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Alterations to Sustain Unfavorable Conditions

Edited by Diah Ratnadewi and Hamim

PLANT GROWTH AND REGULATION - ALTERATIONS TO SUSTAIN UNFAVORABLE CONDITIONS

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Meet the editors

Dr. Diah Ratnadewi is an academic member of the Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Indonesia. Her scientific work focuses on plant physiology, particularly plant cell growth and development and cell/tissue cultures for secondary metabolite production. She obtained her doctorate degree from the University of Montpellier II, France, in 1987. During 2002–2004 she pursued a postdoctoral research program in Plant Biotechnology Laboratory under the direction of Prof. Dr. Ralf Reski at the Albert-Ludwig University of Freiburg. Her postdoctoral works are related to the functional genes of plant tolerance to abiotic stresses. She has published several peer-reviewed articles in international journals, and is frequently invited to review many manuscripts for publication and research proposals. In 2017, she successfully published a chapter on alkaloids in plant cell cultures, which is part of the book entitled *Alkaloids—Alternatives in Synthesis, Modifications and Application*, published by Intech. As an academician, she gives intensive supervision to students at undergraduate as well as magister and doctoral levels. She manages several extramural academic and scientific collaborations.

Dr. Hamim is a lecturer and researcher at Bogor Agricultural University, Indonesia. He presently works as a lecturer and researcher at the Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University. His subject of interest is plant physiology, particularly ecological plant physiology, including plant growth and developments in response to environmental stresses such as drought and heavy metal stresses. He is curious to discover how metal accumulator plants develop mechanisms to sustain their growth under higher metal contents in their body, and if these mechanisms could be transferred to other plants to develop prospective metal-miner plants. Dr. Hamim accomplished his PhD thesis at the University of Essex, United Kingdom, in 2003. He is well versed in university teaching, presentation, as well as research during his carrier in Bogor Agricultural University. As an addition to his experience in multinational institutions such as FAO, IFAD and WFP, he was appointed as Agriculture Counselor in Rome, Italy, from 2011 to 2014. He has published several peer-reviewed articles and one chapter in the book *Values in Sustainable Development*, published by Routledge. Dr. Hamim is also interested in developing joint research with other experts to support undergraduate as well as postgraduate student exchange programs among universities.

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Preface

During growth and development, from embryo to senesence, a plant undergoes a series of changes at the genetic, metabolic, and cellular levels. Internal and external factors involve and influence those processes, directly or indirectly. Irregularity of climate and weather causes increasing environmental stress conditions, which alter a plant's metabolism, resulting in anatomical and morphological changes. Plants modify their genetic expression, metabolic exertions, and structural properties to resist unfavorable conditions to sustain their life. Better knowledge of ultracellular and even molecular traits has led researchers to apply genetic as well as metabolic engineering with the aim of improving plant adaptability to unfavorable growing conditions.

Plant Growth and Regulation—Alterations to Sustain Unfavorable Conditions contains research results along with deep thought provided by several eminent scientists. This book is divided into two focuses: the first three chapters describe the morphophysiological changes within plants when interacting with the environment, and the following three chapters elaborate the importance of a plant's organelle (chloroplast) to support its existence under natural as well as environmental changes. Chloroplast is not only equipped with pigments but also specific genes that have many important roles in plant production as well as plant adaptation to the environment.

Environmental signals perceived by plant cells induce multiple processes, from the expression of certain correlated genes to biochemical and physical traits. A plant under certain stress conditions behaves diversely, leading to physiological, anatomical, and morphological adjustments. Global environmental changes also influence growth, and the morphological and physiological responses of arid and semi-arid grassland plants. Long dry and warm seasons reduce plant height, leaf area, and other growth parameters, but high temperature with precipitation favors photosynthesis, respiration, and chlorophyll content. The functional traits in plants are regulated and driven by hormones and various growth regulators that ultimately are expressed in plant morphology and plant production. Another unfavorable condition, i.e. excessive metals in the soil, causes cellular and ultrastructural alteration of plant roots. A high concentration of metal deviates root structure to avoid toxic effects and maintain cellular integrity to minimize damage. The alterations are a manifestation of the root mechanism to develop tolerance to metal toxicity, involving series of cues including secondary metabolites. Some secondary metabolites are considered as plant growth regulators, in addition to the conventional hormones. They are synthesized in an upregulated or downregulated fashion depending on the type and severity of stress encountered by the plant. Furthermore, these substances are induced by hormones, indicating a close affinity between plant hormones and secondary metabolites, particularly under stress conditions.

Chloroplast, which contains chlorophylls and several other pigments, has its own DNA. Structure, function, and characterization of chloroplast pigments, and how they assemble, are valuable knowledge. Gene transfer from chloroplast to the nuclear genome occurs naturally and creates a continuous process of plant evolution. There are factors influencing the rate of DNA transfer from chloroplast to the nucleus, and the nuclear integrants of plastid DNA undergo progressive evolution in their new eukaryotic environment. Cytonuclear interactions with chloroplast genomes and the impact of hybridization and allopolyploidy on such interactions are also well described in this book. Since chloroplast genomes are highly conserved, with relatively low rates of mutation compared to the plant nuclear genome, genetic engineering of chloroplastic genes has been intensively applied, which provides advantages over the nuclear genome. Gene transfer into the chloroplast genome presents a number of preferences, such as the absence of epigenetic effects and uniparental inheritance of the transgenes, overexpression of foreign protein, and the ability of multiple transgenes to express in operons.

We are very honored to have been asked to be editors of a book by a respectable publisher, IntechOpen. We do hope this book, with contributions from well-respected authors, will be of value to the development of science and knowledge. The rapid progress of science and technology of living organisms, especially plants, will help humankind be able to cope with global environmental changes.

We greatly appreciate the hard work of all the authors and the kind assistance of the IntechOpen staff that together have made this book possible.

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Chapter 1

The Plant Functional Traits of Arid and Semiarid Grassland Plants under Warming and Precipitation Change

Dan Li

Additional information is available at the end of the chapter

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Abstract

In recent years, climate change has produced a trend of temperature rising and fluctuation of annual precipitation in arid and semiarid grassland ecosystem, which has an impact on grassland plant functional traits including plant morphology, photosynthesis, and respiration efficiency. The dominant species in China's arid and semiarid grassland are *Stipa krylovii*, *Stipa breviflora*, *Leymus chinensis*, and other common steppe species. This study summarized the results of temperature and precipitation change test in the growing season in China's arid and semiarid grassland and analyzed the change rule of plant functional traits of typical species. According to several test results, the plant functional traits generally changed as follows: (1) plant height showed declined trend under warming and dry condition, but plant total leaf number and leaf area increased, and root-top ratio decreased as temperature and precipitation went up. (2) Chlorophyll content and plant photosynthesis and respiration efficiency grew under warming and high precipitation. (3) Hydrothermal conditions had synergistic effect on plant functional traits; increased precipitation helped grassland species to resist high-temperature stress. This study contributed to an understanding of plant morphology and physiological response to climate change of dominant species in China's arid and semiarid grassland.

Keywords: grassland plants, plant functional traits, plant morphology, photosynthesis and respiration efficiency, climate change

1. Introduction

Greenhouse gases such as CO_2 released by human activities contribute to the increase of atmospheric temperature [1, 2]. Sea level rise [3] and global precipitation fluctuation [4, 5] caused by global climate change have great impacts on terrestrial ecosystem. Forest, grassland, and farmland ecosystems show their responses to temperature and precipitation change on microscale, appearing as the relationship between plant morphology and physiological characteristics and climate factors [6–9]. As an important basic part of the ecosystem, plants become an ideal indicator in climate change simulation research. Plant functional traits refer to a series of plant physiological and ecological indicators reflecting plants absorb, use, and maintain resources [10]. Plant functional traits show the functional characteristics of the ecosystem and the change of the environment [11]. In this chapter, plant functional traits include plant morphology, plant photosynthesis, and respiration indicators. Summarizing plant functional traits change rules under warming and precipitation changes contributes to the indication analysis of grassland plants on global climate change and provides scientific basis to explore the response of grassland ecosystem to climate change on microscale.

Grassland is the largest terrestrial ecosystem in China, accounting for 41.7% of China's land area [12]. It is an important ecosystem in northern and northwestern China. The constructive species in China's arid and semiarid grassland are mostly *Stipa krylovii*, *Stipa breviflora*, *Leymus chinensis*, and dozens of other xerophyte grassland plant species. They have curled leaves, inside porosity, narrow leaf area, and strong root system to help them to be resistant to dry climate. In spring, summer, and autumn, the grass leaves are perfect forage for livestock. According to research, since the 1980s, the average temperature in China's Inner Mongolia grassland region experienced an obvious increasing trend; the rate of warming was 0.4°C/10a, which was far more than the average warming rate of mainland China. In addition, the average annual precipitation of Inner Mongolia grassland region in the last 50 years continually decreased with a rate of 4.5 mm/10a [13].

As an important environmental factor of grassland ecosystem, the effects of climate change on the physiological and ecological characteristics of grassland plants are significant. The change of the key environmental factors such as temperature and precipitation will influence the grassland ecosystem dominant species through disturbing their physiological and ecological processes, such as plant morphology, evapotranspiration, decomposition, photosynthesis, respiration, etc. The increase of the concentration of CO_2 in the atmosphere causes warming and precipitation change and has a significant impact on plant functional traits and causes grassland ecosystem adaptations in different scales, grassland communities' productivity, composition, and ecological system change with climate change [14].

The response of grassland ecosystem to climate change is summarized in three scales: species, community, and ecosystem (**Figure 1**). A large number of research focus on the community structure [15], community productivity [16, 17], and the assessment biodiversity of different grassland ecological systems [18, 19]. Most of the plant photosynthesis and respiration research also provide results on ecosystem scale, which discuss the contribution of photosynthesis and respiration to carbon balance [20]. However, there is less research in species scale, focusing on the dominant species of Chinese grassland such as *Stipa* and *Leymus* in the face of the temperature increase and rainfall decrease condition, analyzing the plant morphology,

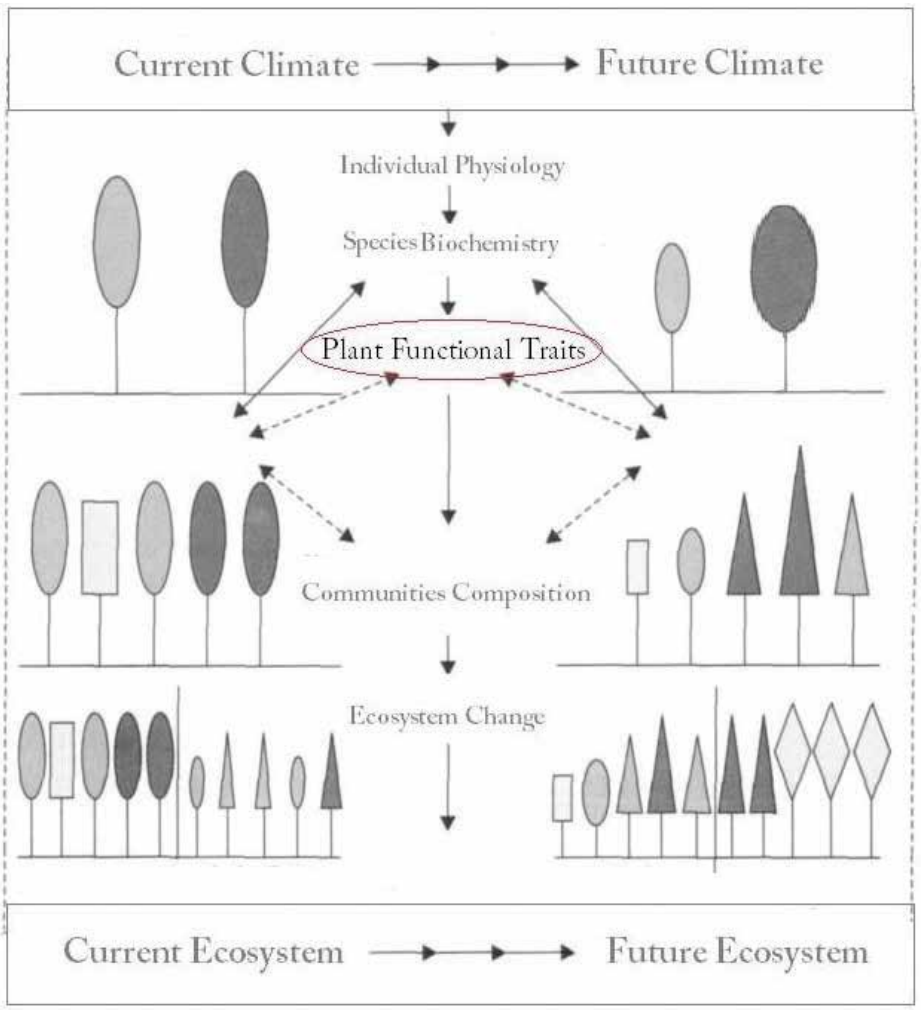

Figure 1. Influence of climate change on ecosystem in different scales.

photosynthesis, and respiration efficiency and other physiological-ecological responses and the adaptive strategies. Therefore, this chapter summarized plant morphology and photosynthetic and respiratory efficiency changes of typical arid and semiarid grassland under different warming and precipitation conditions, showed the response mechanism of typical grassland plant functional traits to climate change, and explained the adaption of China's arid and semiarid grassland ecosystems to climate change on microspecies scale.

2. The influence of warming and precipitation change on plant morphology

Plant morphology refers to the change of plant appearance and structure, including ground parts (leaf, flower, and seed) and underground parts (roots). Environmental changes may

result in different structures of plants with the same genes, such as different plant height and leaf area. The expression of plant physiological changes affected by the environment is shown in the form of plant structure. Therefore, it is helpful to understand the adaptation mechanism of plant morphology under temperature and precipitation change. The responses of plant morphology to temperature and precipitation change were summarized in **Table 1**.

2.1. Effects of warming

The ground part of plant morphology includes plant height, branch, canopy, etc. These characteristics play an important role in the obtaining and use of sunlight energy. The influence of warming on plant structure shows indeterminacy in different species and regions. The herbaceous species in arid region may increase individual growth rate under warming condition, which leads to a shorter growth cycle. As a result, the time for "carbon fixed" and biomass accumulation before seed production is reduced, leading to the formation of shorter individuals by the plant [21].

Leaves are the main plant photosynthetic organs; among all of the plant organs, the leaves have the largest morphological diversity and flexibility under environmental change. The influence of temperature on leaf morphology is indefinitely according to different grassland species [22]. Han et al. [23] found that the leaf thickness of C4 plant such as *Leymus* increased with temperature rise, but the leaf thickness of C3 plant such as *Stipa* did not show the same trend. The influence of temperature on plant total leaf area also indicates a large variability. In general, plant leaf area increases with temperature rise [24]. Under the same temperature range, the increasing rate of annual herb SLA on grassland is larger than woody plant SLA.

The root morphology is the spatial distribution structure of root system. It shows the dynamic response to temporal and spatial variation of nutrient supply and other related constraints

Plant morphology	Warming	Precipitation increased
Plant height	↓	↑
Leaf thickness	C4↑, C3→	Uncertain
Total leaf area	↑	↑
Single leaf area	Uncertain	→
Leaf number	Uncertain	↑
Leaf nitrogen	Uncertain	↓
Root length	↑	↓
Root diameter	↑	Uncertain
Root-top ratio	↑	↓

↑ means positive correlation, ↓ means negative correlation, → means invariability, "uncertain" means insufficient research evidence.

Table 1. The change rule of plant morphology under warming and precipitation change.

[25, 26]. Plant root system has an important role in plants' life course. However, there is little research that focus on root structure and morphological characteristics, and most of them are qualitative description rather than quantitative measurement. In general, warming condition increases the root length and activity, improves the nutrient obtained and rate of the unit root length, and thus affects the growth and morphology of the whole plant root system [27]. The present research suggested that with sufficient nutrition and sunlight conditions, when plants grew below the optimum temperature, warming would promote the expansion of the root, increased the diameter of the fine root, and changed the root branching pattern [28]. When the temperature is higher than optimum, the increase of temperature will limit the growth of plants. The effect of warming on root-top ratio of different grassland species is different. Research on the grassland plants in an arid area showed that the temperature rise had no significant effect on the root-top ratio of herbaceous plant [29, 30], but Hou et al. [31] made a conclusion from the study of four herbaceous plants in grassland that *Stipa* and *Carex* species root-top ratio decreased as temperature rise.

2.2. Effects of precipitation

Water and precipitation are the main limiting factors affecting plant growth and survival in arid and semiarid regions. The adaptation characteristics of desert plants are all related to the use of water resources. Limited precipitation directly restricts the expression of grassland plant morphology, and plants are confronted with the balance of resource allocation in growth, reproduction, and maintenance [32, 33]. The response of grassland plant morphology to precipitation is the results of plants' long-term adaptation to drought.

Precipitation has positive correlations with plant height, total leaf area, plant tiller number, and leaf number but has no obvious influence on single leaf area [34]. The change of total leaf area is mainly because of leaf number change. Leaf nitrogen content and chlorophyll content are positively correlated with precipitation. In China temperate grassland, the *Stipa* and *Leymus* species' leaf nitrogen apparently increases in low precipitation condition, which is the strategy of plant to tolerate water limitation [35], but severe drought decreases wheat nitrogen content [36]. The influences of precipitation on plants have a great extent and depth. Grassland plants have several sensitivity and variable indicator response to precipitation change. Research showed that under less rainfall, *Stipa* plant height, biomass, and seed weight decreased, and leaf length and root-top ratio increased. The *Stipa* species improved water use efficiency by reducing leaf number, spending more on root system to absorb moisture from soil as much as possible [37, 38]. *Stipa* becomes a dominant species in the drought environment in China's Inner Mongolia arid and semiarid grassland. Due to long-term adaptation to the drought environment, *Stipa* adapts to low precipitation by increasing root-top ratio in greater degree, reducing the growth of plant organs—for example, lessen plant height and leaf number—and giving priority in maintaining the growth of root system to ensure the moisture absorption. It is proved that grassland plants have strong drought tolerance ability [39].

Generally speaking, under abundant precipitation, grassland plants have larger total leaf area, plant height, tiller number, and leaf number. However, in the drought environment,

plants adapt to drought conditions with a number of growth strategies, including reducing the ground biomass, plant height, and leaf number, slowing down growth rate, and increasing root-top ratio [40].

3. The influence of warming and precipitation change on plant photosynthesis

Photosynthesis is the process of plant absorbing light energy and compounding organic matter through CO_2 and water (H_2O) synthesis and releasing O_2 at the same time. It is the basis of material circulation and energy flow of land ecosystem. Photosynthesis is easily affected by the growth of plants [41–43] and environmental conditions (such as illumination, temperature, and moisture) [44, 45]. Temperature and precipitation are the most important climatic factors affecting plant photosynthesis. The responses of plant photosynthesis to temperature and precipitation change were summarized in **Table 2**.

3.1. Effect of Warming

Plant photosynthesis is a chemical reaction consisting of a series of enzymatic reactions. Photosynthesis has the highest efficiency under optimum temperature range; temperatures fall too far below or above this range will make photosynthesis efficiency decrease. And the decrease rate shows differences between different species [46]. On the one hand, cold climate inhibits photosynthesis because the low temperature leads to membrane lipid phase change, chloroplast ultrastructure damage, and the passivation of enzymes. On the other hand, high temperature causes thermal denaturation of membrane lipid and enzyme protein and enhances plant light respiration and dark respiration which reduce the net photosynthetic efficiency. C4 plants' optimum temperature of photosynthesis is 40°C, and C3 plants' optimum temperature of photosynthesis was around 25°C [47] (**Figure 2**). Moderate warming could increase photosynthetic efficiency and water use efficiency and raise other photosynthetic parameters of plants [48, 49], but excessive warming will repress photosynthetic efficiency [50].

Temperature affects plant leaf photosynthesis in many ways, such as the influence of temperature on stomatal conductance, chlorophyll content, intercellular CO_2 concentration, thylakoid

Plant photosynthesis	Warming	Precipitation increased
Leaf stomatal conductance	↓	↑
Chlorophyll content	↑	↑
Intercellular CO_2 concentration	↑	↑
Water use efficiency	↓	↓
Photosynthesis efficiency	Uncertain	↑

↑ means positive correlation, ↓ means negative correlation, "uncertain" means insufficient research evidence.

Table 2. The change rule of plant photosynthesis under warming and precipitation change.

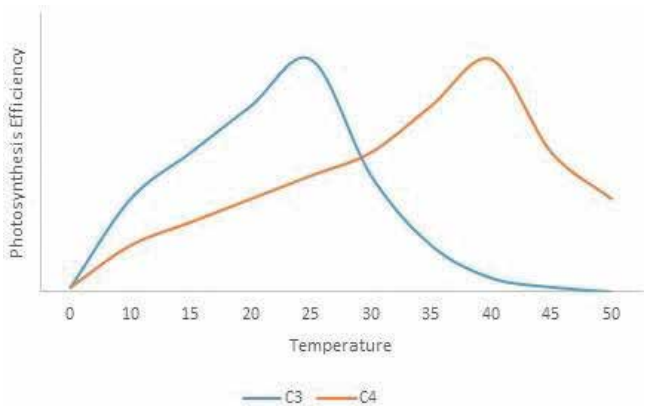

Figure 2. Correlation between temperature and photosynthesis efficiency [47].

membrane, electron transfer, and rubisco activity. It is generally believed that as the temperature increases, leaves' stomatal conductance and transpiration decrease, whereas saturated vapor pressure difference and water use efficiency increase [51, 52]. Hikosaka [53] compared the main parameters of photosynthesis affected by temperature, including the intercellular CO_2 concentration, the maximum rate activation energy of ribulose-1,5-bisphosphate(RuBP) catalyzes (Eav), and maximum rate activation energy of RuBP regeneration ability (Eaj). He pointed out that the Eav and Eaj were the most sensitive parameters to temperature changes and dominant factors to determine the optimal temperature of photosynthesis. At present, the influence of temperature on photosynthetic efficiency of plants is still controversial. The increase of temperature is conducive to plant photosynthesis [54, 55], or inhibitive [56], or no significant effect [57, 58]. This may relate to different geographical locations, vegetation types, and different temperatures, which need to be verified by field simulation results in specific regions.

3.2. Effect of precipitation

Water is an important ecological factor to maintain plant growth and reproduction. Water is not only an important component of plant and metabolism reaction substance but also the solvent of material absorption and transport for plant. However, water consumed by plant photosynthesis only accounts for a small percentage of the plant's absorption from the soil (about 1%), most of the rest is lost by transpiration [59].

In arid and semiarid regions, the influence of precipitation change on photosynthesis is larger than warming [60], because water in this area is the main restrictive environmental factor affecting plant growth and productivity [61, 62]. Research showed that increasing precipitation promoted the photosynthetic rate, stomatal conductance, and transpiration rate of plants but led to the decrease of plant water use efficiency [63, 64], while water stress had the opposite influence [65]. Plant leaf chlorophyll content and photosynthetic rate decrease significantly after precipitation reduces. Even if rainfall increases again, it still could not reverse the downward trend of chlorophyll content at the beginning [66]. The effect of soil water

increased and high temperature together has compensated effects on plant photosynthesis [56]. However, excessive soil moisture content leads to the decrease of soil aeration, interferes with the root activity, and indirectly reduces photosynthesis efficiency.

4. The influence of warming and precipitation change on plant respiration

Respiration is the process by which the organic matter in cells is oxidized and decomposed under the action of a series of enzymes. Respiration provides most of the energy needed for plant life. Part of the energy released during respiration is stored in the form of high-energy compound adenosine triphosphate (ATP). The energy released by ATP hydrolysis process is supporting plant life and activities in the body, such as cell division, plant growth, the absorption of mineral elements, etc. Other energies convert into heat energy and lose in the air. The intermediate product of oxidation provides raw materials for many biosynthesis processes. The emission of CO_2 in respiration is an important physiological process, which affects the carbon balance of plants and ecosystem. The responses of plant respiration to temperature and precipitation change were summarized in **Table 3**.

4.1. Effect of warming

Since respiration is an enzymatic reaction, the factors that affect the activity of enzymes also affect respiration. According to the correlation curve of temperature and enzyme activity, plant respiration has three temperature basis points, the highest, the optimum, and the lowest point (**Figure 3**). Exceeding the lowest and highest temperature point causes the destruction of the cell protoplasmic structure and eventually causes the plant death. The optimum temperature for respiration refers to a temperature range which can maintain a high level of respiration efficiency. For most of the grassland plants, the lowest respiration temperature is about −10 to approx. −20°C. Some cold-resistant plants can survive as low as −25°C. The highest respiration temperature is around 45°C. According to *Stipa* grassland control experiments, the largest respiration rate appears in 40°C, but the duration is very short and quickly decreases. However, at 25°C, although the respiration rate is lower than the rate of 40°C at the beginning, it can maintain a high level for a relatively long time [67, 68]. In addition,

Plant respiration	Warming	Precipitation increased
Leaf stomatal density	*Stipa*, *Leymus*↑ *Carex*→	Uncertain
Metabolic heat release rate	↑	Uncertain
CO_2 output rate	↑	Uncertain
Respiration efficiency	Optimum range↑	Generally ↑, soil moisture saturated or below 60%↓

↑ means positive correlation, ↓ means negative correlation, "uncertain" means insufficient research evidence.

Table 3. The change rule of plant respiration under warming and precipitation change.

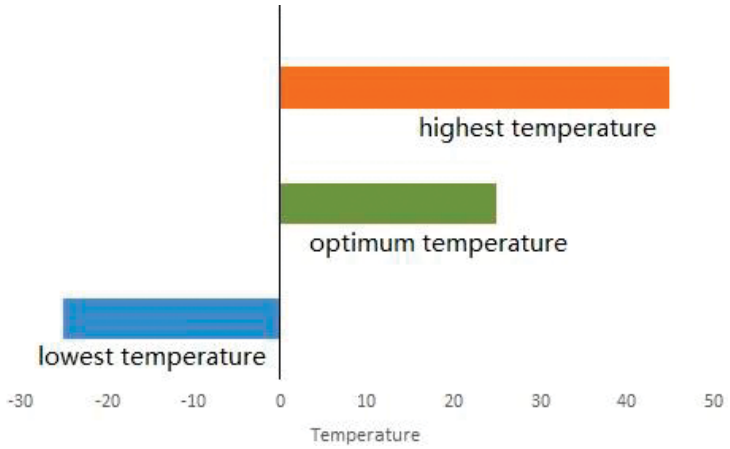

Figure 3. The three basis points of respiration temperature.

the effect of temperature on the respiration efficiency depends on the coordination of other climate conditions. With different conditions, the optimum temperature range of respiration also changes. The plant physiological activity change is the combined contribution of many climate factors. For example, the increasing temperature will improve transpiration of plants and evaporation of soil moisture, promote the mineralization of nitrogen, and cause changes in the activity of enzymes associated with respiration [69, 70].

Some studies confirmed that moderate increase in temperature could promote respiration efficiency [71, 72]. High temperature might cause total leaf area increase [68], and the added leaf stomata increases the cell oxygen partial pressure and therefore promotes the respiration efficiency [73]. The stomatal density of *Leymus* and *Stipa* leaves increases with the high temperature, while the stomatal density of *Carex* does not change with temperature [74]. Under the rising temperature, the metabolic heat release rate (Rq) and CO_2 output rate (RCO_2) of *Ceratoides* are gradually increasing. The differences of Rq and RCO_2 between various *Ceratoides* species increase gradually above 25°C. Compare with the species in Inner Mongolia cold regions, the increasing rate of *Ceratoides* species in relatively warm regions such as Xinjiang, China, shows a greater increase. However, after more than 35°C, the respiratory metabolism of *Ceratoides* is restrained under high-temperature stress.

4.2. Effect of precipitation

Plant respiration is less sensitive to desiccation than photosynthesis. However, the response to desiccation of photosynthesis efficiency and respiration efficiency is roughly the same. In a wide range of moisture in soil from 70 to 90%, the respiration rate maintains relatively stable. But in the range of lower moisture in soil below 60%, the respiration rate decreases with the soil water content [75].

Precipitation can affect the water filling degree of plant cells, and the water saturation degree of protoplasmic cells also has a strong influence on respiration. For plants in the seed stage,

the respiration efficiency increases with the increase of water content. For plant in the growth stage, water and respiration rates are also positively correlated. Nevertheless, when water shortage causes plant leaves to become yellow, their respiration rate will also be extremely active for a short time. It is explained from the perspective of plant material and biological characteristics that swelling of dry seeds and fresh tissue dehydration both can cause enzyme activity transfer to hydrolysis and greatly increase the number of respiratory substrate and reinforce respiration [76]. High-grass steppe experiences plant respiration stagnation when the soil moisture content is saturated or grassland plants are wilted for a long time [77]. In arid and semiarid grassland regions, precipitation is the main limiting factor affecting plant growth and reproduction and also has a great influence on respiration efficiency.

5. Synergistic effects of warming and precipitation on plant functional traits

Temperature and precipitation are important ecological factors of plant growth [78]. Their exclusive or composite change has important effects on plant. Plant functional traits' sensitivity and adaptability to warming and precipitation change determine the degree of the climate change impact on their ecosystem.

In the future climate change scenario, temperature and precipitation are co-changing, and the multifactor effect is significantly different with the simple combination of single-factor effect [41]. The existing simulation experiments on the effects of climate change on plants are mostly isolated temperature or precipitation changes [42], and the range of temperature and precipitation is relatively small. Generally, precipitation is divided into different degrees of drought [79], and the temperature change refers to 2–3°C of warming [13]. Research showed that plant growth has an optimum range for temperature and water changes and growth is inhibited if it exceeds or below the optimum range [80, 81]. If hydrothermal range set in the research is too small or deviated from the optimum range, it is unable to gain a comprehensive understanding of the process of plant responses to hydrothermal change and limits the threshold value and adaptation range of plant response to hydrothermal change. Thus, it has a disadvantage in understanding the adaptation mechanisms of plants to warming and precipitation changes.

5.1. Synergistic effects on plant morphology

Higher temperature promotes plant growth and increases plant productivity [82]. Raising temperature at 3°C can make the mixed grassland underground biomass increase by 11.6% [83], and the increase of water content can promote the net primary productivity of high-grass grasslands [84] and increase the total leaf area [85]. Mild drought does not affect plant growth, while excessive drought inhibits plant growth [86]. The simultaneous increase of temperature and precipitation will significantly increase net primary productivity (NPP) of terrestrial ecosystems, while temperature and precipitation decrease at the same time will

significantly reduce NPP [84]. The above ground net primary production (ANPP) of grassland plant responds to the annual average temperature in logistic relations [87], and the response to precipitation is linear [88].

The effect of precipitation on the *Stipa* plant height is greater than that of temperature, but the effect of temperature on the total leaf area is greater than that of precipitation. The plant morphology response of *Stipa* under synergistic effects of warming and precipitation is different from the exclusive effects. Synergistic effects do not show a significant effect on *Stipa* leaf number but has a significant influence on root length. The synergistic effects have no significant effect on *Stipa* plant height [89], while it has a significant effect on the plant height of *Leymus* [90]. It is showed that the response of different plant morphologic characteristics to hydrothermal synergy is different. The influence of hydrothermal synergy to different plant functional traits should be taken into account, and sensitivity indicators are selected to indicate the response of grassland dominant species to hydrothermal synergistic effects.

5.2. Synergistic effects on plant photosynthesis

Plant photosynthesis is susceptible to internal rhythms and external environmental factors (sunlight, temperature, water, etc.). When temperature changes within the optimum range for plant photosynthesis, the effect of temperature on photosynthesis is positive [91]. Most plants' photosynthesis optimum temperature has a great difference in different environmental conditions. In high-latitude and high-elevation grassland ecological system, the low temperature is often the key limited factor of the plant growth, and warming can promote the plant photosynthesis efficiency in that area [92].

However, the increase temperature causes changes in other environmental factors such as soil moisture. These growing environment changes together with increased temperature will jointly affect plant photosynthesis. In the arid and semiarid grassland, precipitation is the main limiting factor of plant growth [62]. Increasing temperature will reduce plant photosynthesis [52]. The possible reason is that in the whole growing season, daytime air temperature largely increases, and warming causes increasing plant transpiration rate and reduces the soil moisture [58], even leading to dry land. According to the research by Chaves et al. [93], plants under drought stress usually reduced carbon assimilation which consequently limited plant growth. In addition, soil drought after warming is often the main reason that affects photosynthetic physiology of plants. Farquhar and Sharkey [94] believed that factors influencing plant photosynthesis were not only stomatal opening or closing but also other non-stomatal factors. For example, increasing temperature reduces soil moisture and chlorophyll content of *Stipa krylovii*; these changes lead to the decline of photosynthesis efficiency.

In typical arid and semiarid grasslands, effective water is the most important factor controlling plant functional traits. The photosynthesis efficiency of plants decreases under drought condition. According to research by Niu et al. [64], increased precipitation significantly promoted the photosynthetic efficiency of *Stipa krylovii*, especially under soil moisture deficiency caused by temperature increasing. Precipitation may raise phosphorus regeneration ability in photosynthetic phosphorylation process and the chlorophyll content.

5.3. Synergistic effects on plant respiration

The respiration rate is not only related to the environment but also related to plant growth. Under environmental conditions, respiration rate of the same plant varies with its age and growth stage. For example, the young organ with strong activity of meristem has the strongest respiration rate. As plants grow, respiration rate slows down. There are two peaks of respiration during the growth of individual plants: one is the germination period; the other is the flowering period. In the arid and semiarid grassland, the highest respiration rate of *Stipa* appeared in the late spring, with a slight dropped in summer and a rise in fall, until the lowest in winter [95].

The daily change of plant respiration efficiency is mainly affected by temperature, which is the highest in the afternoon and lowest in the night [95]. Seasonal variation is mainly affected by the interaction between temperature and rainfall, and it is related to the limiting factors of the ecosystem. Precipitation change in Inner Mongolia grassland is a decisive factor in the change of respiration rate of *Stipa krylovii* [96]. It is found that excessive high temperature can reduce the respiration rate, but increasing precipitation will lead to a higher respiration rate. However, at the high-temperature conditions, increasing precipitation can reverse the respiration inhibition effects caused by warming. It is suggested that the changes of respiration rate depend on the matching relationship between soil moisture and heat factor [97]. In arid and semiarid grassland, there is a significant positive correlation between temperature and plant respiration rate [98], and in spring and summer when the temperature raise above 15°C, soil moisture will replace the temperature and build a strong positive correlation with respiration rate [99, 100].

6. Conclusion

Under the background of climate change, the response of plant functional traits to temperature and precipitation change directly affects the function and stability of the ecosystem. Plant morphology, photosynthesis, and respiration process have an optimum temperature range, below or above which the growth of photosynthetic and respiration rates will be inhibited. Precipitation change also plays an important role on plant growth and physiological and ecological process. Water is the main limiting factor affecting plant growth in arid and semiarid grassland. In arid and semiarid grasslands, the proper ratio of warming and precipitation is the key factor to regulate the plant functional traits, including plant morphology, photosynthesis, and respiration characteristics.

Drought is an important factor that results in the decrease of grassland ecosystem productivity, biodiversity loss, and ecosystem degradation. In particular, the increase in temperature leads to further aggravation of drought, which seriously affects the growth of grassland plants. Enhanced precipitation could increase the grassland plants' optimum growth temperature, which means that improving precipitation could contribute to resist high-temperature stress. Temperature and precipitation increased synergistic effects have positive effect on plant growth.

In the past 40 years, the annual mean temperature and annual precipitation change increased in arid and semiarid grassland in China, and precipitation suffered reduction. In the future, the temperature will rise in China grassland area, and precipitation will have a weakly reducing trend. A slight increase in temperature and decrease in precipitation will probably promote the growth of underground part of grassland plants. However, if precipitation decreases by more than 30%, the increase in temperature may inhibit the growth of grassland plants. Therefore, climate change in the future will be mainly manifested as temperature rise, and it would make arid and semiarid grasslands in China develop further drought trend, which will be detrimental to the carbon budget and ecological health of grasslands.

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Chapter 2

Cellular and Ultrastructure Alteration of Plant Roots in Response to Metal Stress

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Additional information is available at the end of the chapter

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Abstract

Metal stress is among the important environmental stresses, which influences the growth and development of plants and crops in many areas in the biosphere. Root is an important gate for the absorption of water and mineral nutrition which in many types of lands is also accompanied by a higher concentration of metal elements, either essential (such as Fe, Mn, and Cu) or non-essential metal elements or heavy metals (such as Al, Pb, Hg, Cd, and Ag). In response to metal stress, plant roots sometimes develop a cellular structure to prevent excessive concentration of metal components to avoid toxic effects and cellular damage. Physiological and biochemical responses at the cellular level, which result in ultrastructure changes may occur due to or to avoid the negative effect of metal toxicity. In many cases it was followed by the reduction of root growth followed by discontinuing entirely plant growth. On the other hand, the structural changes are an important part of root mechanism to sustain the plant from metal toxicity. In this chapter, different changes in the cellular ultrastructure resulting from toxic damage or indicating tolerance response to metal stress will be elucidated.

Keywords: metal stress, cellular ultrastructure, root anatomy, heavy metal, metal toxicity

1. Introduction

In nature, plants will face diverse environmental circumstances including unfavorable conditions due to the presence of toxic compounds such as metal elements at toxic concentrations. On the one hand, plants as autotroph organisms require several essential elements from their environment which are mostly metal elements such as Cu, Zn, Mn, Fe, Mo, Co, and Ni but

in small amounts as microelements (trace elements). These elements are essential for crucial biological processes and developmental pathways [1]. But in excessive amounts they will be toxic [2]. On the other hand, their environment sometimes also contains non-essential metallic elements, such as Al, which are normally abundant in the soils with lower pH or even heavy metals such as Pb, Cd, Hg, and Cr on post-mining lands as well as contaminated lands from industrial waste [3, 4]. The existence of these elements causes plants to experience stress, which consequently inhibits the growth of the roots and canopy and can even cause death.

Metal stress occurs due to the absorption of metal elements that exceeds the required concentration threshold which in turn leads to toxicity. For non-essential metallic elements such as Pb, Cd, Cr, and Hg, even at low concentrations, if they are absorbed by plants, they can be toxic for them. The toxic effects of these elements include decreased photosynthesis rate, cell division inhibition, free radical formation, or the inhibition of water absorption rate, which finally cause root growth and plant canopy to be strongly inhibited [5, 6]. Growth is the most easily recognizable morphological parameter of plants undergoing metal stress, where root growth is commonly the most affected. Furthermore, slow growth will result in low crop production if it occurs in cultivated plants.

Some plant species may become resilient to those conditions which allow them to live in environments with higher levels of metals. Some plant species are even able to absorb large amounts of metals in their body that are known as hyper-accumulators such as in *Alyssum bertolonii* and *Berkheya coddii* [7, 8] and *Camellia sinensis* [9]. There are several mechanisms that allow plants to keep growing well in environments with high metal content, including: (a) plants having the ability to keep metal ions from entering into cells, (b) plants having the ability to absorb metals in high concentrations and allocating them certain tissues/organs, and (c) plants having mechanisms that allow metals to be detoxified so that they do not to disrupt plant growth. There are several evidences to show that metal toxicity have a direct effect on growth inhibition of many species, either in roots or in shoots, but the detailed discussion on this response, especially on the perspective of cellular growth, is still rarely found. This will discuss the general feature of growth inhibition of roots in response to metal toxicity and the tentative mechanisms of tolerant plants which are able to sustain their growth under higher metal concentration. This chapter is prepared to present the simple and holistic concept of plant response to metal stress especially in the context of plant growth extracted from newer references and advance researches. The scope is restricted in growth because the initial stage that can be recognized is the inhibition of growth, especially root growth, followed by other morphological and physiological parameters depending on the tolerance level of the plants.

2. Metal source and contaminants in nature

In nature, the abundance of metal elements comes from several sources: (a) from natural parent rocks [10], (b) environmental conditions that influence metal elements to dissolve and cause toxicity to plants such as flooded lands with lower pH [3], and (c) anthropogenic factors, derived from human activities such as mining, industry, and intensive farming activities. Some areas of the Earth have high metal content [11, 12]; one example is the ultramafic bedrock

in Sulawesi, Indonesia, which contains magnesium, iron, and nickel in high quantities [13]. Such soils usually have extreme characteristics because the macronutrient content such as nitrogen, phosphorus, potassium, and calcium is very low while the micronutrient content such as nickel is so high that it is difficult for plants to grow well because of toxicity [14].

Environmental conditions may have set up the abundance of metal elements due to acidified soil. Acid sulfate soil is an example of this which is characterized by an excess of potentially acidic pyritic material over acid-neutralizing free carbonate, adsorbed base, and easily weatherable minerals [15], which cause the accumulation of H^+, Al^{3+}, Fe^{2+}, and organic acid that are toxic to plants [16].

Human activities have influenced the dispersion of metal elements including heavy metals such as Pb, Cd, Ag, Hg, and Cr due to several activities including traditional and mining activities, and intensive agricultural practices such as pesticide and fungicide applications have increased the contamination of metal elements [17–19]. Therefore, heavy metals, especially, have been addressed as critical substances concerning human health and environmental issues due to their high occurrence as contaminants, low solubility in biota, and some heavy metals also have been classified as having carcinogenic and mutagenic effects [20, 21].

Based on plant requirements, metal elements are divided into two groups, essential and non-essential metal elements. Some metal elements such as copper, iron, zinc, manganese, molybdenum, and nickel have important roles in a wide range of physiological processes in plant organs, especially for enzyme activities, which are also known as essential micronutrients or trace element [6]. However, at higher concentrations, they can also be toxic to the plants [22, 23]. Another group of metals such as chromium, arsenic, cadmium, mercury, and lead are non-essential and potentially very toxic to the plants even under lower concentrations [22]. Metal toxicity can inhibit photosynthesis and water absorption, disturb carbohydrate metabolism, and initiate the secondary stresses such as oxidative stress, which influences plant growth and development [24].

3. Growth inhibition due to metal stress

Plant growth is among the morphological characteristics, which is normally inhibited by metal stress, and root growth is the most affected, and therefore root growth sometimes becomes an important parameter to analyze plants tolerant to metal stress [25]. The inhibition of roots in several species in response to metal stress has been reported by many authors, species such as rice [26, 27], soybean, sorghum [28], and wheat [29] in higher aluminum concentrations; Brassica species [30] and soybean [31] in Zn toxicity; as well as tea plants [32] and tomato-sensitive as well as tolerant genotypes [33] in cadmium toxicity.

In *Vigna unguiculata*, Al exposure caused great root inhibition even only 5 h after treatment, even though after 18 h the growth recovered with a higher rate for tolerant genotypes while it was lower in sensitive genotypes [34]. **Figure 1** also shows an example of root inhibition in sensitive, transgenic, and tolerant rice in response to aluminum exposure of 15 ppm at lower pH [27]. Lower pH (4.5) decreased the root length of tolerant (HB), transgenic (TS34, TS13-5,

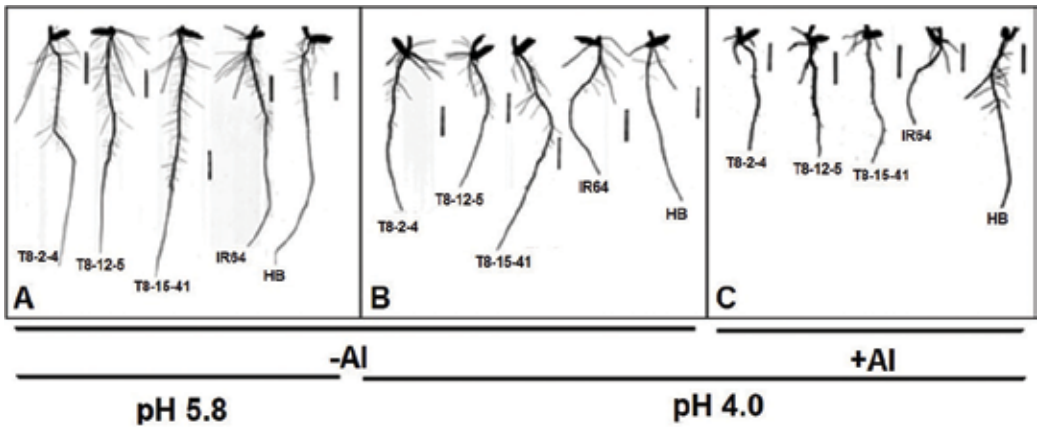

Figure 1. Root growth responses of five rice genotypes to low pH and 15 ppm Al stress. Rice seedlings were grown on nutrient solution at (A) pH 5.8, (B) pH 4.0, and (C) pH 4.0 + 15 ppm of Al. +Al = 15 ppm Al; −Al = 0 ppm Al (control); HB = Al-tolerant rice; IR64 = Al-sensitive rice; T8-2-4, T8-12-5, and T8-15-41 = T4 generations of transgenic lines of IR64. Bar = 1000 mm (After [27]).

TS 15-41) and sensitive rice (IR64) altogether, but Al treatment at 15 ppm caused root inhibition more severe with sensitive variety (IR64) had the lowest root length (**Figure 1**).

At the tissue level, metal toxicity may cause damage to certain tissues such as epidermis, cortex, as well as vascular tissues. The damage of epidermis and cortex tissues was observed when rice seedlings were treated with a high concentration of cadmium [35]. A greater number of nucleoli and vacuoles and enlarged vacuoles were observed in transgenic cotton cultivars exposed to cadmium [36].

At cellular level, metal toxicity has a direct as well as indirect effect on plant physiology and biochemical mechanisms which result in growth inhibition. The direct effect of metal toxicity can be categorized as membrane damage, the alteration of enzyme activity, and the inhibition of root growth, while the indirect effect of metal toxicity can be the disturbance of hormone balance, the deficiency of essential nutrients, the inhibition of photosynthesis, changes in photo-assimilate translocation, the alteration of water relations, and so on, which further enhance metal-induced growth reduction [22]. Therefore, root growth inhibition is sometimes followed by damage to root cells that can be observed from cellular ultrastructure as shown in **Figure 2**. Aluminum-sensitive plant roots treated with a concentration of 15 ppm experienced ultrastructural damage and the cells underwent plasmolysis and had irregular shapes, while the transgenic plant cell structure was still intact with a normal tetrahedron shape (**Figure 2**).

Growth restrictions, especially in roots of plants that undergo heavy metal stress, are caused by two fundamental reasons: (a) inhibition of cell division and (b) decrease of cell expansion (**Figure 3**). During the process of growth, cell division in meristematic tissues is an initial stage that must go, by which if cell division is disturbed, the growth will slow down. Higher cellular activity in the meristematic region of the root tip is a key factor that may be disrupted by abiotic stress including metal stress. The inhibition of cell division or cessation of mitosis due to metal stress has been documented in many species, such as cowpea plant (*Vigna*

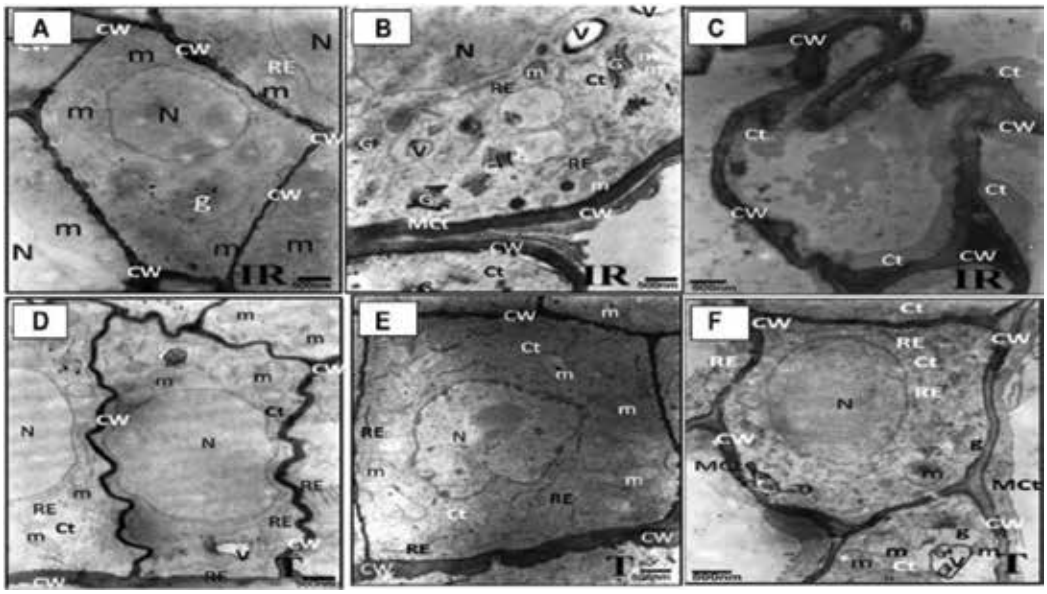

Figure 2. Root tip cell structure after treated with and without 15 ppm Al treatment for 72 h using TEM. (A and D) control treatment without Al pH 5.8; (B and E) control treatment without Al pH 4.0; and (C and F) treatments with 15 ppm Al pH 4.0. Ct = cytoplasm; Cw = cell wall; G = golgi apparatus; IR = IR64; M = mitochondria; MCt = membrane of cytoplasm; N = nucleus; RE = reticulum of endoplasm; T = transgenic rice; TEM = transmission electron microscope; V = vacuole. Magnification 10,000×. Bar = 500 mm (After [27]).

unguicalata) exposed to Al stress [34], *Zea mays*, and *Lemna minor* exposed to Pb [37, 38]. The disorder of cell division often occurs when the basic material for the formation of new cells such as carbohydrates, lipids, and nucleic acids (DNA) is disrupted. Damage to proteins and DNA is one of the effects of metal stress that occurs in many plant species such as in *Urtica dioica* [39]. In addition, some heavy metal such as Pb has caused microtubule disruption in *Zea mays* which caused mitosis inhibition [38].

In addition to cell division, the capacity of plant growth is also determined by cell enlargement and expansion. Cell expansion is an important aspect of cellular growth. During cell expansion, cell wall stress relaxation occurs and results in a decrease in cell water potential and turgor pressure, creating the necessary water potential gradient for water uptake and the irreversible process of cell wall expansion [40]. The process of cell expansion involves important aspects including cell wall loosening or wall stress relaxation, followed by the absorption of water by cells which enlarge and stretch the cells [41, 42]. Therefore, the decrease of cell expansion is mostly triggered by several factors: (1) decrease in cell wall extensibility and elasticity, (2) inhibiting proteins that work in cell wall loosening, (3) decreasing water absorption, (4) the disruption of hormone work, especially auxin which plays an important role in the growth processes, and (5) the decrease of photosynthesis. Wolf et al. [43] suggested that environmental stresses such as salt, heavy metals, osmotic stresses, microbial enzymes, or mechanical injury can threaten the integrity of the rearranging carbohydrate and glycoprotein networks. There are a lot of papers that have explained that some metals including Al are

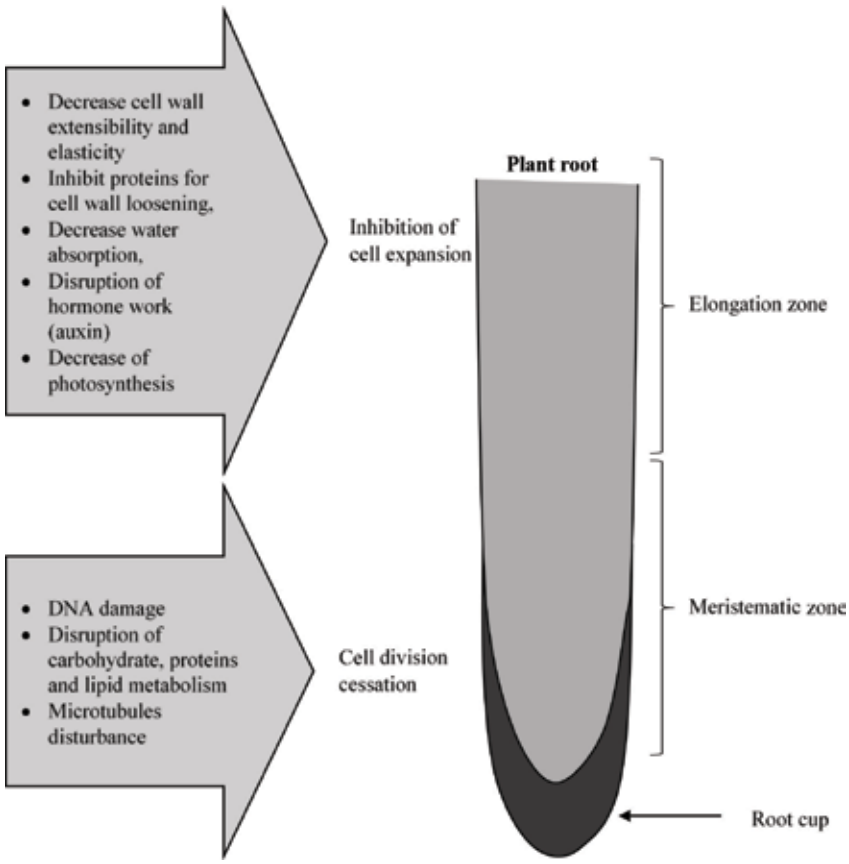

Figure 3. Effect of metal toxicity on roots cell growth involving multifaceted physiological inhibition and disruption including inhibition of cell division in meristematic tissues and inhibition of cell expansion. Cell division cessation may be caused by DNA damage, disruption of carbohydrate, protein and lipid metabolism, and microtubule disturbance. Inhibition of cell expansion could be caused by decrease of cell wall extensibility, inhibition of proteins that work in cell loosening, decrease water absorption, disruption of hormone work and decrease of photosynthesis.

bound to the cell wall such as in algal cells like *Chara coralline* [44], okra hypocotyl [45], and tobacco cells [46], which in turn caused decreased cell wall extensibility and consequently root growth inhibition [45, 47].

Cell wall loosening is a direct cause and an initial part of cell wall expansion which subsequently results in cell growth [48]. Cell wall loosening during cell expansion also involves a group of proteins known as expansins which catalyze the pH-dependent extension and stress relaxation of cell wall [6]. Under normal conditions the decrease of pH in the cell wall will initiate cell wall loosening and cell relaxation. Expansins have the ability to non-enzymatically trigger a pH-dependent relaxation of the cell wall, which loosens and softens it, thus enabling cell expansion. This group of proteins is required in almost all plant physiological developmental aspects, from germination to fruiting, by reducing adhesion between adjacent wall polysaccharides [48]. Some experiments indicated that metal stress caused the inhibition of this group of proteins significantly [49]. In broad beans, some expansin family was also inhibited by Cu and Cd toxicity [50].

Decreased water absorption is one of general effects of metal toxicity, especially generated by heavy metal stresses such as Cd and Hg [51]. The interference with water absorption is partly due to the inactivation of water channel proteins by heavy metals [25]. In addition, the decrease in water potential was probably due to decreased cell wall extensibility or elasticity by cross-linking the pectin carboxyl groups in the walls with heavy metals [22]. In addition to the interference with the absorption of water, metal stress is also suspected to cause the hampering of plant hormones, especially auxins [52]. Although indirect, the decline of photosynthesis also affects cell enlargement, considering that this process will produce the needed materials to form new cell walls. In this phase, photosynthesis also has an important role, so the decline of the photosynthetic rate will result directly in the occurrence of cell division barriers. Data suggest that metal stress results in a decrease in photosynthesis rates such as Cd and Cr [51] and excessive Cu [53, 54].

4. Physiological responses and oxidative stress induced by metal toxicity

In response to metal toxicity, there are several physiological mechanisms exhibited by plants involving biochemical processes as well as cellular and ultrastructural changes (**Table 1**). These mechanisms may be species specific and are associated with its characteristics and tolerance levels to metal toxicity, which comprise two basic mechanisms: (1) retaining metal elements out of cellular cytoplasm through cell wall component binding or active transport excluding the cell and (2) detoxification of metals using chemical compounds such as phytochelatines and metallothioneins and accumulating them in vacuoles (**Figure 4**), which are also known as avoidance and tolerance types [55].

To keep metal elements out of the cytoplasm, cell wall has an important role, because cell wall is a complex structure composed of cellulose microfibrils and non-cellulosic neutral polysaccharides embedded in a physiologically active pectin matrix, cross-linked with structural proteins and sometimes with lignin [56]. The ability of the cell wall to bind divalent metal cations depends on the number of functional groups such as –COOH, –OH, and –SH occurring in cell wall compounds containing cellulose, hemicellulose, and pectin, which are able to bind metal elements [57, 58]. In higher plants, the most significant role is especially determined by polysaccharides abundant in the carboxyl group homogalacturonans (HGA) [59, 60]. In addition to polysaccharide compounds, other compounds such as proteins, amino acids, and phenolics also take part in metal element binding [55].

Accumulation and secretion of organic acids was observed in many species exposed to metal stress, especially Al, Cd, and Pb [9, 61–64]. This organic acid accumulation is associated with the inhibition and avoidance of metals from entering the metabolic-active cellular part through forming metal–organic acid complexes in the cytosol or at the root-soil interface [9]. Cell wall thickening and lignification are also important histological responses of the plants to avoid metal toxicity [35, 42, 63, 65].

It has been well known that plants exposed to heavy metal stresses undergo oxidative stress specified by producing higher free radicals [82–84]. At the cellular level, the generation of reactive

Metal elements	Plant species	Physiological responses	References
Al	*Camellia sinensis*	Malate secretion	[9]
	Triticum aestivum	Citrate secretion	[61]
	Phaseolus vulgaris	Citrate secretion	[66]
	Zea mays	Citrate secretion	[67]
	Glycine max	Oxalate secretion	[68]
	Colocasia esculenta	Oxalate and citrate	[62]
	Brassica napus	Oxalate and citrate	[69]
	Avena sativa	Oxalate and citrate	[70]
	Raphanus sativus Secale cereale	Oxalate and citrate	[71]
	Fagopyrum tataricum	Lower pectin in cell wall	[72]
	Pea (*Pisum sativum*)	Lower pectin in cell wall of tolerant cultivar	[73]
	Rice	α-expansins involved in the root cell wall loosening	[49]
	Medicago sativa	exogenous IAA improve tolerance	[2]
Cd	Rice	Cell wall thickening	[35]
	Cotton	Greater number of nucleoli and vacuoles and enlarged vacuoles	[36]
	Maize	Lignin accumulation and the role	[64]
		apoplastic collenchyma and phloem lignification for metal new bound site	
	Brassica napus	Induced phytochelatin and glutathione	[74]
	Tomatoes genotypes	Induced proline and antioxidant enzymes (APX, GR, CAT)	[75]
	Avena strigosa	Induced antioxidant enzymes and phytochelatines	[76]
	White lupin	Induced Phytochelatines	[77]
	Sedum alfredii	Induced Phytochelatines	[78]
Cd and As	Rice	Disturb IAA biosynthesis	[52]
		Alter the lateral root primordia	
Cd and As	*Pteris vittata* (fern)	Metabolite deposition in intercellular space	[63]
		Induced GSH and phytochelatines (Pcs)	
		Cell wall thickening in epidermis and Increase cuticle	
Cu and Cd	*Broad bean*	Inhibition of a phytochelatin synthase and/or a member of the α-expansin family	[50]
Fe	Wheat	Regulation of phytosiderophore and induction of antioxidative enzymes (CAT, POD, GR) and elevated glutathione, cysteine, and proline.	[79]
Hg	Maize	Induced lipid peroxidation and proline content	[80]
Pb	*Dianthus carthusianorum*	The development and role of pericyclic tissues	[81]
Pb	*Paraserianthes falcataria*	Citrate secretion	[64]

Table 1. Physiological and ultrastructural changes in response to metal toxicity.

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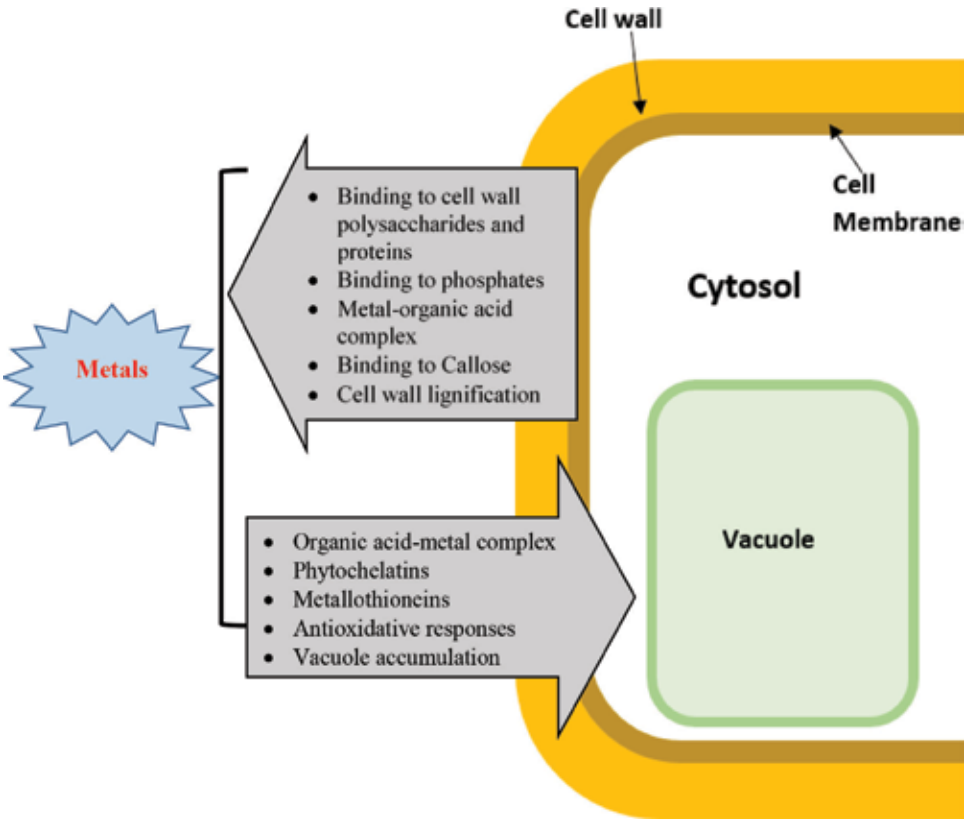

Figure 4. The role of root cells to mitigate metal toxicity involving (1) cell wall barriers such as polysaccharides and proteins binding sites, phosphate binding sites, callose development and cell wall lignification to prevent metals enter to the cells; and (2) cellular resistance mechanism including metal efflux assisted by ATPase-based transporter, phytochelatines, metallothioneins, enzymatic as well as non-enzymatic antioxidant mechanism and accumulation in vacuole.

oxygen species (ROS) which includes superoxide anion (O_2^-), hydroxyl radical (*OH), alkoxyl (RO*), peroxyl (ROO*), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and so on due to metal stress results in oxidative damages to lipids, proteins, and fatty acids which disrupt biomembrane, ultrastructural cellular components, DNA, and causes programmed cell death [85, 86].

Oxidative damage is among the cause of growth inhibition of roots as well as shoots. These reactive oxygen species (ROS) react with lipids, proteins, pigments, and nucleic acids which led to the occurrence of lipid peroxidation, membrane damage, and inactivation of enzymes, thus destroying cell viability [32]. Lipid peroxidation is the general indicator of oxidative stress which is recognized by the accumulation of malondialdehyde (MDA) in the cells or tissues when the plants are under stress [87], and MDA content is often used as an indicator for the extent of oxidative stress [88, 89]. Some experiments showed that cadmium exposure caused gradual the increase of MDA and H_2O_2 content in the leaves as well as roots of resistant as well as sensitive tomatoes [33]. In *Camellia sinensis*, the application of cadmium up to 400 μM caused a linear increase of MDA content, while it caused a significant decrease of chlorophyll and protein content [32]. The significant increase of MDA content was also observed in sensitive rice IR64 treated with 15 mM of Al, while the increase was moderate in tolerant varieties [27].

The plants have specific mechanisms to overcome oxidative stress which in general involves (a) antioxidant enzyme activities and (b) non-enzymatic antioxidant processes. Antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT), glutathione peroxidase (GPX), and dehydroascorbate reductase (DHAR) are among the enzymes that have important roles in cellular scavenging from ROS [82, 90–92]. In *Camellia sinensis*, for example, transcription levels of glutathione reductase (GR), an enzyme involved in the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), showed up-regulation on cadmium exposure [32].

In addition to antioxidant enzymes, to deal with the oxidative stress caused by metal toxicity, the plants sometimes accumulate some non-enzymatic antioxidant compounds such as organic acids, glutathione, tocopherol, phytochelatin, metallothionein, and non-protein thiol [9, 51, 63, 77, 78]. These compounds are important in protecting the cells from the damage caused by heavy metal stress so that plants that have the ability to accumulate such compounds are tolerant to heavy metal stresses [82, 93]. The indication of oxidative stress induced by heavy metals was also demonstrated by the application of several agents such as ascorbic acid, oxalic acid, citric acid, and malic acid [9]. Using Al-sensitive wheat (cv. Scout 66), Ma et al. [29] showed that Al exposure at 10 μM caused a substantial decrease of the roots' elongation of wheat. However, the application of malate, oxalate, and citrate gradually recovered the inhibition of Al to the root elongation as compared to the control, without organic acid application, even though the most effective treatment was using citric acid [29]. Data show that organic acid has an important role in metal toxicity especially Al with different specificities among plant species. Organic acid accumulation including oxalic acid, malic acid, citric acid and glycolic acid was also observed in tea plants treated by high concentrations of aluminum until 2 mM, even though they were decreased when the plant was treated with 4 mM of Al [9].

Glutathione (GSH) is also an antioxidant compound that is known to alleviate the plant from environmental stress, including metal toxicity [51, 94]. GSH is very important because it involves cell protection from free radicals generated from heavy metal toxicity including H_2O_2. In many species, the increase of GSH concentration in the cell has been observed in response to heavy metal treatments, since this compound is known as the precursor of phytochelatin (PC), a typical metal chelator found in plants that facilitates metal sequestration into vacuoles [95], and this has been believed to be part of heavy metal tolerance [96]. Interestingly, the exogenous application of glutathione was also able to alleviate the toxic effect of metal stress especially from Hg toxicity [93]. He explained that the exogenous glutathione application effectively prevented mercury absorption by roots and improved plant tolerance to mercury toxicity by significantly decreased H_2O_2 and O_2^- levels and lipid peroxidation, while it improves the chlorophyll content of *Arabidopsis thaliana*, tobacco, and pepper in the presence of Hg. He also suggested that GSH is a potent molecule capable of conferring Hg tolerance by inhibiting Hg accumulation in plants [93].

Interestingly, the exogenous application of H_2O_2 on *Brassica napus* was able to reduce oxidative stress induced by cadmium application indicated by the decrease of MDA and H_2O_2 accumulation in the plant and the increase of antioxidant enzyme activities, such as APX, DHAR,

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catalase, GR, and GST as well as ascorbate and glutathione content significantly [97]. In this regard, H_2O_2 may become an important substance required to induce antioxidant enzyme activities in the plants when the plant undergoes stress.

5. Accumulator plants are resistant to metal toxicity

Although heavy metals cause plant toxicity, there are some groups of plants that have the ability to accumulate large quantities of metal elements which are known as accumulator plants. These plants are not only able to grow in the area with high metal concentrations but also even able to grow better under high metal contents, even though some plants have slower growth rate. Tea plants (*Camellia sinensis*), for example, have the ability to accumulate aluminum in higher amounts. In his experiments Li et al. [9] showed the growth of *C. sinensis* plant on the medium with Al content ranging from 0, 0.1, 0.4, 2, to 4 mM for 4 weeks, and the best growth was shown by plants treated with Al 0.4 mM. He also showed that even when the plants received Al treatment up to 2 M concentrations they had better growth than control plants [9]. This shows that *C. sinensis* has a high tolerance to Al. Several plant species such as *Alyssum bertolonii*, *Brassica juncea*, *Eichhornia crassipes*, and *Iberis intermedia* have been recognized to accumulate metals in higher concentrations and therefore have been considered to be used in the phytomining of Ni, Co, Tl, Ag, and Au [7, 8, 98, 99]. In an ultramafic area in Tuscany, Italy, *Alyssum bertolonii* was able to extract nickel till 0.7% of its dry weight [7], a very high value of metal component that was there in the plant. Another species *Brassica juncea* was also grown using similar methods that accumulated Au up to 57 mg/kg dry mass [99]. Therefore these plants are categorized as hyper-accumulator plants.

Plants may have ultrastructure modification in shoots as well as root cells in response to metal stress to anticipate the binding or deposition of the metal element when they enter into the cell of accumulator plants. Krzesłowska [55], for example, presented the TEM ultrastructure analysis of poplar root protonema apical cell exposed to lead of 32 μM, and she found that in the cell wall there were extremely large crystalline-like deposits of Pb which thickened the cell wall. She also found internalization of Pb deposits together with pectin in the protonema apical of *Funaria hygrometrica* exposed to 1000 μM of lead.

In maize leaves, the increase of the transversal area occupied by collenchyma in the foliar nervure as well as of the cell wall lignification was pronounced in response to cadmium treatment in combination with lime, even though collenchyma's lignification was not found in the treatment without lime [64]. Another example is cotton, where ultrastructure analysis found cadmium in the form of crystals and electron-dense granules both in the vacuoles and attached to the cell walls, which reveals that the sequestration of cadmium was possibly facilitated by binding with the non-functional parts of the cell, and the increase in number and size of vacuoles and greater number of nucleoli might be important characters of tolerant genotypes to cadmium toxicity in cotton plants [36]. Data show that the accumulation of metals for accumulator or even hyper-accumulator plants may be facilitated by both the capacity of the

cell wall to bind particular metals and the ability to detoxify and have a safer metal-transport mechanism to cell vacuole or other non-active organs. This response may be supported by the dynamic modification of physiological, anatomical, and even ultrastructural changes which allow the plant to sustainably grow under metal stress.

6. Conclusions

Metal toxicity is one of the conditions plants face in the growing environment. Essential trace elements such as Cu, Zn, Fe, and Mn are important to support metabolic processes in the plant, but under high concentrations, they can result in metal toxicity. The presence of non-essential metals such as Al, Pb, Cd, Cr, and Hg in plant media is very toxic to plants even at lower concentrations. Common responses of plants to metal poisoning are the inhibition of growth, chlorosis and necrosis at the leaves, decreased photosynthesis, and even death. The plants have mechanisms to avoid metal toxicity which can be divided into two processes: (1) by avoiding metal elements entering into the cell involving metal-cell wall binding or preventing metal insertion by the chelation mechanism facilitated by organic acid or active exclusion pump and (2) by producing compounds that are able to neutralize the damage when the metal element enter the cell through phytochelatine or metallothionein compounds as well as antioxidant mechanisms before being deposited into vacuole. Ultrastructure changes and cell wall thickening and lignin formation are among the cellular responses that have been observed in many species, while the other phenomena including the increase in the number and size of vacuoles and vesicles inside the cells containing crystalloid-metal elements were also detected.

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Conflict of interest

We declare that they have no conflict of interest.

Nomenclature

MDA	Malondialdehyde
ROS	Reactive oxygen species

CAT	Catalase enzyme
GR	Glutathione reductase
POD	Peroxidases enzyme

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Chapter 3

Chloroplast Pigments: Structure, Function, Assembly and Characterization

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Additional information is available at the end of the chapter

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Abstract

Chlorophyll and carotenoid are vital components that can be found in the intrinsic part of chloroplast. Their functions include light-harvesting, energy transfer, photochemical redox reaction, as well as photoprotection. These pigments are bound non-covalently to protein to make pigment-protein supercomplex. The exact number and stoichiometry of these pigments in higher plants are varied, but their compositions include chlorophyll (Chl) *a*, Chl *b*, lutein, neoxanthin, violaxanthin, zeaxanthin and β-carotene. This chapter provides introduction to the structure and photophysical properties of these pigments, how they assemble as pigment-protein complexes and how they do their functions. Various common methods for isolation, separation and identification of chlorophylls and carotenoid are also discussed.

Keywords: carotenoid, chlorophyll, core complex, ESI-MS/MS, high-performance liquid chromatography, light-harvesting antenna, photosystem, fluorescence spectrum

1. Introduction: Why do we bother about pigments in chloroplast?

Chlorophyll and carotenoid are important pigments that have been used as intrinsic optical molecular probes to observe plant performance during different phases of development. Chlorophyll and carotenoid are biosynthesized in chloroplast and their metabolism is closely related with the chloroplast development. Chlorophyll biosynthesis begins with the formation of 5-aminolevulinic acid (ALA) from glutamate (Glu) via Glu-tRNA synthetase, Glu-tRNA reductase (GluTR) and Glu-1-semialdehyde aminotransferase (GSA-AT) [1]. Eight molecules

of ALA are condensed, eventually forming the symmetric metal-free porphyrin, protoporphyrin IX (Proto IX), which is a common precursor of haem and chlorophyll. The biosynthesis of chlorophyll continues by insertion of Mg^{2+} into Proto IX and followed by several steps in the chlorophyll cycle to create protochlorophyllide.

Further, reaction is one of the most interesting steps because this is the first step in chlorophyll biosynthesis that requires light: the NADPH:protochlorophyllide oxidoreductase converts protochlorophyllide into chlorophyllide. This reaction is then continued to produce chlorophyll (chl) *a* and *b*. So, when dark-grown etiolated seedlings are exposed to light, protochlorophyllide is immediately converted to chlorophyllide and then further to synthesis of chl. Once chl *a* and *b* are formed and properly incorporated into the thylakoid membranes and associated photosystems, chloroplast is fully functional to do photosynthesis [2].

Plant carotenoids are synthesized and accumulated exclusively in plastids, most importantly, chloroplast and chromoplast [3]. There are two types of plant carotenoid: carotene, which is cyclized and uncyclized hydrocarbons, and xanthophylls, which are oxygenated derivatives of carotenes. Carotenoid synthesis is initiated by the formation of C_{40} compound phytoene by the head-to-head condensation of two molecules of geranylgeranyl diphosphate (GGDP) by phytoene synthase and then to a series of 4 sequential desaturation reactions, by two separate enzymes to produce lycopene, which has 11 conjugated double bonds [4]. Lycopene is then cyclized to α-carotene or β-carotene, which is then further hydroxylated to produce colorful xanthophylls such as lutein, β-cryptoxanthin, zeaxanthin, antheraxanthin, violaxanthin and neoxanthin. The biosynthesis and accumulation of carotenoids in the dark-grown etiolated seedling are essential for the assembly of membrane structure and benefits the development of chloroplast when seedlings emerge into the light [5]. Understanding the relationship between structure and photophysical properties of these pigments can provide insights into a better study of how photosynthesis works at the molecular level in chloroplast.

2. Structure, function and photophysical properties

The photophysical properties and functions of chlorophyll and carotenoid reside in their chemical structure. Chlorophylls are defined as cyclic tetrapyrroles carrying a characteristic isocyclic five-membered ring that are functional in light-harvesting or in charge separation in photosynthesis [6]. The chemical structure with IUPAC numbering scheme of chl *a* is shown in **Figure 1**. It is a squarish planar molecule, about 10 Å on a side. An Mg atom in the center of the planar portion is coordinated to four nitrogen atoms. The five rings in chlorophylls are lettered A through E, and the substituent positions on the macrocycle are numbered clockwise, beginning in ring A. Chlorophyll has two molecular axes: *y*-axis is defined as passing through the N atoms of rings A and C and *x*-axis passing through the N atoms of rings B and D. The delocalized π electron system extends over most of the molecule, except for ring D, in which the C-17—C-18 double bond is reduced to a single bond. The tail is formed by condensation of four isoprene units and is then esterified to ring D. It is often called phytol tail, after the polyisoprenoid alcohol precursor that is attached during biosynthesis. Because of the reduced ring D, plant chlorophylls such as chl *a* and *b* are classified as chlorins rather than porphyrins. These types of pigments have (in organic solvents) absorption bands around the blue and red

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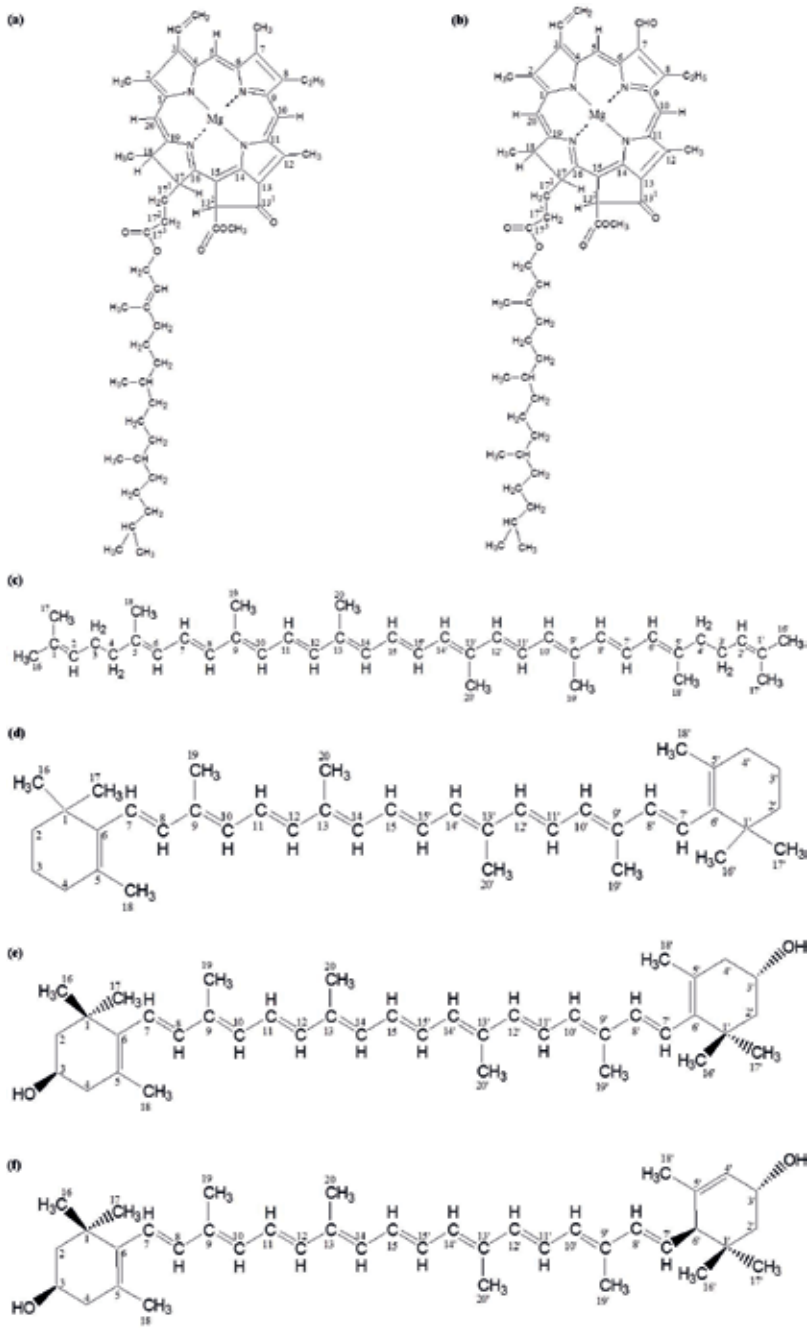

Figure 1. Chemical structure of Chl a (a), Chl b (b), lycopene (c), β-carotene (d), zeaxanthin (e) and lutein (f) with IUPAC numbering system.

spectral regions (**Figure 2a**), which are called B (or Soret) and Q bands, respectively, and arise from $\pi \rightarrow \pi^*$ transition of the four frontier orbitals [7, 8]. One band each pair is polarized along the *x*-axis (B_x, Q_x) and other along *y*-axis (B_y, Q_y). The strong absorption band at the maximum

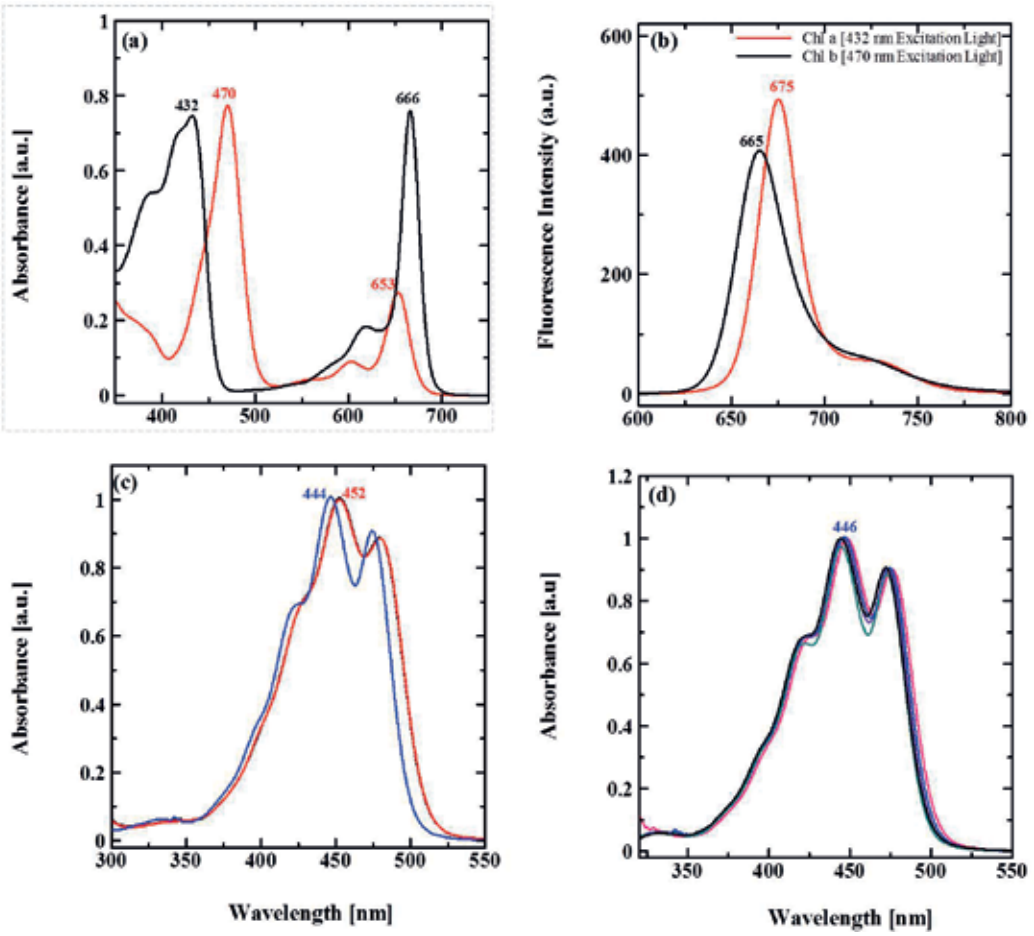

Figure 2. (a) UV–Vis absorption of Chl *a* (black) and Chl *b* (red) in MeOH, (b) fluorescence emission spectra of Chl *a* (red) and Chl *b* (black) in MeOH, (c) β-carotene (red), zeaxanthin (black) and lutein (blue) in EtOH, (d) lutein in several organic solvents; MeOH (black), acetone (pink), diethyl ether (purple), hexane (light blue), EtOH (blue).

absorption wavelength (λ_{max}) 660 nm is called Q_y transition band, which corresponds to the electronic transition polarized along the *y*-axis. The Q_x-band of chl *a* shows a weak band near 550 nm, while the two overlapping Soret (B) bands show at about 430 nm. The chemical structure of Chl *b* is identical to chl *a* except at the C-7 position, where a formyl group replaces the methyl group. This structural change results in a shift of the Q_y maximum absorption to shorter wavelength. The fluorescence spectrum of chlorophylls peaks at slightly longer wavelengths than the absorption maximum. The fluorescence emission (**Figure 2b**) is polarized along the *y* molecular axis, as it is emitted from the Q_y transition. Shift of the emission to the longer wavelength side of the main transition is known as Stokes shift. In light reaction, chlorophyll plays as key pigment in the collection of light energy in the light-harvesting complexes and to carry out reversible photochemical redox reaction (Krasnovsky reaction) in the reaction centers.

Structure of carotenoid is characterized by a linear chain of conjugated π-electron double bonds (**Figure 1**). In oxygenic organisms, carotenoid usually contains ring structures at each

end, and most carotenoids contain oxygen atoms, usually as part of hydroxyl or epoxide groups. The primary molecular factor that gives rise to their strong absorption bands in the visible spectral region is the number of π-electron conjugated double bonds, N. The position of the absorption maxima is affected by the length of the chromophore, the position of the end double bond in the chain or ring and the taking out of conjugation of one double bond in the ring or eliminating it through epoxidation. Progressive movement to longer wavelengths (bathochromic shift) is illustrated by the absorption spectra of the acyclic carotenoid of increasing chromophore length. Carotenoids show different optical characteristics in various solvents, depending on the polarizability of the solvent [9, 10]; however, generally they have a typical three-peaked absorption spectrum with well-defined maxima and minima (fine structure) (**Figure 2a**). A ring closure as in β-carotene produces a less-defined fine structure. The introduction of a carbonyl group in conjugation with the polyene system produces a bathochromic shift and the loss of fine structure [4]. The influence of other substituents such as OH is negligible, for example, β-carotene, cryptoxanthin and zeaxanthin all have very identical absorption spectrums. Owing to the double bonds in the molecule, all carotenoids exhibit *cis-trans* isomerization (stereomutation). A *cis* double bond implies a configuration with the highest-priority group on the same side, whereas in the *trans* configuration they are on opposite sides. The absorption spectrum of a *cis* isomer presents a subsidiary peak in the near-ultraviolet, the *cis* peak; generally, it is located 143 nm from the longest wavelength maximum. For example, *cis* peak will appear at 330 nm if the longest wavelength maximum is 473 nm. In photosynthetic systems, carotenoid has essential functions. First, carotenoid is an accessory pigment in the collection of light energy in the spectral region which chl does not absorb and in transferring energy to a chl pigment [11, 12]. Second, carotenoid functions in a process called photoprotection by quenching triplet state of chl before it reacts with oxygen to form singlet oxygen species (ROS) [13, 14]. Third, carotenoid regulates energy transfer in the light-harvesting antenna through a process called xanthophyll cycle, to avoid over-excitation of the photosynthetic system by safely dissipating excess energy [15, 16].

3. Pigment assembly in pigment protein complexes

In the chloroplast interior, there are four main constituents in plant thylakoids, that is, photosystem II (PSII), cytochrome b_6f, photosystem I (PSI) and the ATP synthesis. Chlorophylls and carotenoids are embedded in PS II and PSI, large pigment-protein clusters, the structures of which are perfectly adopted to ensure that almost every absorbed photon can be utilized to drive photochemistry. Both PSII and PSI consist of two moieties, that is, core complex or the reaction center that is responsible for charge separation and light-harvesting antenna complexes that surround the core complex and have functions to increase the capture of light energy and energy transfer to the reaction center in the core complex.

One can detect chlorophyll and carotenoid bound in PSII and PSI in chloroplast by measuring their absorption and fluorescence spectra. **Figure 3a** (solid red line) shows the absorption spectrum of diluted chloroplast that is indicated by red shift of Chl *a*, Chl *b* and carotenoid's bands because these molecules are bound as pigment-protein complexes in chloroplast. The Soret band of chl *a* in the complexes was detected at 438 nm while in the MeOH it was found

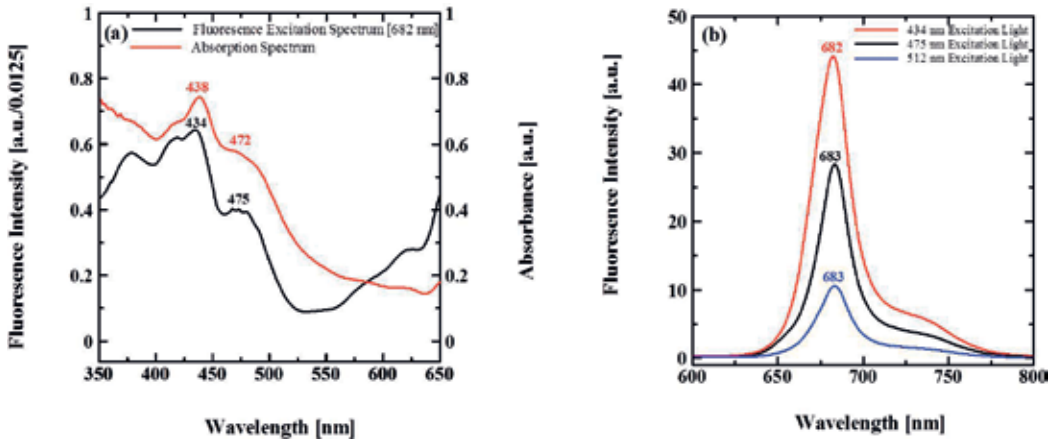

Figure 3. (a) Overlaid of UV–Vis absorption (red) and fluorescence excitation (black) (λ_{em} = 682 nm) spectra of chloroplast and (b) emission spectra of chloroplast with excitation at 434 (black), 475 (red) and 512 (blue) nm. Measurements were conducted at ambient temperature. The isolation of chloroplast was carried out as follows: 20 g of suji leaves (*Pleomele angustifolia*) were washed with running water and cut. The leaves were then homogenized in 200 mL ice-cold isolation buffer (300 mM sorbitol, 50 mM HEPES-KOH pH 7.5, 2 mM EDTA, 80% acetone, 0.1% BSA) for 10 min in a cold environment, followed by filtration using cloth. Centrifugation was conducted in 2 steps, to discard cell debris at 200 g, 4°C, 20 min and to harvest chloroplast pellet at 3000 g, 4°C, 20 min. Final chloroplast pellet was collected and subjected to spectrum UV–VIS (Shimadzu UV-1700)and fluorescence measurement (Jasco FP-8500).

at 432 nm (**Figure 2a** black line). The fluorescence emission spectra (**Figure 3b**) indicate a strong emission band of PSII complexes with maximum wavelength at (λ_{max}) about 682 nm and weak emission band of PSI complexes with λ_{max} at about 730 nm. It is shown here that Chl *a* acts as the main contributor to the excitation band at 434 nm and it shows that excitation at 434 nm (Soret band) produces stronger emission intensity, while the excitation at 475 and 512 nm, correspond to Chl *b* Soret band and carotenoid, respectively, produces weaker emission intensity. If we monitor the emission at 682 nm and measure the excitation spectrum, it shows that the PSII emission at 682 nm is the result of contribution from Chl *a*, Chl *b* and carotenoids (**Figure 3a** solid black lines) with bands at λ_{max} about 414, 434, 475 nm, respectively.

The current high-resolution structural models of antenna complexes have been obtained only for LHCII (2.72 Å) and recently for CP29 (2.8 Å) from PSII of spinach [17, 18]. Here we focus more on the LHCII structure. LHCII shows trimeric structure. Each monomeric contains three transmembrane α-helices, a, b and c (**Figure 4a**). One monomeric subunit contains eight chlorophyll (Chl) *a* pigments, six Chl *b*, two luteins (Lut), neoxanthin and one additional xanthophyll [17, 19]. The 14 chlorophylls are non-covalently attached in the protein cavity. Four carotenoid binding sites per monomer have also been characterized, but in this case the type of carotenoid bound can vary. Typically, two lutein molecules are in groves on both sides of helices *a* and *b* and have been likened to a cross-brace. A third carotenoid, 9-*cis* neoxanthin, is located in the Chl *b*-rich region near helix *c*. The fourth carotenoid is located at monomer-monomer interfaces in the trimer. It has been suggested that this site accommodates carotenoids that can participate in the xanthophyll cycle. It depends on the external stress level of the plant; the fourth carotenoid is either violaxanthin (no or low stress) or zeaxanthin (high stress) [20]. In this structure, the carotenoids are in van der Waals contact with the chlorophylls [9]. This is essential as carotenoids in LHCII act as accessory light-harvesting pigments

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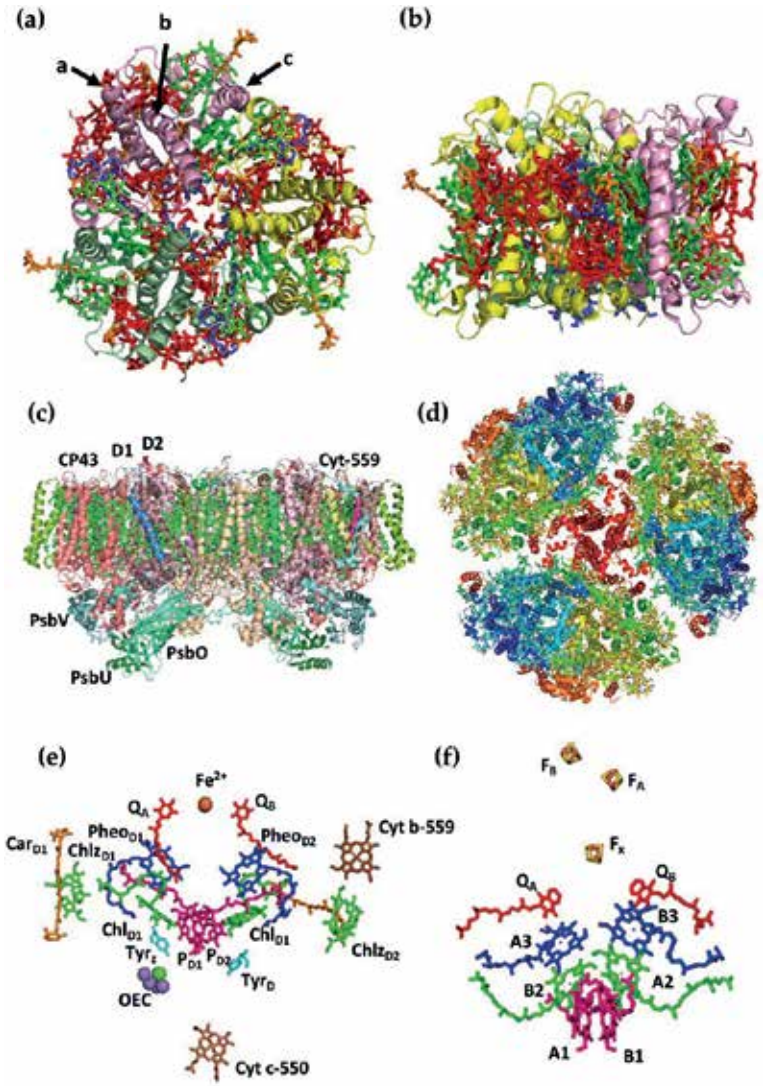

Figure 4. (a) A view looking down on the top of trimeric complex of LHCII structure from spinach. Each monomer is colored magenta, yellow and pale green. The three-transmembrane helices (a, b and c) present in a monomer are labeled and are easily visible. Chl *a* molecules are in red, Chl *b* colored green and carotenoids colored orange. (b) Side-view of LHCII structure shows chlorophyll and carotenoid molecules are packed densely and close to each other (within van der Waals contact), enabling the crucial photo-protective role of these molecules to function by quenching triplet chlorophyll excited states. (c) Structure of PSII from *Thermosynechococcus elongatus* [28], a side-view representation of the overall dimer perpendicular normal with the pseudo-twofold symmetry axis. (e) PSII core reaction center is shown; component co-factors of the electron transport chain viewed along the membrane plane. The two branches are related by the pseudo-twofold symmetry axis. The respective pairs of pigments on the branches are labeled to indicate whether the Mg^{2-} is coordinated by D1, D2. (d) Structure of PSI from *Synechococcus elongatus* [29]; overview of the complete trimer looking along the membrane normal from the stromal side with each polypeptide of the trimer colored differently and chlorophyll molecules given in green. The two main proteins that comprise a monomer are PsaA (yellow) and PsaB (magenta). The electron transport chains are in the center of each monomer. (f) PSI core reaction center component co-factors of the electron transport chain are viewed along the membrane plane. The two branches are related by the pseudo-twofold symmetry axis. The representative pair of chlorophyll molecules on the branches are labeled A or B indicating whether the Mg^{2+} is coordinated by PsaA or PsaB. The iron–sulfur center of F_x involves residues from both PsaA and PsaB, while F_A and F_B are located in an extrinsic subunit called PsaC.

and photoprotectors. The accessory light-harvesting function represents singlet-singlet energy transfer from the carotenoid to the chlorophylls. Since the singlet excited state lifetime of the carotenoid is quite short, approximately 200 fs, the carotenoid must be in close distance to a chlorophyll molecule if the energy transfer is to be efficient. Photoprotection function represents the quenching of triplet excited state of chlorophylls and so preventing the formation of singlet oxygen. This triplet-triplet exchange reaction also requires the carotenoid to be in close contact with the chlorophylls. Regarding CP29, it binds 3 carotenoids and 13 chlorophyll molecules [18]. The position of some chlorophyll binding sites in CP29 differs from LHCII.

The current high-resolution crystal structure of PS II and PSI core complexes is limited to that from cyanobacteria and from pea, respectively [21, 22]. The core of PSII is a multi-subunit complex. Most of the chromophores involve light harvesting as well as electron transfer reaction and are bound to four main subunits, that is, D1, D2, CP43 and CP47. When the core of PSII and PSI reaction center structures is compared, the arrangement of the pigments and other electron transfer co-factors is also very similar (**Figure 4c** and **d**). Here, first we look at the PSII core reaction center. The core of reaction center of PSII is made from two major polypeptides called D1 and D2; each contains five membrane-spanning α-helices. These two helices clasp each other like two cupped hands holding on to each other. The redox cofactors are arranged into two arms that lie on either side of the point where the two groups of helices interact. This arrangement of the helices and the cofactors introduces a pseudo two-fold symmetry axes that runs through the center of reaction center normal to the plane of the membrane. In **Figure 4e**, it is seen that the electron transport pathway in PSII begins with a pair of chlorophyll molecules called P680 (P_{D1} and P_{D2}). Then each arm contains, in order, one monomeric chlorophyll molecule, one pheophytin (a chlorophyll derivative) and one plastoquinone molecule. Here, only the D1 arm is active in electron transport. Upon excitation P680 becomes oxidized and one electron is injected out and passes down the active branch to the quinone Q_A. P680 is re-reduced by electron transfer from a special tyrosine residue called Z (Tyr_z). A second turnover of P680 delivers a second electron to the plastoquinone and the secondary quinone Q_B is now reduced to Q_BH_2. The hole on Tyr_z is filled by electron transfer from the manganese cluster, the oxygen evolving complex. Every four turnovers of P680 stores four positive charges in the manganese cluster that are then used to oxidize water and evolve oxygen. While in CP43 and CP47, there are a total of 49 Chl *a* molecules that are bound and that function as internal antenna and allow excitation energy transfer from the peripheral antenna system to the reaction center.

Unlike PSII, in PS I, the same single polypeptides contain both antenna complexes (Lhca) and the reaction center core. The 3.3 Å resolution crystal structure of PSI from pea showed that plant PSI binds at least 173 Chl *a* and *b* molecules [22]. At this resolution of the crystal structure, it is not possible to identify the Chl species, but biochemical analysis of purified PSI indicated that it has a Chl *a*/*b* ratio in a range of 8.2–9.7 [23, 24]. A large number of Chl *a* and *b* molecules are bound to the Lhca protein, only about 100 Chl *a* are bound in the core complex, and the rest of Chl *a* and *b* are between these moieties. The latter represent the so-called "linker" chlorophylls which are located between Lhca monomers and "gap" chlorophylls (between Lhca and PSI core). The linker chlorophyll molecules probably play an important role in excitation energy transfer between Lhca antennas and from Lhca to the PSI core [20, 25, 26]. Based on biochemical analysis, PSI was reported to bind approximately

33/34 carotenoids, that is, about 12 carotenoid molecules are bound to Lhca, at the interface between Lhca and the core complex, and about 22 β-carotene are bound to the core [20, 23, 26]. Based on these biochemical analysis, it can be estimated that PSI-LHCII supercomplex contains about 215 chlorophyll and 45/46 carotenoid molecules.

The core complex of PSI is composed of smaller number of subunits (15 subunit) than PSII. The large PsaA and PsaB subunit, which contain 11 trans-membrane helices each, forms a heterodimer that binds ~80 Chl *a* and ~20 β-carotene as cofactors for light harvesting as well as 6 Chl *a*, 2 phylloquinones and a 4Fe-4S cluster as cofactors for electron transfer reaction, with the exception of terminal electron acceptors (Fe-S clusters F_A and F_B) which are bound to the PsaC subunit [25]. At closer look (**Figure 4f**), the redox co-factors in the core reaction center are arranged into two arms that are located on either side of the region where two groups of helices interact with each other. Two chlorophylls form P700 and then each arm contains two monomeric chlorophyll molecules (the second one being in the equivalent position to the pheophytin present in photosystem II) followed by one quinone molecule. When P700 is oxidized, both arms of the electron transport pathway are able to work as it was reported that the electron can pass either down the B-branch or the A-branch [27].

4. Chromatographic isolation and identification

Chlorophyll and carotenoid can be isolated as free pigments, detached from the pigment-protein complexes, by organic solvent extraction. Important aspects such as the choice of organic solvents, light exposure and working temperature should be considered while isolating pigments. Based on the structure, chlorophyll is characterized with polar macrocycle ring with non-polar hydrocarbon tail. The structural difference between Chl *b* and Chl *a* is by having an aldehyde group in place of the methyl group at the macrocycle side group. This change is effecting the polarity of Chl *b* to be more polar in comparison to Chl *a*. In the case of carotenoid, structural difference can be seen from the number of conjugated double bonds and the presence of oxygen atoms. Considering these characteristics, mixtures of miscible polar and semi/non-polar solvents are used commonly to extract plant pigments. The mixture of solvent has double functions, that is, penetrating tissues/matrixes and extracting pigments from their lipophilic surrounding. During extraction, exposure of light should be avoided to reduce photodamage of the pigments. Temperature is also important. It is recommended to conduct extraction at lower temperatures, for example, on ice or using liquid nitrogen, to minimize activity of enzyme (e.g. chlorophyllase) that will catalyze breakdown. Antioxidant agent can be also added during extraction to avoid any unwanted oxidation.

After successful isolation, liquid chromatography has been widely used as an effective technique to separate individual type of pigments and for further purification. In this technique, the pigment separation is based on the polarity which depends on the interaction of pigment with the stationary and mobile phases. Elution method either normal phase or reversed phase is chosen according to the type of pigment to be separated. In addition, the choice of liquid chromatographic methods, namely thin layer chromatography (TLC), column chromatography (CC) and high-pressure liquid chromatography (HPLC), is referred to the speed,

resolution and quantity of sample [30]. Currently, ultra-fast liquid chromatography (UFLC), a recent development of HPLC, has been used as a standard for liquid chromatography to achieve high-resolution data with low time consumption [31]. Purification with non-chromatographic method has also been developed, that is, purification method using dioxane has been effective to separate chlorophyll from most of the carotenoids and some lipids [32].

Various types of column absorbents used for chromatographic separation of plant pigments have been well reviewed [30]. Here, we used a silica C30 column attached to UFLC analytic to achieve well separation of carotenoids from *Pleomele angustifolia* leaf using elution gradient program with mixture of water, methanol and methyl tert-butyl ether to separate, at least, 7 dominant pigments within 25 min. (**Figure 5**). The detailed identification of pigments, based on the chromatographic, spectrophotometric and mass properties, is summarized in **Table 1**. Chlorophyll a and chlorophyll b, α- and β-carotenes and violaxanthin are found to be the main chlorophylls and carotenoids, respectively, while the presence of lutein and zeaxanthin in this chloroplast is in low amount.

Larger-scale separation of Chl *a* and *b* can be achieved by CC using Sepharose CL-6B as the stationary phase and a mixture of 2-propanol (IPA) and hexane as the mobile phase. Chl *a* could be eluted using 1.5% IPA in hexane and Chl *b* with 10% IPA in hexane [33]. To achieve a pure, free carotenoid, saponification step is sometimes necessary to eliminate contamination of lipids and chlorophylls. Moreover, carotenoid ester can be hydrolyzed to produce parent carotenoid by using this method [34]. CC is usually used for carotenoid isolation in high quantity of pigment extract. Generally, the purpose of CC is to separate mixtures into carotenoid fractions which are either having high purity to be processed to crystallization or low purity to be extensively separated with further chromatography, that is, HPLC [35].

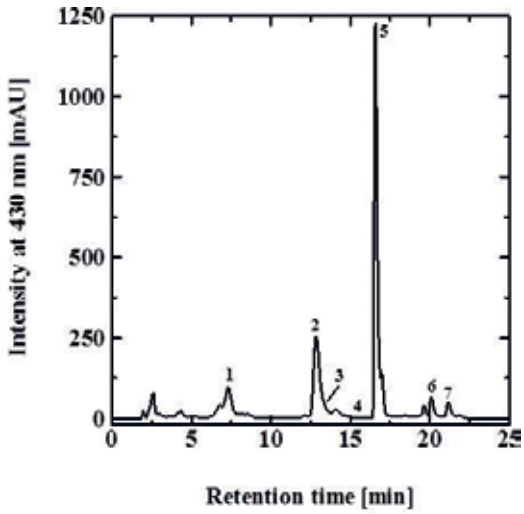

Figure 5. UFLC chromatogram of pigment extract from chloroplast of *Pleomele angustifolia* detected at 430 nm. The UFLC separation condition was as follows: Pigment separation was performed using UFLC equipped with PDA (Shimadzu) on C30 column (150 × 4.6 mm I.D; YMC) with a gradient elution program of water, methanol and methyl tert-butyl ether (MTBE) at the flow rate of 1 mL/min at 30°C.

Peak No	t_R [min]	λ_{maxs} [nm]				Molecular ion	Fragment ions [*m*/*z*]	Identification
		HPLC eluent	Hexane	Ethanol	Acetone	species [*m*/*z*]		
1	7.3	412,436,464	—	—	—	—	—	Violaxanthin
2	12.8	470,601,650	451,595,642	465,601,649	458,596,646	907.7 $[M]^+$	881.7 $[M - COH]^+$	Chlorophyll *b*
							855.7 $[M - COH - Mg]^+$	
3	13.4	422,445,472	422,444,473	–,446,474	–,448,476	568.4 $[M]^+$	551.4 $[M - OH]^+$	Lutein
							476.4 $[M - 92]^+$	
							430.3 $[M - 138]^+$	
4	15.3	–,451,477	425,449,478	425,451, 478	428,454,481	568.6 [M]+	476.4 $[M - 92]^+$	Zeaxanthin
5	16.6	431,618,664	427,613,661	430,616,664	431,617,662	893.5 $[M]^+$	871.5 $[M - Mg]^+$	Chlorophyll *a*
							615.2 $[M - phytyl]^+$	
6	20.1	421,446,473	421,445,474	421,446,476	422,445,473	536.6 $[M]^+$	445.4 $[M + H - 92]^+$	*α*-carotene
7	21.2	–,452,478	–,451,479	–,453,480	–,454,482	536.6 $[M]^+$	444.5 $[M - 92]^+$	*β*-carotene

Table 1. Chromatographic, spectrophotometric and mass properties of pigments separated from the chloroplast of *Pleomele angustifolia*.

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Silica and alumina are frequently used as the absorbent in the CC with the normal phase elution to separate the distinct carotenoids; however, it is not easy to use this method to separate carotenoid isomers, that is, geometrical isomers, diastereoisomers, and so on. In this case HPLC/UFLC can be used to overcome the difficulty in the separation of carotenoids by CC. Turcsi et al. (2016) revealed that the polar carotenoids including optical isomers, and region and geometrical isomers as well as non-polar carotenes, could be well separated by HPLC on C18 and C30 columns, respectively [36]. High purity of isolated pigment can be achieved by HPLC and crystallization processes. UFLC analysis of the purified zeaxanthin shows that this carotenoid had a high purity of around 99.3% (**Figure 2**, left). All purified pigments have purity higher than 95% (**Figure 6**).

Chromatographic, spectrophotometric and mass properties of pigment are minimum requirements for pigment identification [35]. These properties for all purified pigments are shown in the **Table 1**. In **Figure 7** (right), absorption spectra of the purified chlorophyll a and the purified β-carotene in acetone have the same maximum absorption wavelength (λ_{max}) and other spectral properties, such as the fine structure and spectrum shape, compared to these pigments in the references [37, 38]. Absorption spectrum of chlorophyll a in acetone shows typical Soret (431 nm), Qx (617 nm) and Q_y (662 nm) bands, while two well-defined peaks in the absorption spectrum of β-carotene are found at 454 and 482 nm. This pigment analysis based on the results of spectrophotometer UV–Vis could support the advance pigment analysis using HPLC/UFLC equipped with photodiode array detection and coupled with the mass spectrometry. The LCMS technique has provided a power tool for pigment identification [39, 40]. Tentative identification for zeaxanthin peak separated by HPLC/UFLC analysis with PDA revealed that zeaxanthin has similar retention time (t_R), maximum absorption wavelength (λ_{max}) and the shape of absorption spectrum (data not shown) compared to the isolated

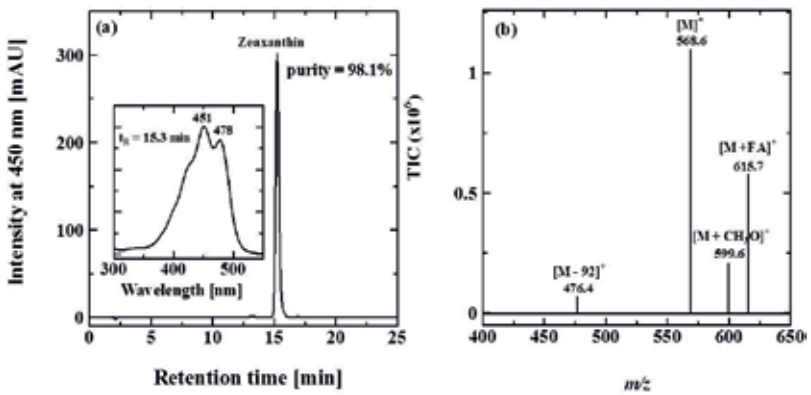

Figure 6. Purification of zeaxanthin: (a) chromatogram detected at 450 nm. Insert figure is UV–Vis spectrum measured by UFLC diode array detector in the eluent and (b) ESI-MS/MS spectrum identification. The conditions of UFLC and ESI-MS/MS analysis were as follows: UFLC analysis of the purified zeaxanthin was performed using UFLC equipped with PDA (Shimadzu) on C30 column (150 × 4.6 mm I.D; YMC) with a gradient elution program of water, methanol and MTBE at the flow rate of 1 mL/min at 30°C. The purified zeaxanthin was directly analyzed to LCMS 8030 (Shimadzu) with an isocratic elution of 0.1% formic acid (FA) in water (10%) and 0.1% FA in methanol (90%) at the flow rate of 0.3 mL/min. MS analysis was operated under the following conditions: (1) heat block temperature = 400°C; (2) desolvation line temperature = 250°C; (3) nebulizing N2 gas flow = 3 L/min; (4) drying N2 gas flow = 15 L/min; (5) interface voltage = 4.5 kV; (6) interface current = 0.1 μA; (7) mass range 400–700 m/z; (8) ionization mode = positive and negative.

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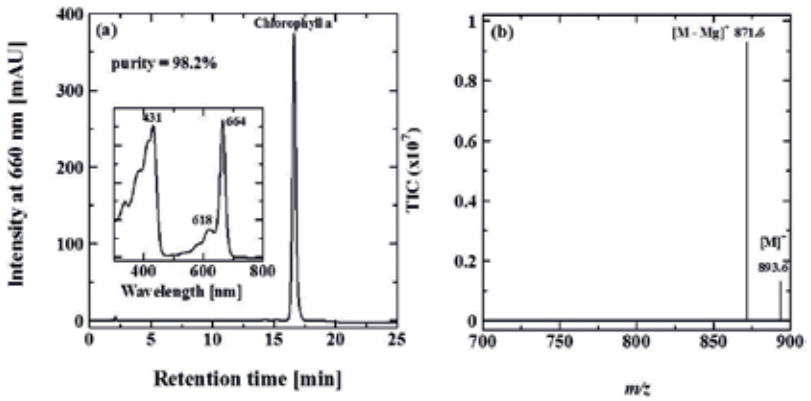

Figure 7. Purification of Chl: (a) chromatogram detected at 660 nm. Insert figure is UV–Vis spectrum measured by UFLC diode array detector in the eluent and (b) ESI-MS/MS spectrum. The condition of UFLC and ESI-MS/MS analysis was as follows: UFLC analysis of the purified chlorophyll a was performed using HPLC equipped with PDA (Shimadzu) on C30 column (150 × 4.6 mm I.D; YMC) with a gradient elution program of water, methanol and MTBE at the flow rate of 1 mL/min at 30°C. The purified chlorophyll a was directly analyzed to LCMS 8030 (Shimadzu) with an isocratic elution of 0.1% formic acid (FA) in water (10%) and 0.1% FA in methanol (90%) at the flow rate of 0.3 mL/min. MS analysis was operated under the following conditions: (1) heat block temperature = 400°C; (2) desolvation line temperature = 250°C; (3) nebulizing N2 gas flow = 3 L/min; (4) drying N2 gas flow = 15 L/min; (5) interface voltage = 4.5 kV; (6) interface current = 0.1 μA; (7) mass range 400–1000 m/z; (8) ionization mode = positive and negative.

zeaxanthin from corn which is a well-known source of zeaxanthin [41]. In addition the mass analysis provides the precursor and fragment ions at the specific *m*/*z* and characteristic fragmentation pattern for pigment identification. Mass spectrum of Chl *a* indicated the molecular ion $[M]^+$ detected at m/z 893.6 and a fragment ion $[M\text{-}Mg]^+$ at m/z 871.6 related to the loss of magnesium as the central metal of chlorophyll (**Figure 1**). This mass spectrum of Chl *a* agrees with the result that was reported [42].

5. Conclusions

Chlorophyll and carotenoid are chloroplast pigments which are bound non-covalently to protein as pigment-protein complex and play a vital role in photosynthesis. Their functions include light harvesting, energy transfer, photochemical redox reaction, as well as photoprotection. The exact number and stoichiometry of these pigments in higher plants are varied, but their compositions include Chl *a*, Chl *b*, lutein, neoxanthin, violaxanthin, zeaxanthin and β-carotene. Liquid chromatography methods are well developed to separate and purify different types of pigments. Identification and characterization of pigments can be well observed by spectroscopy methods such as UV–Vis absorption, fluorescence and mass spectrometry.

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Chapter 4

The Intertwined Chloroplast and Nuclear Genome Coevolution in Plants

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Additional information is available at the end of the chapter

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Abstract

Photosynthetic eukaryotic cells arose more than a billion years ago through the engulfment of a cyanobacterium that was then converted into a chloroplast, enabling plants to perform photosynthesis. Since this event, chloroplast DNA has been massively transferred to the nucleus, sometimes leading to the creation of novel genes, exons, and regulatory elements. In addition to these evolutionary novelties, most cyanobacterial genes have been relocated into the nucleus, highly reducing the size, gene content, and autonomy of the chloroplast genome. In this chapter, we will first present our current knowledge on the origin and evolution of the plant plastome in the different Archaeplastida lineages (Glaucophyta, Rhodophyta, and Viridiplantae), focusing on its gene content, genome size, and structural evolution. Second, we will present the factors influencing the rate of DNA transfer from the chloroplast to the nucleus, the evolutionary fates of the nuclear integrants of plastid DNA (*nupts*) in their new eukaryotic environment, and the drivers of chloroplast gene functional relocation to the nucleus. Finally, we will discuss how cytonuclear interactions led to the intertwined coevolution of nuclear and chloroplast genomes and the impact of hybridization and allopolyploidy on cytonuclear interactions.

Keywords: endosymbiosis, plastome evolution, functional gene transfer, nuclear integrant of plastid DNA (*nupt*), nucleo-cytoplasmic interactions

1. Introduction

Photosynthetic eukaryotic organisms harbor a chloroplast genome (also called 'plastome') within their cells. This genome derives from the endosymbiosis of a prokaryotic organism, which was then gradually converted into the chloroplast. With the increased number of sequences within

publicly available databases and the emergence of very sophisticated phylogenetic and phylogenomic analyses, we can infer much more precisely the origin of this primary endosymbiotic event. In addition, these comparative analyses allow for investigation of plastome evolutionary dynamics in the different plant lineages and the extent of nuclear influence over the chloroplast genome. Overall, plant plastomes harbor a very low gene content compared to their prokaryotic ancestor, which appears to result from either gene loss due to redundant functions in both chloroplast and nuclear genomes or functional transfer and relocation of chloroplast genes into the nucleus. The relocation of thousands of chloroplast genes from the chloroplast to the nucleus was rendered possible due to the massive transfer of DNA from the chloroplast to the nucleus. However, chloroplast genes that have been integrated into the nucleus are not immediately functional and have to adapt to their new eukaryotic environment by acquiring various regulatory elements (i.e., promoter, polyadenylation signal, and target peptide). Despite most of these functional transfers occurred soon after the endosymbiotic event, some clever real-time experiments (using a selectable marker) have allowed for understanding how easily and by which molecular mechanisms DNA is transferred from the chloroplast to the nucleus. Such experiments have also permitted the study of the subsequent evolution of chloroplast DNA in the nuclear genome, and how a chloroplast gene becomes functional in the nucleus.

2. Chloroplast origin and evolution

Photosynthetic eukaryotic cells arose through the engulfment of a cyanobacterium that was then converted into the chloroplast, enabling plants to use sunlight to fix carbon. This major functional innovation allowed for eukaryotes to transition from heterotrophy to autotrophy. This primary endosymbiotic event is at the origin of the astonishing biodiversity visible today in plants, including the Glaucophyta, Rhodophyta, and Viridiplantae lineages (**Figure 1**). With the advent of next-generation sequencing technologies, the number of fully sequenced plastomes has hugely expanded, providing insight into chloroplast evolution in the different plant lineages. In this part, we will present our current knowledge on chloroplast origin and what has been unraveled on the chloroplast genome evolution, regarding genome size, gene content, structure, and mutation rate.

2.1. Primary endosymbiosis event and origin of chloroplasts

The first hypothesis of the endosymbiotic origin of chloroplasts is commonly credited to Russian botanist K. Mereschkowsky, who observed similarities between cyanobacteria and chloroplasts of plants and algae [1]. This hypothesis was then reaffirmed by Margulis in the 1970s. The origin of this primary endosymbiosis event is still debated. While fossil-based phylogeny estimated the origin of chloroplasts to be around 1.4–1.7 billion years ago [2], gene-based approaches dated it around 0.9 billion years ago [3]. Different phylogenetic analyses aimed at determining the cyanobacterial lineage from which the chloroplast was derived and revealed that chloroplasts were closely related to the nitrogen-fixing cyanobacteria Chroococcales, *Nostoc* sp., and *Anabaena variabilis* [4, 5].

It is now widely accepted that this primary endosymbiotic event has a single origin [6–8]; however, it is still unclear how long it took for the conversion of the bacterial endosymbiont into a fully integrated organelle. This transition from endosymbiont to organelle surely involved many

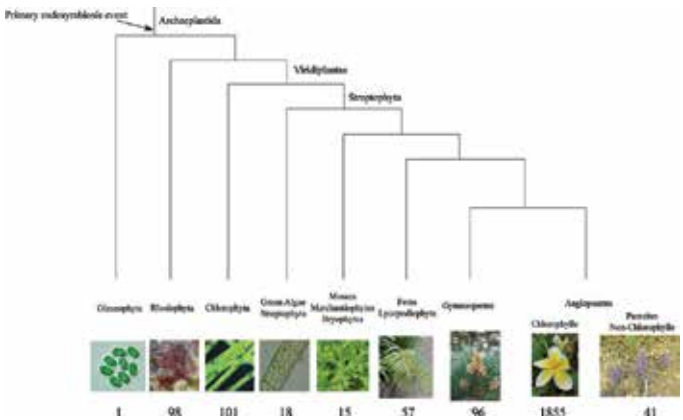

Figure 1. Phylogenetic relationships of the different plant lineages formed after the primary endosymbiosis of a cyanobacterium by an ancestor of the Archaeplastida. The number of available genomes on GenBank is indicated under the image. For simplicity, "Mosses, Marchantyophytes and Bryophytes" on one side, as well as "Ferns and Lycopodiophyta" on the other side, were grouped together in the tree. Pictures copyright to L. Brient, M.T. Misset, R. Delourme, and J. Keller.

steps. The first steps corresponded to the loss of the bacterial wall and the early acquisition by the endosymbiont of a transport system to transfer proteins and metabolites from the cytosol to the chloroplast. This latter step is constituted by two protein complexes: translocon of the outer (TOC) membranes of the chloroplast and translocon of the inner (TIC) membranes of the chloroplast [9–11]. The TIC/TOC complexes allow for transportation of the pre-proteins (proteins with a cleavable chloroplast target peptide) from the cytosol, where they are synthetized, to the chloroplast, where the target peptide is cleaved (reviewed in [11]). The presence of the same protein import apparatus in the different Archaeplastida lineages is the best evidence of the single origin of chloroplasts. Finally, the transition also necessitated the gradual functional transfer of endosymbiont genes to the nucleus [12], leading to the massive reduction of plastome size and gene content.

2.2. Evolution of chloroplast genomes

2.2.1. An unequal sequencing effort

Most of our current knowledge of the conversion from endosymbiont to organelle has been obtained by comparing contemporary Archaeplastida organelles with their closest bacterial relatives. During the last few years, advances in high-throughput sequencing and bioinformatic methods greatly facilitated the assembly, analysis, and publication of complete plastomes. To date, more than 2300 plastomes are fully assembled and deposited in the GenBank database. This number of plastomes actually doubled in the last 2 years. However, the number of sequenced plastomes varies greatly between the different Archaeplastida lineages. Indeed, almost 80% of them belong to Angiosperms. Thus, there is an important inequality in the sequencing effort. The poor level of plastome sequencing in plant lineages outside of the Angiosperms needs to be improved to fully understand chloroplast genome evolution in plants. Some efforts to fill this gap have been performed in the last 2–5 years, but they are still insufficient. In the Glaucophyta, only one chloroplast genome is available (NC_001675), and another is sequenced but not yet published (Lang et al., unpublished). In

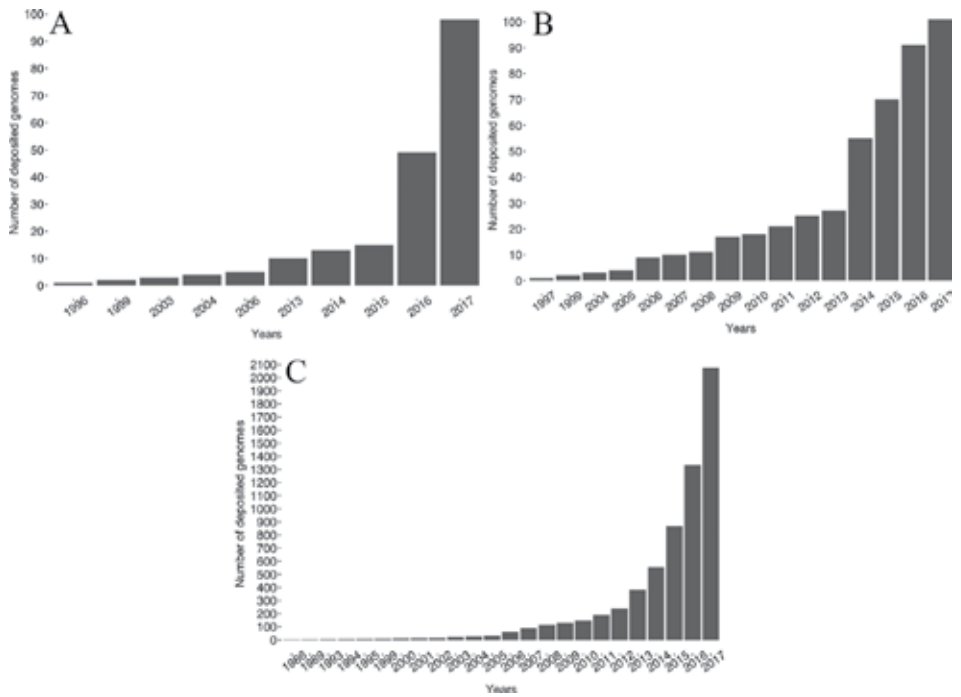

Figure 2. Cumulative numbers of full chloroplast genomes deposited in GenBank for (A) Rhodophyta, (B) Chlorophyta, and (C) Streptophyta.

contrast, the sequencing of Rhodophyta and Chlorophyta (green algae *sensu stricto*) species greatly improved since 2012: from less than 30 plastomes available in 2012 to around 100 in 2017 (**Figure 2A** and **B**).

2.2.2. Gene content evolution

As mentioned previously, the conversion of the cyanobacterial endosymbiont into a chloroplast necessitated the functional transfer or replacement of most cyanobacterial genes into the nucleus. Compared to the thousands of genes (at least 2000) thought to have been once present in the cyanobacterial genome, Archaeplastida plastomes encode a maximum of around 250 genes [13, 14]. This observation indicates that most genes (includes protein coding and structural RNAs) present in the cyanobacterial ancestor have been functionally transferred relatively soon after the endosymbiotic event. Despite gene content among modern chloroplast genomes being relatively well conserved, there are important variations. Thus, Rhodophyta have the highest number of genes (237 in average; minimum 207; up to 266 in *Grateloupia taiwanensis*) compared to the Glaucophyta (195), Chlorophyta (118 in average; minimum 68; maximum 210) or Streptophyta (129 in average; minimum 64; maximum 313), when excluding parasitic and non-chlorophyll species (**Table 1**).

These variations in gene content revealed the divergent evolution of plastomes in the different lineages. As an example, Rhodophyta gene content is characterized by the complete absence of the NADPH dehydrogenase complex [15]. Conversely, some genes are Rhodophyta-specific or rare in other Archaeplastida such as RNase P RNA, tmRNA, or signal recognition

	Glaucophyta	Rhodophyta	Chlorophyta	Green Algae Streptophyta	Masses Marchantiophytes Bryophytes	Ferns Lycopodiophyta	Gymnosperms	Angiosperms	
								Chlorophyllo	Parasites Non-Chlorophylle
Number of plastomes	1	98	101	18	15	57	96	1 855	41
Average genome size (bp)	135 599	183 209	161 975	156 310	129 090	15 004	130 398	152 782	71 736
min/max genome size (bp)		*90,243 / 610,063*	*37,464 / 521,168*	*107,236 / 207,850*	*108,007 / 161,162*	*127,640 / 157,269*	*107,122 / 166,341*	*113,064 / 242,575*	*11,348 / 128,921*
Average number of proteins	149	202	83	97	80	86	82	84	33
Average number of structural RNAs	43	35	35	40	45	42	39	45	30

Table 1. Plastome numbers and characteristics (average size, number of proteins, and structural RNAs) among the Archaeplastida. The minimum and maximum genome sizes are indicated in italic.

particle RNA [16–18]. Rhodophyta chloroplasts generally have a large genome size (see later) characterized by a high number of genes and other features such as the presence of bacteria-like operons, suggesting that Rhodophyta plastomes are phylogenetically closest to the ancestral cyanobacteria genome than any other algae [15]. Gene content variations are also well documented in the Angiosperm family in which multiple independent gene losses have been found such as *infA*, *ycf1*, *rps16*, and *accD* genes, which have been repeatedly lost in several lineages [19]. Within non-parasitic Angiosperms, a few families, such as the Fabaceae and the Campanulaceae, have recently lost various chloroplast genes [19]. In these lineages, recent gene losses from the plastome coincide with the transfer of those genes to the nucleus, providing insight into the underlying molecular mechanisms implicated in such events. For example, chloroplast gene loss may occur through a relaxed selective constraint on the chloroplast copy when a nuclear copy is already functional. This relaxation of selective constraint allows for non-sense mutations that may render the chloroplast copy non-functional [19, 20]. In addition, genes can become non-functional following the loss of their splicing capacity, as observed for *rps16* [21, 22]. The plastome gene content reduction is even more pronounced in non-chlorophyll organisms, such as parasites and obligate symbionts. Among the Angiosperms, 41 plastomes from parasitic plants have been sequenced and showed a great reduction in gene content (with only 63 genes) and size (around 70 kb in average), in line with the progressive loss of photosynthetic abilities. Similarly, plastome reduction is also observed among algae such as in the parasitic *Helicosporium* sp. (green algae) or *Choreocolax polysiphoniae* (red algae). On the contrary, increase in gene number may also be observed but in a lesser extent. In *Pelargonium*, which has among the highest number of chloroplast genes in Angiosperms (more than 180 in *P. transvaalense* and *P. hortorum*), there have been multiple duplication events in 39 genes [23]. Despite the number of coding sequences increased in the species belonging to this genus, this increased number of genes was due entirely to duplications and not to neofunctionalization processes.

2.2.3. Size variation

Among plants, chloroplast genomes range from less than 100 kb to more than 1 Gb, again excluding the non-chlorophyll species that exhibit significantly smaller chloroplast genomes (**Table 1**). The largest chloroplast genome ever sequenced has very recently been found in the red algae *Corynoplastis japonica*. Its genome size goes up to 1 Mb and contains 209 genes [24].

On average, the largest plastomes are found in the Rhodophyta with an average size of about 183 kb (minimum = 149,987 kb, maximum = 610,063 kb, excluding the small 90 kb genome of the parasite of *C. polysiphoniae*), whereas Glaucophyta and Streptophyta have an average chloroplast genome size of between 130 and 160 kb (minimum = 107,236 kb; maximum = 242,575 kb, excluding the parasitic and non-chlorophyll species), respectively (**Table 1**).

Several factors can explain the important size variations found among the Archaeplastida. In the case of the red algae *C. japonica* and *Bulboplastis apyrenoidosa* (more than 1 Mb and 600 kb long plastomes, respectively), the increase of plastome size is due to an expansion of the intron number with more than 200 introns found in these species [24]. In Angiosperms, plastome variations have been observed but in a lesser extent. For example, in *Pelargonium* that encompasses species with the largest chloroplast genomes found in Angiosperms (almost 243 kb), increased size is correlated to the expansion of the inverted repeats (IRs) that can be as long as 75 kb [23, 25]. This has also been observed in the Campanulaceae, *Lobelia thuliniana* [26], and *Musa acuminata* [27]. Expansion of plastomes has been linked to the presence of an increase number of repeats such as in *Trifolium* [28] or the Mimosoid *Acacia* and *Inga* [29]. This increase of plastome size by repeats is presumably the result of a less efficient chloroplast DNA repair mechanism [30, 31]. In contrast, plastome size reductions are also relatively common and can be due to loss of both coding and non-coding regions, especially in the non-chlorophyll species [32] that have an average plastome size of 71,736 bp in Angiosperms (**Figure 2**).

2.2.4. Structural evolution

Among plants, most plastomes seem to exhibit a conserved quadripartite structure, with a large and small single copy separated by two inverted repeats (Palmer 1983). However, multiple rearrangements occurred in diverse lineages, which modified this conserved structure. One of the most striking examples is the loss of one IR that occurred multiple times in the different chloroplast-bearing lineages, such as in the Fabaceae and the Geraniaceae [30, 33, 34]. This has also been reported for different Gymnosperms species such as *Pseudotsuga menziesii*, *Pinus radiata, Cephalotaxus oliveri,* as well as in multiple lineages of Chlorophyta [35–37].

Chloroplast genome structure and gene order are also highly affected by inversions. Many inversions have been described in the literature, especially in legumes, with, for instance, fragments of 50 kb in the Papilionoideae [38], 36 kb in the Genistoids [39]; 29 kb in Sophoreae [40] or 7 kb in *Tylosema esculentum* [41]. Multiple inversions have also been found in Geraniaceae, Campanulaceae (more than 40 inversions detected), and other lineages [25, 42, 43]. Inversions can be caused through flip-flop recombinations between repeat sequences [39, 44].

2.2.5. Evolution rates of plastomes

Chloroplast genomes are known to be highly conserved, with relatively low rates of mutations, especially when compared to the plant nuclear genome. Indeed, the chloroplast genome evolves on average 10 times slower than the nuclear genome [45], with about 1 or less mutation/kb/million years [46] compared with approximately 7 mutations/kb/million years for the nuclear genome [47]. However, there are some exceptions, especially in three Angiosperm families (i.e., Fabaceae, Campanulaceae, and Geraniaceae) that are known to have accelerated evolutionary rates of their plastomes along with multiple structural rearrangements and size

variations [19, 28, 30, 42, 44, 48, 49]. For example, the *ycf4* gene appears to be a hotspot of variation in *Lathyrus*, and this gene evolves 20 times faster than the rest of the chloroplast genome [19]. This localized hypermutable chloroplast region evolves even faster than the nuclear genome. Similarly, faster evolution has been observed in the *clpP* gene in Mimosoid [29]. In *Lupinus* (Fabaceae), two hypervariable regions have been identified (*ycf1* gene and *psaA-ycf4* region) and are characterized by high numbers of indels (with length usually superior to 20 bp) and mutations [22].

To sum up this first section on the origin and evolution of plant plastomes originating from the primary endosymbiosis event, the recent sequencing and bioinformatics progress significantly increased the number of chloroplast genomes available for the scientific community. These advances have greatly improved our knowledge about the evolutionary dynamics of plastomes. Despite the diversity of organisms that harbor chloroplasts, plastomes in general seem to be relatively well conserved among the Archaeplastida (in terms of structure, size, and gene content); however, multiple independent alterations of these features have been observed in the different lineages. In addition, a few plant families (or group of species) seem to present an atypical evolution of the chloroplast genome. It is certain that the continuous effort to sequence much more plastomes (especially in the Glaucophyta and Rhodophyta) will allow the identification of new examples of such atypical evolution and will permit a better understanding of what are the causes and the molecular mechanisms involved in limiting or increasing plastome evolution.

3. Impact of the cyanobacterial endosymbiosis on plant nuclear genome evolution and origin of chloroplast proteins

Since the endosymbiotic event, the host genome (nuclear) has acquired most of the cyanobacterial genes, leading to the gradual loss of autonomy of the endosymbiont and the reduction of its genome. In this part, we will present our current knowledge on the mechanisms as well as the numerous cases of chloroplast DNA transfers to the nucleus and where it is now integrated in the nuclear genome. We will then detail the subsequent evolution and adaptation processes of the chloroplast genome that took place in its new eukaryotic environment. We will also discuss which factors can influence relocation of a chloroplast gene to the nucleus, and how a chloroplast gene transferred to the nucleus may become functional. Finally, we will discuss the important role that transfer of chloroplast DNA to the nucleus plays in the process of diversifying the plant nuclear gene content.

3.1. DNA transfer from the chloroplast to the nucleus

Much earlier than the complete sequencing and assembly of the first chloroplast genome (*Nicotiana tabacum*: [50]), Kawashim et al. [51] observed that the gene encoding the small subunit of the Rubisco chloroplast protein could be transferred by pollen and thus must be encoded in the nucleus. From this early observation arose the question of whether nuclear genes encoding chloroplast proteins were of eukaryotic origin or resulted from transfer of DNA from the chloroplast to the nucleus. The existence of DNA transfer from the chloroplast to the nucleus was discovered a decade later using Southern Blot, by observing the presence of sequences with high homology between spinach (Chenopodiaceae) chloroplast

and nuclear genomes [52, 53], as well as in other closely related Chenopodiaceae species [54]. With the advent of the polymerase chain reaction, Ayliffe & Timmis [54] amplified and sequenced a chloroplast DNA sequence from *N. tabacum* nuclear DNA. This nuclear integrant of plastid DNA (also called '*nupt*') presented more than 99% homology with its homologous chloroplast sequence, indicating that this chloroplast DNA fragment had been transferred to the *Nicotiana* nucleus during the last million year. Using similar techniques, these authors also observed that the tobacco nuclear genome contained long tracts of chloroplast DNA at different locations. These different *nupts* may be as large as the whole chloroplast genome (about 150 kb) and the different *nupts* did not consist of the same sequence homology to the chloroplast homologous sequence, indicating that chloroplast DNA had been transferred at multiple times to the nucleus during plant evolution [54]. To decipher how frequently chloroplast DNA is transferred to the nucleus, experiments using an antibiotic resistance gene tailored for nuclear expression (i.e., nuclear promoter and terminator) were performed [55, 56]. After introducing this selectable marker (antibiotic resistance gene) into *N. tabacum* chloroplast genome and obtaining homoplastomic lines, it was demonstrated that DNA transfer occurred once in about 16,000 pollen grains [55] or once for every 5 million somatic cells [56], highlighting the high rate of DNA transfer from the chloroplast to the nucleus. This deluge of DNA transfer may be even higher in the presence of environmental stresses, such as mild heat [57] or cold stress [58]. It is important to note that in these experiments, the reported transfer rate may be underestimated as only the transfer of the selectable marker (about 2 kb) from the chloroplast genome could be identified. The higher rate of transfer observed in reproductive tissue (from pollen grains) compared to somatic cells may be explained by the higher degree of degradation of chloroplast DNA during pollen development (since chloroplast genomes are maternally inherited) than in somatic cells (more stable plastids). This hypothesis was supported by the observation of a much lower frequency of DNA transfer from female germlines (about 1 every 270,000 ovules) [59]. Some of these newly transferred chloroplast sequences were characterized and demonstrated that integration occurred by non-homologous end joining [60] and predominantly in open chromatin [61]. Surprisingly, it has also been demonstrated that DNA fragments from various plastome regions may insert simultaneously at the same nuclear location [60].

3.2. Short-term and long-term evolution of chloroplast DNA transferred to the nucleus

Some of the chloroplast DNA fragments that were experimentally shown to insert in the nuclear genome were characterized [55, 60] and were often large in size (usually greater than 10 kb in length). Considering the massive transfer of chloroplast DNA to the nucleus, one would expect that some of these *nupts* would be deleted to avoid a rapid increase of the nuclear genome size. This hypothesis was tested by studying the fate of these newly integrated chloroplast fragments [62]. Half of the lines presented an unstable inheritance of the *nupts*, after only one to two generations. Most lines presented a varying level of instability between the different areas of the same plant, and the loss of the *nupt* most often occurred during somatic cell division. However, it was also observed that some *nupt* loss occurred during meiosis [62]. Thus, even if constantly and massively integrated into the nucleus, at least some of these novel *nupts* may be rapidly removed and likely an even larger number

may be deleted over longer evolutionary time scales. Many *nupts* have been identified in various sequenced plant nuclear genomes [63–69], by fluorescent in situ hybridization using a chloroplast DNA probe [70] or using PCR-derived methods [71, 72]. Using the nuclear genome sequences of 17 plant species, the number, size, and genomic organization of *nupts* were studied [65]. They found a positive correlation between nuclear genome size, organelle numbers in cells, and cumulative lengths of *nupts*, as previously observed from a smaller number of plant nuclear genomes [64, 67, 73, 74]. To date, the largest identified *nupt* was found in the rice nuclear genome (131 kb) and corresponds to almost the entire chloroplast genome size (97.4%). A detailed analysis of the *nupts* presents in the rice genome revealed that *nupts* were mainly integrated within the pericentromeric regions [68]. Thereafter, they were rapidly fragmented, vigorously shuffled, and 80% of them were eliminated in the million years following their integration. Accordingly, the largest *nupts* were found to be the youngest, whereas the smallest *nupts* were found to be older. Most of the *nupts* identified in rice were less than 1 million years old (myo), whereas only a few were older than 5 myo. The recently integrated *nupts* were assumed to be decaying over evolutionary time into smaller fragments [64]. In rice, the half-lives of *nupts* were evaluated to be 0.5 myo for fragments whose length is superior to 1.6 kb and 2.2 myo for fragments with length inferior to 1.6 kb [68]. This result differs from those obtained experimentally in *N. tabacum,* where several old *nupts* (up to 6 myo) were larger than 2 kb [71]. The evolutionary fate of *nupt* sequences was scrutinized and revealed the prevalence of G:C → A:T transitions, which partly resulted from the deamination of methylcytosine [71]. However, over-representation of these transition types was similar to what was observed in the *Arabidopsis* nuclear genome, indicating that *nupts* evolved in a nuclear-specific manner. Similarly, the fate of potential protein-coding sequences and non-coding sequences presented within *nupts* was similar and evolved both neutrally, in accordance with the non-functionality of almost all *nupts*.

3.3. Functional replacement of hundreds to thousands chloroplast proteins in the nucleus

Following endosymbiosis, the symbiont to organelle transition involved many steps. This includes the loss of the bacterial cell wall, the acquisition of a protein machinery that transfers nuclear-encoded proteins from the cytosol to the chloroplast (also known as the TIC and TOC complexes [75, 76]), and finally, the functional relocation of most chloroplast genes to the nucleus. As detailed below, a chloroplast gene may be replaced either only after its functional transfer to the nucleus, or directly substituted by a gene of a mitochondrial or eukaryotic origin.

Since the endosymbiosis event, thousands of genes have relocated within the nuclear genome. Indeed, cyanobacterial genomes encode a minimum of 2000 proteins, whereas current plant plastomes encode only 80–200 proteins, although 800 to more than 2000 proteins have been found in some algae and plant chloroplasts [77], respectively. Apart from some genes that presented redundant functions in both chloroplast and nuclear genomes, most chloroplast genes have been functionally relocated to the nucleus with their proteins targeted back to the organelle. Thus, the spectrum of proteins required for function and biogenesis of the cytoplasmic organelle did not greatly evolve since its creation.

3.3.1. Functional transfer and relocation of a chloroplast gene to the nucleus

The current plastome of most plants encodes a maximum of 200 proteins [78] whereas more than 2000 proteins in the chloroplast, suggesting the functional gene transfer and relocation of most chloroplast genes to the nucleus. As chloroplast genes are of prokaryote origin, they are not readily functional in the nuclear genome. To function in this novel environment, a chloroplast gene has to acquire or hijack nuclear gene regulatory elements (eukaryote promoter and terminator), as well as a transit peptide to target the protein back to the chloroplast [60, 79]. However, the acquisition of all these nuclear elements does not have to take place right after the transfer of the chloroplast gene to the nucleus, as they can retain their open reading frames for several million years [71]. In addition, some chloroplast genes can be relatively easily functional as a few chloroplast promoters (i.e., *psbA* and *16S rrn* [80, 81]) were shown to be functional in the nucleus. Similarly, some transit peptides may be of cyanobacterial origin [82] and the AT-richness of 3'UTR chloroplast gene regions may mimic a polyadenylation signal.

To date, the number of chloroplast-encoded proteins (about 80) is relatively well conserved among flowering plants. However, a few chloroplast genes have been independently lost in various plant lineages [19], allowing to understand how they became functional. Such chloroplast gene losses were most particularly observed in the Fabaceae, for which the plastome has been extensively reorganized and contains localized accelerated mutation rates [19]. Some of these genes, such as *rpl22* [83] and *accD* [19], have been shown experimentally to have been functionally transferred to the nucleus. Similarly, recent functional transfers of chloroplast genes, such as *rpl32* [84] or *infA* [85], have been demonstrated. In addition, the functional relocation of *infA* and *accD* genes to the nucleus occurred several times independently [19, 85, 86]. Indeed, after the functional transfer of a chloroplast gene to the nucleus, two genes present in two different cellular compartments will encode for the same chloroplast protein. On one hand, the retention of the chloroplast copy is favored as the chloroplast genome evolves slower than the nuclear genome. On the other hand, even if the nuclear copy loses its functionality, the whole process can be repeated again.

3.3.2. Functional replacement of a chloroplast gene by a gene of mitochondrial (prokaryotic) or eukaryote origin

The functional replacement of a chloroplast gene does not necessarily necessitate its functional transfer from the chloroplast to the nucleus. In the case of the chloroplast RPS16 protein, the chloroplast *rps16* gene has been replaced by a nuclear *rps16* gene of mitochondrial origin [22, 83]. This nuclear *rps16* of mitochondrial origin had been functionally transferred to the nucleus soon after the formation of the mitochondria [22], and it acquired a dual target peptide to transfer the RPS16 protein to both chloroplasts and mitochondria [20]. Such functional replacement is not so surprising and many more similar functional transfers may have occurred as the prokaryote ancestors of chloroplast and mitochondria may encode similar proteins.

Another evolutionary mechanism enabling the functional replacement of a chloroplast gene may occur *via* the acquisition of a chloroplast transit peptide by a eukaryotic gene presenting the same function. Such event was observed for the chloroplast *accD* and the eukaryote *aac* genes,

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which both encode an acetyl-CoA carboxylase. In *Arabidopsis*, the nuclear *acc* gene has been duplicated in tandem, and one copy has acquired a chloroplast targeted protein and thus also encodes a chloroplast ACCD protein [87].

The continuous deluge of organellar DNA to the nucleus has facilitated the functional transfer of almost all chloroplast genes to the nucleus, reducing extensively the plastome size. Additionally, this organellar DNA was not only used to replace organellar genes but also enabled diversifying the plant nuclear gene content [77].

3.4. Importance of chloroplast DNA transferred to the nucleus in diversifying the plant nuclear gene content

Chloroplast gene sequences transferred to the nucleus may present different fates. As presented in the two previous sections: (i) they may remain non-functional, decay, and ultimately be lost; (ii) they may acquire all the necessary elements to conserve the same function and have the protein targeted back to the chloroplast; or (iii) they may acquire new subcellular locations and functions. As mentioned earlier, Martin *et al*. [77] extrapolated that about 18% of *Arabidopsis thaliana* genes were acquired from the cyanobacterial ancestor of plastids and that more than half of these cyanobacterially derived proteins were not targeted to the chloroplasts, suggesting either that they conserved their function but in another cellular localization or that they acquire a new function. These proteins are involved in many different functional categories that are not typically cyanobacterial, such as disease resistance and intracellular protein routing, indicating that they served as a rich source of genetic raw material and led to functional novelties. Similar analyses were performed in the glaucophyte *Cyanophora paradoxa* [88–90] and the green alga model *Chlamydomonas reinhardtii* [91]. Compared to what was observed in the flowering plant *A. thaliana,* only 6–7% of genes were inferred to be of cyanobacterial origin. Of these genes of cyanobacterial origin, 90% were inferred to be targeted back to the chloroplast in *C. paradoxa* [88], indicating that the impact of *nupts* on creating novel genes (new function or new cellular location) varies between plant lineages. We can speculate that many factors could explain these differences, such as the nuclear genome size and its structural evolutionary dynamics. Another major evolutionary impact of *nupt* on plant proteome evolution was determined by observing that *nupts* can generate novel nuclear exons encoding proteins with a different function to the preexisting organellar coding sequence. Additionally, Noutsos et al. [92] found that the Ka/Ks ratios (non-synonymous substitutions/synonymous substitutions) were higher than 1, reflecting a non-neutral evolution of *nupts* and their involvement into innovative functions.

4. Cytonuclear interactions, coadaptation processes, and incompatibilities

The conversion of the cyanobacterial endosymbiont into the chloroplast partly results from the gradual transfer of hundreds to thousands of endosymbiont genes to the nuclear host. Across all lineages, more than 90% of the plant chloroplast proteins are now encoded in the nucleus. Within the few chloroplast-encoded proteins, about 40% of them are involved in

chloroplast protein complexes that are made up of proteins encoded in both the chloroplast and the nucleus. These complexes exhibit important functions that are vital for the plant, such as photosystems I and II. One can only wonder how the stoichiometry between those two compartments is maintained. Indeed, one cell might contain hundreds to thousands of chloroplast copies compared to only one copy in the nucleus. Furthermore, chloroplast inheritance is often maternal, whereas nuclear bi-parental inheritance occurs in angiosperms during sexual reproduction. Therefore, coevolving interactions between cytoplasmic and nuclear genomes have been necessary and have resulted in significant coadaptation processes. When these fine-tuned coevolutionary interactions are disrupted, after intra-interspecific hybridization and/or genome doubling, for instance, incompatibilities and deleterious phenotypes can be observed. These evolutionary processes will be discussed in the light of previous work on synthetic and natural hybrids, as well as in polyploid species.

4.1. Hybridization and cytonuclear intergenomic complexes

Several evolutionary scenarios can explain coadaptation between chloroplast and nuclear genomes after intraspecific hybridization. First, cytoplasmic genomes lack sexual reproduction and are more susceptible to fix and accumulate deleterious mutations by genetic drift [93]. Only positive selection for compensatory nuclear alleles will allow for regaining of optimal organelle function [94]. This mechanism of *compensatory coadaptation* has been shown in several plant species with photosynthesis dysfunction (reviewed in [95]). One of the best examples with detailed genetic studies comes from the genus *Oenothera* [96], where three basic haploid nuclear genomes can be associated with five different chloroplast haplotypes. Of the 30 possible combinations, only 12 produce a green viable phenotype, whereas the 18 remaining associations lead to various degrees of cytonuclear incompatibilities, from reduced phenotypic capacity to embryo lethality [97]. Subsection *Oenothera* has apparently separated into three distinct evolutionary lineages (represent by the three basic haploid genomes A, B, and C) that have coevolved with chloroplast haplotypes I, III, and V, respectively [97]. Recent molecular work suggests that the radiation within this subsection started approximately 1 million years ago [98]. Thus, these results suggest that, in *Oenothera*, cytonuclear incompatibilities and associated coadaptation mechanisms have rapidly lead to strong post-zygotic barriers after only 1 million years apart [99].

Second, some mutations in the organelles could also be adaptive in specific environments and fixed in the population by natural selection. Subsequently, coadaptation process may favor specific nuclear variants to preserve intergenomic interactions. This mechanism is called *adaptive divergence*. However, experimental studies in the genus *Helianthus* are giving some hints of the effects of extrinsic selection on cytonuclear interactions. Exchange of the common sunflower cytoplasm with closely related species' organelles leads, just as in *Oenothera*, to deleterious phenotypes (from altered biomass to reduced seed weight and pollen unviability), suggesting, again, a role of cytonuclear incompatibilities in establishing reproductive barriers between populations [100]. Additional study demonstrated the contrasting adaptive potential of two cytoplasmic genomes in two alternative ecological environments. Sambatti et al. [101] have performed reciprocal transplant experiments of *H. annuus* and *H. petiolaris* and all possible backcross combinations of nuclear and cytoplasmic

genomes into two contrasted ecological environments. The authors elegantly showed that each cytoplasm of *H. annuus* and *H. petiolaris* exhibits higher fitness in mesic and xeric habitats, respectively, and is therefore differentially adapted to these two contrasting habitats. More recently, authors have benefited from the model system *A. thaliana* to investigate the contribution of cytonuclear interactions into plant fitness variation [102]. In this study, a field experiment has been set with 56 different cytoplasmic lines (based on eight natural accessions of *A. thaliana*) combining the nuclear genome of one parent with the organelle genomes of another. Using 28 adaptive phenotypic traits (such as germination, phenology, and fecundity), authors showed that a large proportion of those traits are affected by interspecific cytonuclear interactions. However, the genetic factors and molecular interactions underlying such phenomenon are still to be elucidated.

As mentioned above, the examples for intergenomic coadaptation and incompatibilities are scarce, and we are still very far from unraveling the molecular processes underlying such interactions. Applications of genome-wide studies in association with high-throughput sequencing would greatly improve our understanding of cytonuclear coevolution.

4.2. Effects of whole genome doubling and interspecific hybridization on cytonuclear complex stability

As shown above, cytonuclear interactions are extremely fine-tuned coevolved molecular processes that are still largely understudied. However, in recent years, efforts have been made, especially in neo-polyploid plant species (natural and resynthesized) to better apprehend the consequences of whole genome duplication (WGD) and interspecific hybridization on cytonuclear interactions and stability. In this last section, we will review our knowledge on such systems and elaborate on the many future issues to address.

Although completely overlooked, it is astonishing to envision the numerous and drastic consequences of a WGD event on copy number variation and stoichiometry on those cytonuclear complexes. Impacts of WGD on genomic structure and functional changes have been extensively studied in a large variety of plant systems. Genome redundancy can lead to changes in epigenetic patterns (including transposable element dynamics), altered gene expression (changes in global gene expression but also possible biased contribution of redundant copies), and fractionation processes (gene loss, homologous and non-homologous exchanges). However, to date, very few studies have investigated how the duplication of nuclear genes would affect the assembly dynamics of the multi-subunit cytonuclear complexes [103]. Different hypotheses predict the fate of nuclear and cytoplasmic genes implicated in cytonuclear complexes. They are based on the prediction that selection will favor compensatory mechanisms to maintain coordinated expression between cytoplasmic and nuclear genes leading *in fine* to a functional complex. Immediate impacts of WGD could therefore lead to downregulation of nuclear genes and/or upregulation of cytoplasmic genes. Additionally, another path to achieve the same outcome would be for the cell to enhance organelle biogenesis and produce a larger number of chloroplasts. This has been shown in cotton and alfalfa polyploids, which exhibit larger chloroplast size and higher chloroplast number per cell relative to their diploid progenitors [104, 105]. For instance, chloroplast number in guard cells

is increased by 25, 72, and 102% in triploid, tetraploid, and hexaploid cottons, respectively, compared to diploids [105]. Consequently, it is hypothesized that larger chloroplasts could carry more genome copies per organelle. In maize, only chloroplast number per cell (and not chloroplast size) is accentuated with ploidy [106]. However, it seems that chloroplast proliferation might be more correlated to cell size than nuclear ploidy [107]. Indeed, a positive relationship exists between nuclear genome size and cell size [108], but the direct impact of WGD and presence of redundant genomes have yet to be elucidated.

Only a handful of studies have looked at the consequences of WGD on a longer time scale, in that case, occurrences of subfunctionalization and pseudogenization of duplicated copies are to be expected. Coate et al. [109] stated that there might be a considerable influence of cytonuclear complex sensitivity to gene dosage imbalance and thus the need to return to single copy status or stay in duplicates. More specifically, Coate et al. [109] demonstrated that in *Glycine max*, *Medicago truncatula,* and *A. thaliana* photosystem gene families are preferentially retained as duplicates after WGD. This trend is likely explained by the high dosage sensitivity of these cytonuclear complexes. The authors hypothesized that if one of the duplicated gene copies implicated in the same cytonuclear complex is lost, it will cause gene dosage imbalance between genes, and the complex will not function properly. On the contrary, other complexes are apparently less affected by gene dosage imbalance and tolerate different copy numbers among genes (of the same complex).

All of these processes could be enhanced through allopolyploidy, where divergent parental species first hybridized before genome doubling. In that case, the nuclear genome is redundant and a mixture of two, more or less, divergent parental genomes, whereas the organelles have (usually) a uniparental origin. Therefore, as chloroplast inheritance is usually maternal, selection should favor maintenance of maternal nuclear copies over the paternally inherited homoeolog as to preserve pre-existing coadaptive cytonuclear interactions. In allopolyploids, different scenarios leading to pseudogenization of paternal copies can be envisioned and were tested in a limited set of genes and species. The first scenario involves downregulation and relaxed selection of the paternally inherited homoeolog. An alternative scenario involves preferential gene conversion to the maternal homoeolog resulting in the loss of the paternal-like copy. It is important to note that both scenarios are not exclusive but could be part of a dynamic and gradual process, with first overexpression of the maternal copies leading to paternal homoeolog pseudogenization and maternally biased gene conversion. These hypotheses have only been tested in the Rubisco nuclear-encoded gene *rbcS* in various allopolyploids. In cotton, an ancient allopolyploid formed 1–2 MYA (progenitors diverged 5–10 MYA), and it has been shown in five different allopolyploid species that putative events of gene conversion occur between subgenomes but not in synthetic hybrids [110]. Interestingly, maternal homoeologs are preferentially expressed in wild and cultivated allopolyploids as well as in the synthetic F1 hybrid (whereas no such bias is observed between the diploid progenitors) [110]. These patterns have been shown also in other polyploid models. Following the same methods, concerted evolution is reported between homoeologous genomes of *Arabidopsis suecica*, *Arachis hypogaea,* and *N. tabacum* [111]. Additionally, there is preferential

occurrence of maternal to paternal gene conversion in signaling and regulatory domains of the *rbcS* gene copies. In those polyploids, preferential expression of paternal homoeologs carrying the maternal-like gene conversions has also been described [111]. In contrast, the allotetraploid *Brassica napus* showed no sign of homoeologous exchanges or bias expression probably because of either recent (compared to the other models) divergence time between diploid parental species (only 4 MYA). In the same way, resynthesized reciprocal hybrids and allotetraploids formed between *Oryza sativa indica* and *japonica* (that diverged around 9000 yr. ago) did not exhibit biased expression of *rbcS* alleles or homoeologs and also no biased gene conversion toward maternal gene copies [112]. In *Tragopogon miscellus*, a very recent neo-allopolyploid formed only 80 years ago, homoeolog gene loss and biased expression were limited, occurring only in 12 and 16% of individuals coming from two naturally and repeatedly formed polyploid populations [113]. However, the bias was mainly toward maintaining the maternal nuclear copy of *rbcS* (in 7 of 10 cases of homoeolog loss). Therefore, although parental genomes of the neotetraploid *T. miscellus* polyploid are quite divergent [114], very little evidence for functionalization and homogenization of duplicated copies is visible in the polyploids. This might be due to the recent formation of such polyploids (less than 100 years ago) and the lack of time for such events to take place. Thus, in the cases of allopolyploid formation, divergence between parental species and age of polyploids seems to be important factors driving cytonuclear coevolution processes.

These few studies already highlight the complexity of the different model systems that can be highly influenced by various evolutionary processes such as pre-existing coadaptive mechanisms, natural selection, and divergence between parental individuals (different populations to different species). As all Angiosperms have experienced at least one round of genome duplication and most of them multiple WGDs (Triticum and Brassica), paleopolyploid species are perfect candidates to elucidate the long-term impact of diploidization and biased genome fractionation on rates of asymmetric gene loss and pseudogenization. Additionally, it seems essential to integrate plant families that have contrasted rate of chloroplastic evolution (such as in Geraniaceae, Campanulaceae, and Fabaceae) and paternally inherited chloroplast genomes (such as in Actinidia, Medicago, and most Conifers). Finally, life history features such as reproductive strategy (perennial vs. annual), mating system (selfer vs. outcrosser), population level dynamics, and effective population size will also impact fixation rate of mutations.

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Conflict of interest

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Chapter 5

Biotechnological Applications of Plastid Foreign Gene Expression

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Abstract

Chloroplast is responsible for the major metabolic process photosynthesis. These organelles have their own genome and in the last three decades, the chloroplast genome has been broadly studied and manipulated through genetic engineering tools. The transfer of genes into chloroplast provides advantage over the insertion of transgene into nuclear genome, including overexpression of foreign protein, no positional effects, absence of epigenetic effects and uniparental inheritance of the transgenes, the ability to express multiple transgenes in operons and the possibility of eliminate the marker gene after the transformation and integration of the foreign gene. Now more than 100 transgenes have been reported stably integrated into the chloroplast genome including genes encoding enzymes with industrial value, biomaterials, biopharmaceuticals, vaccines and genes with agronomic trails. The chloroplast genetic tools have been implemented in several important crops. So, the chloroplast engineering technology has been positioned as the most important for the production of proteins and metabolites with biotechnological applications.

Keywords: chloroplast engineering, foreign protein, biotechnological applications

1. Introduction

1.1. Chloroplasts: from cyanobacteria to bioreactors

The chloroplasts are organelles that allowed obtaining electrons from water, as part of the basic photosynthetic machinery that have evolved from cyanobacteria to flowering plants. In most

groups of plants, they are indispensable due to its role in metabolic process as photosynthesis, biosynthesis of fatty acids, amino acids, pigments and vitamins and any interruption of its normal metabolism can cause lethality in plants [1, 2].

All functions that occurs in the chloroplast are regulated by a genome that comprises around 120 genes grouped in a double stranded of ~150 kb; these genes have implications on photosynthesis, replication, transcription and translation processes; the organization of the chloroplast genome allow the site-directed gene integration without altering the integrity of endogenous genes; this characteristic has allowed the expression of genes codifying enzymes with industrial value, antibodies, antibiotics, vaccines, antigens and also genes of environmental importance. Due to the genetic organization in operons, chloroplast genes can be expressed together and with the advantage of minor suppression effects, where chloroplast expression yields until 70% of total soluble protein (TSP), which has improved the vegetal biotechnology in agricultural, food, medicine and environmental areas [3, 4].

Currently, different industries require metabolites for improving production processes; in this sense, several technologies based on microorganisms has been used as source of such metabolites; nevertheless, they could not satisfy demands at the rate they are needed; on the other hand, overexpression of several proteins in chloroplast has proved its usefulness for specific protein production, although this technology force to know the metabolic route itself by the presence of multiple isozymes in the metabolic route what would considered a disadvantage currently. Chloroplast engineering has been efficiently used in the expression of omega-3 genes, desaturases of omega-6, tocopherols and tocotrienols, flavodoxin, carotenoid or vitamins in crops as tomato, cauliflower, cabbage, rape, poplar, beet, potato and eggplant [5, 6].

2. Applications for the agronomic traits

The increases of agriculture, industry and globalization have given the opportunity to transgenic crops production and trade; but chloroplast engineering gives the opportunity of generating transgenic crops with the possibility of containing the transgene flow to minimize the outcrossing transgenes related to weeds or crops; this characteristic has allowed the development of crops expressing genes with useful agronomic traits via the chloroplast genome [7]. In this sense, the requirements in the agriculture have focused in crop protection based on insect, herbicide resistance, drought and salt tolerance and phytoremediation. For example, the expression of 'cry2Aa2' operon bio-insecticidal protein from *Bacillus thuringiensis* known as Bt, which confers resistance to pests, was accumulated to up 45% of total soluble protein. Additionally, this technology has also been used to protect crops against bacteria and fungi when antimicrobial peptides are expressed [8, 9]. Also, the chloroplast engineering has impacted in the agriculture and environmental issues, by conferring crop tolerance to salinity through the expression of betaine aldehyde dehydrogenase gene or maximizing heavy metals removal through increasing efficiencies of absorption by the roots, shoots translocation and volatilization [10, 11].

The chloroplast engineering still has some obstacles because the transgene expression depends of factors such as the specie, the regulatory regions used (promoters, 5′-, and 3′-UTR) and efficient methods of tissue culture and regeneration protocols [12, 13]; also, recent studies

indicate that protein overexpression can interact with intermediaries of metabolic pathways causing mutant phenotypes [14]; this coupled to somatic embryogenesis which cannot be achieving a homoplasmic state in monocotyledons is one of the biggest obstacles for plastids engineering in agronomic crops [15]. Despite not having a viable method for transgene integration to all crop species, currently transformations has been successfully done in tomato, cauliflower, cabbage, rape, poplar, beet, alfalfa, potato, carrot, cotton, oilseed rape, petunia, rice, soybean, sugar beet and eggplant [5, 6, 16, 17]. The efficient use of this technology in crops has shown that possibly in the future these could interact with the biopharmaceutical sector [18].

3. Enzymes to cellulose degradation

Plant lignocellulosic biomass is mainly compounded of polymeric sugars as cellulose, hemicellulose, pectin and polyphenolics (lignin); this complexity avoids its use because the residues are joined by forming crystalline microfibrils that are highly resistant to enzymatic hydrolysis [14, 19].

Nevertheless, despite this, cell wall constituents can be degraded with consortiums of enzymes such as pectin lyase capable of degrading the pectin through α-(1 $\rightarrow$ 4) bond hydrolysis of polygalacturonic acid [20, 21]. To degrade lignin residues imbibed in plant biomass an enzyme cocktail is required and usually includes laccase 'Lac', lignin peroxidase 'LiP' and manganese peroxidase 'MnP'; interestingly, all ligninases exhibit high homology in their primary sequence [22, 23]. The ligninases are implicated in the removal of an electron of phenol moiety of lignin which is stabilized by organic acid chelators facilitating the degradation of phenolic compounds in the presence of H_2O_2; as lignin is the most important source of aromatic polymers in nature, its decomposition is also necessary for carbon recycling [24, 25].

Pectin and lignin constituents are not the only ones present in the cell walls, also the cellulose residues represent 40% of plant biomass, which placed it as the highest carbohydrate synthesized by plants, and it is formed of 100–14,000 residues of glucose with β-1-4 linkages forming a crystalline cellulose which needs synergistic action of several highly specialized enzymes to release glucose units, these are known as endo-β-glucanases (1,4-β-D-glucan-4-glucohydrolase 'EC 3.2.1.4') and hydrolyzed random internal glycosidic linkages, resulting in a decrease in polymer length and a gradual increase in the reducing sugar concentration [26, 27]; subsequently, a exoglucanases (1,4-β-D-glucanglucohydrolase 'EC 3.2.1.74' and 1,4-β-D-glucancellobiohydrolase 'EC 3.2.1.91') hydrolyse cellulose chains by removing cellobiose from the reducing or non-reducing ends and finally β-glucosidases (β-D-glucoside glycohydrolase 'EC 3.2.1.21') hydrolyse cellobiose to release D-glucose [28, 29].

However, the degradation mechanism of celluloses is different in several organisms; for example, the amorphous or soluble cellulose is degraded by the action of endocellulases alone, while crystalline cellulose first requires an exocellulase and then a cellobiase to release two glucose moieties and sometimes glucohydrolase may act as a component of the exoglucanase releasing glucose and not cellobiose from the non-reducing end of poly- and oligosaccharides [30]; also the cellobiose can be oxidatively degraded for a cellobiose-quinone oxidoreductase to

cellobionic acid [31–33]. In symbionts of termites and fungi, endo-1,4 glucanases cleave inner-1,4-glycosidic bonds and generate oligosaccharides, then the cellobiohydrolases split off cellobiose from the non-reducing end of the oligosaccharide chain; whereas in aerobic bacteria, cellulose hydrolysis has been attributed to action of two types of enzymes that act like fungal endocellulases and cellobiases [32–34]. Exocellulases also have been found in few bacteria and the cleavage of crystalline cellulose is done by an intra-molecular synergism of bacterial endocellulases [35, 36]. However, despite the mechanism which the cell wall components are degraded, currently there are no enzymes with high capacity of degradation and the need for them increases [26, 37].

To supply the necessity of efficient enzymes in the industry, the microorganisms are the first potential source of enzymes to be used in genetic engineering [26, 38] and currently, from them it has been possible to obtain endoglucanases, xylanases, cellobiohydrolases, exoglucanases, glycosyl hydrolases, glucuronoyl esterases, ferulic acid esterase and acetylesterases [19]. However, the enzymatic activity obtained from microorganism's expression is limited by the enzymatic capacity inherent to own system and the protein overexpression is only possible with the change of expression system; to supply the high quantities of proteins required by industry, currently, the chloroplast genetic engineering has been used to express several hydrolytic enzymes genes such as cellulases (*bgl1C, cel6B, cel9A, xeg74, celA, celB, bgl1, Cel6, Cel7, EndoV, CelKI, Cel3, TF6A, Pga2, Vlp2 peroxidase* genes), pectinases (*PelA* genes), ligninases (*MnP-2* genes) and xylanases (*xyn, xynA* genes) [14, 39–43].

With the expression of hydrolytic enzyme in chloroplast genome, positive results have been observed in the expression of β-glucosidase (*bgl1 gene*) [41] when plants with longer internodes (150% of height) and increasing leaf area (resulting in 190% more biomass) and mature transplastomic plants with earlier flowering were obtained. The expression of β-glucosidase also increased twofold the levels of gibberellic acid precursor (GA_{53}, GA_{44}, GA_{19}, and GA_{20}), (GA_1) hormone, and catabolite (GA_8) and in the case of indolacetic acid and zeatin hormones had more than 200% of increases. Surprisingly the glandular trichomas density resulted up to 10-fold with more production of natural sugar esters that shown to be effective biopesticides against a range of insect species [44] in the β-glucosidase expression plants, they showed 18-fold less aphid and whitefly population by a toxicity increase in exudates of plants; but, there are report when the transplastomic plants that expressed lacasses showed slightly retarded vegetative growth with a light green leaf color may be attributable to copper deficiency induced by ligand chelation related to produced laccase [45]. In plants with xylanases overexpressed, a 60% increase of xylanase (*xynA* gene) was observed and the accumulation of the fungal enzymes was more than 10-fold higher levels but the transplastomic plants displayed pale-green-to-white leaf color and severely retarded growth [46]. In other researches using *bgl1C, cel6B, cel9A* and *xeg74* as a cocktail genes, all transplastomic lines displayed strong mutant phenotypes showing severe pigment deficiency, slow growth in soil or even they did not survive due to their insufficient photosynthetic performance [14]. With manganese expression, the transplastomic plants showed mild phenotypic effects in green house with some leaves turning pale as they matured [47]. On the other hand, there are reports where transgenic plants are morphologically and agronomically indistinguishable from the untransformed plants. These open new avenues for large scale production of several other industrially useful cellulolytic enzymes through chloroplast expression [39, 48, 49].

Despite different results obtained in several researches; currently, enzymes with high capacity to degrade the plant biomass have been notoriety in industry. And due to extensive processes, that are involved as cotton processing, paper recycling, detergent enzymes, juice extraction and as animal feed additives, they are positioned as the third most common enzymes used [26, 37]. Today, enzyme treatments of plant biomass are considered more cost-effective compared with mechanical processes, resulting in 20–40% energy savings [50]; although, the costs associated with the production of microbial enzymes are expected to be high [51]. For this, the chloroplast technology can provide an inexpensive source of active cellulases, which is critical to efficient and cost-effective conversion of lignocellulosic biomass into the different industries [26, 38].

4. Factors involved in protein plastid expression

To achieve the gene expression successfully, several factors are involved in the gene regulation since their integration in the chloroplast genome until the concomitant modification of sequence to improve the translation.

4.1. Homologous recombination sequence

The recombination is a fundamental conserved process in all spices and is essential not only in the conservation of genome stability, facilitate the genetic diversity, but also is involved in the reparation, replication, maintenance and segregation of DNA [52, 53]; the principal role of recombination restores the DNA damage caused by photooxidation and other environmental stresses. It has proposed that the *rec*A system regulate the recombination and that the mechanism is limited by enzymes availability more than substrates. Although recombination in chloroplast is well documented, its molecular mechanism is not well defined. However, the recombination is an advantage in chloroplast engineering because genes of interest and selectable marker genes flanked by homologous native chloroplast DNA sequences can be inserted in the chloroplast genome via homologous recombination [54].

4.2. Marker genes

The transplastomic lines and the gene of interest integration in the chloroplast genome must be confirmed with selectable marker genes that confer resistance to biotic or abiotic stress. The first selection marker gene was mutant from rRNA 16S that conferred the spectinomycin resistance [55]; however, the stable integration of a marker gene was reported by Goldschmidt-Clermont [56] with the *aadA* gene expression that confers spectinomycin and streptomycin resistance and this resulted 100-fold more that the previously obtained by Svab and Maliga [57]. In 1993, the *neo* gene (kanamycin-resistance) was used as an alternative of selection, but in 2002, the *aph*A6 gene was reported with the same resistance with high efficiency of transformation [58]; however, Kumar et al., [59] achieve results eightfold that *aph*A6 with a 'double barrel' from *aphA6* and *nptII* genes, also, it has been activated other selection markers as *aacC1* gene that confer gentamycin resistance or *bar* gene to phosphinothricin; multiple mechanisms have been used as selection method as *cod*A gene used as negative marker [60]; recently, it has

been used the *cat* gene to chloramphenicol resistance with low resistance compared with *aadA* gene, but without spontaneous resistant's mutant [61]. Furthermore, Nuccio et al. [62] used the *badh* gene that confer salinity resistance and this selection marker gene was tested in chloroplast transformation of carrot by Kumar et al. [59] showing as a selection alternative against disadvantages of marker genes with antibiotics resistance.

The selection markers are chosen according to cellular autonomy and portability; thus some selection markers are dominant like the *aadA* gene, while other genes are recessives like the punctual mutation in the RNAr genes (*rrnS* and *rrnL*); the dominant markers are important for transformations of the highly polyploid plastomes because they increase the transformation frequency due to its effect at early stages of selection despite that may only integrate in the minority of the plastomes; on the another hand, recessive markers have lower efficiency in the transformation and only are efficient in random segregations if the plastids have sufficient copies of transformed genome [63, 64]. However, the selection markers and genes of interest can be added under one promoter because the polycistronic RNAs are efficiently translated in plastids [65]; also, it is possible to obtain a transductional fusion between a resistance gene and reporter gene like *gfp* gene avoid phenotypic marking in transformed cells with more results in the selection process from transformed tissue [66].

4.3. Promoter

To obtain high levels of protein expression in plastids, first, the levels of stable mRNA must be increased and then use a promoter with high efficiency [67]. To realize the protein expression, the plastomes of algae and higher plants account with a polymerase RNA cyanobacteria type usually named 'PEP' (plastid-encoded RNA polymerase) constituted by enzymatic cores encoded by *rpoA*, *rpoB*, *rpoC1* and *rpoC2* genes [68]. The PEP recognize promoters type σ^{70} and account with conserved region −10 (TATAAT) and −35 (TTGACA) which is responsible of start the transcription of 5–7 nucleotides downstream of −10 element of promoter; also, the PEP is associated at 8 auxiliary protein that regulate the transcription as kinase of plastid transcription (PTK) is regulated by a phosphorylation factor [69, 70].

The eubacteria enzymatic machinery has acquired a second polymerase type phage (NEP, nuclear-encoded plastid RNA polymerase), which is active in plastids; however, a nuclear gene encoded the catalytic core of NEP [71], which recognizes three different promoters: NEP type I with recognition in the region ∼15 nucleotides upstream (−14 to +1) from start codon (+1), a subtype promoter NEP Ib with a conserved motif to 18–20 nucleotides upstream of YTRa motif designed as 'box II' or 'GAA box' [72, 73] and promoter NEO type II that recognizes downstream region of start codon (−5 to +25) [74]. Several genes and photosynthetic operons have a PEP type promoters, the genes non-photosynthetic are transcribed as PEP as NEP, whereas the only a few genes are transcribed exclusively by NEP promoters [74].

The promoters recognized by PEP/NEP are regulated differentially which is tested with the expression level and their efficiency can be related with upstream sequences of start codon [73].

Recently, it has been reported with *gfp* expression that *rrn* promoter is 90-fold strong that *psbA* or of *trc* promoter, indicating that the transcription is more efficient in P*rrn* [75]; also, it has been recorded that other promoters such as P*clp*P-53 with high efficiency related with NEP non-dependent of enzymatic core of PEP [76]; several reports have ideated the analysis of the capacity of promoter from operon RNA (P*rrn*), which can be fused with control sequences to improve the protein expression [77]. In this sense, the expression levels of a protein are determined by their sequence or their promoter and different promoter can be used to improve the gene expression in chloroplast genome [78].

4.4. 5′ and 3′ UTR sequences

The transgene expression in the plastid genome required sequences that can be recognized by transcriptional machinery from the own plastid; however, the role and mechanism of the regulatory elements and flanked sequences is not well defined [16]. The protein expression in plastid depends of mRNA stabilization; also, the translation and accumulation efficiency of protein expressed by chimeric transgenes are determined by 5′3′UTR regions and the interaction of mRNA-rRNA [79]. The loss of regulatory elements 5′ and 3′UTR leads to fast degradation or low transcripts accumulation [80]. The start of translational activation is currently unknown, but it is possible that the ribosomal protein *S1* media the mechanism of affinity from 5′UTR [81].

The 5′3′UTR elements are recognized by proteins that protect the mRNA against exonucleases 5′3′ and processional endonucleases 5′3′; the eukaryotic cells used this mechanism in the cytoplasm to remove defective mRNA capable of originate truncated proteins, and this shows how the untranslated region has influence in the stability and degradation of plastid mRNA [82]. The 5′UTR region is a rich A + T region and contains *cis* elements that determine the mRNA stability by interaction with the α subunit of RNA polymerase [83]. It has been reported that mutations in 5′UTR drastically decreased the protein expression [84]. On the contrary, the 3′UTR is considered as a stabilizing region of mRNA being able to be similar or different to Shine-Dalgarno sequences; however, the upstream region of ATG are highly dependent from individual mRNA; in this way, these sequences are critic to start translation. The 3′UTR region from plastid mRNA is an inverted repeat sequence that can be folded as stem and loop in involved structures in the termination of prokaryotes transcription [85]. It has been suggested that the 3′UTR is of less importance to mRNA stability [86]; however, the mRNA levels are mostly determined by 3′UTR more than 5′UTR [87]. Recently, it has been reported that *clpP* can be used as potential source to obtaining regulatory sequences based on results on potato [88].

4.5. Shine-Dalgarno sequences

The plastid gene expressions are controlled in post-transcriptional stage, especially, during the translation [89]. The prokaryotic mRNA normally contains with a 5′ untranslated region (5′UTR) and Shine-Dalgarno (SD) sequences as ribosome binding site [90]. The translational

regulation is to be determined by untranslated region upstream at start codon that sometimes is specific to each mRNA [91, 92]. The localization of SD upstream start codon is 4–9 nucleotides as the optimum form [79]; however, several sequences has a SD at different distances; in these cases, specific proteins added at 5′UTR and redirectioned the 30S subunit to start codon or next AUG downstream [90].

The plastids and *E. coli* have an identical anti-SD sequence in 3′end from 16S rRNA (5′-TGGATCA**CCTCC**TT-3′), because of this the SD sequence 'GGAGG' of plastids can be recognized in *E. coli* and vice versa; it has been reported that bacterial SD can be used to improve the expression of genes in plastid [93]; although, still it is unknown as the SD are recognized and how the used determine the efficiency translational [94]; in this sense, the cistron can be processed in both system, but the translation efficiency is major in plastids, this may be due at the presence of plastid ribosomal proteins 'PSRPs', and although this proteins are not well defined, they can be explained why this proteins were acquired in the evolution [94, 95].

It has been reported that is possible used multiple SD sequence upstream at the start codon to improve the translation; however, adjacent SD sequence are unrecognized by multiple ribosome at the same time. The organization of four SD sequence with sufficient spacer length (39-nt) that allow the incorporation of ribosomal units 30S can improve the expression until 71-fold hence attracting more ribosome; however, if there is a start codon, stop codon or mini ORF between two SD, the translation is low [94]. It is not well known the correlation between 5′UTR, SD and start codon number to the mRNA stability but the genes with polycistronic expression can contain SD canonic sequences upstream of each cistron indicating that initiation of internal translation can occur; sometimes, the polycistron does not make intercistronic cleavage and is efficiently translated and it is unknown as the SD downstream are recognized, but it is speculated that regulatory proteins are involved in the process [91, 92, 94].

5. Transformation methods

The plastid transformation is widely used in basic research and into biotechnological applications; initially developed in *Chlamydomonas* and tobacco, currently is feasible in several plant species [96]. This technology require the transfer of genes into chloroplast genome; now, the strategies more common to transgenes insertion in chloroplast genome are biolistic and polyethylene glycol (PEG); the biolistic has been tested with gold and tungsten particles; this method is efficient because there are no limits due to interaction of host-pathogen such as viruses and bacteria; the biolistic has no restriction by cellular species, foreign DNA length, sequence or conformation [97]. The reports suggest that particles of 0.4 μm improve fourfold the transformation efficiency more than 0.6 μm; also, exhibit less cellular damage [98]; it has been shown that charged particles can promote transformation events in isolated genomes as plastid or nuclei at the same time [99]. On the another hand, the PEG method is efficient and with low cost of transformation, eliminates the dependent of particle shots and decreases the risk of cellular death explosion and the dependence of species can be more efficient that particles bombardment; both methods are efficient but the use is crop dependent [100].

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6. Elimination of marker gene

One the biggest impediments in the development of transgenic plants is that the manipulation and expression of multiple genes is difficult of achieve, still when new methods have been developed; nevertheless, when an integration and genetic expression has been achieved, the marker selection genes that allowed the detection of transformed tissue that need to be eliminated from the sequences at troubles associated to high-level protein expression and the possible effect of horizontal transference at microorganism [101]. Multiple strategies have been reported to eliminate the markers genes as co-transformation, transposons, homologous recombination, specific site recombination and even P-DNA [102].

One of the most studied site-specific system is the *Cre/loxP* which included two components: (*a*) two sites *loxP,* each one with 34 pb (inverted repeat) flanking the sequence to eliminate and (*b*) the gene *Cre* (recombinase of 38 kDa) that joins to *loxP* sites and cleavage the sequence between both sites; in this sense, placing *Cre/loxP* system under control of OsMADS45 promoter to improve an autocleavage from marker selection gene improve the efficacy [103]. Although the *Cre/loxP* system has been analyzed in multiple plants including *Arabidopsis, Nicotiana tabacum Zea mays* and *Oryza sativa*, only *Brassica juncea* has been reported complete efficiency until F_2 progeny [103]. Other system to elimination specific site of marker selection genes was reported using phage phiC31 (INT) that media the recombination *attB/attP*, *Flp/frt* from *Saccharomyces cerevisiae*, R/RS from *Zygosaccharomyces rouxii* [104] and *Gin/gix* from Mu bacteriophage [105].

7. Conclusions

Chloroplast genetic engineering is still under development but is emerging as a promising tool in the improvement of agricultural crops, expression of biopharmaceuticals elements, and manufacturing of biomaterials. Although the chloroplast transformation has been achieved in several important crops is not well defined in crops such as cereals due to factors like regenerate through somatic embryogenesis by inefficient methods of tissue culture and regeneration protocols, although there have been some significant progress in this regard [103]. Also currently, the transformation methods for grasses are restricted to nuclear genome by *Agrobacterium*, electroporation, and biolistic [97, 106]. Furthermore, plastid genetic transformation has allowed phylogeny study, which is similar to transformation with emphasis in genetic improvement, requires knowledge of the genome that has not been studied in all species, this also makes a disadvantage. In this sense, although it would have to prepare a vector for each crop, there are reports of transformations using plastid vectors from tobacco in potato and tomato transformations [107], indicating that differences in mRNA processing are determined as a vector that can be used. On the other hand, although the transgenes could not escape through pollen, rare hybrids can be generated if modified crops are pollinated by wild relatives [108]; considering that of the 13 most important crops in the world, 12 of them hybridize with wild relatives [109]; coupled with this, it has been reported the transmission

parental of chloroplasts in tobacco even when considering the strictly maternal inheritance of organelles [110]. Therefore, it should be considered new controls in transgene transmission contention, which makes it the biggest challenge to pursue in the development of genetically modified crops [15].

Conflict of interest

The authors declare that they have no conflicts of interest.

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