is prevented. This mechanism represents a very efficient protection of plants against drought-induced stress.

A lack of water in soil and a leaf water deficit result also in a gradual reduction of photosynthesis and changes in assimilation of carbon and nitrogen (Chavaria & Pessoa Dos Santos, 2012, Zlatev &Cebola Lidon, 2012). Drought-induced decrease in photosynthesis is primarily due to a stomatal closure, which lowers CO_2 availability in the mesophyll, not due to a direct effect on the capacity of the photosynthetic apparatus (Escalona *et al*., 1999). Osmotic stress is a common feature of many abiotic stress factors, that affect grapevines (Gramer, 2010). Some biochemical characteristics, e.g. the stability of chlorophyll, can be used for selection of cultivars resistant to drought conditions (Sinbha & Patil, 1986, Pavloušek, 2011b).

Water-use efficiency (Wue) can be considered for the most important indicator of water management of plants (and also in grapevine). The Wue can be defined as a balance existing between the biomass gain (expressed in kilograms of produced biomass or in mols of assimilated CO_2) and losses of water (expressed as cubic meters of consumed water or mols of transpired water). From the agronomic point of view the Wue can be defined as the volume of yield produced per unit of consumed water (Tomás, et al., 2012). Quality of grapes is very markedly dependent on the amount of water consumed by plants and for that reason an improvement in efficiency of water use represents the major requirement concerning crop sustainability and quality of grapes (Medrano et al., 2012). New aspects of Wue and actual data concerning this indicator were dealt with and studied in many recent studies (Flexas et al., 2010, Schultz & Stoll, 2010, Lovisolo et al., 2010, Tomás et al., 2012, Medrano et al., 2012). The Wue is a key parameter that enables to evaluate the efficiency of water use within the agrarian sector. It is dependent on the total amount of water consumed by plants in the course of the growing season. This sum represents the amount of water used by plants plus water losses caused by transpiration (Flexas et al., 2010).

For that reason it can be expected that there is a relationship between Wue on the one hand and genetic foundations (i.e. genomes) of cultivars or rootstocks on the other. Basing on the knowledge of Wue of individual species, rootstocks or cultivars it could be therefore possible

to recommend them for planting in individual sites/localities with regard to their availability of water.

Some studies dealt with the Wue of individual botanic species and rootstocks. A higher Wue value was found out in *Vitis rupestris* while a lower one in *Vitis doaniana, Vitis californica* and *Vitis candicans* (Padgett-Johnson, et al., 2003). Higher Wue values were also described in *Vitis riparia* (Flexas et al., 1999) and the rootstock 110 R (Pou et al., 2008).

Soar *et al.* (2006) reported that rootstock effect on gas exchange of vineyard-grown grapevines is most likely due to differences in the relative capacity of rootstocks to extract and provide scions with water. Rootstocks have been reported to affect the efficiency of water transport to the shoots via conductivity constrains imposed by the anatomy of xylem vessels (De Herralde *et al*., 2006).

Greenspan (2006) differentiates between terms "drought-tolerance" and "drought-avoidance". Drought-tolerance refers to the ability of the rootstock to support grapevine physiological functions during periods of low soil moisture availability. Rootstocks may exhibit drought-tolerance through several mechanisms:

Maintaining a low hydraulic resistance to water flow, even under dry conditions.

1. Maintaining photosynthetic activity in leaves, even under low water availability conditions.
2. Preventing the abscission of leaves during periods of low water availability.

Drought-avoidance refers to the ability of the rootstock to prevent low vine water status by one or more of many mechanisms, including:

1. Deep or extensive root exploration to fully exploit soil moisture reserves.
2. Conservation of vine water use by inducing closure of the leaf stomatal pores to limit transpiration.
3. Restricting vine vigour, thereby limiting the amount of transpiring leaf surface area.

The relationship existing between the response of plants and the drought-induced stress influences, through physiological reactions of plants, also the development of important qualitative parameters of grapes (Lovisolo et al., 2010):

1. Effects of plant metabolism, above all photosynthesis and transpiration, on accumulation of sugars and secondary metabolites in berries.
2. Consequences at the berry level of both the chemically-mediated long distance signalling between root and shoot (essentially cytokinin and ABA) and the whole-plant hydraulic control via both the xylem and the phloem from root to berry.
3. Adaptation of berry metabolism to a severe osmotic stress existing in berry cells.

ABA, which is present in xylem fluid represents a key signal of root-shoot in plants that are stressed by drought (Schachtmann & Gooder, 2008).

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The grapevine belongs to plants, in which a the existence of a very strong relationship between ABA produced in drought-exposed and stressed roots on the one hand and quality of grapes on the other.

Abscisic acid (ABA) is therefore the most important plant hormone that influences ripening and quality of grapevine berries. The participation of ABA is high at the beginning of development of berries and decrease till the period of berry softening. Its content increases again during the initial stages of accumulation of sugars and reaches the maximum approximately 2–3 weeks later (Davies & Böttcher, 2009). The content of ABA in the skin is higher than in the pulp (Coombe and Hall, 1973). Also grapevine seeds contain more of this acid than pulp (Zhang et al., 2003). ABA participates also in biosynthesis of anthocyanins and, according to Davies & Böttcher (2009) in the accumulation of sugars in berries. Drought-induced stress supports the formation of ABA and show a positive effect on the formation of secondary metabolites, above all of flavonoids, which involve anthocyanins and tannins. Under conditions of water stress, concentrations of anthocyanins and proanthocyanidins in the skin increase independently on the size of berries; this process is dependent above all on the availability of water (Roby et al., 2004).

In grapevine plants suffering from drought-induced stress, the synthesis of reserve substances may be impaired due to an inhibited photosynthesis. This means that the plants are not adequately prepared for overwintering. This stress markedly influences the quality of grapes. Contents of amino acids, organic acids and also sugars are usually reduced. Stressed plants show a decreased uptake of minerals from soil and, thus, lower extract in wine. In extreme cases it is possible to observe negative effects on smell and taste of wine as well as the occurrence of the ATA phenomenon (atypical aging – ATA). Due to a high content of polyphenols, the taste of stressed wines is bitter, disharmonic and „short".

From the viewpoint of quality of harvested gapes, a proper evaluation of the drought-induced stress risk is very important. In white wine varieties, this type of stress may show more negative effects than in varieties used for making of red wines because in this case the synthesis of ABA may show a positive effect on formation of phenolic substances.

4.2. Tolerance to drought in wild species and rootstocks varieties

The use of rootstocks makes it possible to give plants a certain capacity to adapt drought conditions. A good knowledge of tolerance of rootstocks is important above all with regard to the use of these genetic resources in the process of breeding and selection of plants tolerant to drought.

The capability of plants to create a root system efficiently penetrating into the soil is an important factor, which enables them to survive during longer periods of drought and water-stress.

The assessment and evaluation of tolerance of rootstocks to drought represents an important component of the process of selection of suitable rootstocks and for further breeding work. The classification of rootstocks into five groups according to their tolerance to drought is presented in Table 5 (Carbonneau, 1985).

Degree of resistance	Rootstock variety
Highly resistant	R 110, R 140, 44-53,
Resistant	P 1103, 196-17, P 1447, SO4, R 99, 7383,
Less resistant	3309, 7405, 7903, 420 A, Fercal, RSB1, 7921, 5 BB, 161-49, 41 B, Rupestris du Lot, 101-14
Susceptible	Rupestris du Lot, 101-14, EM 333, 7924, Yuga,
Highly susceptible	7542, Vialla

Table 5. Evaluation of drought tolerance of individual rootstock varieties (Carbonneau, 1985).

It is well-known that there are really remarkable differences in tolerance to drought. Some rootstocks (e.g. 101-14 and Schwarzmann) show a low tolerance while in others (e.g. Lider 116-60, Ramsey, 1103 Paulsen, 140 Ruggeri, and Kober 5 BB) this property is better (Sommer, 2009). Also Cirami *et al.*, (1994) observed a good tolerance to drought in rootstocks Ramsey, 1103 Paulsen, and 140 Ruggeri.

Table 6 presents tolerance to drought in some rootstocks varieties after Lavrenčič et al. (2007) and Pouget & Delas (1989).

Rootstock	Tolerance to drought	Reference
3309 Couderc	Low-very sensible	LAVRENČIČ *et al*. (2007)
1103 Paulsen	High	
Riparia Gloire	Low	POUGET & DELAS (1989)
101-14		
161-49		
41 B		
3309 Couderc	Moderate	
Gravesac		
SO 4		
420 A		
Fercal		
110 Richter	High	
140 Ruggeri		
1103 Paulsen		

Table 6. Tolerance of rootstocks to drought (Lavrenčič et al., 2007, Pouget & Delas, 1989).

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Cregg (2004) stated that to compare the relative tolerance among different genotypes, the variables to evaluate are as follows: survival potential, growth capacity, and water use efficiency based of morphological and physiological adaptations that might occur in the plant.

The most drought-tolerant grapevine species are V. arizonica, V. californica, V. champinii, V. doaniana, V. gidriana, and V. longii. The lowest tolerance was observed in V. berlandieri, V. cinerea, V. lincecumii, V. riparia, and V. solonis. V. rupestris showed only a moderate tolerance to drought (Padgett-Johnson, et al., 2003).

V. cinerea can assure not only a complete phylloxera resistance; it also shows a positive influence on scion performance especially in shallow, gravely, and consequently dry soils. Phylloxera-resistant *V. cinerea* hybrids are therefore recommended for vineyards established in sites with generally dry conditions. In dry locations *V. riparia x V. cinerea* hybrids represent a valuable expansion of the range of rootstocks currently available in Germany. Particularly on steep slopes and in seasons with rare rainfall the results obtained with these hybrids were superior (Schmidt *et al*., 2005).

Basing on the evaluation of all traits of tolerance to drought of research in the Czech Republic it is possible to conclude that the highest number of drought-tolerant hybrids originated from the crossing of Binova x Börner so that there is a very good chance to use the rootstock Börner and *Vitis cinerea* for further breeding and selection of rootstock resistant to drought stress. However, hybrids with *Vitis rupestris* and *Vitis amurensis* in their pedigrees show only a medium resistance to drought stress. (Pavloušek, 2011b).

The occurrence of drought is also very closely correlated with the overall soil conditions of the site. For that reason it is recommended to select individual rootstocks with regard to the type of soil and also to contents of loamy, clayey and sandy particles within the soil profile.

White (2009) arranged rootstocks with regard to their drought tolerance and pedological conditions of the site in the following manner (Table 7):

Soil profile characteristics	Vineyard water status	Recommended rootstocks
Soil depth < 20 cm: sand, loam or clay including any root-impeding subsoil	Dry soil	110 Richter, 140 Ruggeri, 1103 Paulsen
	Irrigated soil	110 R, 140 Ru, 1103 P, Ramsey
Soil depth 20-75 cm, sands, loams or clays, with no root-impeding subsoil.	Dry soil	99R, 110R, 140 Ru, 1103P, Ramsey, Kober 5 BB
	Irrigated soil	99R, 110R, Ramsey, Kober 5BB, Teleki 5C, Schwarzmann, SO4, 420A, 101-14 (in loams and clays).
Soil depth"/> 75 cm, uniform or gradational profile of sand, loam or clay.	Dry soil	99R, 110R, 1103P, Ramsey (in sand), Kober 5BB.
	Irrigated soil	SO4, 101-14, Teleki 5C, Schwarzmann, 3306 a 3309 Couderc, 420A.

Table 7. Dependence of tolerance drought and chlorosis of rootstocks on soil conditions (WHITE, 2009).

A good understanding of physiological mechanism that enable plants to adapt themselves to the water deficit and to maintain growth also during stress periods could help within the framework of individual breeding programs to screen and select stress-tolerant genotypes (Winter *et al*., 1988).

5. Conclusion

Regarding climatic changes and a more and more frequent occurrence of periods of drought within the growing season, the problem of lime-induced chlorosis and drought damage of grapevine plants becomes to be more and more important.

Hofäcker (2004) presented a general evaluation of the drought and chlorosis resistance of rootstocks most commonly grown in Europe; results of this analysis are presented in Table 8.

Rootstock	Parentage	Country of origin	*Drought resistance	*Chlorosis resistance
5 BB	*V. berlandieri x V. riparia*	Austria	+++	+++
SO4	*V. berlandieri x V. riparia*	Germany	+++(+)	++++
Binova	*V. berlandieri x V. riparia*	Germany	+++(+)	++++
125 AA	*V. berlandieri x V. riparia*	Austria	++(+)	+++(+)
5C	*V. berlandieri x V. riparia*	Hungary	+(+)	++/+++
Teleki 8B	*V. berlandieri x V. riparia*	Hungary	+++(+)	++++
420A	*V. berlandieri x V. riparia*	France	++++	++(+)
161-49 Couderc	*V. berlandieri x V. riparia*	France	+	+++++
R.S.B.1	*V. berlandieri*	France	+++	+++++
140 Ruggeri	*V. berlandieri x V. rupestris*	Italy	++++	++++
1103 Paulsen	*V. berlandieri x V. rupestris*	Italy	++++(+)	++++
775 Paulsen	*V. berlandieri x V. rupestris*	Italy	++++(+)	++++
Richter 110	*V. berlandieri x V. rupestris*	France	++++	++++
Richter 99	*V. berlandieri x V. rupestris*	France	++++	+++
3309 Couderc	*V. riparia x V. rupestris*	France	+++	+(+)
Schwarzmann	*V. riparia x V. rupestris*	Czech Republic	++(+)	++
101-14 Millardet de Grasset	*V. riparia x V. rupestris*	France	+(+)	+(+)
Cosmo 2	*V. berlandieri x V. riparia*	Italy	+++	+++
Cosmo 10	*V. berlandieri x V. riparia*	Italy	+++	+++

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Rootstock	Parentage	Country of origin	*Drought resistance	*Chlorosis resistance
Riparia Glorie de Montpellier	*V. riparia*	France	+	+++(+)
Rupestris du Lot	*V. rupestris*	France	++	++
Börner	*V. riparia x V. cinerea*	Germany	+++(+)	++(+)
Rici	*V. riparia x V. cinerea*	Germany	+++(+)	++(+)
Cina	(*V. berlandieri x V. riparia*) x *V. cinerea*	Germany	+++(+)	++(+)
Sori	*V. solonis x V. riparia*	Germany	+++	++
1616 Couderc	*V. solonis x V. riparia*	France	+++	++
Gravesac	161-49 C x 3309 C	France	++++	+++
Fercal	(*V.berlandieri* x Colombard) x / *V.berlandieri* x (*V. riparia x V. rupestris x V. candicans*)/	France	++	+++
Sorisil	Sylvaner x 1616 C	Germany	++++	+++(+)
26 G	Trolinger x *V. riparia*	Germany	+++	++++
41B Millardet de Grasset	Chasselas blanc x *V. berlandieri*	France	++++	+++
333 E.M.	Cabernet Sauvignon x *V. berlandieri*	France	++++	++++
Golia	Castel 156-12 x *V. berlandieri*	Italy	+++(+)	+++
Georgikon 28	Kober 5 BB x *V. vinifera*	Hungary	++++	++++

Note: + = Very low, ++ = Low, +++ = Medium, ++++ = High, +++++ = Very high

Table 8. Drought and chlorosis tolerance of the most common European rootstocks (adapted after *Hofäcker, 2004).

Effects of lime-induced chlorosis and drought of grapevine rootstocks are therefore very important, especially in association with a better understanding of effects of these abiotic factors on grapevine on the one hand and the possibility of the use of such a knowledge when breeding and selecting rootstocks on the other.

The aim of this survey of literature is to present a complete overview of rootstock tolerance to two important abiotic factors – lime and drought.

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Chapter 10

Abiotic Stress Tolerance in Plants with Emphasizing on Drought and Salinity Stresses in Walnut

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Additional information is available at the end of the chapter

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1. Introduction

Drought and salt stress, together with low temperature, are major problems for agriculture because these adverse environmental factors prevent plants from realizing their full genetic potential. Salt stress afflicts agriculture in many parts of the world, particularly irrigated lands [4]. Compared to salt stress, the problem of drought is even more pervasive and economically damaging [1; 3]. Temperature and precipitation are key determinants of climate. The Koppen Climate Classification System recognizes five major climatic types: A, Tropical Moist Climates; B, Dry Climates; C, Moist Mid-latitude Climates with Mild Winters; D, Moist Mid-latitude Climates with Cold Winters; and E, Polar Climates. The Dry Climates are easily recognized (a desert is after all a desert) but water-limited environments can be difficult to classify precisely [30]. Meigs [30] developed a widely used system for classifying water-limited environments based upon mean precipitation. Extremely arid lands have at least 12 consecutive months without rainfall, arid lands have less than 250 mm of annual rainfall, and semiarid lands have a mean annual precipitation of between 250 and 500 mm.

Drought stress signaling certainly merits separate treatment. Nevertheless, most studies on water stress signaling have focused on salt stress, primarily because plant responses to salt and drought are closely related and the mechanisms overlap. Salinity is detrimental to plant growth, causing nutritional constraints by decreasing uptake of phosphorus, potassium, nitrate and calcium, ion cytotoxicity and osmotic stress. Under salinity, ions like Na^+ and Cl^- penetrate the hydration shells of proteins and interfere with the function of these proteins.

Uptake of abundantly available Na^+ and Cl^- therefore, offers a comparatively cheap way to lower the tissue-osmotic potential. To avoid the risk of ion toxicity associated with this strategy, Na^+ and Cl^- are generally compartmentalized in the vacuole and/or in less sensitive tissues [228]. In parallel, adjustment of the cytoplasmic compartment is achieved via production of

compatible osmolytes such as, proline, mannitol, sorbitol, and glycine betaine. The latter also acts as an antioxidant and thus detoxifies reactive oxygen species (ROS) [227]. Ionic toxicity, osmotic stress, and nutritional defects under salinity lead to metabolic imbalances and oxidative stress. From a practical point, salt stress can be imposed more easily and precisely in laboratory settings. Although the importance of salt and drought stress signaling was recognized long ago, few molecular components were known until recently.

Also, drought, salinity, extreme temperatures and oxidative stress are often interconnected, and may induce similar cellular damage. For example, drought and/or salinization are manifested primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell [5-6]. Oxidative stress, which frequently accompanies high temperature, salinity, or drought stress, may cause denaturation of functional and structural proteins [7]. As a consequence, these diverse environmental stresses often activate similar cell signaling pathways [8] and cellular responses, such as production of stress proteins, up-regulation of anti-oxidants and accumulation of compatible solutes [9-11]. Compatible solutes are small organic metabolites that are very soluble in water and are non-toxic at high concentrations. Therefore, breeding for drought and salinity stress tolerance in agronomy and horticultural crops (for food supply) and in forest trees (a central component of the global ecosystem) should be given high research priority. Molecular control mechanisms for abiotic stress tolerance are based on the activation and regulation of specific stress related genes. These genes are involved in the whole sequence of stress responses, such as signaling, transcriptional control, protection of membranes and proteins, and free-radical and toxic-compound scavenging. Recently, research into the molecular mechanisms of stress responses has started to bear fruit and, in parallel, genetic modification of stress tolerance has also shown promising results that may ultimately apply to agriculturally and ecologically important plants [180].

Persian walnut (*Juglans regia* L.) is one of the most economically valuable tree species of northwest, northeast and central regions of Iran. Natural distribution of this species is quite sensitive to site water status [213]. Walnut trees need large amounts of water for optimum growth and productivity and are among the more sensitive plants to abiotic stresses [14]. The majority of walnut trees in the world are propagated by seed or by grafting onto seedling rootstocks [101]. Hence, there is huge genetic diversity among rootstock traits. For example, there are many old Persian walnut trees in Iran that have been planted on the banks of rivers. The survival of these trees for hundreds years may indicate possession of valuable stress resistance genes that help them cope with unfavorable environmental conditions [101].

Finding genetic resources tolerant to abiotic stress at different growth stages is important for such arid and semiarid regions. In studies of fruit trees, half-sib progeny of several species (for example, 'Serr' walnut, 'Texas' almond, 'Lovell' and 'Missouri' peach) have been used for producing rootstocks [180]. Because half-sibs are individuals that have one parent in common and differ in the other parent, the mean genotypic value of the group of half-sibs is by definition half the breeding value of the common parent. This reduces the number of seedlings of half-sib families needed as replicates for studying tolerance genes in rootstock breeding programs [213; 219].

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The various parts of a walnut tree differ in their needs during the year for photosynthate for respiration, growth of new plant parts, and developing nut crop. Depending on the cultivar, heavy crop loads may adversely impact the following year's crop by reducing female flower initiation and the amount of stored carbohydrates. The processes associated with nut production appear to be under strong genetic control; thus annual heavy nut production will require selection of seedlings of walnut cultivars exhibiting multiple leaf layers to maximize photosynthetic production, tendencies toward lateral bearing, good resistance to anthracnose, and efficient use of photosynthesis for tree growth and nut production.

The genus *Juglans* consists of four sections. Three of these, Rhysocaryon (black walnuts native to the Americas), Cardiocaryon (Japanese, Manchurian and Chinese walnuts, including selections known as heartnuts) and Trachycaryon (the butternut of eastern North America), exhibit thick shells and non-dehiscent hulls [234]. The fourth section, Juglans, is comprised of a single species, *Juglans regia* L., distinguished by a dehiscent hull which separates from the shell at maturity [234]. *J. regia*, the Persian walnut, is native to central Asia and grows as a wild or semi-cultivated tree in a wide area from south-eastern Europe and the Caucasus to Turkey and Iran, through southern portions of the former Soviet Union into China and the eastern Himalayas. It has been cultivated for its nut crop for at least several thousand years and was probably introduced into European commerce and agriculture by the ancient Greeks. It was prized by the Romans as Jovis glans and was utilized in medieval Europe as an herbal medicine, particularly for brain and scalp ailments. Since its introduction into North America it has commonly been referred to as the English walnut to distinguish it from the American black walnut while the correct name is Persian walnut [15; 217].

Species of walnut are distributed in temperate and subtropical areas of the Northern Hemisphere, mainly in mountain forests. Walnuts are distributed in three separate regions, Mediterranean, East-Asian Himalayas, and North American. Walnut is deciduous, monoecious, and wind-pollinated. Walnut trees are sharply differentiated from other fruit trees by their size and vigor, tree height, and crown diameter, often reaching 30 m and trunk diameters as large as 2 m. Trees may have single trunks or be multi-stemmed [13].

The pedigree of major seedlings of walnut cultivars and advanced selections in the breeding program in California is shown in Figure 1.

Walnut (*Juglans spp.*) is generally very sensitive to specific ion toxicities [218]. but genetic variation in growth indices and morphological, physiological, biochemical and cellular responses to water stress, especially in germination and early growth of walnut seedlings, have been studied to some extent. There are clear economic incentives for identification of drought-adapted walnut genotypes that can be used successfully in extensive arid and semi-arid regions. Our preliminary work has identified some walnut seedlings that are very tolerant to drought and especially to salt stresses at the germination stage [192; 212]. Mechanisms of adaptation and tolerance in selected walnut rootstocks of walnut were also investigated.

To facilitate breeding for improved water use and drought resistance, a number of questions should be addressed: (1) What physiological traits contribute to efficient water use and high drought resistance and how do they interact with traits of rapid growth? (2) What is the range

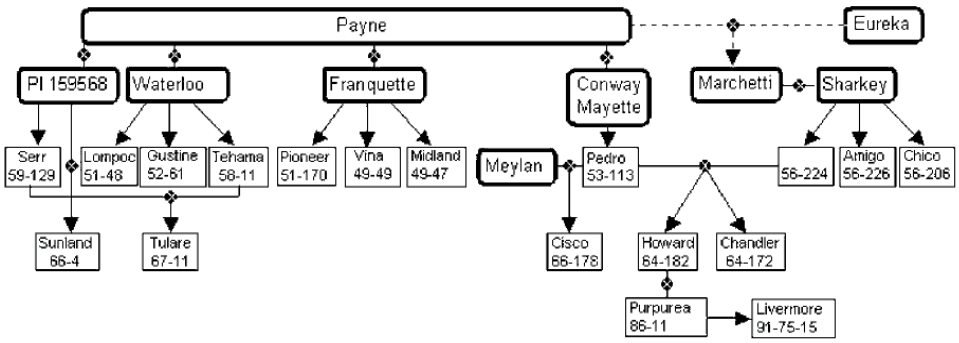

Figure 1. Pedigree of major seedlings of walnut cultivars and advanced selections in the breeding at the California, Davis, [Courtesy Dandekar et al., 2004]

of variation among walnut varieties in critical traits setting the potential for breeding? (3) Can diagnostic tools be developed for identification of critical traits that could serve as selection tools in breeding programs? In this chapter we review and discuss the available literature and current knowledge regarding abiotic stress in walnut. Following we explain the morphological, physiological and molecular aspects of abiotic stress responses in plants emphasizing on wlanut in three separate sections and summarize related research in each case.

2. Morphological responses to abiotic stresses

2.1. Soil-root interface and water absorption

The efficiency of any tree in terms of water relations depends on its ability to absorb water at a rate able to prevent internal water deficits during periods of high transpiration. Water supply to trees implies two major steps: absorption and transport of water (i.e. ascent of sap), both driven by transpiration. The efficiency of soil water absorption in trees depends on both spatial extension and density of their root system [18]. Spatial extension: water uptake by individual trees depends on fine root exchange surface, i.e., on their cumulated length or biomass. Both vertical root distribution and seasonal root growth dynamics depend closely on physical soil properties (mainly texture like clay content, bulk density, content of coarse elements etc.) and the physiological constraints on root survival and development (water table, oxygen supply, nutrients, aluminum or manganese toxicity, soil pH). Climate itself could also influence fine root dynamics [19]: data from a literature survey support the view that rainfall is one of the major environmental factors controlling fine root biomass [20].

Surprisingly, an exceptionally small fine root biomass was detected in a dry beech stand when compared to five other stands with higher rainfall [20]; this could be due to a large mortality of fine roots during peak drought. It is also well established under continental [21-22] or like mediterranean climates [21] that soil water uptake displays a gradual downward shift as the

soil dries out, and that a small fraction of total fine roots, growing deeper into the soil, ensures the overnight recovery of the soil to tree water potential equilibrium [22], and supports a fraction of tree transpiration during periods of stomatal closure. At least this small fraction of root systems enables survival of the trees by providing the unbearable amount of water. Most of the studies reported the occurrence of two periods of active root growth in fruit trees, namely during spring and early autumn. The two periods of slowest root growth occur during winter and summer, and coincide with lowest soil temperatures and with lowest soil moisture, respectively [21-22].

With its large canopy and expansive root system, the walnut tree (*Juglans regia*) has more specific soil and water requirements than most other temperate-zone fruit trees. However, its cultivation has been extended more recently to marginal lands with inappropriate soil quality and limited water resources, aggravating the problems of nutrition and irrigation management. A study conducted during 1999-2000 in a 13-year old walnut orchard established on a shallow flood plain with stratified sand and loam soil was undertaken to diagnose the main factors that adversely affect walnut growth and yield. Results showed that high boron (B) concentration in irrigation water (>300 ppm) was the main factor inhibiting tree growth, reducing expected yield by 94 % and adversely affecting nut quality. Shallow soil, light soil texture, and deficiency in micro and macro elements also adversely affect orchard establishment and lead to poor yield and low nut quality [180].

2.2. Strategies for water economy

Homoiohydric plants have evolved a hierarchy of protective mechanisms that maintain favorable protoplasmic water content or modify the deleterious effects of stress on cellular constituents. In contrast, poikilohydric plants are unable to control water loss to the environment with the result that cellular water content fluctuates in concert with external water availability. The prefixes homo- and poikilo- are widely used in terminology related to eukaryotic physiology. For clarity they are defined, by the Oxford English Dictionary (http://dictionary.oed.com), as 'of the same kind' and 'variegated', respectively. However, we maintain that no plants are homoiohydric in the strict definition of the term because plants are incapable of maintaining their water content at a fixed value. Plants cannot create water where none exists, and ultimately all plants are unable to control water loss to the environment [12].

In the dry season, plants with deep root systems are believed to take water from the deep soil layers, thereby avoiding or minimizing water stress [28]. However, detailed studies of soil water status, root distribution, water resource derivation and shoot water stress development under natural, varied moisture conditions during the same time period were lacking for walnut until 2011. Of interest is that deep water resources can compensate for drought in the air and upper soil layers. For example, *Juglans regia*, which has an extensive root system, has a wide distribution in the mountainous regions of northern, central and west Iran and northern China. This could show that the ecophysiological responses of the aboveground shoots of *J. regia* in response to drought in their natural habitat, as well as under controlled greenhouse conditions, have been well studied and the mechanisms underlying these shoot responses are well understood [16; 121]. Also, detailed studies on

the role of below-ground root structures of *J. regia* during the development of water stress in field environments and variation in soil water uptake and its effect on plant water status during dry and wet seasons have been published [17].

In recent years, hydrogen and oxygen isotopic application has contributed significantly to tracing and understanding below-ground processes [23-24]. During water transport between roots and shoots, the isotopic composition of xylem water remains unaltered from that of the soil [23]. Therefore, it is reasonable to analyze the branch xylem water to determine the water source [25]. Soil water is also a key factor in restoring forest ecosystems in arid and semi-arid zones [26], while the efficiency of soil water uptake by trees could be the ultimate determining factor in their productivity [26-27]. Therefore, knowledge of root distribution and mechanisms of soil water extraction and transport by trees is indispensable for successfully restoring ecosystems.

Walnut root growth differs in dry and wet seasons [17]. Mean root length in both the upper (0-30 cm) and deep (30-80 cm) soil layers shortened when the soil water content and relative humidity of the air were lower [17]. After rain events, re-watering, or irrigation, the total root length increased compared with dry periods [17]. The abundance of new roots significantly increased in both the upper and deep soil layers in response to the rain and rewatering events. The growth of new roots was greater in the upper soil profile than in the deep soil profile. Dead root length in the upper soil layer was significantly greater in the wet season than in the dry season, while no difference in dead roots has detected in the deep soil layer between the seasons and diameter of the roots did not significantly change by season [17]. Water supply to trees involves two major steps: absorption and transport of water (i.e. ascent of sap), both driven by transpiration. The efficiency of soil water absorption in trees depends on both spatial extension and density of their root system [18].

There is significant variation in the vertical distribution of roots among different walnut varieties [17; 212]. Roots are the most abundant at 10-30 cm depth, followed by 0-10 cm depth. Root biomass decreases with depth below 30 cm. Generally, most of the root surface area, root length density and root biomass were confined to the upper soil layers (0-30 cm), accounting for 61, 62.5 and 79% of the total root measurements from the 0-80 cm soil layers, respectively [17]. Walnut roots were mainly distributed in the upper soil layers at our study sites and likely in the whole region. Soil moisture was a key factor regulating root growth and water uptake efficiency of the roots [17; 212]. The shallow roots had reduced efficiency in water uptake in the dry season, and therefore *J. regia* was compelled to extract a greater ratio of water from the deep soil layers. However, the shift was not able to prevent water stress on the plants, which were characterized by increased pre-dawn branch xylem PLC, reduced pre-dawn leaf water potential and transpiration with soil drying [17]. In addition to serving as an indicator of water sources, changes in the stable-hydrogen isotope (δD) values in walnut branch xylem water reflected plant water status and the severity of soil drought.

2.3. Excess water supply

On soils subject to flooding or with shallow restrictive layers, excess soil moisture can also be a problem. Excess soil moisture during the growing season leads to decreased oxygen in the

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soil and death of roots needed to absorb adequate soil water during periods of high transpiration. On soils with restrictive layers in the walnut rooting zone, soil water accumulates above the restrictive layer leading to a perched water table during the dormant season. Walnut roots within the perched water table die from a lack of oxygen. If these roots are not replaced during the growing season, it results in a reduced capacity to absorb soil moisture during the following growing season, followed by stomatal closure from moisture stress and subsequent decreases in the rate of photosynthesis [14].

2.4. Deficient water supply

Insufficient available soil moisture causes stresses that can lead to wilting and premature defoliation under extreme conditions. Under less extreme conditions, the stomata close to decrease the rate of transpiration. When this occurs, carbon dioxide can no longer enter into the leaves through the stomata and photosynthesis decreases. If walnut orchards are not going to be irrigated, then soil depth and water holding capacity become very important during site selection for the walnut orchard. The water held within the rooting zone determines if adequate soil moisture is available during dry spells. In the central hardwood region, droughts usually occur in late summer when there is a high demand for photosynthesis to fill the developing nuts. Lack of adequate soil moisture in late summer can also affect the physiological condition of the tree and suppress the initiation of female flowers necessary for the following year's crop [58].

2.5. Germination under abiotic stress conditions

The germination percentage of walnut seeds of eighteen cultivars decreases significantly in response to decrease in (more negative) water potentials and increases of salinity level. Decreasing the water potential to –1.0 MPa reduced the germination of all varieties to less than 50% and at –1.50 MPa, the germination decreased to less than 25% [192]. The drought and salt stress treatments were unaffected by the size of seeds or seed weight and there was not a significant correlation between percent germination and either seed or kernel weight [212]. Seedlings of walnut cultivars showed differential responses to salt stress under greenhouse conditions. Increase in salinity levels decreased root and shoot length, diameter, and fresh and dry mass, especially those of shoots. Seedlings of 'Lara' and 'Chandler' were most and least affected by salt stress, respectively. The increase in salinity levels was accompanied by a substantial decrease in root RWC (relative water content) [212]. Seed germination rates were generally more rapid in control (no salt stress) than in salt containing media. The FGP (final germination percentage) values were significantly lower at higher salinity levels and there were also differences in FGP among species [212].

The mean germination time differed both among different treatments and cultivars and also a significant interaction was found between these two factors under salt stress condition [212]. For all the seedlings of walnut cultivars studied, the mean germination time was shorter in the control than in the other treatments [212]. There were also differences in the mean germination time among cultivars. At high salinity levels (200 and 250 mM), the average mean germination

time for 'Chandler' and 'Panegine20' was 2.3 to 5.2 d and 2.6 to 5.4 shorter, respectively, than those observed in the other cultivars [192; 212].

In the study of 18 walnut cultivars, the lengths of nuts were varied from 2.07 (± 0.47) cm for 'Lara' to 4.08 (± 0.78) cm for 'K72'. Seed size could be a factor affecting germination in stressed media [212]. Many studies have shown that various seed sizes and weights may behave differently in terms of germination under stress conditions [180; 212]. It is generally believed that large seed sizes have a higher propensity for germination in saline and dry media. However, some previous studies, found a negative relationship between seed size and germination capacity in *Trianthema triquetra* L. Within the range of seed sizes studied, we did not observe any significant differences in the germination response and analysis failed to show any relationship between percent germination and seed weight under both salt and drought stress.

2.6. Wilting

The amount of water lost before visible leaf wilting varies by species. Temporary wilting is the visible drooping of leaves during the day followed by rehydration and recovery during the night. During long periods of dry soil, temporary wilting grades into permanent wilting. Prolonged permanent wilting kills trees [14; 212; 220]. The relation between water loss from leaves and visible wilting is complicated by large differences among species in the amount of supporting tissues leaves contain. Leaves of black cherry (Prunus), dogwood (Cornus), birch (Betula), and basswood (Tilia) wilt readily. Leaf thickness and size do not prevent wilting. Rhododendrons are also extremely sensitive to drought with leaves that curl, then yellow and turn brown. By comparison, the leaves of holly and pine are supported with abundant sclerenchyma tissue (i.e. tough, strong tissue) and do not droop readily even after they lose considerable water.

2.7. Leaf shedding

In normal abscission, an organized leaf senescence process, which includes the loss of chlorophyll, precedes leaf shedding. With severe drought, leaves may be shed while still full of valuable materials [220]. For example, sycamore (*Platanus*) sheds some leaves, and buckeye (*Aesculus*) may shed all of its leaves, as drought continues. On the other hand, leaves of dogwood (*Cornus*) usually wilt and die rather than abscise. Many times these leaves are stunted [220]. Walnut is also known to shed leaves in response to drought [61]. Sometimes drought-caused leaf shedding may not occur until after rehydration. Abscission can be initiated by water stress but cannot be completed without adequate water to shear-off connections between cell walls. The oldest leaves are usually shed first [220].

Injury to foliage and defoliation are most apparent in portions of the crown that are in full sun. These leaves show drought associated signs of leaf rolling, folding, curling, and shedding. Over the past 20 years, our knowledge of the hydraulic architecture of trees has increased and some hypotheses have been raised to explain how trees might be designed hydraulically to help them cope with period of drought [220]. Hypotheses have generally invoked a mechanism

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that permits plants to shed expendable distal components of its shoots while preserving other parts that represent years of carbon investment. Leaf shedding is a potentially cost-effective way for plants to deal with drought stress by a plant segmentation mechanism.

2.8. Growth inhibition

Growth of vegetative and reproductive tissues of walnut is constrained by cell initiation shortages, cell enlargement problems, and inefficient food supplies. Cell enlargement depends upon hydraulic pressure for expansion and is especially sensitive to water stress. Cell division in generating new cells is also decreased by drought.

2.9. Shoot growth

Internal water deficits in trees constrain the growth of shoots by influencing development of new shoot units (nodes and internodes). A period of drought has a carry-over effect in many species from the year of bud formation to the year of expansion of that bud into a shoot. Drought also has a short-term effect by inhibiting extension of shoots within any one year. The timing of leaf expansion is obviously later than that of shoot extension. If shoot extension finishes early, a summer drought may affect leaf expansion but not shoot extension [220].

Shoots of some trees elongate for only a few weeks in late spring. This growth form is called fixed or determinant growth. Other species elongate shoots over a period of several months which is called multiple flushing or continuous growths. A late July drought may not affect current-year shoot elongation in species with fixed growth, like oaks. Oak shoots expand only during the early part of the growing season [220]. A late July drought can inhibit expansion of shoots from multiple flushing species, like sycamore, which elongate shoots during much of the summer. Spring and summer droughts damage both types of trees. In the southern pines, late summer droughts will influence expansion of shoots in the upper crown to a greater extent than those in the lower crown [212; 220]. This is because the number of seasonal growth flushes varies with shoot location in the crown. Shoots in the upper crown normally exhibit more seasonal growth flushes than those in the lower crown. Buds of some lower branches may not open at all [220].

In walnut, the maximum decrease in shoot fresh weight was observed after 4 days of osmotic stress treatment [192; 212-213]. Response of half-sib families differed as the severity of water and salt stress increased. Under severe osmotic stress (–1.50 MPa), offspring of 'Panegine20' and 'Chandler' produced the greatest shoot fresh weight [212].

Available water, more than any other resource, determines the annual growth potential of individual trees. Variations in water availability account for up to 80% of the inter-annual variability in size increment in temperate stands. Tree water deficits dramatically reduce height and radial growth as well as bud production [168]. Abiotic stress experiments on one and two year old trees of promising walnut varieties showed the same trends [192; 212-213]. Twig growth patterns are affected during several years, as demonstrated by Fulton and Buchner [14] for Persian walnut in California. Recovery from the previous drought was still not complete when the next drought began, and induced even further

growth suppression. A similar reduction of twig growth over several years after drought was also seen in black walnut [214-215].

2.10. Cambial growth

Cambial growth slows or accelerates with rainfall. Cambial growth is constrained by water supply of both the current and previous year. Last year's annual growth ring of wood affects growth material supply on this year's growth [220]. This year's drought also will affect next year's cambial growth. Such a delayed effect is the result of drought impacts upon crown development, food production, and tree health. Drought will produce both rapid and delayed responses along the cambium [220]. Shoot thickness of seedlings of sensitive and semi-tolerant walnut genotypes decreased significantly in response to increased osmotic stress [212-213].

The stem of a woody plant comprises several different cell/tissue layers [222], from the periphery and inwards: the protective outer bark; the inner bark with the phloem responsible for sugar transport from leaves to roots; the vascular cambium responsible for growth of new phloem outwards and new xylem inwards; and the mature xylem responsible for water transport [222]. Transport occurs in conduits, comprising separate cell elements, interconnected by pores in their walls and/or series of cell elements forming vessels; all water conducting cell elements die after completion of secondary cell wall growth and are then filled with water. Zwieniecki et al. [37; 88] suggested that the interconnecting pores have a variable diameter, since pectin is present in the pores and acts as a hydrogel in response to variable ion concentration in the transported water.

2.11. Root growth

When roots are exposed to drought, the allocation of food to root growth may increase [220]. This provides more root absorptive area per unit area of foliage and increases the volume of soil colonized. Extended drought leads to root suberization to prevent water loss to the soil. Good water absorbing ability, coupled with a low transpiration rate for the amount of food produced (high water-use efficiency), allows trees a better chance to survive drought conditions [220]. The annual root system (absorbing roots) takes up a majority of the water in a tree. Annual roots are not the woody roots seen when a tree is dug. Large woody roots have bark. Any bark crack or damage is quickly sealed-off so little water flows through these areas. It is the young roots, the roots easily damaged by drought, which are the major absorbers of water and essential elements in a tree [220].

In a study on walnut, under drought and salt stress, root length and dry weights for the seedlings of many genotypes decreased significantly in response to increased osmotic stress levels. Albeit under high osmotic pressure due to drought or salt stress root length was greatest in the most tolerant varieties, 'Chandler' and 'Panegine20' [212-213]. Root dry weight of most genotypes decreased significantly in tolerant genotypes vs. non-tolerant ones. Tolerant genotypes ('Chandler' and 'Panegine20' and relatively 'Hartley'), had more or less similar trend in term of root length and dry weight and did not show significant differences at high Ψs.

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Generally, the root component accounts for 20 to 90% of the total resistance (reciprocal of conductance) of the plant [38]. This variability largely reflects differences in the proportion of roots, their anatomy and the depths at which they grow [39; 41]. The resistance to water transport in roots is initially relatively high as water has to pass a complex anatomical structure before reaching the conduits of the xylem [40; 42].

The importance of roots for plant water relations increases with the onset of drought for several reasons. First, root growth is typically favored over leaf growth early on during drought, thus growth of the organ exploiting the most limiting resource is favored [43]. Second, under more severe conditions of drought, root layers may shrink or lateral roots may die from dehydration causing deteriorated contact with soil particles holding water, thus increasing the resistance of hydraulic water transport from soil to roots [44; 46]. Third, roots seem to be particularly prone to suffer cavitation of conduits. In many species, including poplar [45; 47], willows and walnut [212] roots are more vulnerable to xylem cavitation than shoots.

2.12. Root and shoot water content

Tissue water content may be expressed in several ways, including the amount of water per unit dry or fresh weight and per unit weight of water at full hydration. Fresh weight seems to be the less accurate of them to measure tissue water content because is highly influenced by changes in tissue dry weight [213]. Sometimes decreases in tissue water content may be more important than decreases in water potential or pressure potential in terms of influencing growth.

The vast majority of land plants, including all major horticultural plants, would be classified as drought avoiders. Although vascular plants do produce specialized structures capable of withstanding severe stress (e.g. pollen, seeds and spores), few species can survive substantial loss of water from their vegetative tissues [34 -36]. Tolerance is the ability to withstand a particular environmental condition. Under water-limiting conditions, plants will experience a net loss of water to the environment and cells will dehydrate (i.e. Ψw and relative water contents, RWC, will decline). Land plants can be classified based upon how they respond to this water deficit. Drought-avoiding plants strive to maintain elevated Ψw. Drought-tolerant plants are able to tolerate extended periods of water deficit. However, both drought-avoiding and drought-tolerant plants will reach a 'permanent wilting point' where Ψw has declined to such a degree that the plant cannot recover upon rewatering.

Under stress condition, derangement in the leaf water potential and its components takes place [31]. It is reported that the water relation and transpirational parameters are closely correlated, and in the laboratory, where equipment to quantify plant water potential are not available, determination of the RWC is still a valid parameter to quantify the plant water status [32-33].

RWC is a measure of the relative cellular volume that shows the changes in cellular volume that could be affecting interactions between macromolecules and organelles. As a general rule, a RWC about 90-100% is related to closing of the stomata pore in the leaf and a reduction in the cellular expansion and growth. Contents of 80-90% are correlated with changes in the

composition of the tissues and some alterations in the relative rates of photosynthesis and respiration.

Under salt and drought stress, different seedlings of walnut cultivars show significant differences in RWC content. Semi-tolerant ('Hartley') and tolerant ('Chandler' and 'Panegine20') cultivars of walnut have moderate and high levels of TWC and RWC at osmotic stress level [212]. RWC below 80 % usually implies a water potential on the order of -1.5 MPa or less, and this would produce changes in the metabolism, reduced photosynthesis, increased respiration and increased proline and abscisic acid accumulation.

2.13. Root biomass

Walnut root growth differs significantly between the dry season and wet season. Mean root length in both the upper (0-30 cm) and deep (30-80 cm) soil layers was shortest in early July when the soil water content and air relative humidity were lower [17]. After rewatering events, the total root length in late August and early October increased by 128% and 179%, respectively, compared with that in early July [17]. The abundance of new roots significantly increased in both the upper and deep soil layers in response to the recovery events. The growth of new roots was greater in the upper soil profile than in the deep soil profile. Dead root length in the upper soil layer was significantly higher in the wet season than in the dry season, while no difference in dead roots was detected in the deep soil layer between the seasons. In walnut, the diameter of the roots did not significantly change by season [17]. The increase in osmotic drought level was accompanied by a substantial decrease in root relative water content and differences between genotypes at different osmotic levels were highly significant [213].

There was a significant variation in the vertical distribution of roots under stress condition (Table 1). Roots were the most abundant at 10-30 cm depth, followed by 0-10 cm depth. Root biomass decreased with depth below 30 cm. Generally, most of the root surface area, root length density and root biomass were confined to the upper soil layers (0-30 cm), and accounting for 60.9, 62.2 and 78.9% of the total root measurements from the 0-80 cm soil layers, respectively.

2.14. Leaf architecture and position

Annual heavy nut production will require selection of seedlings of walnut cultivars with multiple leaf layers to maximize photosynthetic production, tendencies toward lateral bearing, good resistance to anthracnose, and efficient use of photosynthates for tree growth and nut production [212].

Leaves are extraordinarily variable in form, longevity, venation architecture, and capacity for photosynthetic gas exchange. Much of this diversity is linked to water transport capacity [17]. The pathways through the leaf constitute a substantial (≥30%) part of the resistance to water flow through plants, and thus influence rates of transpiration and photosynthesis. Leaf hydraulic conductance (Kleaf) varies more than 65-fold across species, reflecting differences in the anatomy of the petiole and the venation architecture, as well as pathways beyond the xylem through living tissues to sites of evaporation.

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Time	Depth (cm)	Surface area (cm^2)	Average diameter (mm)	Root length density (cm/dm^3)	Weight (g)
May	0-10	65.4±19.4 b	0.8± 0.2 b	466.3± 52.6 b	1.3± 0.4 ab
	10-20	103.0± 17.9 a	1.0± 0.2 a	541.7± 93.6 a	2.3± 1.1 a
	20-30	121.3± 11.6 a	1.0± 0.2 a	257.3± 26.5 c	1.8± 0.5 ab
	30-40	61.1± 16.5 b	0.8± 0.2 ab	201.9± 61.2 cd	1.2± 0.6 b
	40-50	54.0± 14.5 b	0.8± 0.1 b	179.0± 61.4 cd	0.8± 0.3 bc
	50-60	39.9± 3.8 c	0.7± 0.1 b	152.6± 24.8 d	0.6± 0.1 c
	60-70	23.7± 3.3 d	0.6± 0.1 bc	112.7± 27.4 de	0.3± 0.1 d
	70-80	13.5 ±1.8 e	0.4± 0.1 c	81.0 ±18.0 e	0.2 ± 0.1 d
October	0-10	82.1± 32.6 b	0.7± 0.2 c	539.9± 78.6 b	2.3± 0.7 ab
	10-20	142.6± 53.3 a	0.8± 0.2 bc	720.7± 82.5 a	2.9± 0.7 a
	20-30	131.8± 35.1 a	1.3± 0.4 a	408.7± 52.9 c	3.6± 1.3 a
	30-40	76.1± 20.4 b	1.0± 0.4 ab	344.9± 39.5 cd	2.0± 1.1 ab
	40-50	60.7± 15.9 bc	1.0± 0.3 b	230.2± 25.4 e	1.3± 0.7 b
	50-60	40.5± 12.0 c	0.8 ±0.2 bc	190.2± 31.4 e	0.7± 0.5 bc
	60-70	37.5 ±13.5 cd	0.9± 0.2 bc	171.3± 22.1 e	0.5± 0.3 c
	70-80	25.0± 9.3 d	0.8± 0.2 c	120.5± 20.9 f	0.2± 0.2 d

Table 1. Vertical root distribution in different soil layers. Roots from four different distances (50, 100, 150 and 200 cm) from the tree trunk in the same soil layer from each sampling location were pooled. Three sampling locations were used. Means and SD are shown (n= 3). Different letters refer to significant difference at the $P \leq 0.05$ level within the same sampling time [Courtesy Sun et al., 2011].

Angle between main stem and lateral branches, lateral branches and petioles of leafs are the most suitable morphological markers for cultivar screening in walnut [213]. Under stress, the angle between the main stem and lateral branches and especially angle between lateral branches and petioles of leaves showed significant decreases [213].

Drought during the year of bud formation decreases the number of new leaves formed in the bud and the number of new stem segments (internodes) present. These phenomena were observed for several walnut varieties during the first and second years of growth [212]. Drought then influences the number of leaves, leaf surface area, and twig extension the following year when those buds expand [212]. Summer droughts can greatly reduce shoot

elongation in species that exhibit continuous growth or multiple flushing. Drought may not inhibit the first growth flush that usually occurs before peak drought intensity, but may decrease the number of nodes formed in the new bud that will then expand during the second (or third, etc.) flush of growth. If drought continues, all growth flushes will be affected [212].

As a consequence, severe drought limits leaf area production by reducing the number and viability of leaf buds and thus the tree's ability to recover an efficient crown development after resuming normal water availability [212]. At the stand level, leaf area index may be reduced by as much as 2–3 the year following a severe drought [212-213], without any tree mortality, and the recovery of LAI to pre-drought levels may require several years. Leaf area index of walnut stands may also decreases after severe drought, due to an abnormal shedding of older leaves. Such a reduction in tree leaf area has also been reported from crown transparency observations, as used for tree vitality assessment in European forest condition monitoring and in walnut stands of Iran [169; 213]. When too much or too little water is applied repeatedly over the life of the orchard, it may be at the expense of overall productivity and orchard longevity [14].

3. Physiological responses to abiotic stresses:

3.1. Plant water status

During stress by water deficit, the water status of the plants plays a key role in the activation of defense mechanisms. Contrasting results under the same experimental conditions can be related to difference in species, growth conditions, and stage of the plants [221]. Decline in relative water content in the walnut seedlings at different osmotic potentials was paralleled by a substantial decrease in water potential (Ψw), especially in tolerant genotypes (Figure 2). Values of Ψw decreased during the day and subsequently recovered and re-equilibrated at night, showing a pattern of progressive decline during the drought treatment. During the last day (29^{th} day) of the drought treatment, Ψw decreased in all plants subjected to drought stress. But in 'Panegine20' and 'Chandler' progeny, there was a quick reduction in Ψw from –1.8 MPa in control plants to –4.9 at –2.0 MPa of osmotic treatments (Figure 2). So these genotypes have mechanisms (like ion homeostasis, osmotic regulation) to keep an osmotic potential gradient in leaf and stem tissues and are tolerant to osmotic stress [213].

The water status of a plant is a function of uptake (by roots) and loss (via stomata and cuticle) of water. Water status in walnut under stress conditions was investigated in several previous studies. Parker and Pallardy [214] demonstrated genetic variation in the drought response of leaf and root tissue water relations of seedlings of eight sources of black walnut (*Juglans nigra* L.) using the pressure-volume technique. Tissue water relations were characterized at three stages of a drying cycle during which well-watered plants were allowed to desiccate and then were re-irrigated. Sources varied both in the capacity for, and degree of, leaf and root osmotic adjustment, and in the mechanism by which it was achieved. A decrease in osmotic potential at the turgor loss point (Ψπp) of 0.4 MPa was attributable to increased leaf tissue elasticity in seedlings of four sources, while seedlings of an Ontario source exhibited a 0.7-0.8

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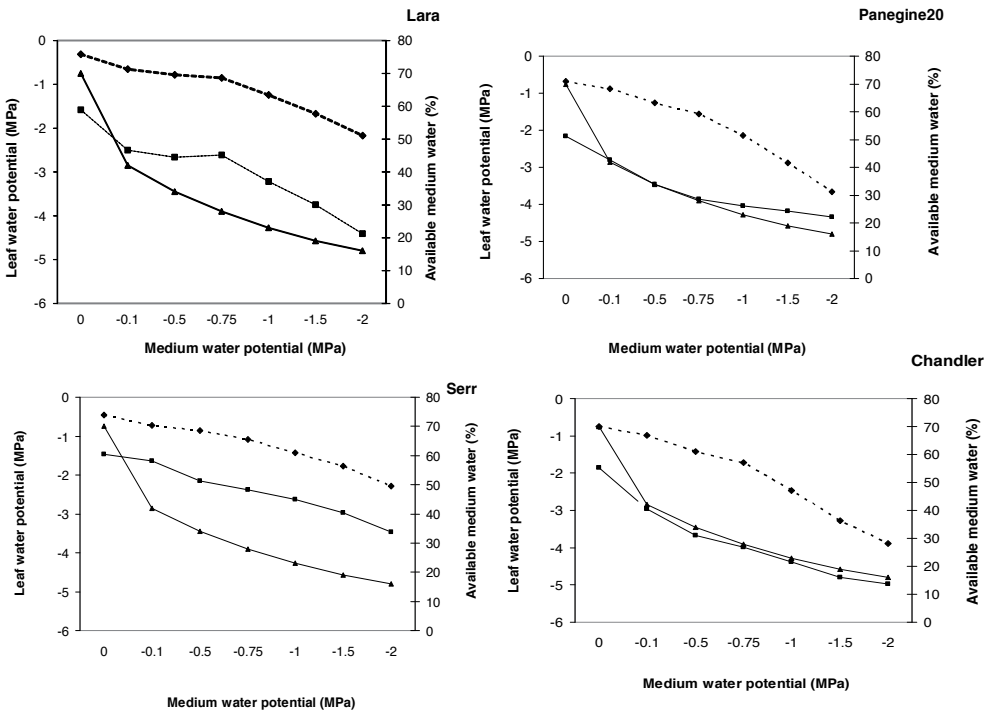

Figure 2. Patterns of predawn leaf water potential (◆), midday leaf water potential (■)and soil available water (▲) measured in walnut seedlings during drought treatments [Lotfi et al., 2010].

MPa decline in Ψπp as a result of both increased solute content and increased leaf tissue elasticity. Seedlings of a New York source showed no detectable osmotic adjustment [214].

They concluded that in roots, decreased Ψπo (osmotic potential at full hydration) and Ψπp were observed under drought. Sources that exhibited significant leaf osmotic adjustment also generally showed a similar response in roots. Tissue elasticity and Ψπo of roots were higher than those of shoots, whereas Ψπp of the two organs was similar for most sources. Because of greater elasticity, roots exhibited a more gradual decline in turgor and total water potential than did leaves as tissue relative water content decreased [214].

Cochard et al. [29] focused their analysis on some of the endogenous physiological parameters likely to be altered during a water stress and reported in the literature to be associated with stomatal responses. These parameters are the Ψ_{soil} (soil water potential), the R_{soil} (soil resistance), the R_{root} (root hydraulic resistances), and the R_{shoot} (shoot resistance); all of these parameters are strongly correlated under natural drought conditions. The experiments were designed to alter Rplant in very different ways, which probably had a primary influence on different parts of the pathway.

Soil dehydration provoked mainly a drop in Ψ_{soil} and an increase of R_{soil}. The resistance of the interface between the soil and the root, probably increase during drought stress [87]. When R_{root} modified to the extent that the radial flow into the root xylem altered. R_{shoot} probably not

altered, because the level of xylem embolism remained low during these experiments. However, if the ionic composition of the sap changed dramatically as a result of the drought, then R_{shoot} may have varied [37]. Stem pressurization provoked only an increase in R_{shoot} when the pressure exceeded the point of embolism induction (about 2.0 MPa) [89]. If the air was propagated along the xylem flow path significantly beyond the injection point, R_{root} and R_{leaf} (leaf hydraulic resistances) may also have being altered. Therefore, combining the results of all these experiments, it is possible to determine whether g_s and E_{plant} (plant transpiration) were responding specifically changes in Ψ_{soil}, R_{soil}, R_{root}, and/or R_{shoot} or not. Because air humidity, air temperature, and light intensity were maintained constant in many such experiments, leaf to air vapor deficits and leaf boundary layer conductance were also constant. Therefore, the g_s and E_{plant} patterns corresponded in drought stress. The relationship between g_s and hydraulic parameters are likely to depend on these environmental conditions, contrary to the relationships with E_{plant} [90].

The results showed that different experiments significantly reduced E_{plant} and g_s. Therefore, the response of g_s to Ψ_{soil}, R_{root}, R_{soil}, and Rshoot was neither specific nor exclusive. An alternative analysis of the problem is not to consider Ψ_{soil}, R_{root}, R_{soil}, and R_{shoot} individually but rather to examine their combined effect on P_{rachis} or Ψ_{leaf}. The relationship between P, Ψ_{soil}, R_{root}, R_{soil}, E_{plant}, and g_s under steady-state conditions is well described by the Ohm's law analogy [91]:

$$P_{rachis} = \Psi_{soil} - (R_{soil} + R_{root} + R_{shoot}) \,.\, SF\ plant \,.\, g_s \,.\, D \quad (1)$$

where SF plant is the plant leaf area and D the air vapor pressure deficit, two parameters that remained constant during experiments. The gravity term and the xylem sap osmotic potential are assumed negligible in equation 1. A similar relationship is obtained with Ψ_{leaf} if we further include the leaf blade hydraulic resistance. The dependency of g_s or E_{plant} on P_{rachis} (water pressure in the leaf rachis xylem) and Ψ_{leaf} were similar whatever the experiments. This would suggest that, g_s were not correlated to changes in Ψ_{soil}, R_{soil}, R_{root}, or R_{shoot} per se but rather to Prachis and/or Ψ leaf. An identical relationship was obtained between E_{plant} and Cplant (defined as $[R_{soil} + R_{root} + R_{shoot}] -1$). These results are in agreement with the finding of Saliendra et al. [92], Sperry [93], and Hubbard et al. [94]. Many of the studies [29; 89; 92-94] concluded that combining different experimental procedures, stomata were not responding to changes in Ψ_{soil}, R_{soil}, R_{root}, or R_{shoot} per se but rather to their impact on P_{rachis} or Ψ_{leaf} [29].

Genetic variation in tissue water relations of black walnut under drought was studied in two consecutive years by Parker and Pallardy [214]. Black walnut seedlings of some sources studied in 1983 exhibited osmotic adjustment under drought in both leaves and roots. Significant variation among sources in root tissue elasticity was also evident before drought, but was not observed thereafter. Initial differences in osmotic potential at full saturation were not evident at the point of turgor loss [214].

Walnuts close stomata under high leaf-to-air vapor pressure deficit (VPDl) or low leaf water potential (Ψl) [61], preventing the stem water potential (Ψs) from becoming lower than –1.4 MPa, when cavitation occurs in the xylem [17; 62]. Hence walnut has been defined as a "drought avoider" [61]. Daily course of Ψs and gas exchange was tested in previous studies

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of Rosati et al. [121]. Stem water potential (Ψs) decreased during the day and was lower in droughted than in control trees [121]. The lowest average Ψs values were –1.2 MPa in droughted trees and –0.4 MPa in control trees.

The decline in relative water content in Persian walnut seedlings at different osmotic potential was paralleled by a substantial decrease in water potential (Ψw), especially in tolerant genotypes [213]. Values of Ψw decreased during the day and subsequently recovered and re-equilibrated at night, showing a pattern of progressive decline during the drought treatment. During the last day (29^{th} day) of the drought treatment, Ψw decreased in all plants subjected to drought stress. But in 'Panegine20' and 'Chandler' progeny, there was a quick reduction in Ψw from –1.8 MPa in control plants to –4.9 at –2.0 MPa of osmotic treatments [213].

3.2. Stomata responses to water stress

Foliar conductance to water vapor of mesophytes and crop plants often lie in the range of 10-20 mm s^{-1} under conditions in which stomata are largely open, and these figures fall to values near 0.1 mm s^{-1} or lower-equivalent to the cuticular conductance—when stomata close [164-165]. In xerophytes and many trees, conductance under water stress can fall still lower to values approaching 0.01 mm s^{-1}. Clearly, understanding the factors that control stomatal aperture will be crucial to future developments toward improving vegetative yields in the face of increasing pressure on water resources and arable land usage.

At the same time, the guard cells that surround the stomatal pore have become a focus of attention in fundamental research. The ability of these cells to integrate both environmental and internal signals and their unique situation within the leaf tissue has provided a wealth of experimental access points to signal cascades that link membrane transport to stomatal control.

Stomata have a fundamental role in controlling two of the most important processes in vegetative plant physiology, photosynthesis and ranspiration: they open to allow sufficient CO_2 to enter the leaf for photosynthetic carbon fixation, and they close to reduce transpiration under conditions of water stress [192]. The mechanics of stomatal function are intimately connected with their morphology. On the other hand, as may be expected, estimates of the change in guard cell volume between the closed and open states of stomata vary between species because, even in one species, guard cell size can vary dependent on growth conditions and the age of the plant [192].

A study about stomatal density of leaf samples in different walnut varieties revealed that the shape and volume of stomata significantly differ among varieties [212]. Tolerant and semi-tolerant varieties had a small volume of guard cells and high stomatal density especially in the abaxial epidermis of leaves [212]. So these varieties have a high potential to maximize CO_2 entry to the leaf for photosynthetic carbon fixation and they close quickly to reduce transpiration under conditions of abiotic stress [212].

3.3. Xylem embolism under abiotic stresses

A certain degree of water stress is generally experienced by plants irrespective of life cycle and habitat [57]. Particularly in trees, the decrease in water potential may be greater, since hydraulic

resistance increases through embolism in the xylem. The plant water content recovers at night, equalizing to the soil water potential and allowing the plant to reach its highest water potential just before dawn. Trees are even more sensitive to changes in atmospheric humidity [58-59], however, and stomates close as the vapor pressure deficit between the leaf and the air increases [57]. Hydraulic conductivity of the soil and root-soil contact is potentially important in limiting water flux to roots in drying soil [60]. The xylem water potential necessary to induce this cavitation varies widely among plants [48-49] and has been shown to correlate with the lowest xylem water potentials normally experienced under natural conditions [50]. Plants tend to control stomata such that the xylem water potential does not fall below cavitation inducing pressures [51-52]. As soil moisture or humidity declines, either transpiration is reduced or leaf-specific hydraulic conductivity is increased. In this way, plants balance the demand for transpirational water loss and carbon uptake by leaves with allocation to root absorption or stem-conducting tissue [53-54; 209-210]. There is only a modest negative relationship or trade-off between the hydraulic conductivity and the susceptibility to drought cavitation for the wild-land species that have been examined to date [55]. This may be because susceptibility to cavitation is more a function of vessel and tracheid pit anatomy than conduit size [56].

Walnuts close stomata under high leaf-to-air vapor pressure deficit (VPDl) or low leaf water potential (Ψl) [61], preventing the stem water potential (Ψs) from becoming lower than –1.4 MPa, the point at which cavitation occurs in the xylem [29]. Many species have been found to operate very close to the point of embolism. Stomata controls both plant water losses and sap pressure and thus may actively control the risk of xylem embolism [63].

Many hypotheses have been raised about xylem embolism and cavitation in walnut. Rsoil, Rroot, Rshoot, and Ψsoil have been used to identify hydraulic parameters associated with stomatal regulation during water stress and test the hypothesis that stomata control embolism during water stress [29]. Clear hydraulic segmentation was reported in a few species like walnut trees (*Juglans regia*) [212-213]. In these species, petioles disconnect the leaves from the stem through massive cavitation during drought and avoid irreversible damage to perennial parts of the tree. Nevertheless, this is not a general trend; some species showing more vulnerable twigs than petioles. Fewer data are available for root vulnerability than for branches but roots were found to be less vulnerable. [47-48].

At elevated CO_2, the decreased osmotic potential, symplasmic water fraction and rate of water transport, increased the modulus of elasticity and no changes in the formation of xylem embolism were found in tolerant walnut varieties [83; 213]. We postulate here that embolism and cavitation are important factors which influence the tracheid volume in stressed environments in walnut species [17].

3.4. Leaf water potential and branch xylem embolism at pre-dawn

Predawn leaf water potential varies by season with a significant difference in pre-dawn embolism of walnut between dry and wet seasons. The pre-dawn embolism of walnut branches was found to be 23.20% and 26.60% on 2 July and 15 August, respectively, higher than the 17.56% and 16.25% observed on 27 August and 6 October, respectively (Figure 3b) [17]. As drought progressed, the water potential reached a minimum of −1.51 MPa on 15 August. After

rain events, the water potential rapidly increased and was significantly higher than in the dry season (Figure 3). The embolism level increased with xylem δD. Similar analyses were performed between xylem δD and leaf pre-dawn water potential, leaf transpiration and photosynthesis. The former two parameters had significantly negative and linear correlations with xylem δD, while photosynthesis was not significantly correlated with xylem δD (Figure 4b–d) [17].

The daily sap flow varied significantly between seasons and was mainly determined by the daytime sap flow. Generally, in summer, even in early October, the flow was higher than in spring (17 April). On 15 August, at the point of lowest soil moisture, the daily sap flow was also restricted. The daily sap flow was significantly correlated to transpiration demand and also to mean air temperature. For all individuals, the sensors showed negligible night-time sap flow with lowest values on 15 August [17].

The leaf transpiration rate exhibited similar dynamics to the pre-dawn water potential in the growing season [17]. There was a significant difference in transpiration between the dry and wet seasons. During the dry season, the transpiration rates ranged as 0.9–1.6 mmol m^{-2} s^{-1}, significantly lower than the range of 2.1–2.5 mmol m^{-2} s^{-1} observed in the wet season. The assimilation rate did not completely follow the dynamic pattern of transpiration (Figure 4d). Photosynthetic rate was lowest on 15 August, when the soil moisture was lowest in the growing season; however, the highest photosynthetic rate occurred on 17 April, when the soil moisture was not highest [17] (Table 2).

	Regression equation	R value	R^2 value	P value
Transpiration	Y = -1.770 + 0.398x	0.951	0.904	0.013
Air temperature	Y = -2.141 + 0.541x	0.894	0.800	0.041

Table 2. Relationships between the daily sap flow and transpiration and air temperature in walnut [Courtesy Sun et al., 2011].

3.5. Vulnerability to cavitation

Cavitation occurs when negative sap pressure exceeds a threshold value defined by anatomical characteristics [62-65]. Many species have been found to operate very close to the point of embolism. Therefore, stomata control both plant water losses and sap pressure and, thus, may actively control the risk of xylem embolism [63-69].

Vulnerability curves (VCs) were constructed by plotting the changes in the percentage loss of xylem conductance (PLC) versus xylem pressure were demonstrated by Cochard et al at [29]. Significant differences were found between organs. Leaf rachises were the most vulnerable, roots the least vulnerable, and leaf veins and shoots intermediate. Turgor pressure (P_{leaf} 0) at full turgor averaged 0.93 ± 0.06 MPa (n = 5, ±SE) and the turgor loss point averaged -1.53 ± 0.04 MPa. When plants were continuously exposed to a constant and high light intensity for 1 week, a higher level of water stress was obtained. Eplant and g_s dropped close to zero whereas

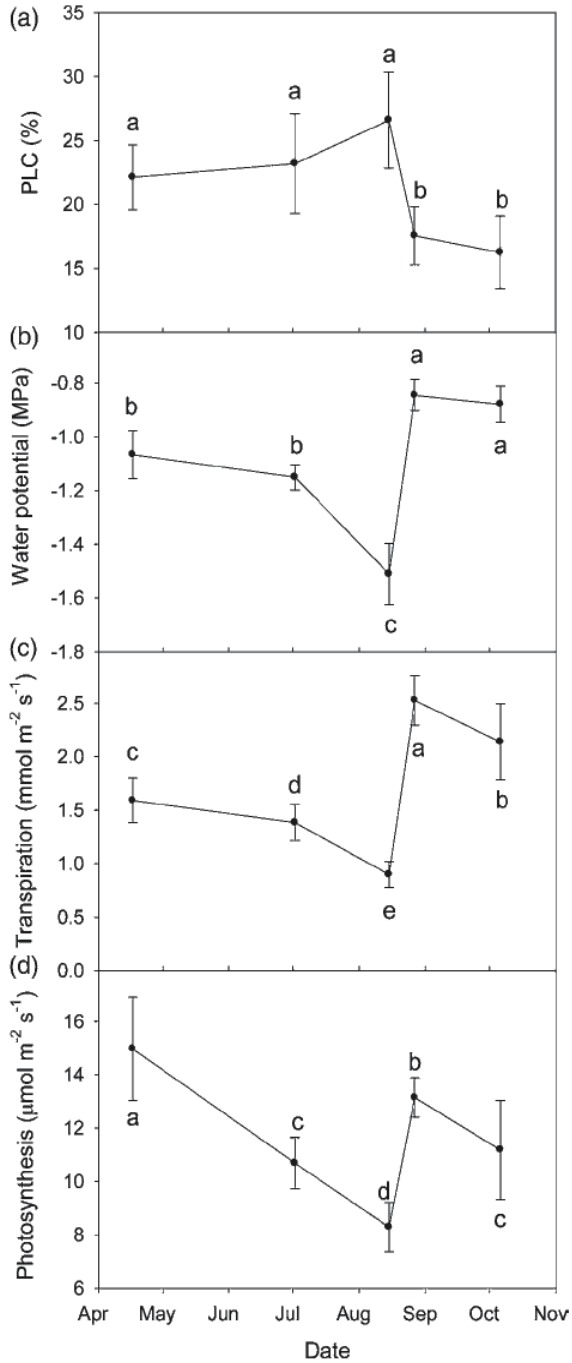

Figure 3. Variations in (a) pre-dawn branch xylem PLC, (b) pre-dawn leaf water potential, (c) leaf transpiration and (d) assimilation over the growing season. Means and SD are shown (n = 6). Different letters above the bars refer to significant difference at the P ≤0.05 level [Courtesy Sun et al., 2011].

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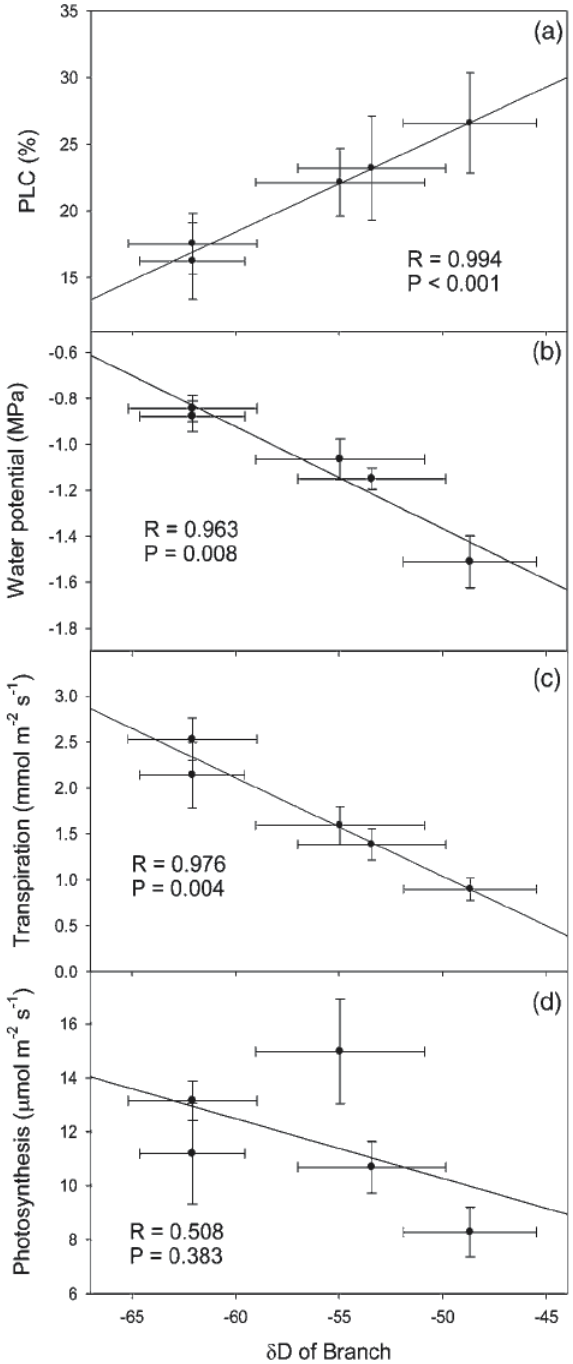

Figure 4. a) Pre-dawn branch xylem PLC, (b) pre-dawn leaf water potential, (c) transpiration and (d) assimilation as a function of the δ*D* values of branch xylem water. Linear Pearson's correlation was performed between these measurements. The error bars refer to SD [Courtesy Sun et al., 2011].

P_{rachis} leveled at approximately -1.4Mpa. At this point, the degree of xylem embolism in the leaf rachis was still less than 10 PLC [29].

The relationship between cavitation vulnerability and climate has been investigated in several tree species. Conifer seedlings originating from the most mesic populations were found to be the most susceptible to water-stress-induced cavitation [76]. Walnuts, native to dry zones, are less susceptible to drought-induced cavitation than species native to well-watered areas [75].

3.6. Evidence for a stomatal control of xylem embolism in walnut

Effect of stomatal closure is to maintain P_{rachis} above a threshold value around -1.4 MPa and Ψ_{leaf} above approximately -1.6 MPa. To further understand this behavior, it is necessary to identify a major physiological trait associated with a stomatal closure that would threaten plant integrity at lower P_{rachis} and/or Ψ_{leaf} values [74]. The answer to this question is obviously very complex, because many traits are probably involved and correlations between them probably exist [29; 213]. Cochard et al argues that, xylem cavitation is correlated with the stomatal closure [29; 70-73]. A physiological trait associated with a stomatal closure during water stress should meet at least the following three main conditions. First, its impairment should represent a serious threat to plant functioning. This results from the consideration that the reduced carbon gain, reduced growth, reduced reproductive success, etc. So the gain associated with the regulation should overcome the loss. Cavitation is a serious threat for plants because it impairs the xylem conductive capacity and may eventually lead to leaf desiccation and branch mortality [95]. Indeed, leaf desiccation was not observed in some studies as long as the xylem integrity was maintained. Leaf desiccation was noticed only when high levels of embolism were measured in the leaf petioles [29]. The gain associated with stomatal closure was thus the maintenance of leaf vitality, which largely overcomes the drawbacks cited above.

The second condition is that the impairment of the trait should be water deficit dependent because the effect of stomatal closure is precisely to prevent excessive leaf dehydration. The mechanism of water stress-induced cavitation has been well documented [138]. Air is sucked into the xylem lumens through pores in the pit wall when pressures in the sap exceed the maximum capillary pressures that can sustain the pores. Therefore, the likelihood of cavitation occurrence is directly determined by the degree of water deficit in the xylem, more precisely by P_{rachis}. The maintenance of leaf turgor above cell plasmolysis is another physiological trait that might also satisfy these first two conditions.

The third condition is that the impairment of the trait should have the same water deficit dependence as stomata. Stomata were completely closed in walnut trees when P_{rachis} reached about approximately -1.4 MPa and Ψ_{leaf} about approximately -1.6 MPa. The impairment of the trait associated with stomatal closure should therefore occur at comparable P_{rachis} or Ψ_{leaf} values. The leaf rachis was the most vulnerable organ along the sap pathway in the xylem and was also exposed to the lowest xylem pressure values. Leaf rachis is therefore the Achilles' heel of the walnut tree sap pathway. Segmentation in xylem vulnerability to cavitation has been demonstrated for several other species [29; 94]. A lot of variation exists between species, and occasionally the roots appear to be the most cavitation sensitive organs in the plant [96]. The dependencies of leaf rachis xylem embolism and transpiration on water deficit were very

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similar. Stomata were completely closed at the incipience of xylem embolism in the leaf rachis. Variations of E_{plant} and leaf turgor pressure (P_{leaf}) were concurrent with bulk Ψ_{leaf}. It is also clear from this graph that stomata were completely closed at the incipience of leaf cell plasmolysis (turgor loss point). The maintenance of xylem integrity and leaf turgor was closely associated with stomatal closure during water stress in walnut [29]. Stomatal closure was rather pre-emptive in avoiding cavitation. This behavior might be explained by the potential for "catastrophic xylem failure" [51]. There is a feedback between xylem conductance and xylem pressure during cavitation. Cavitation decreases xylem conductance, which in turn decreases xylem pressure and thus provokes more cavitation. Tyree and Sperry [51] and Jones and Sutherland [63] have computed that catastrophic xylem failure occurs at the expense of some xylem conductance and at a critical transpiration rate (E_{crit}) only slightly greater than the actual maximum E. The hypothesis of a stomatal control of catastrophic xylem failure was evaluated with a hydraulic model of a walnut tree explicitly taking into account the feedback between xylem pressure and xylem conductance. Our simulations confirmed the results of Sperry et al [64] and Comstock and Sperry [65]. Transpiration was maximized (E_{crit}) at the expense of all conductance in the distal leaf rachis segment. E_{crit} was therefore much higher than the actual E_{plant}. Using the same model, they have computed E_{plant} provoking 1% (E_{1PLC}) and 10% (E_{10PLC}) loss of rachis conductance. The onset of tree water loss regulation occurred when E_{plant} reached E_{1PLC} and E_{plant} tracked E_{10PLC} when plant conductance was further reduced. This model suggests that the risk of catastrophic xylem failure was not associated with the stomatal regulation in walnut. g_s was not maximized at the expense of all xylem conductance. Rather, xylem conductance was maximized at the expense of all g_s. To experimentally validate these computations, we have tried, without success, to feed stressed plants with fusiccocine, a drug supposed to promote stomatal opening. The use of mutants lacking efficient stomatal regulation is probably a better way to test such hypotheses [66].

These experiments demonstrate that stomatal closure caused by soil drought or decreased air humidity can be partially or wholly reversed by root pressurization [29].

3.7. Recovery of conductivity after drought-induced embolism

Recovery from drought-induced embolism is rarely reported in trees when the xylem has experienced low water potentials. More often, the conductivity is restored only the following year by the formation of a new ring of functional xylem. For tree species generating positive xylem sap pressure in the roots during spring, like walnut, the recovery of conductivity is partially achieved by flushing embolised vessels with pressurized sap and full recovery of the transport ability occurs usually only after the new year ring has been developed [77]. Recovery of xylem conductivity after embolism can occur during spring due to xylem pressure generated by starch hydrolysis [78] or during transpiration, as has been reported for *Laurus nobilis* which is able to recover despite predawn leaf water potential remaining as low as –1 MPa [81]. Similar refilling events have been reported for a range of species [79-80]. Nevertheless, the reality of such refilling of embolised vessels in transpiring trees is still a matter of debate and although several models have been proposed to explain it, there is a clear need for further research in this area [82]. Regardless of mechanism, embolism repair after drought remains a costly

process requiring metabolic energy to generate the necessary positive pressure. Cavitation avoidance is probably a much more efficient way to cope with reduced soil water, and stomatal control of transpiration probably plays a major role in this respect.

3.8. Biological lag effects

Drought and salt stress can also produce chronic symptoms such as shoot die-back, crown and root rot, tree decline and eventual death [14; 213]. In some seasons and in some field settings too much water is the result of uncontrollable natural phenomena such as excessive rainfall, high water table, and flooding. In other situations, too much water may be the result of water management decisions such as starting the irrigation season too soon, applying too much water per irrigation, irrigating too frequently, operating irrigation systems that apply water non-uniformly, or exposing sensitive parts of the tree such as the root crown to excessive water [14; 213]. Conversely, too little water may result from starting the irrigation season too late, applying too little water per irrigation, irrigating too infrequently, or operating irrigation systems that apply water non-uniformly. When too much or too little water is applied repeatedly over the life of the orchard, it may be at the expense of overall productivity and orchard longevity [14; 213].

In 1986, Dreyer and Mauget [22] tested immediate and delayed effects of summer drought on development of young walnut trees (*Juglans regia*). Two treatment periods were defined: in spring, after the first shoot growth flush, and at the end of summer, following complete cessation of shoot elongation. These treatments induced both immediate effects (halted growth, reduction of leaf area) and significant delayed effects appearing at resumption of watering. During summer, many normally quiescent buds resumed growth on trees submitted to drought after rewatering. Winter dormancy of buds was reduced by late summer drought. Unlike other trees, walnut trees showed no detectable residual effect on the timing of spring bud burst the following growing season.

3.9. Gas exchange

Light-saturated net CO_2 assimilation rate (A_{max}) and stomatal conductance (g_s) are closely related in many species [85; 107-108]. However it is not clear whether the reduction in carbon fixation is due to closing of stomata or changes in leaf biochemistry. In walnut, A_{max} decreases at high temperatures [109-110], but it is not clear whether temperature has a direct effect on photosynthesis, or just affects g_s. Another hypothesis is that A_{max} and g_s are co-regulated under water stress [111-112]. While g_s is, at times, correlated with VPD_l [113], an increasing body of literature suggests that g_s depends on leaf water status [72 -74; 84], possibly leaf or turgor pressure potentials [85-86]. Thus, while both water status and VPD_l affect g_s, the mechanisms of such responses are not clear.

In an attempt to answer this question, Rosati et al. [121] studied diurnal changes in the water status and gas exchange of droughted [50% crop evapotranspiration (ETc)] and fully irrigated (100% ETc) walnut trees, over 2 d. Stem water potentials (Ψs) ranged from –0.5 MPa in the morning to –1.2 MPa in the afternoon under drought, and from –0.1 MPa to –0.4 MPa under

full watering. Net CO_2 assimilation (A_{max}) ranged from 15 μmol CO_2 m^{-2} s^{-1} in the morning to 3 μmol CO_2 m^{-2} s^{-1} in the afternoon under drought, and from 25 μmol CO_2 in the morning to 10 μmol CO_2 mm^{-2} s^{-1} in the afternoon under full watering. At these times, stomatal conductance (g_s) varied from 0.2 to 0.02 mol H_2O m^{-2} s^{-1} and from 0.7 to 0.2 mol H_2O m^{-2} s^{-1}, respectively.

Drought reduced the internal CO_2 concentration (C_i) by about 55 μmol mol^{-1} on day 1, and by about 100 μmol mol^{-1} on day 2 and increased leaf temperature (T_l) by about 2–5 °C. The reductions in g_s and C_i with drought suggest that lower photosynthesis was associated with stomatal closure [121]. However, in each treatment, A_{max} decreased during the day, while C_i was stable, suggesting that photosynthesis was also reduced by a direct effect of heat on leaf biochemistry. Both A_{max} and g_s correlated with T_l and with the leaf-to-air vapor pressure deficit (VPD_l), but with different relationships for droughted and control trees. However, when stomatal limitations to photosynthesis were accounted for (i.e., based on the assumption that under stomatal limitation photosynthesis is proportional to C_i), a single relationship between A_{max} and T_l described all the data (R^2 = 0.81). Thus, photosynthesis was limited by both the closing of stomata under drought and by a direct effect of heat on leaf biochemistry. These results suggest that hot and dry weather reduces photosynthesis and potential productivity in walnut in the absence of a soil water deficit [121].

To test the hypothesis that A_{max} was limited by both T_l and g_s, we corrected A_{max} for the g_s (i.e., C_i) limitation and plotted the corrected A_{max} ($A_{maxCorr}$) against T_l. A single fit described all the data, suggesting that CO_2 assimilation responded directly to T_l, and that the rest of the variation in A_{max} was due to additional g_s limitations (i.e., low C_i), especially under drought. Given the close correlation between T_l and VPD_l, $A_{maxCorr}$ was also closely correlated with VPD_l [121]. Stomatal conductance is probably more related to Ψl [72-74; 84] and not Ψs, but these two parameters are closely related in droughted walnut [29]. If g_s was limited by water status at low Ψs, rather than by VPD_l, then it remains unclear why g_s was also closely related to VPD_l (R^2 = 0.85) under drought (i.e., low Ψs), although with a different relationship than for the controls [121]. This was probably due to the strong link between Ψs and VPD_l [180]. A strong relationship between Ψs and VPD_l or VPD (i.e., vapor pressure deficit in the air) has been found in several species, and is commonly used to explain variation in Ψs for fully-irrigated trees [106].

Also stomatal patterns of A, g_s, C_i and E were studied for irrigation treatments under salt stress conditions by Girona et al [215]. All of the traits studied were highly affected by salt stress. Gas exchange parameter seasonal patterns showed three groups of responses: A) less affected plants, B) moderately affected plants and C) highly affected plants [215].

3.10. Relationship between variation in water source partitioning and plant water status

Comparison of the δD values in plant stem water and soil water at different depths demonstrated that *J. regia* was compelled to take a higher ratio of water from the deep soil layers in the dry season. However, measurements of water relationships indicated that the larger water uptake from deep soil was not able to prevent water stress on the plants. Deep soil water resources may allow plants with deep root systems to survive in dry seasons [104]. Also, deep

soil water supplementation could maintain the hydraulic conductivity of roots in the nutrient-rich upper soil throughout the dry season [46], keeping roots ready to extract water when moisture becomes available in the upper soil. Otieno et al [105] found that *Quercus suber*, with a deep root system, took up most of its required water from the deep soil layers during drought to maintain good water status, but no growth was recorded during this time. Water in the upper soil layers, however, seemed to play a more important role in tree productivity. Values of δD trace the ratio of water sources, but not the absolute amount of water. Lower δD values suggest that xylem water has a higher ratio of water from the deep soil layers, but cannot be automatically translated into greater water uptake from the deep soil layers. Such a finding could also indicate reduced water uptake from the upper soil layers or a mixture of reduced water uptake from the upper layers and increased water uptake from the deep layers. Thermal dissipation probe transpiration measurements indicated that the daily sap flow decreased by around 30% on the driest day in comparison with 2 July and 27 August, suggesting that the highest xylem δD on 15 August would be mainly attributed to reduced water uptake from the upper soil layers. Additionally, the δD values in xylem water were significantly correlated with the shallow soil layers (0–20 and 20–40 cm depths), but not so significantly correlated with the deeper soil layers (40–60 cm depths) [17], suggesting that water uptake by walnut would tend to be mainly determined by water supply of the upper soil. During water transport between roots and shoots, the isotopic composition of water remains unaltered; therefore, it is reasonable to believe that water in sap flow was also mainly provided by the upper soil [17]. Many studies with stable isotopic hydrogen and oxygen on seasonal changes in water sources investigated the water source shift from upper to deep soil layers with decreasing precipitation, and the results sometimes imply that water uptake from deep soil, where water is available, could solve the drought problem. Deep water can help but not always enogh to avoid serious stress.

Walnut roots were mainly distributed in the upper soil layers [212]. Soil moisture was a key factor regulating root growth and water uptake efficiency of the roots [17]. The shallow roots had reduced efficiency in water uptake in the dry season, and therefore *J. regia* was compelled to extract a greater ratio of water from the deep soil layers. However, the shift was not able to prevent water stress on the plants, which was characterized by increased pre-dawn branch xylem PLC, reduced pre-dawn leaf water potential and transpiration with soil drying. In addition to serving as an indicator of water sources, changes in the δD values in walnut branch xylem water reflected plant water status and the severity of soil drought [17].

In previous studies, comparison of the δD values of plant stem water and soil water at different depths revealed the existence of different water source partitioning patterns between different soil moisture conditions in a planted walnut forest for example in northern China [17]. The δD values showed that plants mainly used water from the upper soil in the wet season, while upper and deep soil water more or less equally contributed to plant xylem water in the dry season. The result is consistent with that of previous studies. McCole and Stern [102] reported a change in juniper water use from a predominantly deep water source during summer, when it was hot and dry, to a predominantly upper soil source during winter, when it was cool and wet. *Pinus edulis* and *Juniperus osteosperma* largely use monsoon precipitation during the

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monsoon period, but use of this precipitation declines sharply with decreasing summer rain input [103]. No other water source was available for trees in this system. However, the roots might penetrate through the dense gravel layers and may be in contact with groundwater. Therefore, the influence of groundwater on xylem isotopic signature cannot be completely excluded, although Williams and Ehleringer [103] found that plants did not use groundwater in the pinyon–juniper ecosystem of the southwestern USA, a site similar to this study region. Nevertheless, it should be noted that the seasonal change of water resource partitioning was based on the two measured depths.

Rosati et al. [121] studied Kaolin applications to mitigate the negative effects of water and heat stress on walnut physiology and productivity. Kaolin applications were found to improve A_{max} in apple but only under high temperature and vapor pressure difference [122]. Other authors found no effect or even a reduction in yield, A_{max} or both [97-98; 122]. Little data are available for other tree species: kaolin improved A_{max} and stomatal conductance (g_s) in citrus at mid-day but not in the morning [99] and no effect was found on pecan [100].

A_{max} for walnut was highest in the early morning and decreased throughout the day, for both the water-stressed (S) and the well-irrigated (W) treatments [121]. A_{max} was always lower for the S treatments, especially in the afternoon. Kaolin application reduced A_{max} (by up to 4 mmol CO_2 m^{-2} s^{-1}) within each irrigation treatment, especially in the morning when A_{max} was high, whereas in the afternoon this effect tended to disappear in the W treatment and disappeared completely in the S treatment [123; 125]. The average reduction in A_{max} during the day was minor compared with the reduction due to water stress and was 1-4 mmol CO_2 m^{-2} s^{-1} in the S treatments and 2-4 mmol CO_2 m^{-2} s^{-1} in the W treatment [121].

Also in this study, intercellular CO_2 concentration (Ci) was greatly reduced with water stress in walnut while the irrigated walnut and the almond trees had similar Ci values [121]. Kaolin application increased Ci in all cases except for two out of five measurements in the S treatment in walnut. The average daily increase in Ci with kaolin was 28 mmol mol^{-1} in the S and 19 mmol mol^{-1} in the W treatments for walnut and 10 mmol mol^{-1} for almond [121]. As a result they concluded that Kaolin application reduced leaf temperature (T_l) and leaf to- air vapor pressure difference (VPD_l), but not sufficiently to compensate for the increase in Tl and VPDl with water stress in walnut. The kaolin-induced reduction in Tl and VPD_l did not mitigate the adverse effects of heat and water stress on Amax. Kaolin application did not affect g_s and Ys. The prevailing effect of kaolin application appeared to be the shading of the leaves and the consequent, albeit minor, reduction of Amax, except at very low A_{max} [121].

3.11. Delayed consequences of drought

Irreversible drought-induced damage leads to organ dysfunction, but it seldom results in direct and immediate tree decline and mortality. Drought induces short term physiological disorders like decreased carbon and nutrient assimilation, and sometimes even a breakdown of the photosynthetic machinery itself. These tissues have to be repaired before normal processes can resume. The tree must allocate existing stored reserves among the demands for repair, maintenance, growth and defense. As a consequence, tree ring width or leaf area is frequently smaller during several years following a severe drought [166-167]. Moreover,

physiological disorders increase tree vulnerability to secondary stresses like insect damage, frost or another drought [168].

4. Molecular responses to abiotic stresses:

4.1. Mineral composition and Ion homeostasis under abiotic stress

Perhaps the most significant change in plant electrophysiological studies, beginning about 25 years ago, was a shift in focus from more basic electrical and biophysical properties of plant membranes to pursuing an understanding of the physiological and cell biological functions of individual plant ion channel types [114]. In the 1990s, ion channels were characterized as targets of upstream signal transduction mechanisms, and in the later 1990s powerful combined molecular genetics, patch clamp, and plant physiological response analyses further manifested the importance of ion channels for many biological and stress responses of plants [114]. Essential metals and ions in the intracellular and intraorganellar spaces of plant cells contribute to the activities of regulatory proteins, signal transduction, and to the maintenance of turgor pressure, osmoregulation, toxic metal chelation, and membrane potential control. A large number of studies on mineral nutrition have sustained the profitable cultivation of plant growth and development and provided important knowledge on mechanisms of mineral absorption from soils [114]. Lotfi et al. [180] tested the mineral composition of promising walnut varieties under both salt and drought stress. Their results showed that differences in the range of sodium accumulation were minimal as compared with other minerals at different salt and drought stress levels. In control plants, the average sodium content ranged from 0.34 to 1.82 mg g^{-1} dry weight (DW), whereas the shoots of the sensitive cultivars (Lara, Vina, and Serr) had significantly higher sodium contents than other cultivars [212].

In salt-treated plants, the average sodium content was higher than in control plants (nearly twice) and ranged from 0.52 to 7.92 mg g^{-1} DW [212], and the Chandler seedlings had significantly less sodium content than the others. Sodium levels in roots were higher than in the shoots in almost all varieties, especially in the tolerant and semi-tolerant varieties [212]. In contrast, the increase in sodium content was more evident in shoots of sensitive and semi-sensitive varieties.

Results of mineral composition analysis showed that the calcium and potassium accumulations were increased by the increase in salt and drought stress levels, especially in shoots of semi-sensitive and tolerant cultivars [212]. Also, variations of magnesium accumulation in root and shoot samples were significant at all stress levels and were dependent on cultivar [212].

Several classes of Ca^{2+} permeable channels have been characterized in the plasma membrane of plant cells, including depolarization-activated Ca^{2+} channels [139; 140] and hyper polarization-activated Ca^{2+} influx channels [114]. In general, plant Ca^{2+} channels are not entirely Ca^{2+} selective but also show permeability to other cations [166]. However, the genes encoding plasma membrane Ca^{2+} channels remain less well-clarified. Two gene families are likely to provide possible candidates. One family includes 20 genes in the Arabidopsis genome and

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encodes homologs to "ionotropic" glutamate receptors, which encode receptor ion channels in animal systems [115].

Calcium ions act as a second messenger in intracellular signal transduction during ABA signaling [132]. In-flow of calcium ions into the cytosol from the vacuole and extracellular space increases the cytosolic concentration of calcium ions in ABA-treated guard cells. The level of calcium ions oscillates at intervals of several minutes. This increase in calcium concentration is not observed in the ABA insensitive mutant's abi1 and abi2 [133]. Calcium ions suppress inward potassium channels and activate inward anion channels; thereby playing a central role in stomatal closure [134]. Also, the active oxygen species formed activate the calcium ion channel to increase the cytosolic concentration of calcium ions.

Uptake and distribution of sodium ions within the root is to a large extent connected with the effects of potassium, since Na^+ efflux in root cortex cells is stimulated by K^+ influx, which is related to the K/Na root selectivity [135]. The presence of potassium (and calcium) ions has been shown to decrease Na+ influx into plant cells (e.g. [136]). Potassium promotes cell elongation and maintains osmoregulation. Potassium promotes photosynthetic rate and controls the rate of transport of photosynthates from source to sink. Potassium is also essential for protein synthesis and activates nearly 45 enzymes involved in various metabolic processes [222].

We observed differential responses in the uptake of sodium and in the pattern of germination in seedlings of walnut cultivars which could account for the differences in response to salinity. The ability to maintain low sodium concentration in leaves and in growing shoots is crucial for plant growth in saline media. The salt tolerance in species that exclude salts is achieved by changes between sodium and calcium ions, rather than changes in osmotic potential, since adsorption of calcium ions on membranes of root cells leads to reduced penetration of monovalent cations [124]. This was demonstrated for wheat where inhibition of non-directional Na^+ influx occurred following the addition of external Ca^{2+} [137]. Involvement of both Ca^{2+} sensitive and Ca^{2+} insensitive pathways (regulated mainly by non-selective cation channels) in the control of Na^+ entry into the root has been proposed [138]. When sodium accumulates in the cytoplasm of shoot or leaf cells, it can lead to tissue necrosis and leaf abscission; thus, the photosynthetic apparatus is impaired and plant growth is hindered. The accumulation of sodium in shoots was significantly different in the three salt tolerance classes, but they presented distinct responses to the increasing concentration of NaCl. Similarly, Sixto et al [116] observed differences in leaf sodium content among *P. alba* cultivars from different Spanish origins subjected to salt stress. Possibly the halfsib seedlings of 'Chandler', which accumulated significantly less sodium in shoots, has mechanisms for sodium exclusion at the root level, which reduces sodium uptake and its translocation to the shoot tissues. Mechanisms for sodium exclusion in roots are well studied in *P. euphratica* [117] which is the most salt-tolerant poplar species. In *P. alba*, the ability to maintain lower sodium content in leaves has also been associated with less severe symptoms of salinity stress [116]. Our results confirm a negative relationship between sodium accumulation in the shoots and its effects on shoot growth in 'Chandler'. The negative effect of long-term salt stress on shoot growth of 'Lara' is probably more due to sodium toxicity than to osmotic effect. The excess sodium can be both

actively accumulated in the vacuole or be excreted into apoplast. Sodium compartmentation in the vacuole is an adaptation mechanism typical of halophytes [118]. Ottow et al. [118] observed that *P. euphratica* could tolerate increasing sodium concentration by apoplastic accumulation of salt in the leaves' cell wall regions but not in the vacuole. A similar mechanism for apoplastic localization of sodium could operate in *P. alba* and accounts for the different behavior observed among the cultivars studied. These hypotheses need to be tested by further studies to determine the exact site of sodium localization at histological, cellular, and subcellular levels.

The results of our previous study suggest that seedlings of different walnut cultivars differ in tolerance to salinity and drought stress. Results demonstrated variability in germination ability and seedling growth in saline and drought habitats, implying that it might be possible to select walnut seedlings for salt and drought tolerance in germination stage [180; 212-213]. Salinity treatments caused a net K^+ uptake, which is likely to be the result of osmotic adjustment in tolerant cultivars. Net Na^+ uptake by sensitive cultivars was noticeably higher than in tolerant cultivars. Interestingly, in control plants, the sodium content in shoots of cultivars that belong to the sensitive groups was significantly higher than in the shoots of the other halfsib progeny. This suggests a constitutive ability of these cultivars to accumulate more sodium in the leaves. This feature could contribute to osmotic adjustment in response to salinity or drought as has also been observed in *P. euphratica* plants exposed to salt stress, in which the osmotic adjustment was mainly resulted from sodium accumulation [118]. In the tolerant and semi-tolerant groups, roots had higher potassium contents than shoots. This could reflect differences in the membrane transport properties of cells in different stress-tolerant groups [119]. The amount of calcium accumulation was increased by increase in salinity stress levels, especially in shoots of tolerant and semi-sensitive cultivars. Calcium is an essential plant nutrient that is required for its structural roles like in membrane integrity, as a counterion for inorganic and organic anions in the vacuole, as an intracellular messenger in the cytosol, and as an enzyme activator [120]. In conclusion, different strategies for adaptation to salinity or drought have been observed in seedlings of walnut cultivars with different climatic origins when grown in a greenhouse trial. Thus, a different genetic basis underlies the different behaviors observed under salt and drought stress. The degree of variation in salinity and drought tolerance in these cultivars could be linked to their different abilities in sodium exclusion at the root level or to different regulation of ion transport across shoot cell membranes. Our results suggest that the cultivars Chandler and Panegine20 could also be suitable models to be used for the study of the physiology and genetics of abiotic stress tolerance in walnut [212].

The higher content of seed nutrients is of vital importance for germination, but salinity and drought suppresses their role in the metabolism of seeds and the production of seedlings [144]. During germination of walnut seeds, a higher content of potassium, calcium, phosphorus, and nitrogen was partitioned into the plumule and radicle as a strategy of tolerance to salinity [213]. Guerrier [145] attributed the reduced salt tolerance of tomato to its inability to accumulate and transport lower amounts of calcium and potassium. The SOS pathway (salt overly sensitive) is triggered by a transient increase of cytosolic Ca^{2+} as a first effect of salt stress. The increase in Ca^{2+} concentration is sensed by a calcium binding protein (SOS3) [212].

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4.2. Seed germination and ion homeostasis under abiotic stress

The initial events in stem propagule germination may differ in some respects those of seeds but bud activation, elongation, and establishment events are similar. Germination of sugar cane sets (stem cuttings) exhibited significant reduction in the rate and percentage of germination due to NaCl damage [150]. These plants had an enhanced content of Na^+ and Cl^-, a concomitantly reduced content of potassium, calcium, nitrogen, and phosphorus and reduced elongation and dry matter of seedlings.

Citrus rootstocks used to raise plantlets had a negative correlation of Cl+ with certain nutrients [146]. Resting buds of salt-stressed poplar plant, grown in vitro, did not accumulate glycinebetaine and proline and thus had reduced growth of seedlings [2]. Similarly, tubers of hydrilla showed signs of salt damage and reduced germination [147-148]. There remains a shortage of information particularly about the salt tolerance of propagules during germination.

Exposure of seeds or seedlings to salinity results in the influx of ions with the imbibition of water, which exerts an adverse effect on the growth of embryo [141; 143]. This may lead to a marked decrease in the internal potassium concentration [143], a vital nutrient for protein synthesis and plant growth [149]. Seedlings exposed to salinity are highly prone to excessive ions, sometimes leading to their death shortly after emergence [142; 150]. The ability of plants to cope with ion toxicity is principally related to the greater transport of ions to shoot [143-144]. Grasses show a strategy of salt tolerance by storing toxic ions in the mesocotyl up to a certain limit [151-152]. This has significance in that the epicotyls and hypocotyl avoid ion toxicity, thus ensuring better growth [141].

4.3. Regulation of Na^+ homeostasis in roots and shoots in tolerant walnut varieties

The fine-tuned control of net ion accumulation in the shoot involves precise *in planta* coordination between mechanisms that are intrinsically cellular with those that are operational at the intercellular, tissue or organ level [125, 157]. Several processes are involved, including the regulation of Na^+ transport into the shoot, preferential Na^+ accumulation into the shoot cells that are metabolically not very active and the reduction of Na^+ content in the shoot by recirculation through the phloem back to the root [125-126].

Ions loaded into the root xylem are transported to the shoot largely by mass flow, driven by the size of the transpirational sink [124-127]. A control response is to lower transpiration by a reduction in stomata aperture; however, this is only effective as a short-term response because plants need to maintain water status, carbon fixation and solute transport [157]. Controlling ion load into the root xylem restricts accumulation in the shoot to a level where cells in this organ can be effective ion repositories by vacuolar compartmentalization [125, 157]. In our studies, tolerant walnut varieties showed such trends under both salt and drought stress [213]. Endodermal cells constitute a major control point in radial ion transport from the soil solution to the root xylem since the Casparian strip is an impermeable barrier to apoplastic solute movement [128]. However, bypass systems that function through 'leaks' in the Casparian strip barrier or movement through areas of the root where the specialized endodermal cells are not fully developed may be additional major entry points [129-130]. Regardless, vacuolar com-

partmentalization in cells that form the interconnected network between the soil solution and the root xylem progressively lowers the content of ions that are entering the transpirational stream. It is presumed that NHX-like cation/H^+ transporters have a major function in this process [131; 157].

4.4. Osmotic homeostasis: Compatible osmolytes

Osmotic balance in the cytoplasm is achieved by the accumulation of organic solutes that do not inhibit metabolic processes, called compatible osmolytes. These are sugars (mainly sucrose and fructose), sugars alcohols (glycerol, methylated inositols), complex sugars (trehalose, raffinose and fructans), ions (K^+), charged metabolites (glycine betaine) and amino acids such as proline [156; 157]. 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 [157]. These solutes also function to protect cellular structures through scavenging ROS [6; 10; 157]. Salt tolerance requires that compatible solutes accumulate in the cytosol and organelles where these function in osmotic adjustment and osmoprotection [187]. With exceptions like K^+, most compatible osmolytes are organic solutes. Genes that encode enzymes that catalyze the biosynthesis of compatible solutes enhance salt and/or drought tolerance in gain-of-function strategies [155].

Proline occurs widely in higher plants, and normally accumulates to large quantities in response to environmental stresses [205]. In addition to osmotic adjustment, it is involved in prevention of protein denaturation and preservation of enzyme structure and activity [187]. Most of research on proline as an osmoregulatory compound has been carried out on the vegetative parts of the plants. Little attention has been paid to the reproductive organs, especially seeds. Recently, information has been published on osmotic adjustment of seeds under stress conditions. Salt stress increased proline accumulation in the cotyledons and roots of germinating ground-nut seeds [162]. Proline accumulated in the endosperm and radicles of germinating barley seeds with increasing NaCl concentrations in the growing media [163-164]. This proline probably originated from the degradation of stored protein in the endosperm. Walnut seeds average 15-25 g protein per 100 g of kernel and the proline content of seeds varies with genotype, ranging from 1100 to 1500 mg/100g kernel. A high amount of proline was detected in embryonic axis and leaves [181]. Our previous study revealed that the amount of proline in seeds of different genotypes of walnut, especially in semi-tolerant and tolerant genotypes, is high [212]. Even at the beginning of a drought period, the machinery for proline accumulation was most activated in the tolerant genotypes ‘Chandler’ and ‘Panegine20’ of walnut [180]. These initial differences in proline content, observed among the genotypes at day zero, prior to application of WI, and notably high in ‘Panegine20’ and ‘Chandler’, could be due to the natural adaptation to abiotic stress of the germplasm from which these genotypes were derived. Proline content of both ‘Chandler and ‘Panegine20’ were elevated and similar to each other early in the drought period, but at the end the proline content of ‘Panegine20’ was higher than that of ‘Chandler’ [180]. Proline appears to be a major osmotic regulator in ‘Panegine20’ and ‘Chandler’ under drought stress. Also, our previous study demonstrated

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that in 'Panegine20', contrary to 'Chandler', "ion osmosis" is another important osmotic regulator under drought and salt stress [212].

Proline content increases significantly in relation to the severity of drought stress, in particular in roots of tolerant walnut genotypes [180]. In two and three years old walnut seedlings proline content of both roots and shoots was elevated initially and increased significantly with length of drought stress in tolerant genotypes [180]. During 16 days of water stress, root proline content increased 1.48 fold in 'Panegine20' and 1.38 fold in 'Chandler' seedlings [180]. Similarly, leaf proline content increased 2.07 times in 'Panegine20' and 1.50 times in 'Chandler' seedlings compared to the control plants [180]. The increase in proline content was greater in 'Panegine20' than in 'Chandler' and greater in roots than in shoots [180].

4.5. Total soluble sugars and starch variation under abiotic stress

Salinity and drought cause the accumulation of soluble sugars, free proline, and soluble proteins [141; 154]. Parida and Das [177] reported that lower osmotic potential allows leaves to withstand a greater evaporative demand without loss of turgor. This requires an increase in osmotica, either by the uptake of soil solutes or by the synthesis of metabolically compatible solutes [138]. These findings appear to apply to olives since Tattini et al. [179] showed a correlation between leaf glucose and increasing levels of salinity in the root zone.

Drought and salt stress significantly increased the total soluble sugar content of roots and leaves only in 'Panegine20' and 'Chandler' varieties [180]. Leaf soluble sugar content increased 1.39 times in 'Panegine20' and 1.59 times in 'Chandler' compared to the controls. The increase in sugar concentration may result from the degradation of starch [202]. Soluble sugar content was elevated initially and increased progressively in drought stressed tissues of the tolerant genotypes. Sugars may act directly as osmotica or may protect specific macromolecules and thereby contribute to the stabilization of membrane structures [197]. In general, soluble sugar content tends to be maintained in the leaves of drought-stressed plants even though rates of carbon assimilation are partially reduced. In this study, observed increases in soluble sugar concentration coincided with decreases in starch content as the water potential dropped.

Metabolites may prove to be beneficial to germination, first by reducing osmotic inhibition and second by providing substrates for the growth of embryonic tissues [150; 153]. Imposition of different polyethylene glycol treatments on promising genotypes of walnut seedlings significantly increased total soluble sugar content [180]. Compared to the control, a drastic increase was observed in shoots and roots. Root content soluble sugar increased 1.65 times in 'Panegine20' progeny and 1.70 times in 'Chandler', and shoot soluble sugar content increased 1.73 times in 'Panegine20' and 1.60 times in 'Chandler' relative to control plants. Starch content significantly decreased in roots and shoots of both genotypes. Total starch content of roots decreased 49.46% in 'Panegine20' and 38.18% in 'Chandler'. This decrease was 52.79% in 'Panegine20' and 47.42% in 'Chandler' relative to the control plants [180].

Ability of LEA proteins to act synergistically with non-reducing sugars to form a glassy matrix, and thus confer drought protection, is an attractive hypothesis [170]. This hypothesis is supported by the abundance of LEA proteins and reducing sugars in desiccation-tolerant plant

tissues [171]. Several factors appear necessary to confer desiccation tolerance. Evidence implicates the accumulation of soluble sugars, especially sucrose and raffinose family oligosaccharides [172-173]. However, such sugars have also been detected in immature desiccation-intolerant embryos of maize and wheat [174]. Other factors, such as heat-stable late embryogenesis abundant proteins, may be involved [175], but some of these have been identified in recalcitrant (desiccation-intolerant) seeds [176]. Hence, examining the drought response of desiccation tolerant and intolerant seeds fails to provide conclusive evidence of a role in desiccation tolerance for either soluble sugars or heat-stable proteins. Soluble sugars and heat-stable proteins were equally likely (or unlikely) to be involved in the development of seed quality [178].

4.6. Chlorophyll pigments and photosynthetic activity under abiotic stress

It is clear from numerous similar studies of water and salt relations that turgor maintenance alone does not assure continued leaf expansion [196]. It may be that photosynthetic capacity is insufficient to provide carbon for both wall synthesis and ''turgor-driven cell expansion". Or it may be that some higher level controls operate to limit expansion in spite of the available potential [221]. The Chl a and Chl b contents as well as the photosynthetic electron transport rate in leaves of stressed 'Lara' and 'Serr' seedlings decreased significantly at all drought and salt periods tested, but stressed 'Panegine20' and 'Chandler' seedlings did not differ significantly from the controls in regards to these traits at any time during the applied stress. The decreases were more apparent with longer drought exposure time [180]. The Chl a/b ratios remained constant in all cases and there were no significant differences observed within genotypes [180].

The stability of chlorophyll content and chlorophyll a/b ratio in 'Panegine20' and 'Chandler' seedlings suggests that the pigment apparatus is comparatively resistant to dehydration in these tolerant walnut cultivars. Drought and salt stress can directly or indirectly reduce the photochemical efficiency of PS2 due to either inefficient energy transfer from the light-harvesting complex to the reaction centre, or to inability of the reaction centre to accept photons as a result of structural alterations in the PS2 complex [201; 210]. The results obtained indicate that abiotic stress like drought and salt affects both the light-harvesting complex and the reaction centre of PS2. Also Rosati et al [121] revealed that Kaolin application in walnut under water stress did not affect dark respiration rate, nor Amax2500, but significantly reduced Amax2000/Amax2500 and apparent quantum yield, while compensation point was significantly increased. The modeled leaf photosynthetic response to PAR was different for the kaolin-coated and the control leaves [121]. Assuming that only 63% of the PAR incident on the kaolin-coated leaves actually reached the leaf surface, the modeled curves for the two treatments overlapped perfectly at any PAR [121].

Drought reduces photosynthesis in walnut (*Juglans regia* L.), but it is not known whether this is mainly due to the closure of stomata, or to possible effects on leaf biochemistry. In an attempt to answer this question, Rosati et al [121] studied diurnal changes in the water status and gas exchange in droughted [50% crop evapotranspiration (ETc)] and fully irrigated (100% ETc) walnut trees, over 2 d. They resulted that stem water potential (Ψs) ranged from –0.5 MPa in

the morning to –1.2 MPa in the afternoon under drought, and from –0.1 MPa to –0.4 MPa under full watering. Net CO_2 assimilation (Amax) ranged from 15 μmol CO_2 m^{-2} s^{-1} in the morning to 3 μmol CO_2 m^{-2} s^{-1} in the afternoon under drought, and from 25 μmol CO_2 m^{-2} s^{-1} in the morning to 10 μmol CO_2 mm^{-2} s^{-1} in the afternoon under full watering. At these times, stomatal conductance (g_s) varied from 0.2 to 0.02 mol H_2O m^{-2} s^{-1} and from 0.7 to 0.2 mol H_2O m^{-2} s^{-1}, respectively. Drought reduced the internal CO_2 concentration (Ci) by about 55 μmol mol^{-1} on day^{-1}, and by about 100 μmol mol^{-1} on day^{-2} and increased leaf temperature (Tl) by about 2–5°C. The reductions in g_s and Ci with drought suggest that lower photosynthesis was associated with stomatal closure. However, in each treatment, Amax decreased during the day, while Ci was stable, suggesting that photosynthesis was also reduced by a direct effect of heat on leaf biochemistry. Both Amax and gs correlated with Tl and with the leaf-to-air vapor pressure deficit (VPDl), but with different relationships for droughted and control trees. However, when stomatal limitations to photosynthesis were accounted for (i.e. based on the assumption that, under stomatal limitation, photosynthesis is proportional to Ci) a single relationship between Amax and Tl described all the data (R_2 = 0.81). Thus, photosynthesis was limited by the closing of stomata under drought, and by a direct effect of heat on leaf biochemistry. These results suggest that hot and dry weather reduces photosynthesis and potential productivity in walnut in the absence of soil water deficit.

Under normal physiological conditions, electron transport is directed toward sequential and fully coordinated reduction of intermediate electron acceptors PS2 and PS1. However, drought and high temperature can provoke a state of hyper-reduction in the electron transport chain, enhancing generation of superoxide radicals as has been shown in cotton [183-184] and rice [186]. Theoretically, high photosynthetic efficiency can increase water-use efficiency as more carbon is assimilated per unit water transpired. In walnuts, a positive correlation was reported between photosynthesis and stomatal conductance—an important determinant of water use efficiency [121; 223]. The effect of salinity stress on the photosynthetic enzyme activities is postulated to be a secondary effect mediated by the reduced CO_2 partial pressure in the leaves caused by the stomatal closure [224]. The present review also reveals that in all the walnuts grown in non-saline and desiccated soils, an increased rate of assimilation is coupled with increased stomatal conductance [180].

4.7. Total phenols and PPO activity under abiotic stress condition

Walnut nuts have high amount of phenolic compounds. Walnut kernels are rich in oils composed of unsaturated fatty acids, such as linoleic and oleic acid, and are susceptible to oxidation. However the content of a-tocopherol, an antioxidant, is lower in walnut than in other nuts such as almonds, hazelnuts, peanuts [212]. This implies that the nut contains antioxidants inhibiting lipid auto-oxidation. Recently, a walnut extract containing ellagic acid, gallic acid, and flavonoids was reported to inhibit the oxidation of human plasma and low density lipoproteins (LDL) in-vitro [158]. Although the presence of ellagic acid suggests the occurrence of its bound forms, ellagitannins, there are some reports on the tannin constituents of walnut [233-235]. Muir et al. [235] demonstrate that a shikimate pathway enzyme, SDH (shikimate dehydrogenase), is directly responsible for GA [gallic acid]

production in both plants and bacteria when shikimic acid (SA) or 3-DHS were used as substrates and $NADP^+$ as a cofactor. Finally, they showed that purified *E. coli* and *J. regia* SDH produced GA in-vitro. Also, they proposed that the C-terminal, AroE/SDH domain of the plant enzyme is the region of the protein responsible for GA production [235]. Because of the importance of GA as an antioxidant in plants, controlling its production and accumulation in plants could significantly increase the nutritional value and tolerance of walnut for abiotic stresses. Further expression studies using fragments of the walnut gene(s) will be performed to verify the activity of each individual domain in GA production [235]. Anderson [158] examined antioxidative tannins and related polyphenols in foods and nuts, isolating 16 polyphenolic constituents including three new hydrolysable tannins, along with adenosine and adenine, from commercial walnuts. Under abiotic stress, the profile of total phenols and PPO activity was similar in both roots and leaves of all genotype seedlings subjected to salt and drought stress, but amounts of phenolics and levels of PPO activity were higher in leaves than in roots [213]. A significant increase (25.3% and 38.4%) in total phenolic concentration was observed within 20 d of water deficit treatment in leaves of both 'Chandler' and 'Panegine20' [213] in contrast to a small and not significant increase in total phenolic concentration in seedlings of some varieties, especially in root tissues [213]. The reverse pattern was observed for PPO activity, with 'Chandler' and 'Panegine20'showing a slight decline in root and leaves while PPO activity in 'Lara' and 'Serr' increased sharply during drought in both tissues. Among the antioxidative enzymes analyzed, PPO was the only one clearly down-regulated under WI conditions. 'Chandler' and 'Panegine20' leaves showed a marked decline in PPO activity during water deficit stress but in roots PPO activity decrease were less sharp [213]. A significant increase in PPO activity in water stressed leaves of 'Lara' and 'Serr' (112% and 76%) was apparent after 7 d of drought [213]. In these varieties, PPO activity linearly increased until the Ψw was -1.84 MPa or more (during the 7th d of drought period) and then remained at a similar level [213]. The antioxidant properties of plant phenolic compounds are well-documented [206]. These are synthesized de novo [207] and can influence auxin metabolism, membrane permeability, respiration, oxidative phosphorylation, and protein synthesis [199] and their activity also has been related to the occurrence of physiological injury [208]. The changes in phenolic production and PPO activity observed in drought-stressed walnut seedlings show that some varieties, namely 'Lara' and 'Serr', are more sensitive to drought than the tolerant varieties 'Panegine20' and 'Chandler' [213].

4.8. Effects of salt and drought on Malondialdehyde (MDA) content

Earlier, Jouve et al. [225] found that the endogenous level of MDA did not vary in control and in the salt stressed aspen (*Populus tremula* L.) plants. This indicates that the level of lipid peroxidation was similar in stressed and non-stressed plants. Likewise, MDA concentration changed with increasing salt concentration in the shoots of tolerant walnut varieties, decreased slightly at 100mM, while increased at 200 and 250mM salt stress which suggests that walnut shoots are better protected from oxidative damage under salt stress [213]. Changes in the MDA content of leaf tissues subjected to drought and salt were well documented by Lotfi [213]. Application of drought for 20 d caused a linear increase in the

MDA content of 'Lara' and 'Serr' seedlings with the MDA content peaking at ~143 nmol g^{-1} FW on the 16th d. in comparison with control plants at ~ 67 nmol g^{-1} FW, a trend similar to PPO. Seedlings of 'Panegine20' and 'Chandler' showed significant decreases in MDA content under the same conditions [213]. Under most oxidative conditions, malondialdehyde (MDA) is a product too often considered as a marker of peroxidative damage. It is important to interpret such measurements with caution, since there are a lot of drawbacks linked to the thiobarbituric acid (TBA) test for MDA determination [160-161]. MDA is produced when polyunsaturated fatty acids in cell membranes undergo peroxidation. The results reported here show that accumulation of MDA was higher in seedlings of sensitive varieties, especially in 'Lara'. The lower levels of MDA observed in 'Panegine20' and 'Chandler' suggests that less membrane damage occurs in droughted seedlings of these varieties, contributing to their tolerance [213].

4.9. Effects of drought and salt on peroxidase (PAO) activity

PAO activity peaked in leaf tissues of walnut 'Lara' and 'Serr' seedlings on 5th day of WI (24.56 and 19.78 mmol guaiacol/mg protein/min) simultaneously with increasing of PPO activity in these varieties [213]. 'Panegine20' and 'Chandler' seedlings showed insignificant increases in PAO activity [213]. In our study, the generation of ROS was tightly linked in sensitive genotypes to catabolism of PAs by PAO and decreased PAO activity coincided with accumulation of proline. PAO activity in drought tolerant seedlings under water stress was significantly lower than in sensitive seedlings [213], likely accounting for the higher accumulation of PAs in tolerant seedlings. The function of PAO is oxidation of Spermidine (Spd) to pyrroline, 1,3-diamine propane (DAP) and H_2O_2, and spermine (Spm) to aminopropylpyrroline, DAP and H_2O_2 [182, 200]. Enhanced H_2O_2 production as a result of PAO activity may be important in signal transduction leading to programmed cell death (PCD) and in expression of defense genes involved in responses to drought tolerance [185]. Low PAO activity in the tested tolerant cultivars, and probable resulting polyamine accumulation, likely reflect a protective response to abiotic stress.

Our results also show that low PAO activity and subsequent accumulation of endogenous PAs increased the activity of peroxidase (POD) and catalase (CAT), along with proline production in 'Panegine20' and 'Chandler' seedlings under WI [213]. Our results are in agreement with the results reported by Seki et al. [188]. DAO and PAO are also considered to be important controllers of the ABA signaling pathway involved in stomatal regulation [213]. In drought tolerant cultivars a decreases in PAO activity was observed relative to sensitive ones, indicating the ABA signaling pathway integrates PA, DAO and PAO activity in regulating H_2O_2 production [191]. The high activity of antioxidants observed in this study in roots and shoots of 'Panegine20' and 'Chandler' seedlings suggests these may convey drought tolerance that can be a first step to protecting the plant leaves. The maintenance of root and leaf PA concentrations, along with low PAO activity, suggest that a balance between their biosynthesis and oxidation cannot be excluded as a further specific feature of 'Panegine20' and 'Chandler' two-phase responses under drought conditions. Further studies are needed to determine the specific PAs present in these cultivars.

4.10. Effects of salt and drought on LOX activity

Both enzymatic and non-enzymatic lipid peroxidation have been previously implicated in ROS perception. Oxylipins resulting from enzymatic oxidation via lipoxygenases (LOX) might function in leaf senescence [159]. LOX activity in leaves of sensitive genotypes increased markedly by the 5th d of WI and then continued to rise slightly. Also under salt stress conditions the same trends were observed for walnut seedlings. Leaves were the most affected by water deficit, showing a four-fold increase in LOX activity over control seedlings [213]. LOX activities in root tissues were 1.7 and 1.6 times the control values at the maximum drought stress [213]. LOX activity of controls did not change significantly during the full 20 d of WI. There were no significant increases in LOX activity in seedlings of 'Panegine20' and 'Chandler' [213]. LOXs are a family of enzymes that catalyze the oxygenation of polyunsaturated fatty acids (PUFAs) into lipid hydroperoxides (LOOHs) which are involved in responses to stresses [190]. Plant LOXs may be involved in growth and developmental control processes through the biosynthesis of regulatory molecules and volatile compounds [198]. The high degree of lipid peroxidation observed could produce lipid derivatives acting as secondary messengers capable of activating some drought stress associated genes by means of specific transcription factors, triggering plant responses to desiccation [212]. Increase in LOX activity can be due to an increased amount of enzymatic protein [204]. However, in this study a lower amount of the enzymatic protein was found in drought-stressed seedlings of 'Panegine20' and 'Chandler' than in controls [213].

4.11. Effects of salt and drought on antioxidant defense systems

A recent comprehensive study revealed that both salt and drought stresses led to down-regulation of some photosynthetic genes, although most of the changes were small, possibly reflecting the mild stress imposed. Compared to drought, salt stress affected more genes and more intensely, possibly reflecting the combined effects of dehydration and osmotic stress under salt-imposed conditions [194]. Desingh and Kanagaraj [226] pointed out that photosynthetic rate and RuBP carboxylase activity decreased with increasing salinity but some antioxidative enzymes significantly increased. An important consequence of salt stress is the excessive generation of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radicals (OH^-), particularly in the chloroplast and mitochondria [195].

In plant cells, ROS are generated in high amounts by both constitutive and inducible routes, but under normal situations, the redox balance of the cell is maintained via the constitutive action of a wide range of antioxidant mechanisms that have evolved to remove ROS [194]. ROS are produced during photosynthesis and respiration, as by-products of metabolism, or via dedicated enzymes. Cells are equipped with a range of efficient antioxidant mechanisms to remove ROS. Changes in the cellular redox balance result from exposure to various abiotic and biotic stresses, with induction of both ROS generation and removal mechanisms. Enzymatic ROS scavenging mechanisms in plants include SOD (superoxide dismutase), present in many cellular compartments; catalase, located in peroxisomes; and the ubiquitous ascorbate-glutathione cycle. SOD catalyses the dismutation of superoxide to H_2O_2, and is thus one of the

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primary mediators of H_2O_2 production from intracellular sources of superoxide. Unlike most organisms, plants have multiple forms of the different types of SODs encoded by multiple genes [216]. According to our previous study, SOD activity in water-stressed 'Panegine20' walnuts increased 58% and 29% relative to controls in leaves and roots respectively. In 'Chandler' seedlings this increase was 51% and 33%, respectively [213]. In 'Serr' seedlings, the decline was 54% and 42% in the different tissues, respectively and in 'Lara' walnuts; the decline was 67% and 53% in leaves and roots. For all cultivars, the increase in SOD activity under WI conditions in roots was less than those recorded in leaves [213].

Increasing SOD activity induces a higher tolerance to oxidative stress under salt or drought stress [205]. Metallo enzyme SOD, which is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress, is the most effective intracellular enzymatic antioxidant [184]. This enzyme provides the first line of defense against the toxic effects of elevated levels of ROS. Yang et al. [193] found that under drought conditions and high light SOD activity increased significantly relative to low light.

The patterns of SOD, CAT, POD and APX activities were roughly parallel in all the tissues examined, showing a significant increase under salt and drought-treatment conditions in tolerant walnut varieties [213] but at different levels among genotypes and plant tissues. 'Panegine20' and 'Chandler' seedlings of walnut showed the highest levels of antioxidative enzyme activity. The increases in APX and CAT activities in 'Panegine20' seedlings were significant in leaves and roots tissue and activity was greater in leaves than roots. In 'Chandler' seedlings, the increase in APX activity under WI was significant only in leaves [213]. In 'Panegine20' and 'Chandler' seedlings APX activity increased more than SOD or CAT activity under WI conditions [213]. Activities of SOD, CAT and APX peaked at the 7^{th} d of WI, but POD activity climaxed on the 5^{th} d and was higher in 'Panegine20' than 'Chandler' [213].

Abiotic stresses, such as drought stress, cause molecular damage to plant cells, either directly or indirectly, through the formation of AOS. In this study, the plants exposed to abiotic stress showed a significant increase in CAT, APX, SOD and POD activity. MDA is regarded as a biomarker of lipid peroxidation and stress-induced damage to the plasmalemma and organelle membranes [189]. In this study, the amount of MDA in tolerant varieties decreased with increasing drought stress. CATs are tetrameric heme-containing enzymes with the ability to directly convert H_2O_2 into H_2O and O_2 and are indispensable for ROS detoxification under stress conditions [204]. APX is thought to play an essential role in scavenging ROS and protecting cells by scavenging H_2O_2 in water-water and ASH-GSH cycles and utilizing ASH as the electron donor.

APX has a higher affinity for H_2O_2 (μM range) than CAT and POD (mM range) and may have a more crucial role in the management of ROS during stress. As expected, the activities of all these enzymes changed significantly in walnut seedlings under water stress. The observed greater increase in APX activity in leaves of water-stressed plants than in roots could be due to localization of APX in chloroplasts. The significant increase in APX activity seen in leaves could be a mechanism developed by walnut trees for protection of chloroplasts, which under stress conditions develop sustained electron flows and are the main producers and targets of ROS action [195]. The increase in CAT activity in leaves of water-stressed plants may be an

adaptation aimed at scavenging photo respiratory H_2O_2 produced during drought stress [203]. The reduced PPO activity in stressed walnut seedlings could be a response to increase the abundance of antioxidative phenols. PPO could also be involved, through proteolytic action, in removing proteins damaged by oxidative stress effects [211].

The increased POD, APX and CAT activities observed in the more drought and salt-tolerant 'Panegine20' and 'Chandler' seedlings, relative to 'Lara' and 'Serr' seedlings, underline the effectiveness of 'Panegine20' and 'Chandler' antioxidative enzyme systems in protecting the cellular apparatus under water deficit conditions. Furthermore, the higher proline accumulation observed in 'Panegine20' and 'Chandler' seedlings under WI was accompanied by higher activities of SOD, APX, POD and CAT. These results suggest that proline accumulation could activate the antioxidative defense mechanism in walnut trees as has been suggested by Yang et al. [193] in salt-stressed soybean plants.

In conclusion, genotypic differences were observed among walnut seedlings in leaf water status, photosynthetic performance, pigment content, proline accumulation and antioxidative enzyme activity. The close relationship observed between photosynthetic rate (Pn) and proline content points to an important role of this osmolyte in the maintenance of photosynthetic activity and therefore in drought tolerance. These literature reviews show that differences in SOD, APX, POD, PPO, LOX, PAO and CAT activities among walnut genotypes could be attributed to differences in the mechanisms underlying oxidative stress injury and subsequent tolerance to abiotic stress. Varietals differences in pigment content could be related to differences in antioxidative enzyme activity. Notably, the 'Panegine20' and 'Chandler' seedlings, which exhibit higher drought tolerance, also showed higher antioxidative enzyme activity than other walnut seedlings. Seed of the later cultivars should be considered high-risk for planting in dry areas. In addition, these results show that seedling genotypes with the higher photosynthetic activity ('Panegine20' and 'Chandler') also had higher proline content and antioxidative enzyme activity. This supports an interaction between proline and the antioxidative defense system as suggested by Yang et al [193]. To verify this hypothesis, we suggest further studies focusing on the effects of exogenous application of proline and paraquat on the activities of protective enzymes in walnut trees would be of interest.

4.12. Biotechnology and abiotic stress engineering in walnut

Breeding for drought and salinity tolerance in crop plants should be given high priority in plant biotechnology programs. Molecular control mechanisms for abiotic stress tolerances are based on the activation and regulation of specific stress-related genes. These genes are involved in the whole sequence of stress responses such as signaling, transcriptional control, protection of membranes and proteins, and free-radical and toxic-compound scavenging. A major objective of walnut rootstock breeding is vigour, in order to promote rapid growth of the scion under a variety of soil and environmental conditions and to quickly establish a full-sized bearing canopy. Other objectives include resistance to diseases and pests, most notably Phytophthora, nematodes and crown gall, and tolerance of soil-related problems including waterlogging, salt accumulation and cold. There is interest in controlling tree size but not at the cost of vigour. In walnut, breeding for abiotic stress tolerance or resistance has been limited

at best. One of the first attempts is transformation of somatic embryos of Persian walnut with a gene isolated from a cyanobacter. This gene controls expression of flavodoxin. The role of flavodoxin in response to salinity and osmotic conditions is known [229].

Ferredoxins are very ancient proteins widely used by anaerobic organisms for many metabolic pathways. Ferredoxin (Fd) is up-regulated by light, indicating that under autotrophic growth, Fd is the normal electron carrier [230]. As a replacement for Fd, Flavedoxin gene (fld) is induced under various environmental sources of stress including oxidative stress in enterobacteria and salinity stress in cyanobacteria [231]. Results showed that transgenic plantlets of walnut harboring the fld gene clearly grow better at 200 mM NaCl than the non-transgenic controls. The control plants did not produce any callus and turned brown and died after 10 days, while transgenic lines showed no brown symptoms, produced callus, and continued their growth for up to 45 days on 200 mM NaCl [229]. Compared to salt stress, the decrease in evaluated parameters of transgenic and non-transgenic SEs caused by PEG-induced stress was relatively lower. At the 1.5% PEG, the number of cotyledonary embryos was significantly increased in both transgenic and non-transgenic somatic embryos (SEs) [229]. With increasing concentrations of PEG in culture medium to 5% and 10%, significant differences between transgenic and non-transgenic SEs for most of the evaluated parameters were observed. The results showed that transformants reduced stress in both salt and osmotic stress conditions and the degree of response was greater to salt than to PEG. Over-expression of the fld gene in transgenic lines of Persian walnut partially decreases some of the hostile effects of salinity stress. Production of callus and new shoots by transgenic plants expressing this gene and grown on stress-inducing media is in agreement with previous reports in tobacco [232]. All findings reported show clearly that expression of cyanobacterial proteins can be a powerful tool to enhance the stress tolerance of some plants.

5. Conclusions and perspectives

- Cavitation avoidance is a likely physiological function associated with stomatal regulation during abiotic stress in walnut. This suggests that stomata are responding to leaf water status as determined by transpiration rate and plant hydraulics and that P_{rachis} might be the physiological parameter regulated by stomatal closure during water stress, which would have the effect of preventing extensive developments of cavitation during water stress.
- Hydraulic segmentation for walnut trees (*Juglans regia*) by petioles displaying a large vulnerability to abiotic stresses in sensitive genotypes. This phenomenon disconnects leaves through massive cavitation during stress and avoids irreversible damage to perennial parts of the tree.
- Photosynthesis is limited by stomatal closing during drought and by direct effects of heat on leaf biochemistry. This suggests that hot and dry weather reduces photosynthesis and potential productivity in walnut even in the absence of soil water deficit. But, some promising varieties show the sufficient net assimilation rate and photosynthesis under abiotic stress conditions.

- Walnut roots are mainly distributed in the upper soil layers. Soil moisture is a key factor regulating root growth and water uptake efficiency of the roots. The shallow roots lose efficiency in water uptake during the dry season and the shift to uptake by deeper roots does not fully compensate for the loss of uptake by shallow roots and is not able to prevent water stress, which is characterized by increased percentage loss of xylem conductance (PLC) in pre-dawn, reduced pre-dawn leaf water potential and transpiration during abiotic stresses.
- Understanding the ability of genotypes to absorb essential elements is indicative of their ability to withstand stress.
- Differences in antioxidative enzymes (such as SOD, APX, POD, PPO, LOX, PAO and CAT) activities among walnut genotypes could be attributed to differences in the mechanisms underlying oxidative stress injury and subsequent tolerance to abiotic stress.
- Higher proline accumulation observed in tolerant seedlings of walnut to osmotic stresses was accompanied by higher activities of antioxidative emzymes (e.g. SOD, APX, POD and CAT). These results suggest that proline accumulation could activate the antioxidative defense mechanism in walnut trees.
- The degree of stress tolerance found in seedlings of some walnut varieties has been characterized at various stages of growth. Identified stress-tolerant genotypes are candidates for further studies under longer periods of drought and field studies to determine their suitability for areas with adverse environmental conditions, and eventually for use as drought-tolerant rootstocks.
- Application of biotechnology tools for increasing tolerance to abiotic stresses in walnut is underway. Some promising results have been reported under in-vitro conditions.

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Chapter 11

The Role of Transcription Factors in Wheat Under Different Abiotic Stresses

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Additional information is available at the end of the chapter

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1. Introduction

Abiotic stresses such as drought, salinity and low temperature adversely affect the growth and productivity of plants. The development of stress-tolerant crops will be essential for agriculture in the many regions in the world that are prone to such stresses [48].

Wheat (*Triticum aestivum* L.) is one of the four major cereals in the world. As one of the most important agricultural crops, wheat is a staple food crop for a large portion of the world's population [83]. It is grown under both rain-fed and irrigated cultivation and thus under conditions subjected to many environmental stresses [68]. Unfortunately, its production is severely affected by adverse environmental stresses. Therefore, the identification and functional study of stress responsive genes will elucidate the molecular mechanisms of the plant stress response and tolerance, and will ultimately lead to improvement of stress tolerance in wheat [58, 83]. Abiotic stresses such as drought and high salinity lead to wide range of biochemical, physiological and morphological, responses in plants in the process of adaptation to these adverse conditions. These adaptations require a large number of changes in gene expression. Many of the differentially expressed gene products protect plant cells from damage, such as dehydrins, enzymes for the synthesis of osmolytes and enzymes for the removal of reactive oxygen species (ROS) [3]. The production of these functional proteins is widely regulated by specific transcription factors [58, 65].

Transcription factors (TFs) are considered to be the most important regulators that control genes and gene clusters [49]. Many families of transcription factors have been demonstrated to play a role in stress responses in plants. Among them, the bZIP [69], WRKY [43], AP2 [64], NAC [78] and C2H2 zinc finger [31] families comprise a high proportion of abiotic stress-responsive members [58]. One TF gene can control the expression of a broad range of target genes through binding to the specific cis-acting element in the promoters of these genes, also

referred to as regulon [49]. Transcription factors (TFs) provide a possibility for plants to overcome and respond to biotic and abiotic stresses and are also involved in modulating developmental processes [45, 83].

Until now, several major regulons involved in response to abiotic stress have been identified in *Arabidopsis*. Recent studies have demonstrated that DREB1/CBF, DREB2, AREB/ABF, and NAC regulons have important functions in response to abiotic stresses in rice [49]. Significant advances have been made in recent years towards identifying regulatory genes involved in stress responses which confer abiotic stress tolerance in plants [18].

In this review, we provide an overview of the functions of different TF family members with particular emphasis on the role of bZIP, bHLH, WRKY, MYB, and NAC TFs and their involvement in abiotic stress responses in wheat.

2. bZIP transcription factors

Basic region/leucine zipper (bZIP) TFs possess a basic region that binds DNA and a leucine zipper dimerization motif. The bZIP domain comprises two structural features located on a contiguous α-helix: a basic region of about 16 amino acid residues with a nuclear localization signal, an invariant N-x7-R/K motif to contact the DNA as well as a heptad repeat of leucines or other bulky hydrophobic amino acids located exactly nine amino acids towards the C-terminus, to create an amphipathic helix. When binding to DNA, two subunits adhere through interactions of the hydrophobic sides of their helices, which create a superimposing coiled-coil structure (zipper). The capability to form homo- and heterodimers is governed by the electrostatic attraction and repulsion of polar residues adjacent to the hydrophobic interaction surface of the helices. Proteins with bZIP domains are present in all eukaryotes analyzed to date and bZIP proteins typically bind to DNA sequences with an ACGT core. Plant bZIPs bind to the A-box (TACGTA), C-box (GACGTC) and G-box (CACGTG), but there are also reports of nonpalindromic binding sites for bZIPs [24]. Based on the sequence similarities of common domains, 75 bZIP protein members have been divided into ten subgroups in *Arabidopsis* [24, 35]. In plants, bZIP transcription factors present a divergent family of TFs which regulate processes including light and stress signaling, seed maturation, pathogen defense, and flower development [24, 59].

The plant hormone abscisic acid (ABA) plays an essential role in maturation and germination in seeds, as well as mediating adaptive responses to abiotic environmental stresses. ABA induces the expression of many genes, including late-embryogenesis-abundant (LEA) genes. *HVA1* is one of the LEA genes whose expression is affected by ABA. Analysis of the interplay between ABA and TaABF1 as a bZIP factor in the aleurone cells of imbibing wheat grains by Keyser [32] indicated that the two are not additive in their induction of the *HVA1* promoter. It has been shown that TaABF1 may undergo an ABA-induced posttranslational modification. However, the lack of synergism between ABA and TaABF1 overexpression in *HVA1* induction does not support this conclusion. These findings indicate that the branch of ABA signaling leading to *HVA1* is more complex [32].

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Kobayashi et al. [35] isolated a wheat *lip19* (encoding bZIP-type transcription factors) homologue, *Wlip19* and analyzed its expression in response to cold stress. *Wlip19* expression was stimulated by low temperature in seedlings and was higher in a freezing-tolerant wheat cultivar than in a freezing-sensitive variety. *Wlip19* expression was also activated by drought and exogenous ABA treatment. Heterologous expression of *Wlip19* in tobacco has showed a significant increase in abiotic stress tolerance, especially freezing tolerance.

It was indicated that WLIP19 acts as a transcriptional regulator of Cor/Lea genes in the development of abiotic stress tolerance by enhancement of expression of four wheat Cor/Lea genes, *Wdhn13*, *Wrab17*, *Wrab18*, and *Wrab19*, in wheat callus and tobacco plants. Furthermore, direct protein–protein interactions between WLIP19 and another bZIP-type transcription factor in wheat, the OBF1 homologue TaOBF1, was observed, implying that this interaction is conserved in cereals [35].

Expression analysis of a group of bZIP candidate genes in long term salinity into contrasting cultivars of wheat by reverse northern blot showed that *bZIP1* (CN011839] was up-regulated in a susceptible variety (Chinese Spring) and down-regulated in a tolerant cultivar (Mahouti) during salt stress. Sequence analysis by BLASTX showed that this gene's protein has two homologues in *Arabidopsis* (AtZIP56, E value=$1e^{-2}0$] and wheat (TaABF, E value=$6e^{-}5$]. The results of published work showed that *TaABF* mRNA accumulates together with *PKABA1* mRNA (an ABA-induced protein kinase) during wheat grain maturation and dormancy acquisition and *TaABF* transcripts increase transiently during imbibitions of dormant grains. In contrast to *PKABA1* mRNA, *TaABF* transcripts are seed specific and were not markedly produced in vegetative tissues in response to ABA application or abiotic stress [29, 59].

HY5, another bZIP1 homologue from the H group of *Arabidopsis* bZIPs, is involved in photomorphogenesis regulation. The necessary TF for response to a broad spectrum of wavelengths of light acts as a positive regulator in photomorphogenesis by regulating the expression of downstream genes in response to a light signal. Interestingly, *HY5* integrates both hormone and light signaling pathways. In *hy5* mutants, the expression of hundreds of genes is affected by UV-B or blue light [7]. Another affected bZIP by salt stress in tolerant genotype was bZIP5 (CV765814] from group I bZIPs. The analysis of group I genes from several species indicates that they might play a role in vascular development [24, 59].

3. bHLH transcription factors

Basic helix-loop-helix (bHLH) proteins comprise a group of diverse transcription factors with highly diverse functions and are present in both plants and animals. The bHLH domain, the characteristic of this family, consists of about 60 amino acids with two functionally distinct regions. The basic region at the N-terminal end of the domain is required for DNA binding while the C-terminal HLH region functions as a dimerization domain. These TFs in plants, act as transcriptional regulators required for phytochrome signaling, anthocyanin biosynthesis, fruit dehiscence, carpel, and epidermal development, as well as for stress response. However, the biological function of most members of this gene family in plant has not yet been elucidated [37].

Gene expression analysis by reverse northern blot has shown that two selected candidate wheat bHLHs (*bHLH2*: CA599618 and *bHLH3*: CJ685625] are affected by salt stress in a tolerant wheat cultivar. The BLASTx results showed that both have a homologue in wheat, *bHLH94* (E value=$5e^{-85}$ for bHLH2 and E value=$5e^{-102}$ for bHLH3]. AtAIB was another homologue for bHLH3 from *Arabidopsis* involved in the regulation of ABA signaling in *Arabidopsis* and plays a role in drought tolerance and ABA treatment response [37, 59]. The high homology (E value=$2e^{-5}$1] between these orthologues and the result of reverse northern blot hybridizations in that research indicate that these two bHLH gene may have an important function in tolerance to salt stress in wheat [59].

4. WRKY transcription factors

WRKY transcription factors have been studied in plants extensively in the last two decades. First Ishiguro and Nakamura [23] identified a WRKY protein in sweet potato (*Ipomoea batatas*); since then many other members of this TF family have been cloned and functionally characterized in plants, including wild oats (*Avena fatua*) [62], parsley (*Petroselinum crispum*) [63], tobacco (*Nicotiana tabacum*) [8, 19, 34, 61], *Arabidopsis thaliana* [12], potato (*Solanum tuberosum*) [5, 14], orchardgrass (*Dactylis glomerata*) [2], winter bittersweet nightshade (*Solanum dulcamara*) [22], desert legume (*Retama raetam*) [55], barley (*Hordeum vulgare*) [66], rice (*Oryza sativa*) [41], cotton (*Gossypium arboreum*) [77], and coconut (*Cocos nucifera*) [44]. More recently, WRKY family TFs were also identified in lower plants including ferns (*Ceratopteris richardii*), mosses (*Physcomitrella patens*) [4], a smile mode (*Dictyostelium discoideum*) and the protist (*Giardia lamblia*) [81, 73].

A WRKY domain of about 60 amino acids is a characteristic of WRKY proteins. This domain comprises the absolutely conserved sequence WRKYGQK followed by a zinc finger motif. The WRKY domain binds to the W box ([T][T]TGAC[C/T]) of target gene promoters to modulate transcription [10, 73]. It should be mentioned that in spite of the strong conservation of their DNA-binding domain, the overall structures of WRKY TFs are highly divergent. WRKY TF family members are grouped into three distinct groups based on the number and type of the WRKY domains which might also reflect their different functions [59]. WRKY TFs with two WRKY domains belong to group I and members of group II and group III possess one WRKY domain. Group I and group II have a C2H2 zinc finger motif, while in group III, the WRKY domain contains a C2HC motif. WRKY TFs can then be further classified into different subgroups based on their phylogenetic clades. The WRKY family is one of the TF families for which the regulatory role in biotic and abiotic stresses has been demonstrated in plants. These include infection of bacteria, fungi, oomycetes and viruses, treatment with salicylic acid (SA) or H_2O_2, mechanical stimulation, drought, cold, wounding, high-salinity and UV radiation.

Most WRKY TFs of group III play a role in plant defense signaling pathways. Some members of the WRKY family may have key functions in plant development, such as embryo development, fruit maturation, tannin synthesis in the seed coat, maturation of root cells, morphogenesis of trichomes, senescence, and dormancy. Furthermore, some of WRKY family

members have a role in hormone signaling such as OsWRKY71 and OsWRKY51 which were ABA-inducible and could repress GA signaling transduction in aleurone cells.

Wu et al. [73] obtained sequences for 15 wheat cDNAs encoding putative WRKY proteins. Phylogenetic analysis showed that the 15 WRKY genes classified to three major WRKY groups and expression analysis revealed that most genes were highly expressed in leaves. A few of them such as *TaWRKY10* are expressed in the crown intensively and several genes are strongly up-regulated during the senescence of leaves. Eight isolated genes were responsive to high or low temperature, NaCl or PEG (polyethylene glycol) treatment. In addition, differential expression was also measured between wheat hybrids and its parents, and some genes were more responsive to PEG treatment in the hybrid. The authors concluded that the differential expression of these WRKY genes in the hybrid might contribute to heterosis by improving the stress tolerance in hybrids [73].

Orthologous genes are subjected to similar transcriptional regulation by orthologous TFs, suggesting that the terminal stages of signal transduction pathways leading to defense are conserved, implying a fundamental role of pathogenesis-related genes, such as PR4 genes in plant defense. This suggests that diversification between monocot and dicot plants has most likely occurred after the differentiation of WRKY functions. Proietti et al. [56] reported the ability of TaWRKY78 to bind to a W-box-containing region of the *wPR4e* promoter. Transient expression assays of *TaWRKY78* and *AtWRKY20* showed that both TFs are able to recognize the cognate cis-acting elements present in the *wPR4e* and *AtHEL* promoters [56].

Expression analysis by reverse northern blot hybridizations of a group of putative wheat WRKYs showed that *WRKY1* (CN009320] and *WRKY2* (CJ873146] were up-regulated in a stress-tolerant genotype. AtWRKY75 (E value=$3e^{-4}$2] is a homologue from *Arabidopsis* for WRKY1 which is up-regulated in response to phosphorous deficit stress [15, 17, 59]. This gene also acts as positive regulator in defense responses to pathogens. Functional characterization of the WRKY2 homologue in *Arabidopsis*, AtWRKY33 (E value=$4e^{-1}$8], showed that its expression in response to salt, mannitol (simulated drought) treatment and cold stress in shoots and roots increased but this gene was down-regulated during heat stress. It also appears that its expression is independent of SOS signaling and only partly dependent on ABA signaling, but forms part of plant responses to microbial infections [27, 40, 59].

5. MYB transcription factors

MYB TFs form one of the largest transcription factor families in plants. More than 200 MYB proteins are encoded in genomes of *Arabidopsis* and rice. MYB TFs contain one to four imperfect repeats [50–53 amino acids) in their DNA-binding domain (MYB domain) near to the N-terminus and are classified into four subfamilies [58, 83].

According to the number of repeat(s) in the MYB domain: 4R-MYB has four repeats, 3R-MYB (R1R2R3-MYB) has three consecutive repeats, R2R3-MYB has two repeats, and the MYB-related type usually, but not always, has a single repeat [16, 28, 61]. Typically, the MYB repeat

is 50–53 amino acids in length and contains three regularly-distributed tryptophan (or phenylalanine) residues, which can together form a hydrophobic core. Each MYB repeat forms three α-helices: the two that are located at the C-terminus adopt a variation of the helix–turn–helix (HLH) conformation that recognizes and binds to the DNA major groove at the specific recognition site such as C/TAACG/TG [51, 52].

Since the first plant MYB gene, *C1*, was isolated in *Zea mays* [54], research concerning different aspects of the MYB gene family, including gene number, sequence characterization, evolution, and potential functions, has been widely conducted in plants [9, 16, 72]. So far, large numbers of MYB genes have been identified in different plant species, comprising 204 members in *Arabidopsis*, 218 members in rice, 279 members in grapevine, 197 members in poplar, and 180 members in *Brachypodium* [9, 70, 72].

MYB proteins are involved in many significant physiological and biochemical processes, including the regulation of primary and secondary metabolism, the control of cell development and the cell cycle, the participation in defense and response to various biotic and abiotic stresses, and hormone synthesis and signal transduction [16, 83].

Extensive studies of the MYB gene family in various plant species have provided a better understanding of this gene family; however, little is known about this gene family in bread wheat [83].

We previously analyzed the expression levels of ten MYB TF genes from wheat (*Triticum aestivum*) in two recombinant inbred lines contrasting in their salt tolerance in response to salt or drought stress via quantitative RT-PCR [58]. A potential new MYB gene (*TaMYBsdu*1] was significantly up-regulated in leaves and roots of wheat plants subjected to long-term drought stress. Furthermore, *TaMYBsdu1* showed higher transcript abundance in the salt-tolerant genotype than in the susceptible genotype under salt stress. These data suggested that TaMYBsdu1 is a potentially important regulator for wheat adaptation to both salt and drought stresses [58].

In other work, two putative MYB genes, *MYB2* (DQ353858.1] and *MYB3* (CJ920766] were up-regulated in a tolerant variety (Mahouti) under salt stress conditions but down-regulated in the susceptible cultivar (Chinese Spring), *MYB2*. Sequence analysis with the BLASTx and Plant Gene Ontology assignment showed that MYB2 is a part of TaMYB1 (E value=$6e^{-15}$5]. The results of a study by Lee et al. [36] show that *TaMYB1* is involved in abiotic stresses responses in wheat. The expression of this gene increases during oxygen deficiency (flooding), PEG treatment (drought) and salt increases, especially in roots. In addition, its transcript gradually increases in starting ABA and PEG treatments [36]. In research conducted by Mott and Wang [46] on comparative transcriptome analysis of salt-tolerant wheat germplasm lines using wheat genome arrays, it was found that *TaMYB1* was one of the up-regulated genes with 34 times higher expression levels under stress condition relative to the control. Functional analysis of the MYB2 homologue in *Arabidopsis*, AtMYB44 (E value=$1e^{-5}$9], showed that this gene was up-regulated in response to drought, salt, cold and ABA treatments, especially in stomata guard cells and vascular tissue. Transgenic plants overexpressing this gene showed more tolerance to mentioned stresses compared to wide-type plants [28]. Homology analysis of MYB3 (a

member of R2R3MYB) has shown that there is a high homology between this gene and *AtMYB59* in *Arabidopsis* (E value=$4e^{-6}$0]. It has been shown that *AtMYB59* expression increases in response to phytohormones including jasmonic acid, SA, gibberellic acid and ethylene, especially in leaf and stem tissues [38, 39, 59]. But its expression level in roots and inflorescences was lower than in other organs, showing its role in hormonal signal pathways in response to biotic stresses and plant defense against pathogen attacks [38, 39, 59].

Full-length cDNA is an important resource for isolating the functional genes in wheat. Recently, Zhang et al. [83] analyzed a group of MYB genes that respond to one or more stress treatments. They isolated 60 full-length cDNA sequences encoding wheat MYB proteins. A phylogenetic tree with wheat, rice, and *Arabidopsis* MYB proteins was constructed to examine their evolutionary relationships and the putative functions of wheat MYB proteins based on *Arabidopsis* MYB proteins with known functions. Tissue-specific analysis and abiotic stress response expression profiles were carried out to find potential genes that participate in the stress signal transduction pathway, including the analysis of transgenic *Arabidopsis* plants expressing the MYB gene, *TaMYB32* [83].

Recently, Qin et al. [56] identified a new R2R3-type MYB transcription factor gene, *TaMYB33*, from wheat (*T. aestivum*). This gene was induced by ABA, NaCl, and PEG treatments, and its promoter sequence contains the putative ABRE, MYB and other abiotic stress-related cis-elements. Ectopic over-expression of this gene in *Arabidopsis* significantly enhanced its tolerance to drought and NaCl treatments, but not to LiCl and KCl stresses. The expression of two genes, *AtP5CS* (involved in proline synthesis) and *AtZAT12* (a C2H2 zinc finger transcription factor that is involved in regulating ascorbate peroxidase expression), was induced in the *TaMYB33*-expressing transgenic *Arabidopsis* lines. This suggests that TaMYB33 promotes the ability for ROS scavenging and osmotic pressure balance reconstruction. *TaMYB33* over-expression lines displayed up-regulation of *AtAAO3* along with down-regulation of *AtABF3* and *AtABI1*, indicating that ABA synthesis was elevated while its signaling was constrained. The authors concluded that TaMYB33 enhances salt and drought tolerance partially via an improved ability for ROS detoxification and osmotic balance reconstruction [57].

TaMYB56 (on chromosomes 3B and 3D) in wheat was identified as a cold stress-related gene by Zhang et al. [82]. The expression levels of *TaMYB56-B* and *TaMYB56-D* were strongly induced by cold stress, but slightly induced by salt stress in wheat. Detailed characterization of the *Arabidopsis* transgenic plants that overexpressed *TaMYB56-B* revealed that TaMYB56-B is possibly involved in the responses of plants to freezing and salt stresses. The expression of some cold stress-responsive genes, such as *DREB1A*/*CBF3* and *COR15a*, were found to be elevated in the *TaMYB56-B*-overexpressing *Arabidopsis* plants compared to wild-type [82].

TaMYB3R1 is another MYB gene which has been shown to be potentially involved in wheat response to drought, salt and cold stress. Cai et al. [6] cloned *TaMYB3R1* from wheat (*T. aestivum*). TaMYB3R1 amino acid sequence shares high identity to other plant MYB3R proteins. Subcellular localization experiments in onion epidermal cells proved that TaMYB3R1 was present in the nucleus. Trans–activation assays in yeast cells confirmed that TaMYB3R1 was a TF that required the C-terminal region to activate the expression of reporter gene. DNA-binding tests showed the MSA cis-element-binding activity of TaMYB3R1. *TaMYB3R1*

expression was induced following ABA treatment and gradually increased expression until 72 h after salt or cold treatment. In contrast, PEG treatment lead to an early expression peak at 6 h after treatment, and then gradually decreased [6].

Zhang et al. [84] identified *TaMYB32* as a salt stress-related gene, during the bulk sequencing of full length cDNAs in wheat (*T. aestivum*). The sequences of TaMYB32 were cloned from different varieties of hexaploid wheat and its diploid ancestors. Sequence analysis indicated that two types of sequences existed in the diploid ancestors and four in the hexaploid wheat. One of the sequences was identical in both diploid and hexaploid wheat. This implied that TaMYB32 was conserved during the evolution of wheat. The genomic *TaMYB32* sequences proved to be non-intron genes after comparing with their cDNA sequences. TaMYB32 was mapped onto the homoeologous group 6 of wheat using the electronic mapping strategy, and two copies of the gene were found in each genome of hexaploid wheat. Homologous analysis found that TaMYB32 had a similarity with some R2R3-MYB proteins from rice (*Oryza sativa* L.) and maize (*Zea mays* L.) as high as 72.4% and 73.7%, respectively. The expression of *TaMYB32* in roots, stems, leafs, pistils, and anthers in wheat, was induced by salt stress [84].

6. NAC transcription factors

The first sequenced cDNA encoding a NAC protein was the *RESPONSIVE TO DEHYDRATION 26* (*RD26*] gene in *Arabidopsis* [80]. The NAC domain was characterized based on consensus sequences from Petunia NAM and *Arabidopsis* ATAF1/2 and CUC2 proteins [1]. Many NAC TFs, including *Arabidopsis* CUC2, play important roles in plant development. Some NAC genes mediate viral resistance [48], while others are up-regulated during wounding and bacterial infection [11]

NAC domains mediate transcriptional regulation of various biological processes by forming a helix-turn-helix structure that specifically binds to the target DNA [1]. NAC TFs are quite diverse in their C-terminal sequences which possess either activation or repression activity. More than 100 NAC genes have so far been identified in *Arabidopsis* and rice which can be categorized into six major groups. Phylogenetic analyses suggest that these were already present in an ancient moss lineage. NAC TFs play a range of important roles during plant development and abiotic stress responses [48]. Many plant growth and developmental processes are regulated by NAC TFs, including shoot apical meristem formation, lateral root development, senescence, cell wall development, and secondary metabolism. A large number of NAC TFs are also differentially expressed in responses to abiotic and biotic stresses [74] and transgenic *Arabidopsis* and rice plants overexpressing stress-responsive NAC genes have displayed improved drought tolerance. These studies indicate that stress-responsive NAC transcription factors have important roles for the control of abiotic stress tolerance and that their overexpression can improve stress tolerance via biotechnological approaches [48].

Interestingly, rice plants overexpressing *OsNAC6* possessed enhanced tolerance to abiotic (dehydration, high salinity) as well as biotic stresses (blast disease) [47]. The *Arabidopsis* NAC TF, ATAF2, is induced by salicylic acid (SA) and methyl jasmonate (MeJA) treatments, and is

also differentially expressed following wound stress response [22]. The potato *StNAC* gene shows induced expression in responses to *Phytophthora infestans* infection and wounding treatment [23]. Barley plants with the *HvNAC6* gene knocked down show penetration resistance in epidermal cells when inoculated with virulent isolates of *Blumeria graminis* f. sp. *hordei* [25]. Overexpression of rice *OsNAC4* resulted in hypersensitive response (HR) cell death; and in the *OsNAC4* knocked down lines, HR cell death was markedly decreased in response to the avirulent bacterial strain (*Acidovorax avenae* N1141] [67]. Therefore, it seems that plant NAC TFs play multiple roles in defense responses to pathogen attack as well as exogenous stimuli [74].

Although these transcription factors can bind to the same core NAC recognition sequence, recent reports have shown that the different NAC TFs have different functions in plant development. In addition, NAC proteins can form homo- or hetero-dimers. Stress-responsive NAC TFs can be used for improving stress tolerance in transgenic plants, although the mode of action appears complex in plants. Recent reports support the notion of substantial crosstalk between plant growth and stress responses. In rice, Kikuchi et al. [33] characterized the molecular properties of eight NAC genes (*OsNAC1* to *OsNAC*8].

In contrast to *Arabidopsis*, the NAC regulon may have additional roles in monocot plants. Important future tasks will, therefore, lie in the comparative analysis of gene expression patterns and the identification of their target genes to determine the function of these genes in plant development and tolerance to abiotic and biotic stresses [49].

Xia et al. [74] reported the full-length cDNA sequence of a novel wheat (*T. aestivum*) NAC TF, *TaNAC8*, (using *in silico* cloning, reverse transcription PCR and 3' rapid amplification of cDNA ends PCR methods. TaNAC8 shows strong homology to rice OsNAC8 with an N-terminal NAC domain and a trans-membrane helices motif in the C-terminus. Yeast one hybrid assays confirmed that TaNAC8's C-terminal region acted as transcriptional activator. Inoculation of wheat with an incompatible isolate of the stripe rust pathogen *Puccinia striiformis* f. sp. *tritici* or treatments with MeJA or ethylene led to increased *TaNAC8* transcription in leaves 24 h post inoculation/treatment. However, SA and ABA had no significant effect on gene expression. Abiotic stress treatments, including high salinity, PEG and low-temperature, also induced *TaNAC8* expression, suggesting that TaNAC8 may function as a transcriptional activator involved in wheat defense responses to both abiotic and biotic stresses [74].

Mao et al. [42] obtained a fragment of *TaNAC2* from suppression subtractive cDNA libraries of wheat treated with PEG, and its full-length cDNA was obtained by screening a full-length wheat cDNA library. Gene expression profiling indicated that TaNAC2 was involved in response to drought, salt, cold, and ABA treatment. Overexpression of *TaNAC2* in *Arabidopsis* resulted in enhanced tolerances to drought, salt, and freezing stresses which coincided with enhanced expression of abiotic stress-response genes and several physiological indices [42].

TaNAC4 encodes another NAC TF in wheat high homology with rice OsNAC4 [73]. Functional analysis using onion epidemical cells and yeast one-hybrid assays confirmed that TaNAC4 functions as a transcriptional activator. *TaNAC4* expression was induced in wheat leaves by infection with stripe rust, and also by MeJA, ABA and ethylene treatments. However, SA had no

obvious effect on *TaNAC4* expression. Similar to *TaNAC8*, abiotic stresses such as high salinity, wounding, and low-temperature also induced *TaNAC4* expression, suggesting a role of TaNAC4 as a transcriptional activator during biotic and abiotic stresses responses in wheat [75].

Rahaie et al. [59] have shown that *NAC67* (BU672229], a putative member of the NAC family was up-regulated during salt stress treatment. The encoded protein has a close homologue in wheat (TaNAC69, E value=$2e^{-15}$1] [59, 78]. Xue et al. [78] demonstrated the role of TaNAC69 in response to abiotic stresses including drought, cold and ABA treatments. Expression analysis of three highly homologous *TaNAC69* genes showed that these were up-regulated during the above-mentioned stresses, especially drought stress. Besides their up-regulation by drought, *TaNAC69* genes were expressed at high levels in the root under unstressed conditions. This suggests that *TaNAC69* genes are not just involved in drought stress, but may also be required in normal cellular activities of roots [78]. Over-expression of *TaNAC69* in transgenic wheat leads to enhanced dehydration tolerance and improvement of water use efficiency [79]. AtNAC2 is also a NAC67 homologue in *Arabidopsis* which is involved in salinity stress, ABA, ACC and NAA treatment in *Arabidopsis*, but *AtNAC2* induction by salt stress requires the ethylene and auxin signaling pathways. It has been shown that the expression level of *AtNAC2* in roots and flowers has been higher than in other tested tissues [20, 59].

7. Enhanced abiotic stress resistance by genetic manipulation of a transcription factor linked to crop yield improvement in the field

In the past decade numerous transgenic plant studies have demonstrated that the improvement of abiotic stress resistance can be achieved by genetic manipulation of transcription factors. However, many resistant transgenic lines with constitutive over-expression of a transcription factor exhibit a slower rate of growth under non-stress conditions. Field trials have also shown that some transgenes tend to have a negative effect on grain yield under normal growth conditions [76]. This phenomenon can theoretically result from the following two causes: (i) genes that are induced during stress generally have a negative impact on the growth and yield, and (ii) the energetic cost of the stress-related metabolite accumulation due to over-expression of a transcription factor. Therefore, the expression of a transcription factor needs to be tailored to meet the requirement for plant stress adaptation if the crop yield is concerned. Any reduction of crop yield under normal growth conditions could potentially override a marked yield advantage under stress.

The expression of a transcription factor can be tailored to stress adaptation by using a stress-inducible promoter. For example, transgenic *Arabidopsis* plants carrying a drought inducible promoter-driven *DREB2A* gene exhibit the improved drought resistance with no significant difference in growth rate under normal growth conditions [64]. Other aspects for consideration of minimizing the negative impact of transgene expression on growth and yield include the appropriate expression level of the transgene and cell specificity. Recently, a root-specific promoter has been used for driving expression of drought-upregulated transcription factors for engineering drought tolerance [26, 60]. Most interestingly, a number of transcription factors

have been shown to improve crop yield under field conditions when they are over-expressed in transgenic plants (Table 1). These studies clearly demonstrate that genetic manipulation of stress-responsive transcription factors has potential for improvement of crop yield in the future, including wheat.

Gene description	Host	Expression mode	Acquired traits	Reference
Rice SNAC1 (NAC)	Rice	Constitutive OE	Improved spikelet fertility under drought and reduced transpiration	21
Maize NF-YB2 (NF-YB)	Maize	Constitutive OE	Less wilting, delayed senescence, higher photosynthesis rate and improved yield under drought	50
ZAT10 (C_2H_2 zinc finger)	rice	Drought-inducible or constitutive OE	Improved spikelet fertility and grain yield per plant under drought	76
CBF3 (AP2)	rice	Drought-inducible OE	Improved spikelet fertility and grain yield per plant under drought	76
Rice AP37 (AP2)	Rice	Constitutive OE	Enhanced drought resistance and grain yield under severe drought conditions	53
Rice NAC10 (AP2)	Rice	Root-specific OE	Enhanced drought resistance and grain yield under both normal and drought conditions	26
Rice NAC9 (AP2)	Rice	Root-specific OE	Enhanced drought resistance and grain yield under both normal and drought conditions	60

OE = over-expression

Table 1. Transgenic crops with over-expression of a transcription factor improve yield under field conditions

8. Conclusion and prospective

Abiotic stresses such as drought, salinity and low temperature adversely affect the growth and productivity of plants. Successful breeding of stress-tolerant varieties will be vital to ensure food supply in areas that are prone to such stresses. Recent advances towards identifying potential abiotic stress tolerance genes have been made. Many TFs and other regulatory genes involved in stress responses have been identified, giving rise to the idea that plants have developed flexible molecular and cellular response mechanisms to respond to various abiotic stresses. bZIP, WRKY, bHLH, MYB and NAC transcription factors represent the major groups of regulatory genes of which some members are found to be involved in abiotic stress responses in plants. To date, the functions of a number of abiotic stress-responsive transcription factor genes have been studied in many different species, including wheat. Recent studies have

indicated that certain stress-induced TF genes play significant roles in wheat stress tolerance. These studies enhance our understanding of the mechanisms of responses and tolerance to abiotic stress in wheat. Also, it provides us a collection of suitable candidate genes for over- or under-expression studies in transgenic wheat aiming to achieve increased abiotic stress tolerance.

In the future, a systems biology approach using reverse genetics, functional genomics and proteomics, as well as metabonomics during various developmental stages and stress conditions will provide us with critical information to elucidate the function of the different stress-responsive TFs and their relationship in transcriptional control in wheat.

In the years ahead, the verification of abiotic stress tolerance and agronomic traits of transgenic wheat utilizing stress-responsive TF genes should be evaluated under harsh field conditions over several years. It can be expected that with increases in climatic variations, more robust cultivars that withstand a wide variety of stresses will be superior over those that are high yielding under optimal conditions. To this end, it will be necessary to clarify the differential function of the individual stress-responsive TF genes from different families of TFs for the control of abiotic stress tolerance and other biological processes including biotic stress tolerance, growth regulation, senescence and yield in order to fully utilize the potential of transcription factors.

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Chapter 12

Water Deficit Stress - Host Plant Nutrient Accumulations and Associations with Phytophagous Arthropods

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Additional information is available at the end of the chapter

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1. Introduction

When the availability of water is insufficient to maintain plant growth, photosynthesis, and transpiration, plants become water deficit stressed (Fan et al., 2006), a serious problem that reduces world crop production (Boyer, 1982; Vincent et al., 2005). While drought has profound direct detrimental effects against plants, including rendering otherwise arable regions less, or non-, arable, herbivorous arthropod populations and the injuries they cause can be affected by stress-related changes that occur in the plant. Moderate stress is known to heighten the nutritional value of some plants' tissues and juices, in some instances to reduce concentrations of plant defense compounds, and even to select against predators and parasitoids that otherwise help reduce pest populations to economically tolerable levels, each of which can contribute toward greater pest infestations. Sometimes the injury inflicted on water deficit stressed plants is intensified even if numbers of the pest haven't been affected, as in the instances of honeylocust spider mites, *Platytetranychus multidigituli* (Ewing), on honeylocust trees, *Gleditsia triacanthos* L. (Smitley & Peterson, 1996), and greenbug and flea beetle, *Aphtona euphorbiae* Schrank, on several different crop species (Popov et al., 2006). When the stress associated with water deficit is more severe, however, host plant suitability for utilization by arthropods declines (Mattson & Haack, 1987; Showler, 2012) because of insufficient availability of water for the pest, and from senescence and drying of the plant's tissues. As plants desiccate further, they eventually die and concerns about arthropod pest damage to that crop become moot unless the pests move from unsuitable dead plant material to vulnerable, living crops.

Although severe water deficit stress that causes plant mortality usually renders plants useless to herbivores, chronic lower level or pulsed water deficit stress can enhance the nutritional value of plants to arthropods, resulting in selection preference, heightened populations, intensified injury to crops, and even outbreaks that affect production on area-wide scales. Twospotted spider mite, *Tetranychus urticae* Koch, populations, for example, increase on drought stressed soybeans, *Glycine max* (L.) Merrill (Klubertanz et al., 1990) and populations of the Russian wheat aphid, *Diuraphis noxia* (Morvilko), increased in nonirrigated wheat, *Triticum aestivum* L., fields as compared with fields that received irrigation (Archer et al., 1995). The cabbage aphid, *Brevicoryne brassicae* L., infested water deficit stressed rape, *Brassica napus* L., more heavily than nonstressed plants (Burgess et al., 1994; Popov et al., 2006), and greenbug, *Schizaphis graminum* (Rondani), densities were higher and more injurious to wheat stressed by drought (Dorschner et al., 1986). Water deficit stressed host plants are also known to favor the xerophilic maize leaf weevil, *Tanymecus dilaticollis* Gyllenhall (Popov et al., 2006); scolytid bark beetles infesting trees (Lorio et al., 1995); flea beetles on corn, *Zea mays* L. (Bailey, 2000); and the fall armyworm, *Spodoptera frugiperda* (J. E. Smith), on tall fescue, *Festuca arundinacea* Schreb. (Bultman & Bell, 2003). Under circumstances where water deficit is beneficial to arthropod pests, population growth generally results in further damage to crops that have already been injured or stunted by water deficit stress itself.

Water deficit stress in plants can affect the amounts and composition of volatile compounds, and the concentrations of several kinds of nutrients beneficial to arthropod pests. Its associations with free amino acids and carbohydrates are chiefly described in this chapter because those two kinds of nutrients have been researched to an appreciable extent, permitting some conclusions to be drawn about arthropod host plant selection and levels of infestation.

2. Water deficit, host plant nutrient accumulation, and associations with phytophagous arthropods

Water deficit stress alters plant metabolism and biochemistry (Hsiao, 1973; Beck et al., 2007), and consequent changes to plant physiological processes have been reported as being factors affecting herbivorous arthropod host plant preferences, growth, and development (Mattson & Haack, 1987; Showler, 2012). Although soil dries in association with drought, evapotranspiration rates in affected plants are often maintained (Jordan & Ritchie, 1971) by elevated accumulations of free amino acids, especially proline, and other organic solutes (Janagouar et al., 1983). Osmotic stress in plants involves several interlinked molecular pathways that transmit signals and produce stress-responsive metabolites (Ingram & Bartels, 1996; Zhu, 2002), and gene transcripts associated with signaling can be up- or down-regulated minutes after stress induction (Seki et al., 2001; Showler et al., 2007). Water deficit stressed plants often have diminished osmotic potential (Labanauskas et al., 1981; Golan-Goldhirsch et al., 1989; Bussis & Heineke, 1998), heightened oxidative stress (Becana et al., 1998; Knight & Knight, 2001), and accumulations of osmolytes such as antioxidants, amino acids, carbohydrates, and inorganic ions, altering the attractiveness and nutritional value of the plant (Jones, 1991; Showler & Castro, 2010a). Reduced leaf water content relative to dry

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biomass in water deficit stressed plants, in combination with the increased quantities of nutritional metabolites (White, 1984; Dubey, 1999; Ramanulu et al., 1999; Garg et al., 2001), may contribute toward the increased nutritional value of plants per unit of surface area consumed by arthropods. It is likely that arthropods can perceive cues about host plant suitability from emission of plant volatile compounds, or semiochemicals.

Chemical cues from plants play a major, perhaps decisive, role in host plant selection and utilization by herbivorous arthropods (Schur & Holdaway, 1970; Fenemore, 1980; Waladde, 1983; Burton & Schuster, 1981; Ramaswamy, 1988; Salama et al., 1984; Udayagiri & Mason, 1995). Water deficit stress in plants alters plant metabolism which can affect quantities and combinations of volatile compounds (Apelbaum & Yang, 1981; Hansen & Hitz, 1982; Zhang & Kirkham, 1990). Apple trees, *Malus domestica* Borkh., for instance, emit 29 volatile compounds, some of them in elevated amounts during water deficit stress (Ebel et al., 1995). Many phytophagous arthropods appear to respond to certain blends of volatiles (Miller & Strickler, 1984) that signal the host plant's nutritional value (Mattson & Haack, 1987; Bernays & Chapman, 1994; Showler, 2012). Increased production of volatiles (*e.g.*, ethylene, acetaldehyde, and ethanol) resulting from plant stress (Kimmerer & Kozlowski, 1982) can be attractive to some herbivorous arthropods and repellent to others (Chrominsky et al., 1982; Dunn et al., 1986; Haack & Slansky, 1987; Bernays & Chapman, 1994). Ethylene, for example, attracts the boll weevil, *Anthonomus grandis grandis* Boheman (Hedin et al., 1976), and, in many host plants it can increase susceptibility to the Egyptian cotton leafworm, *Spodoptera littoralis* Boisd. (Stotz et al., 2000), but ethylene deters the fall armyworm from corn (Harfouche et al., 2006) and the olive moth, *Prays oleae* Bern, from olive trees (Ramos et al., 2008). Forest outbreaks of many species of scolytid bark beetles (Hodger & Lorio, 1975; Wright et al., 1979; Vité et al., 1986; Ormeño et al., 2007; Branco et al., 2010) and the western spruce budworm, *Choristoneura occidentalis* Freeman, are related to amounts and kinds of host plant volatiles emitted during conditions of drought (Cates & Redak, 1988).

Once the phytophagous arthropod has found or selected the host plant, contact chemoreceptors on many are important in the acceptance or rejection of a host plant based on the presence or absence of stimulant (*e.g.*, sugars, amino acids, vitamins) or deterrent chemicals, and moisture (Dethier, 1980; Schoonhoven, 1981; Städler, 1984; Otter, 1992; Krokos et al., 2002). Free amino acids, for example, elicit electrophysiological responses from the sensillae of lepidopteran larvae (Städler, 1984; Blaney & Simmonds, 1988). Many free essential amino acids (essential for insect growth and development) accumulate in plant tissues during water deficit stress in crop plants that range from cotton to sugarcane, *Saccharum* species, to pine trees, *Pinus* species (Mattson & Haack, 1987; Showler, 2012). Amino acids were even found to be more important determinants of corn susceptibility to neonate fall armyworms than toxins or other biochemical factors (Hedin et al., 1990). Resistance against the sugarcane aphid, *Melanaphis sacchari* (Zehnter), and the yellow sugarcane aphid, *Sipha flava* (Forbes), involved absence of some free essential amino acids in resistant sugarcane varieties (Akbar et al., 2010). Free amino acids are more available for use by herbivorous arthropods because insects absorb nitrogen through the gut mostly as free amino acids or small peptides (Brodbeck & Strong, 1987). Hence, enhanced foliar nutritional value as a result of water deficit is known to be an important determinant of

neonate lepidopteran performance (Mattson, 1980; English-Loeb et al., 1997; Showler, 2001, 2012; Showler & Moran, 2003; Moran & Showler, 2005; Chen et al., 2008). In terms of water deficit stress, the mealybug *Phenacoccus herreni* Cox & Williams develops and reproduces better on drought stressed than on well watered cassava, *Manihot esculenta* Crantz, in response to greater concentrations and more nutritious combinations of free amino acids (Calatayud et al., 2002). The eldana borer, *Eldana saccharina* Walker, a stalkborer of sugarcane in Africa, prefers water deficit stressed host plants (Moyal, 1995), and the European corn borer, *Ostrinia nubilalis* (Hübner), inflicts up to twice the injury to water deficit stressed corn than to corn under conventional irrigation (Godfrey et al., 1991). Correlations were reported between elevated free amino acid concentrations in phloem sap of water deficit stressed wheat, *Triticum aestivum* L., and barley, *Hordeum vulgare* L., and population increases by the bird oat-cherry aphid (Weibull, 1987) and the cabbage aphid on *Brassica* spp. (Cole, 1997). Similarly, bark beetle outbreaks during times of drought are associated with greater concentrations of amino acids (and soluble sugars) in host plant phloem that likely contribute toward improved scolytid performance (Mattson & Haack, 1987).

In addition to elevated levels of free essential amino acids, free proline, a nonessential amino acid that accumulates in most water deficit-afflicted plants, is a feeding stimulant for many phytophagous arthropods (Mattson & Haack, 1987; Städler, 1984). Dadd (1985) reported that a number of amino acids, particularly glycine, alanine, serine, methionine, histidine, proline, and γ-aminobutyric acid, were phagostimulants to a number of insect species. Amino acids that elicited the greatest response as feeding stimulants to southwestern corn borer larvae were determined to be arginine, histidine, lysine, methionine, phenylanaline, valine (essentials), alanine, glycine, and serine (nonessentials) (Hedin et al., 1990), but not proline.

Water deficit stress has also been associated with increased concentrations of carbohydrates (which have important roles in osmotic adjustment) in many plants (Schubert et al., 1995; Kameli & Lösel, 1996; Massacci et al., 1996; Mohammadkhani & Heidari, 2008). Corn plants with elevated soluble carbohydrate concentrations were preferred by the European corn borer for oviposition (Derridj & Fiala, 1983; Derridj et al., 1986), and styloconic sensilla of larvae and adults of three noctuid species were highly responsive to sugars, especially sucrose and fructose (Blaney & Simmonds, 1988). These two sugars are known to be important feeding stimulants for both life stages (Frings & Frings, 1956; Blom, 1978), and fructose, glucose, maltose, and sucrose have been identified as phagostimulants for other insects (Bernays, 1985). Electrophysiological recordings revealed that the maxillary sensilla styloconica of fifth instar African armyworm, *Spodoptera exempta* (Walker), and the lepidopteran stalkborers *E. saccharina*, *Maruca testulalis* (Geyer), and *Chilo partellus* (Swinhoe), were stimulated by 13 different carbohydrates (Otter, 1992). In an experiment involving fall armyworm larval feeding, sucrose elicited ≥5-fold more feeding response than fructose or glucose (Hedin et al., 1990). Carbohydrates are well known as sources of energy for arthropods, and they are therefore highly important as nutrients (Nation, 2002). Studies on larval rice stem borers, for instance, showed that fructose, glucose, and sucrose are highly nutritious as compared with other carbohydrates based on their growth and development (Ishii et al., 1959; Ishii, 1971). Also, eastern spruce budworm, *Choristoneura fumiferana* Clemens, outbreaks often follow

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droughts (Mattson & Haack, 1987) because water deficit stressed trees accumulate sugar and sugar alcohols (Price, 2002).

3. Water is a nutrient, too

Water deficit affects both the availability of water, which is a nutrient itself, to herbivores as well as the nutritional quality of dietary biochemical components that accumulate as osmoprotectants or for other purposes. When herbivorous arthropods are unable to have access to sufficient amounts of wager, their populations can decline. For example, aphid populations are reduced under conditions of continued and severe host plant water deficit (Showler, 2012). Black bean aphid, *Aphis fabae* Scopali, survivorship was diminished on continuously drought stressed sugar beet, *Beta vulgaris* L., leaves (Kennedy & Booth, 1959), and reproduction and survival were negatively affected for the mustard aphid, *Lipaphis erysimi* (Kalt.) on radish, *Raphanus sativus* L. (Sidhu & Kaur, 1976); the spotted alfalfa aphid, *Therioaphis maculata* (Buckton), on alfalfa, *Medicago sativa* L. (McMurtry, 1962); the greenbug on sorghum, *Sorghum bicolor* (L.) Moench (Michels & Undersander, 1986); the potato aphid, *Macrosiphum euphorbiae* (Thomas), on potato, *Solanum tuberosum* L. (Nguyen et al., 2007); the bird oat-cherry aphid, *Rhopalosiphum padi* (L.), on tall fescue (Bultman & Bell, 2003); and the eastern spruce gall adelgid, *Adelges abietis* (L.), on Norway spruce, *Picea abies* (L.) Karst. (Bjőrkman, 2000). The most likely cause of the host plants' unsuitability for aphids under such conditions is low turgor which reduces the ability of aphids to feed (Levitt, 1951; Wearing & Van Emden, 1967). Turgor facilitates aphid ingestion by forcing fluids out of the plant and through the aphids' stylet lumens (Kennedy & Mittler, 1953; Maltais, 1962; Auclair, 1963: Magyarosy & Mittler, 1987; Douglas & Van Emden, 2007); turgor loss reduces or curtails feeding by aphids despite their cybarial pump. This has been reported to occur for the black bean aphid on different plant hosts (Kennedy et al., 1958); the cotton aphid, *Aphis gossypii* Glover on cotton, *Gossypium hirsutum* L. (Komazaki, 1982); the greenbug on wheat (Sumner et al., 1983); and the pea aphid, *Acyrthosiphon pisum* Harris, on alfalfa (Girousse & Bournoville, 1994). Also, greater concentrations of host plant osmolytes and other biochemicals associated with drought stress increase sap viscosity which resists flow through the stylets (Douglas & Van Emden, 2007), impeding ingestion despite the enriched nutritional quality of the sap (Kennedy et al., 1958).

The greater nutritional quality of water deficit stressed plants can be offset by the condition that causes it: insufficient water. When provided with dried, ground material from water-deficit stressed tomato plants, *Lycopersicon esculentum* Mill., incorporated into a nonnutritive diet, beet armyworm, *Spodoptera exigua* (Hübner), larval growth decreased (English-Loeb et al., 1997). Cecropia moth, *Hyalophora cecropia* L., larvae reared on water deficit stressed wild cherry, *Prunus serotina* Ehrh., leaves grew more slowly than those fed on well-watered plants, but they, and beet armyworm larvae on water deficit stressed cotton leaves, consumed greater quantities of leaf tissue in order to gain access to more water, and possibly in order to supplement body water with water derived from respiration (Scriber, 1977; Showler & Moran, 2003). Under field conditions, fall armyworm; soybean looper, *Pseudoplusia inclu-*

dens (Walker); and beet armyworm larval survivorships increased and development was hastened in soybeans that were irrigated compared with dryland-grown soybeans (Huffman & Mueller, 1983). These observations suggest that soft-bodied lepidopteran larvae that live on plant surfaces exposed to the desiccating effects of direct sunlight and ambient air (unlike lepidopteran stalkboring larvae that live in moist plant interiors) are especially vulnerable to the desiccating effects of insufficient water supply.

4. Some non-nutrient-related associations of water deficit with phytophagous arthropods

Host plant selection among insects also involves visual and physical factors such as leaf shape, color, and size (Ramaswamy, 1988; Renwick & Radke, 1988; Renwick & Chew, 1994; Showler & Castro, 2010b), and both constitutive and inducible plant chemical defenses can vary in response to water deficit stress (Lombardero et al., 2000), but visual and physical cues, and defensive compounds are not considered as being nutritional for the purposes of this chapter (although defensive compounds might loosely be considered as being types of nutrients, they mostly repel, interfere with feeding, or act as toxins). Concentrations of several classes of defensive secondary compounds tend to increase in plant tissues in response to moderate drought, including terpenoids (some of which are attractants (Mattson & Haack, 1987) and alkaloids (Gershenson, 1984; Hoffmann et al., 1984; Sharpe et al., 1985; Lorio, 1986; Mattson & Haack, 1987; Showler, 2012), but intensified drought stress can lead to reductions of these compounds (Mattson and Haack, 1987). Drought can also influence predator and parasitoid guilds that affect phytophagous arthropod populations (Showler, 2012), but plant stress is not directly involved. Other mechanisms that might also contribute toward plant vulnerability to herbivorous arthropods under conditions of water deficit stress have been suggested (Mattson & Haack, 1987), including acoustical cues, detoxification of foods by drought stressed insects, and drought-induced genetic changes in arthropods, but they have not been well substantiated.

5. Multiple effects of water deficit: case study on sugarcane and the Mexican rice borer

The Mexican rice borer, *Eoreuma loftini* (Dyar), and its association with sugarcane is arguably one of the most illustrative examples of how an economically important phytophagous arthropod is affected by limited availability of water. The crambid moth is indigenous to western Mexico (Morrill, 1925; Van Zwaluwenberg, 1926) where it is a major pest of sugarcane, but it had spread by the mid 1970s to Veracruz, San Luis Potosi, and Tamaulipas in eastern Mexico (Johnson, 1984). First detected in the United States in the Lower Rio Grande Valley of Texas in 1980 (Johnson, 1981, 1984; Johnson & Van Leerdam, 1981), the pest dispersed into rice producing areas of east Texas (Browning et al., 1989; Reay-Jones et al., 2008), and in

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2008 it moved into Louisiana (Hummel et al., 2008, 2010). Because the Mexican rice borer was recently determined to prefer corn over other crop plants (Showler et al., 2011), its assumed range might be considerably underestimated (Showler & Reagan, 2012).

Eggs are mostly deposited in clusters within folds of dry sugarcane leaves, although eggs are also laid in folded green living tissue if available (Showler & Castro, 2010b). Van Leerdam et al. (1986) found 96% of the pest's eggs on the basal 80 cm of sugarcane plants where most dry leaf tissue is located. The Mexican rice borer is not so much stress-oriented as it is nutritionally-oriented in that it prefers to lay eggs on dry foliage of plants stressed by limited water and of plants growing in enriched soil (Showler & Castro, 2010a; Showler & Reagan, 2012). Water deficit stress in sugarcane plants, however, unlike over-fertilized plants, offers increased quantities of dry, folded leaf tissue per plant, contributing to the crop's vulnerability (Reay-Jones et al., 2005; Showler & Castro, 2010b). In a greenhouse no-choice cage experiment using sugarcane plants from which all dry leaf tissue was excised and removed from the cages, or placed at the bottom of the cages like a mulch, and intact (dry leaf tissue remained on the plants) sugarcane plants (controls), numbers of eggs and the degree of larval infestation was distinctly greater on the controls (Figs. 1 & 2; Showler & Castro, 2010b).

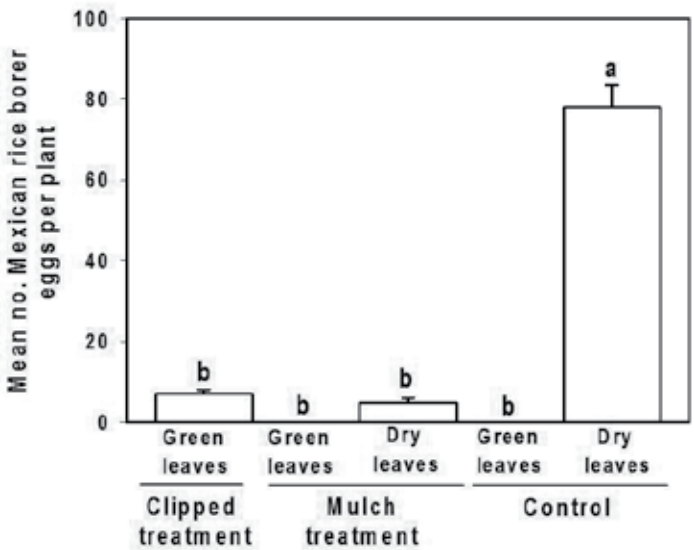

Figure 1. Mean (± SE) numbers of Mexican rice borer eggs on green and dry leaf tissue per sugarcane plant; ANOVA, Tukeys HSD ($P < 0.05$), $n = 7$ replicates per assay (Showler & Castro, 2010b).

Early instars feed on living leaf tissue, under fresh leaf sheaths, and some tunnel into the leaf midrib; later instars bore into the main stalk (Wilson, 2011). Injury from stalk tunneling results in deadheart, decreased sugar production, and stunting or lodging of stalks sometimes so severe that harvest becomes unfeasible (Johnson, 1985; Legaspi et al., 1997; Hummel et al., 2008). Tunnels within host plant stalks are packed with frass, blocking entry of predators and parasi-

toids (Hummel et al., 2008). Pupation occurs within the stalk after mature larvae make emergence holes protected with a thin window of outer plant tissue (Hummel et al., 2008). In the Lower Rio Grande Valley, a life cycle takes 30–45 days, and there are 4–6 overlapping generations per year (Johnson, 1985; Legaspi et al., 1997). Tunneling damage and the insect's prevalence has made it the key sugarcane pest of south Texas, displacing the sugarcane borer, *Diatraea saccharalis* (F.) (Van Leerdam et al., 1984; Legaspi et al., 1997).

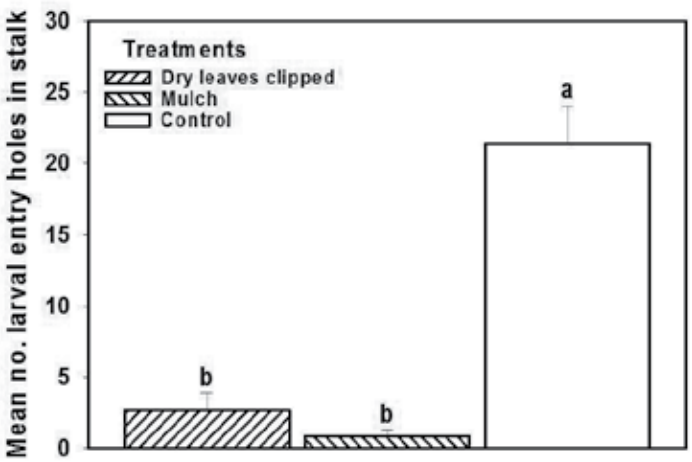

Figure 2. Mean (± SE) numbers of Mexican rice borer larval entry holes per sugarcane stalk; ANOVA, Tukeys HSD ($P < 0.05$), $n = 7$ replicates per assay (Showler & Castro, 2010b).

Approximately 20% of sugarcane internodes are injured by Mexican rice borers in south Texas, and larval entry holes also provide portals for red rot, resulting in additional loss of sugar (Van Zwaluwenberg, 1926; Osborn & Phillips, 1946; Johnson, 1985). On some varieties of sugarcane, up to 50% bored internodes have been reported (Johnson, 1981); Mexican rice borer injury results in losses of US$575 per hectare of sugarcane (Meagher et al., 1994) and US$10–20 million annually (Legaspi et al., 1997, 1999). Projected economic consequences of Mexican rice borer infestation of Louisiana includes US$220 million in sugarcane and US$45 million in rice (Reay-Jones et al., 2008). In corn, stalk boring and secondary infection by stalk rot pathogens can cause shattering, lodging, and complete collapse of stalks (Showler et al., 2011) such that by season's end >50% of stalks of susceptible varieties are destroyed (Showler, unpublished data).

A connection between irrigation practices and severity of Mexican rice borer infestation was first suggested by Meagher et al. (1993), and later studies indicated that drought stressed sugarcane is preferred for oviposition because there is more dry leaf tissue and the nutritional value, at least in terms of a number of important free amino acids, is enhanced (Tables 1 & 2) (Muquing & Ru-Kai, 1998; Reay-Jones et al., 2005, 2007; Showler & Castro, 2010a). Although severe water deficit stress of sugarcane reduces sugar production, some cultivars under moder-

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ate stress accumulate sugars (Hemaprabha et al., 2004), and Mexican rice borer preference among species of host plants (Showler et al., 2011) has been associated with concentrations of fructose (Showler, unpublished data). Differences in oviposition preference were not observed on excised dry leaf tissue regardless of whether the sugarcane plant from which it originated was water deficit stressed or well watered; hence, the expression of sugarcane vulnerability or resistance appears to require the pest's ability to detect nutrients in living leaf tissue (Showler & Castro, 2010b). Although a sugarcane cultivar with some degree of resistance to the Mexican rice borer was still better protected than a susceptible variety under drought conditions, water deficit increased injury to the crop by ≈2.5-fold in each (Reay-Jones et al., 2005). Reay-Jones et al. (2003) also reported that high soil salinity, a stress factor that also heightens free amino acid accumulations in plants (Labanauskas et al., 1981; Cusido et al., 1987), increases Mexican rice borer infestations in sugarcane. Further, relatively high concentrations of organic matter incorporated into soil of the Lower Rio Grande Valley (and conventionally fertilized with nitrogen) resulted in 18% more stalk production per sugarcane stool but this effect was offset by substantial increases in Mexican rice borer infestation, causing stalk weight, length, and percentage brix reductions relative to sugarcane fertilized with conventional nitrogen fertilizer or chicken litter (Showler, unpublished data). The composted soil was associated with greater accumulations of free amino acids and fructose (Showler, unpublished data). These associations reveal that the pest is not responding simply to water deficit, but instead to nutritional enhancement of the plant whether moderated by stress or by other factors.

		Treatment[b]			
Measurement	F, P[a]	L97-128 W	CP70-321 W	L97-128 D	CP70-321 D
Water potential	1,177.41, <0.0001	9.2 ± 0.5b	10.3 ± 0.36b	30.0 ± 0.2a	30.0 ± 0.2a
No. dry leaves	25.16, <0.0001	2.0 ± 1.0b	3.8 ± 0.8b	9.5 ± 0.8a	10.3 ± 0.4a
No. egg clusters	7.26, 0.0025	0.3 ± 0.3b	0.5 ± 0.3b	2.7 ± 0.6a	2.0 ± 0.4a
No. eggs	6.93, 0.0038	4.7 ± 4.7b	5.0 ± 3.2b	48.0 ± 13.7a	29.3 ± 8.5a
No. entry holes	20.33, <0.0001	5.8 ± 0.5b	4.8 ± 0.5b	10.2 ± 1.2a	10.8 ± 1.2a
No. exit holes	12.28, 0.0003	1.7 ± 0.4b	1.5 ± 0.2b	4.7 ± 0.7a	5.0 ± 0.7a

[a] One-way ANOVA, randomized complete block design, df = 3,15.

[b] W, well watered; D, drought stressed.

Table 1. Mean (± SE) water potential (bar), and numbers of dry leaves, Mexican rice borer egg clusters, total eggs, entry holes, and exit holes per stalk of two sugarcane varieties maintained under well watered or drought stressed greenhouse conditions (Showler & Castro, 2010a)

Free amino acids[a]	*F*, *P*	L97-128 W	CP70-321 W	L97-128 D	CP70-321 D
Alanine	3.55, 0.0480	7323 ± 1858ab	3478 ± 1124b	15078 ± 1847a	7857 ± 2167ab
Arginine	6.45, 0.0075	1358 ±347a	462 ± 129b	1272 ± 188a	855 ± 132ab
Aspartic acid	1.34, 0.308	2533 ± 257	2443 ± 157	1996 ± 187	2118 ± 289
Glutamic acid	13.07, 0.0004	6 ± 6ab	149 ± 69ab	29 ± 13bc	860 ± 206a
Glycine	5.36, 0.0142	484 ± 144b	441 ± 81b	1161 ± 87a	653 ± 105ab
Histidine	11.47, 0.0008	424 ± 103b	938 ± 199b	1618 ± 172a	1995 ± 211a
Isoleucine	16.68, 0.0001	703 ± 173c	1263 ± 145b	1916 ± 332a	2858 ± 215a
Leucine	17.75, 0.0001	731 ± 150b	946 ± 127b	1939 ± 285a	2639 ± 285a
Lysine	6.15, 0.0090	708 ± 91a	328 ± 87b	639 ± 92a	514 ± 68ab
Methionine	16.18, 0.0002	384 ± 57b	228 ± 76b	1224 ± 226a	1241 ± 250a
Phenylalanine	19.73, 0.0001	239 ± 59b	229 ± 23b	523 ± 84a	1008 ± 123a
Proline	16.89, 0.0001	421 ± 70b	497 ± 128b	1674 ± 520a	4062 ± 903a
Serine	5.51, 0.0130	3612 ± 809b	4290 ± 892ab	6875 ± 737ab	8875 ± 1125a
Threonine	9.01, 0.0021	1464 ± 251b	2568 ± 484ab	2863 ± 353a	3887 ± 306a
Tyrosine	32.51, <0.0001	209 ± 24c	138 ± 7c	318 ± 31b	515 ± 58a
Valine	12.63, 0.0005	1826 ± 391b	3188 ± 470a	3871 ± 490a	5584 ± 351a
Free essential amino acids	11.02, 0.0009	7841 ± 1248b	10153 ± 1686b	15365 ± 1678a	20585 ± 1474a
Total FAAs	6.92, 0.0059	22432 ± 4034b	21592 ± 3865b	42498 ± 4143a	45528 ± 4159a

Means within each row followed by different letters are significantly different ($P < 0.05$).

[a] Cystine was detectable but not found in the samples.

[b] One-way ANOVA, randomized complete block design, df = 3, 12.

[c] W, well watered; D, drought stressed

Table 2. Mean (± SE) picomoles of free amino acid per µl of sugarcane leaf juice in two varieties, L97-128 and CP70-321, that were well watered or drought stressed (Showler & Castro, 2010a)

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In addition to water deficit stress associations with Mexican rice borer preferences for physical (*i.e.*, dry, curled leaf tissue) and nutritional factors (*i.e.*, amino acids and possibly sugar accumulations), water availability has a strong influence on abundances of a voracious predator, the red imported fire ant, *Solenopsis invicta* Buren, which has already been shown to be an efficient predator of the stalk boring moth, *D. saccharalis*, in Louisiana (Showler, 2012; Showler & Reagan, 2012). Originally from wet habitats of South America, the red imported fire ant entered the United States in 1929 and it spread throughout much of the wet southern states (Lofgren, 1986). To provide another example of the predator's effectiveness against insect pests, red imported fire ant foraging activity accounts for 58% of boll weevil mortality along the relatively wet coastal cotton-growing region of Texas (Sturm & Sterling, 1990), and red imported fire ant predation on immature boll weevils averaged 84% compared with 0.14% and 6.9% mortality caused by parasitism and desiccation, respectively (Fillman & Sterling, 1983). In the drier subtropics of south Texas, however, even in cotton with rank weed growth commonly associated with thriving red imported fire ant populations in wetter regions (Showler et al., 1989; Showler & Reagan, 1991), few or no red imported fire ants were found and boll weevil infestations were not affected by predation (Showler & Greenberg, 2003). While sugarcane in relatively dry regions, such as south Texas, is not protected by red imported fire ants, it is possible that the predator's greater abundance in the more moist sugarcane growing conditions of Louisiana will suppress Mexican rice borer populations (Showler & Reagan, 2012) despite its cryptic larval behavior.

6. Conclusion

Water deficit might initially appear to affect herbivorous arthropod populations because of a single factor, but the associations of the Mexican rice borer with water indicate a more complex relationship that can involve physical, biochemical, and ecological factors. Levels of Mexican rice borer infestation are likely influenced by low water availability in at least three ways, only one of which is directly related to the nutritional status of the crop. Drought changes many environmental conditions relative to arthropods, such as soil condition, leaf size and color, lignification of plant cell walls, secondary protective compounds, and natural enemy activity, but accumulations of nutrients, particularly free amino acids and carbohydrates, unlike the other drought-related conditions, directly result from water deficit stress to the plant. This plant stress response to water deficit influences levels of pest infestations by causing the plant emit volatile semiochemicals and by enhancing the nutritional quality of the plant. Water deficit can also make it difficult for some plant sucking insects (*e.g.*, aphids) to attain water and nutrients, and soft-bodied lepidopteran larvae living on surfaces of water deficit stressed plants ingest insufficient amounts of water to sustain themselves against desiccation despite compensating by consuming greater quantities of plant tissue. While non-nutritional factors are often important under conditions of water deficit, the nutritional status of the plant to herbivorous arthropods is directly modulated by water deficit stress, and host plant nutritional quality is arguably the most fundamental component of plant-herbivore interactions.

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This book is not intended to cover all known abiotic stresses or every possible technique used to understand plant tolerance but, instead, to describe some of the widely used approaches to addressing such major abiotic stresses as drought, salinity, extreme temperature, cold, light, calcareous soils, excessive irradiation, ozone, ultraviolet radiation, and flooding, and to describe major or newly emerging techniques employed in understanding and improving plant tolerance. Among the strategies for plant stress survival, examples of both avoidance and tolerance are presented in detail and comprehensive case studies of progress and directions in several agricultural crops such as apple, walnut, grape and wheat are included.

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