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M.N.V. Prasad *Editors*

Environmental Adaptations and Stress Tolerance of Plants in the Era of Climate Change

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Preface

Any external factor that imposes negative impact on growth and development of the plant is known as stress. Plants often experience abiotic stress like drought, salinity, alkalinity, temperature, UV-radiations, oxygen deficiency, etc. Abiotic stress is responsible for the huge crop loss and reduced yield more than 50% of some major crops. Ion imbalance and osmotic stress is the primary effect of abiotic stress. Prolonged exposure to primary stress causes secondary stress through the generation of reactive oxygen species (ROS). These are deleterious for the plants as it causes oxidative damage by reacting with biomolecules. Plants are able to perceive the external and internal signals and are then used by the plant to regulate various responses to stress. Plants respond the abiotic stress by up- and downregulation of genes responsible for the synthesis of osmolytes, osmoprotectants, and antioxidants. Stress-responsive genes and gene products including proteins are expressed and provide tolerance to the plant. To understand the physiological, biochemical, and molecular mechanisms for abiotic stress, perception, transduction, and tolerance is still a challenge before plant biologists.

The chapters in this book deal with the effect of different abiotic stresses on plant metabolism and responses of the plants to withstand the stress. Chapter 1 describes involvement of different osmolytes, osmoprotectants, and antioxidants during abiotic stress. Chapter 2 deals with the role of halophytes in understanding and managing abiotic stress. Chapter 3 addresses the effect and defense mechanisms in plants under UV stress. Chapter 4 throws light on the potassium uptake and its role under abiotic stress. Chapters 5–7 deal with the effect of temperature (heat, chilling) on plants and their responses. Chapter 8 deals with the formation and function of roots under stress. Chapter 9 is concerned with role of ROS and NO under abiotic stress. Chapter 10 throws light on nitrogen inflow and nitrogen use efficiency (NUE) under stress. Chapter 11 addresses Am symbiosis and soil interaction under abiotic stress. Chapter 12 deals with the role of small RNA in abiotic stress. Chapter 13 describes the involvement of transcription factors (TFs) under abiotic stress. Chapters 14–17 deal with the involvement of different signaling molecules (Ca^{2+}, H_2O_2, and phytohormones) under abiotic stress. Chapter 18 covers the role of ethylene and plant growth-promoting bacteria under environmental stress. Chapter 19 throws light on new approaches about metal-induced stress. Chapters 20 and 21 address the role of sulfur and salicylic acid in

alleviating heavy metal-induced stress. Chapters 22 and 23 cover the bioremediation of organic contaminants and utilization of different weeds in removal of heavy metals. We hope that this volume will provide the background for understanding abiotic stress tolerance in plants.

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Abiotic Stress Responses in Plants: An Overview

1

Hans-Werner Koyro, Parvaiz Ahmad, and Nicole Geissler

Abstract

Plants are more and more affected by environmental stresses, especially by the devastating consequences of desertification and water scarcity which can be seen and felt all over the world. About 3.6 billion of the world's 5.2 billion hectares of dryland used for agriculture have already suffered erosion, soil degradation, and salinization. Desertification can hinder efforts for sustainable development and introduces new threats to human health, ecosystems, and national economies. This problem is catalyzed by global climate change which exacerbates desertification and salinization. Therefore, solutions are desperately needed, such as the improvement of drought and salinity tolerance of crops, which in turn requires a detailed knowledge about tolerance mechanisms in plants. These mechanisms comprise a wide range of responses on molecular, cellular, and whole plant levels, which include amongst others the synthesis of compatible solutes/osmolytes and radical scavenging mechanisms. Regarding global change, elevated atmospheric CO_2 concentrations can enhance salt and drought tolerance because oxidative stress is alleviated and more energy can be provided for energy-dependent tolerance mechanisms such as the synthesis of compatible solutes and antioxidants, thus increasing the suitability of plants as crops in future. A detailed knowledge of the physiological and biochemical basis of drought and salt tolerance and its interaction with elevated CO_2 concentration can provide a basis for the cultivation of suitable plants in regions threatened by desertification and water scarcity under sustainable culture conditions. Even the drylands could offer tangible economic and ecological opportunities.

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The aim of this chapter is to uncover how compatible solutes and antioxidants alleviate environmental stress, especially drought and salt stress, and the role elevated CO_2 concentrations can play in this context, so that early indicators allowing successful breeding can be identified and the potential of plants as crops in a CO_2 rich world can be assessed.

Keywords
Abiotic stress • Antioxidants • Osmolytes • Oxidative stress

1 Introduction

Plants are continuously affected by a variety of environmental factors. Whereas biotic environmental factors are other organisms such as symbionts, parasites, pathogens, herbivores, and competitors, abiotic factors include parameters and resources which determine plant growth like temperature, relative humidity, light, availability of water, mineral nutrients, and CO_2, as well as wind, ionizing radiation, or pollutants (Schulze et al. 2002). The effect each abiotic factor has on the plant depends on its quantity or intensity. For optimal growth, the plant requires a certain quantity of each abiotic environmental factor. Any deviation from such optimal external conditions, that is, an excess or deficit in the chemical or physical environment, is regarded as abiotic stress and adversely affects plant growth, development, and/or productivity (Bray et al. 2000). Abiotic stress factors include, for example, extreme temperatures (heat, cold, and freezing), too high or too low irradiation, water logging, drought, inadequate mineral nutrients in the soil, and excessive soil salinity. As especially drought and salt stress are becoming more and more serious threats to agriculture and the natural status of the environment, this chapter will focus on these stress factors. They are recurring features of nearly all the world's climatic regions since various critical environmental threats with global implications have linkages to water crises (Gleick 1994, 1998, 2000). These threats are collaterally catalyzed by global climate change and population growth.

The latest scientific data confirm that the earth's climate is rapidly changing. Due to rising concentrations of CO_2 and other atmospheric trace gases, global temperatures have increased by about 1°C over the course of the last century, and will likely rise even more rapidly in coming decades (IPCC 2007). Scientists predict that temperatures could rise by another 3–9°C by the end of the century with far-reaching effects. Increased drought and salinization of arable land are expected to have devastating global effects (Wang et al. 2003b). Abiotic stress is already the primary reason of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Bray et al. 2000; Wang et al. 2003b). It will soon become even more severe as desertification will further increase and the current amount of annual loss of arable area may double by the end of the century because of global warming (Evans 2005; Vinocur and Altman 2005). Simultaneously, rapid population growth increasingly generates pressure on existing cultivated land and other resources (Ericson et al. 1999). Population migration to those arid and semiarid areas increases the problems of water shortage and worsens the situation of land degradation in the destination, and in turn causes severe problems of poverty, social instability, and population health threats (Moench 2002). Water scarcity and desertification could critically undermine efforts for sustainable development, introducing new threats to human health, ecosystems, and national economies of various countries. Therefore, solutions to these problems are desperately needed, such as the improvement of salt and drought tolerance of crops, which in turn

requires a detailed knowledge about salt and drought tolerance mechanisms in plants.

The viability of plants in both dry and saline habitats depends on their ability to cope with (I) water deficit due to a low water potential of the soil and (II) restriction of CO_2 uptake. Plants growing on saline soils are additionally confronted with (III) ion toxicity and nutrient imbalance.

Water deficit (I) causes detrimental changes in cellular components because the biologically active conformation and thus the correct functioning of proteins and biomembranes depends on an intact hydration shell. As a consequence, severe osmotic stress can lead to an impairment of amino acid synthesis, protein metabolism, the dark reaction of photosynthesis or respiration and can cause the breakdown of the osmotic system of the cell (Larcher 2001; Schulze et al. 2002). Water deficit can be counteracted by compatible solutes, organic compounds which are highly soluble and do not interfere with cellular metabolism. They serve as a means for osmotic adjustment and also function as chaperons by attaching to proteins and membranes, thus preventing their denaturation. This protective function of compatible solutes can also alleviate ion specific effects of salt stress caused by ion toxicity and ion imbalance such as the precipitation of proteins due to changes in charge or the destruction of membranes caused by alterations of the membrane potential.

Regarding the restriction of CO_2 uptake (II), the negative effects of osmotic stress described earlier force plants to minimize water loss; growth depends on the ability to find the best tradeoff between a low transpiration and a high net photosynthetic rate (Koyro 2006). However, various plant species show a clearly reduced assimilation rate under osmotic stress conditions due to stomatal closure (Huchzermeyer and Koyro 2005). A consequence can be an excessive production of reactive oxygen species (ROS) which are highly destructive to lipids, nucleic acids, and proteins (Kant et al. 2006; Türkan and Demiral 2009; Geissler et al. 2010). However, generated ROS can be scavenged by the antioxidative system which includes nonenzymatic antioxidants and antioxidative enzymes (Blokhina et al. 2003).

Ion toxicity (III) on saline habitats is caused by ion specific effects on membranes and proteins: On the one hand, changes of the ionic milieu lead to alterations of the membrane potential and thus to a destruction of biomembranes (Schulze et al. 2002). On the other hand, the hydration and charge of proteins are negatively influenced, so that their precipitation is promoted, but their activity is reduced (Kreeb 1996). These effects of salt stress can be alleviated by the protective chaperone function of compatible solutes, similarly as explained above for osmotic stress.

When looking at drought and salt tolerance of plants in the face of global climate change, another important aspect should be considered: Compared to salinity and drought, elevated atmospheric CO_2 concentrations have contrary effects on plants: They often improve photosynthesis while reducing stomatal resistance in C_3 plants, thus increasing water use efficiency, but decreasing photorespiration and oxidative stress (Urban 2003; Kirschbaum 2004; Rogers et al. 2004). Furthermore, more energy can be provided for energy-dependent tolerance mechanisms such as the synthesis of compatible solutes and antioxidants. Therefore, the salt and drought tolerance and the productivity of these plants can be enhanced under elevated CO_2 (Ball and Munns 1992; Wullschleger et al. 2002; Urban 2003), increasing their future suitability as crops. Against the background described earlier, this review uncovers how compatible solutes and antioxidants alleviate environmental stress, especially drought and salt stress, and the role elevated CO_2 concentrations can play in this context.

2 Compatible Solutes Which Can Prevent Detrimental Changes Under Environmental Stress

Severe osmotic stress can cause detrimental changes in cellular components. The best characterized biochemical response of plant cells to osmotic stress is the accumulation of high concentrations of either organic ions or other low

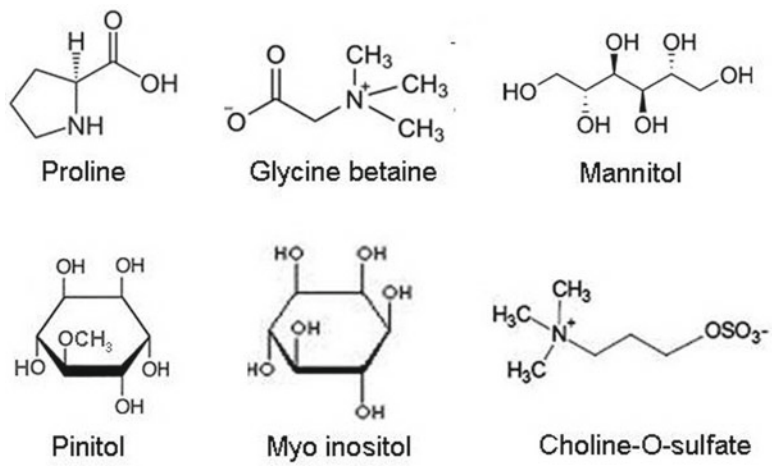

Fig. 1.1 Chemical structure of some important compatible solutes in plants

molecular weight organic solutes termed compatible solutes. These compounds are highly soluble in water, electrically neutral in the physiological pH range, and noninhibitory to enzymes even at high concentrations, so that they do not interfere with essential metabolic (enzymatic) reactions (Rhodes et al. 2002). The structure of some important compatible solutes is shown in Fig. 1.1.

Organic solutes play a crucial role in higher plants grown under dry or saline conditions. However, their relative contribution varies among species, cultivars, and even between different compartments within the same plant (Ashraf and Harris 2004). A wide range of metabolites which can prevent these detrimental changes in cellular components have been identified, including mono-, di-, oligo-, and polysaccharides (glucose, fructose, sucrose, trehalose, raffinose, and fructans), sugar alcohols (mannitol, glycerol, and methylated inositols), quaternary amino acid derivatives (Pro, GB, β-alaninebetaine and prolinebetaine), tertiary amines (1,4,5,6-tetrahydro-2-mehyl-4-carboxyl pyrimidine), and sulfonium compounds (choline-*O*-sulphate, dimethylsulphoniopropionate) (Flowers and Colmer 2008; Vinocur and Altman 2005). The primary function of compatible solutes is to reduce water potential, to maintain turgescent cells, and to ensure balanced water relations (Wang et al. 2003a). In addition, high concentration of compatible solutes exists primarily in the cytosol to balance the low water potentials achieved by high apoplasmic and vacuolar Na^+ and Cl^- concentration (Türkan and Demiral 2009). Recent studies indicate that compatible osmolytes also protect subcellular structures and mitigate oxidative damage caused by free radicals produced in response to salt stress (Slama et al. 2008; Smirnoff and Cumbes 1989). In many halophytes, organic osmolytes such as Pro or GB accumulate at suitably high concentrations to create osmotic potentials even below 0.1 MPa. In contrast to halophytes, in many glycophytes the concentrations of compatible solutes do not seem to be high enough to generate sufficiently low osmotic potentials (Türkan and Demiral 2009). This difference between halophytes and glycophytes can be used as an early indicator for salt resistance. Therefore, in the next chapters, the most important compatible solutes are described in detail.

2.1 Betaines

The quaternary ammonium compounds that function as effective compatible osmolytes in plants subject to salt stress are GB, β-alaninebetaine, prolinebetaine, choline-*O*-sulphate, hydroxyprolinebetaine, and pipecolatebetaine (Ashraf and

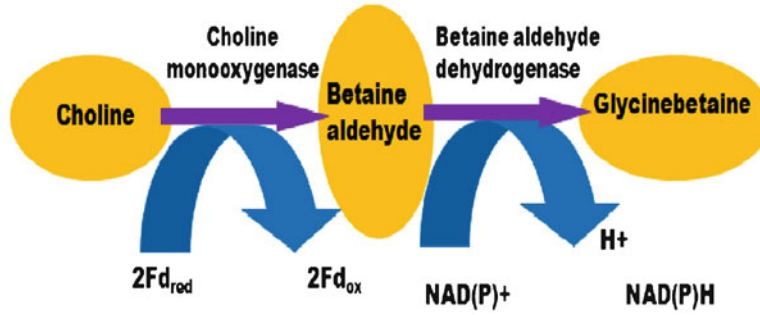

Fig. 1.2 Biosynthetic pathway of glycinebetaine (adopted from Ahmad and Sharma 2008)

Harris 2004). GB occurs most abundantly in response to a variety of abiotic stress conditions by numerous organisms including bacteria, cyanobacteria, algae, fungi, animals, and many plant families such as *Chenopodiaceae* and *Gramineae* (Türkan and Demiral 2009). This metabolite is mainly located in chloroplasts and plays a vital role in the stroma adjustment and protection of thylakoid membranes, thereby maintaining the photosynthetic activity (Jagendorf and Takabe 2001). GB protects the photosystem II (PS-II) complex at high salinity (Murata et al. 1992) and at extreme temperatures or pH (Mohanty et al. 1993). GB also protects membranes against heat-induced destabilization and enzymes, such as RUBISCO, against osmotic stress (Mäkelä et al. 2000). In higher plants, GB is synthesized from serine via ethanolamine, choline by two-step oxidation reactions that were catalyzed by choline monooxygenase and betaine aldehyde dehydrogenase, respectively (Russell et al. 1998; Ahmad and Sharma 2008; see Fig. 1.2). The insertion of serine and glycine can be taken as an indicator for the close relationship of the photorespiration (peroxisomes) to the synthesis of GB. Besides this, recently a biosynthetic pathway of GB from glycine with the involvement of two N-methyl transferase enzymes has been reported (Waditee et al. 2005). Highly tolerant genera such as *Spartina* and *Distichlis* accumulated the highest levels of GB, moderately tolerant species intermediate levels, and sensitive species hardly any GB (Rhodes and Hanson 1993). Genetic evidence that GB improves salinity tolerance has been obtained for many important agronomical crops such as tobacco, tomato, potato, barley, maize, and rice. These plants have long been a potential target for engineering GB biosynthesis pathway and thus for resistance against different environmental stress conditions (Sairam and Tyagi 2004; Türkan and Demiral 2009). The importance of N-methyltransferase for stress tolerance could also be shown for *Arabidopsis*. Genetically modified plants of this genus accumulated betaine to significant levels at different environmental stress conditions and hence improved seed yield (Waditee et al. 2005). A moderate stress tolerance was noted in some transgenic lines based on relative shoot growth in response to salinity, drought, and freezing. Huang et al. (2000) reported metabolic limitation in betaine production in transgenic plants. In fact, *Arabidopsis thaliana*, *Brassica napus,* and *Nicotiana tabacum* were transformed with bacterial choline oxidase cDNA, and their levels of GB were only between 5 and 10% of the levels found in natural betaine producers.

Beyond this, choline-fed transgenic plants synthesized substantially more GB. This result was taken as a hint that these plants require a distinct endogenous amount of choline to synthesize an adequate amount of GB (Sairam and Tyagi 2004).

The protective effect of GB at salinity or drought could also be demonstrated by exogenous application at rice seedlings, soybean, and common beans (Ashraf and Foolad 2007; Demiral and Türkan 2006). GB pretreatment also alleviated salinity-induced peroxidation (oxidative damage) of lipid membranes of rice cultivars. Besides rice,

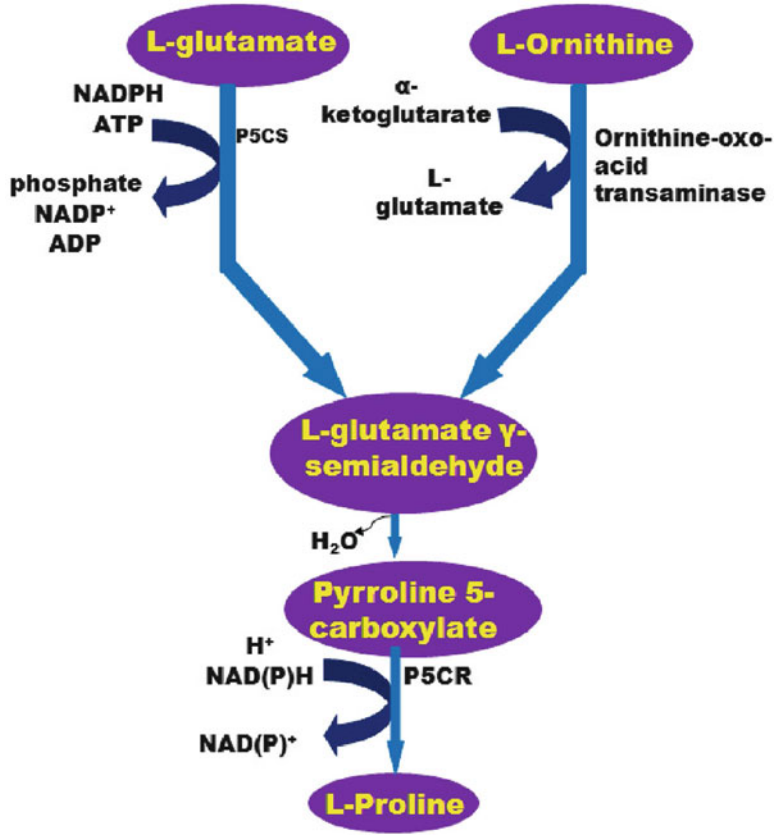

Fig. 1.3 Biosynthetic pathway of proline (adopted from Ahmad and Sharma 2008)

the correlation between the protective effect of GB and the antioxidative defense system has been observed in chilling-stressed tomato (Park et al. 2006), drought- or salt-stressed wheat (Raza et al. 2007), and salt-stressed suspension cultured tobacco BY2 cells (Hoque et al. 2007).

2.2 Amino Acids, Proline, and Amides

It has been reported that amino acids (such as alanine, arginine, glycine, serine, leucine, and valine, the nonprotein amino acids citrulline and ornithine (Orn)), together with the imino acid Pro, and the amides such as glutamine and asparagine are accumulated in higher plants under salinity and drought stress (Dubey 1997; Mansour 2000). Pro is known to occur widely in higher plants and can be accumulated in considerable amounts in response to salt stress, water deficit, and other abiotic stresses (Ali et al. 1999; Kavi Kishore et al. 2005; Koca et al. 2007; Ahmad and Sharma 2008). The Pro concentration is metabolically controlled. This imino acid is synthesized in plastids and cytoplasm while degraded to L-glutamate (Glu) in mitochondria. There are two different precursors of Pro in plants: Glu and Orn (Fig. 1.3). Pro is synthesized from Glu via glutamic-γ-semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylate (P5C). P5C synthase (P5CS) catalyses the conversion of Glu to P5C, followed by P5C reductase (P5CR), which reduces P5C to Pro (Ashraf and Foolad 2007). The other precursor for Pro biosynthesis is Orn, which is transaminated to P5C by a mitochondrial Orn-γ-aminotransferase (OAT) enzyme (Verbruggen and Hermans 2008). In the reverse reaction, Pro is metabolized to Glu in a feedback manner, via P5C and GSA with the aid of Pro

dehydrogenase followed by P5C dehydrogenase (P5CDH) (Wang et al. 2003a).

The contribution of Glu and Orn pathways to stress-induced Pro synthesis differs between species, and it has been shown that stress-tolerant plants are able to accumulate Pro in higher concentrations than stress-sensitive plants. Slama et al. (2008) showed a positive correlation between Pro accumulation and tolerance to salt, drought, and the combined effects of these stresses. Osmotic stress (particularly mannitol stress) led to a considerable increase of the Pro concentration in the obligatory halophyte *Sesuvium portulacastrum*, while the contents in soluble sugars and in Na^+ remained unchanged. In drought-stressed plants, the concentration of K^+, Na^+, Cl^-, and Pro, as well as ornithine-δ-aminotransferase (δ-OAT) activity increased significantly. Inversely, Pro dehydrogenase activity was impaired. Re-watering leads to a recovery of these parameters at values close to those of plants permanently irrigated with 100% of field capacity. The presence of NaCl and mannitol in the culture medium (ionic and osmotic stress) led to a significant increase of the Na^+ and Pro concentration in the leaves, but it had no effect on leaf soluble sugar content. Slama et al. (2007a, b) assumed that the ability of NaCl to improve plant performance under mannitol-induced water stress is caused by an improved osmotic adjustment through Na^+ and Pro accumulation, which is coupled with the maintenance of the photosynthetic activity. Similarly, the Pro concentration in the roots of salt tolerant alfalfa plants rapidly doubled under salt stress and was significantly higher than in salt sensitive genotypes (Petrusa and Winicov 1997). In addition to its role as an osmolyte for osmotic adjustment, Pro contributes to stabilizing subcellular structures (membranes and proteins) by forming clusters with water molecules which attach to proteins and membranes and prevent their denaturation (Koca et al. 2007; Ashraf and Foolad 2007; Lee et al. 2008). Due to its protective function on membranes it can also improve cell water status and ion homeostasis (Gadallah 1999; Gleeson et al. 2005), and it can scavenge free radicals and buffer cellular redox potential under stress conditions (Koca et al. 2007; Ashraf and Foolad 2007; Lee et al. 2008). Pro is also involved in alleviation of cytoplasmic acidosis and sustaining $NADP^+$/NADPH ratios at required levels for metabolism (Hare and Cress 1997), thus supporting redox cycling (Babiychuk et al. 1995).

Transgenic approaches proved an enhancement of plant stress tolerance via overproduction of Pro. For instance, transgenic tobacco (*N. tabacum*), overexpressing the *p5cs* gene that encodes P5CS, produced 10- to 18-fold more Pro and exhibited better tolerance under salt stress (Kavi Kishor et al. 2005). In *Arabidopsis*, the overexpression of an antisense Pro dehydrogenase cDNA resulted in an increased accumulation of Pro and a constitutive tolerance to freezing and a higher salt tolerance (Nanjo et al. 2003). Similarly, Borsani et al. (2005) reported that the *Arabidopsis* P5CDH (Δ^1-pyrroline-5-carboxylate dehydrogenase) and SRO5, an overlapping gene of unknown function in the antisense orientation, produced two types of siRNAs, 24-nt siRNA and 21-nt siRNA. In fact, they compared the levels of salt stress-induced Pro accumulation in various mutant plants (dcl2, sgs3, rdr6, and nrpd1a) which lacked SRO5-P5CDH nat-siRNAs and cleavage of the P5CDH transcript, Pro accumulation was not significantly induced by salt stress or was induced to a lesser extent than in the corresponding wild type. This result is consistent with their inability to downregulate P5CDH under stress, thereby causing a continued Pro catabolism and reduced Pro accumulation. In contrast, the dcl1 and rdr2 mutants, which were able to degrade P5CDH mRNA, had the same Pro level as the wild type under salt stress. The wild-type level of Pro accumulation in dcl1 indicates that although the 21-nt P5CDH nat-siRNAs were not produced, the 24-nt SRO5-P5CDH nat-siRNA alone was sufficient to cause the downregulation of P5CDH (Fig. 1.4).

An alternative approach to improve plant stress tolerance is the exogenous application of Pro, which can lead to either osmoprotection or cryoprotection. For example, in various plant species growing under salt stress, among them the halophyte *Allenrolfea occidentalis*, exogenous application of Pro led to a higher osmoprotection and an increased growth (Yancey 1994).

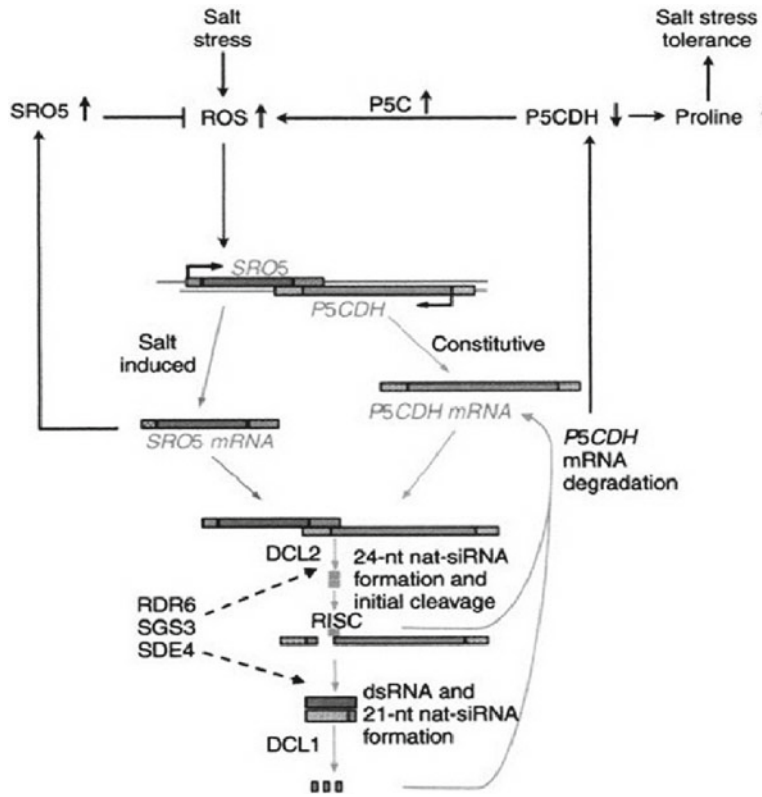

Fig. 1.4 Diagram of phased processing of SRO5-P5CDH nat-siRNAs and its role in a salt-stress regulatory loop (Borsani et al. 2005)

2.3 Sugars and Sugar Alcohols

Several studies have been attempted to relate the magnitude of changes in soluble carbohydrates to salinity tolerance. Parida and Das (2005) found out that carbohydrates such as sugars (glucose, fructose, sucrose, and fructans) and starch are accumulated under salt stress. Furthermore, Megdiche et al. (2007) and Geissler et al. (2009a) proved that *Cakile maritima* and *Aster tripolium* plants accumulate high amounts of total soluble carbohydrates and Pro at high salinity (400 and 500 mM NaCl, respectively). The major functions of sugars and sugar alcohols are osmoprotection, osmotic adjustment, carbon storage, and radical scavenging (Adams et al. 2005; Ashraf et al. 2006; Messedi et al. 2006; Lee et al. 2008; Ahmad and Sharma 2008). Furthermore, there is a discussion about that they serve as molecular chaperones (Hasegawa et al. 2000; Liu et al. 2006).

There is a difference between starch and sugar accumulation in short- and long-term reaction (da Silva and Arrabaca 2004). In short-term water stress experiments, a decrease in sucrose and starch content was observed for *Setaria sphacelata*, a naturally adapted C_4 grass while in long-term experiments, a higher amount of soluble sugars and a lower amount of starch were found. da Silva and Arrabaca (2004) assumed that the shift of metabolism towards sucrose might occur because starch synthesis and degradation are more affected than sucrose synthesis.

Trehalose, a rare, nonreducing sugar, is present in several bacteria and fungi and in some desiccation-tolerant higher plants (Vinocur and Altman 2005). Under various abiotic stresses, the disaccharide trehalose accumulates in many organisms as an osmolyte and osmoprotectant, protects membranes and proteins in cells, and reduces the aggregation of denatured proteins

(Ashraf and Harris 2004). In the transgenic plants, a comparatively moderate increase in trehalose levels lead to a higher photosynthetic rate and to a decrease in photooxidative damage during stress. Trehalose is thought to protect biological molecules from environmental stress (such as desiccation-induced damage), as suggested by its reversible water-absorption capacity (Penna 2003). It was shown that the contents of reducing and nonreducing sugars and the activity of sucrose phosphate synthase increase under salt stress, whereas starch phosphorylase activity decreases (Dubey and Singh 1999).

In general, the sugar alcohols are divided in acyclic (e.g., mannitol) and cyclic (e.g., pinitol) polyols. Polyols can make up a considerable percentage of all assimilated CO_2 and can have several functions such as compatible solutes, low molecular weight chaperones, and scavengers of stress-induced oxygen radicals (Bohnert et al. 1995). Polyols act in two indistinguishable ways, namely, osmotic adjustment and osmoprotection (Parida and Das 2005). In osmotic adjustment they act as osmolytes, facilitating the retention of water in the cytoplasm and enabling the sequestration of sodium into the vacuole or apoplast (cell wall). These osmolytes protect cellular structures by interacting with membranes, protein complexes, or enzymes. For instance, mannitol, a sugar alcohol that accumulates upon salt and water stress can alleviate abiotic stress. Transgenic wheat expressing the mannitol-1-phosphatase dehydrogenase gene (mtlD) of *Escherichia coli* was significantly more tolerant to water and salt stress (Abebe et al. 2003). Consequently, the transgenic wheat plants showed an increase in biomass, plant height, and number of secondary stems (tillers). The cyclic sugar alcohols pinitol and ononitol were accumulated in tolerant species such as the facultative halophyte *Mesembryanthemum crystallinum* when exposed to salinity or water deficit (Bohnert and Jensen 1996). Pinitol can be synthesized from myoinositol by the sequential catalysis of inositol methyl transferase and ononitol epimerase. An inositol methyl transferase (Imt) cDNA was isolated from transcripts in *M. crystallinum* growing under saline conditions (Vernon and Bohnert 1992), and transgenic tobacco for Imt has been obtained (Vernon et al. 1993).

2.4 Polyamines

Under stressful conditions, different plant species respond differently towards levels of polyamines. Some might accumulate polyamines in response to stress, while others do not increase or even decrease their endogenous polyamine contents when exposed to harsh environments. It is proposed that PA play a defensive role during biotic stress responses (Walters 2003a, b). One of the examples is the hypersensitive response (HR) which consists of rapid cell death at the sight of pathogen entry, typically develops in the interaction between tobacco mosaic virus (TMV) and *N* resistance gene carrying *N. tabacum* and leads to enhanced polyamine synthesis and accumulation (Kusano et al. 2008). It is also believed that stress-induced polyamines tend to modulate the activity of a certain set of ion channels to adapt ionic fluxes in response to environmental changes. Many more examples of responses to biotic stress have been quoted by Kusano et al. (2008).

Various abiotic stress conditions have been reported to alter the concentration of polyamines (Bouchereau et al. 1999; Walters 2003a). Exogenous polyamine application and/or inhibitors of enzymes involved in polyamine biosynthesis pointed out a possible role of these compounds in plant adaptation/defense to several environmental stresses (Bouchereau et al. 1999; Alcázar et al. 2006; Groppa and Benavides 2008; Alcázar et al. 2010). More recent studies using either transgenic overexpression or loss-of-function mutants support this protective/adaptive/defensive role of PAs in plant response to abiotic stress (Alcázar et al. 2006; Kusano et al. 2008; Gill and Tuteja 2010). For example, *Arabidopsis* plants overexpressing *Cucurbita ficifolia* Spd synthase gene were tolerant of multistresses (chilling, freezing, salinity, drought, and paraquat toxicity) (Kusano et al. 2007; Tassoni et al. 2010). According to Rhee et al. (2007), the basic principle underlying polyamine adaptive responses appears to be shared by the prokaryotic stringent response and the eukaryotic unfolded protein response (UPR). UPR is triggered when unfolded proteins and uncharged tRNAs accumulate in the endoplasmic reticulum (ER) due to ER stress or nutrient starvation.

As a result of this, cap-dependent translation of many mRNAs is suppressed and the expression of a certain set of genes including the luminal binding protein gene *BiP* is induced. The underlying mechanisms of UPR in yeasts and mammals have been well researched (Rutkowski and Kaufman 2004), although those in plants have not (Kamauchi et al. 2005; Urade 2007; Kusano et al. 2008). Recently, nitric oxide (NO), an endogenous signaling molecule in plants and animals, has gained considerable importance in the PA studies. It is known to mediate responses to biotic and abiotic stresses. It has been reported by Tun et al. (2006) that spermine and spermidine are potent inducers of NO in *Arabidopsis*, but putrescine and its biosynthetic precursor arginine are not. There are many more examples of NO affecting the concentrations of PAs and over the past few years studies on polyamines and NO are gaining attention (Kusano et al. 2008).

3 Oxidative Stress and Antioxidative Responses to Environmental Stress

3.1 Production of ROS

Environmental stresses are responsible for the production of ROS. The production and removal of ROS is thought to be at equilibrium under normal conditions, whereas environmental stress disturbs this equilibrium by enhancing the production of ROS. ROS are very toxic for the organism as they affect the structure and function of the biomolecules. The main source of ROS production in plants is chloroplasts, mitochondria, and peroxisomes (Fig. 1.5).

Mitochondria are responsible for the generation of oxygen radicals and hydrogen peroxide due to the overreduction of the electron transport chain.

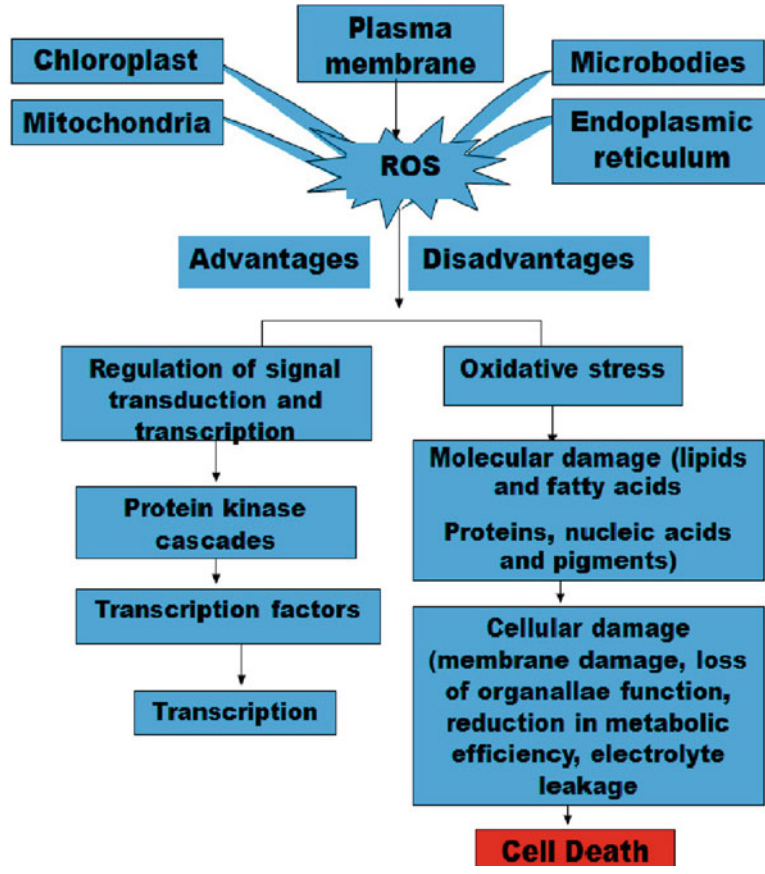

Fig. 1.5 Sites of reactive oxygen species (ROS) and the biological consequences leading to a variety of physiological dysfunctions that can lead to cell death (adopted from Ahmad et al. 2008)

Chloroplasts are found to be the major producer of O_2 and H_2O_2 (Davletova et al. 2005). This is because the oxygen pressure in the chloroplast is higher than in other organelles. Photoreduction of O_2 to $O_2^{\bullet-}$ during the photosynthetic electron transport takes place and is called Mehler reaction. The production of superoxides is due to the reduction of molecular oxygen in the plastoquinone pool. This reduction may be due to the plastosemiquinone, by ferredoxin (Fd) or by sulfur redox centers in the electron transport chain within PSI (Dat et al. 2000). These superoxides are converted to hydrogen peroxide either spontaneously or by the action of the enzyme SOD. Hydrogen peroxide is also responsible for the production of hydroxyl radicals ($OH^{\bullet}$).

The major producer of H_2O_2 in plant cells are peroxisomes. It has been reported that peroxisomes are also responsible for the production of superoxides (O_2^-). In peroxisomes, the production of O_2^- occurs in the peroxisomal matrix and the peroxisomal membrane. In the peroxisomal matrix, the oxidation of xanthine and hypoxanthine to uric acid in the presence of the enzyme xanthine oxidase generates O_2^- radicals (Halliwell and Gutteridge 2000). Peroxisomes have got two pathways for the production of H_2O_2. One is the disproportionation of O_2^- generated in this organelle and the other is a direct pathway. During photorespiration glycolate is catalyzed by glycolate oxidase, yielding H_2O_2. Fatty acid β-oxidation, the enzymatic reaction of flavin oxidases, can also produce H_2O_2 (Baker and Graham 2002; del Rio et al. 2002).

ROS include $^1O_2$, $O_2^{\bullet-}$, $H_2O^{\bullet}$, H_2O_2, $OH^{\bullet}$, $RO^{\bullet}$ organic hydroperoxide (ROOH), excited carbonyl ($RO^{\bullet}$), etc. They cause damage to biomolecules like proteins, lipids, carbohydrates, and DNA, which ultimately results in cell death (Foyer and Noctor 2005). Fortunately, plants are equipped with an antioxidant machinery that scavenges the ROS and helps the plant to tolerate the stress conditions. The antioxidants include enzymatic antioxidants, viz., superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), etc., and nonenzymatic antioxidants like ascorbic acid (AsA), vitamin E (α-tocopherol), reduced glutathione (GSH), etc.

3.2 Enzymatic Antioxidants

3.2.1 Superoxide Dismutase

SOD is one of the ubiquitous enzymes in aerobic organisms and plays a key role in cellular defense mechanisms against ROS. Within a cell, the SODs constitute the first line of defense against ROS. Its activity modulates the reactive amounts of $O_2^{\bullet-}$ and H_2O_2, the two Haber–Weiss reaction substrates, and decreases the risk of OH radical formation, which is highly reactive and may cause severe damage to membranes, proteins, and DNA (reviewed by Ahmad et al. 2010b). SOD was for the first time reported by Cannon et al. (1987) in maize and it catalyzes the dismutation of superoxide into hydrogen peroxide and molecular oxygen.

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

Different types of SOD isoforms have been observed in plants on the basis of metal cofactors attached to the active site. The isozyme containing Mn II at its active site is known as Mn-SOD. Similarly, the isozyme the active site of which contains Cu II and Zn II is known as Cu/Zn-SOD. The third isozyme contains Fe III and is referred to as Fe-SOD. The fourth SOD isoform contains Ni at the active site, is called Ni-SOD and is found in several *Streptomyces* species (Youn et al. 1996) and cyanobacteria (Palenik et al. 2003). Ni-SOD has not been reported in plants yet. Whereas only one type of SOD is found in most organisms, plants have multiple form of each type, which are encoded by more than one gene, indicating that plants have more complex antioxidant defense systems than other organisms.

Several studies have reported enhanced stress tolerance related to overproduction of chloroplastic SOD (Pastori and Foyer 2002). In maize leaves, GR and DHAR were exclusively localized in mesophyll cells whereas most of the SOD and APX were localized in mesophyll and bundle sheath cells. Increased SOD activity was reported in *Radix astragali* under water deficit stress, which varied in three different genotypes (Tan et al. 2006). Chilling stress has a significant effect in the enhancement of SOD activity in cucumber

seedlings (Feng et al. 2003). The increase in SOD activity under drought stress was about 25% in soybean plants (Zhang et al. 2007). SOD activity was doubled in water stressed citrus plants when compared to well-watered control plants during seedling stage (Wu et al. 2006).

SOD activity increased under drought stress in *Euphorbia esula* (Davis and Swanson 2001), maize (Pastori et al. 2000), *Cassia angustifolia* (Agarwal and Pandey 2003), wheat (Singh and Usha 2003), rice (Wang et al. 2005), *P. acutifolius* (Turkan et al. 2005), and *Camellia sinensis* (Chen et al. 2011), and the SOD activity was higher under salinity stress in *C. roseus* (Jaleel et al. 2008) and *Morus alba* (Ahmad et al. 2010a). While subjecting higher plants to water deficit stress SOD activity increases (Reddy et al. 2004). Koca et al. (2007) have shown that elevated SOD activity is accompanied with an increase in the activity of major H_2O_2 scavenging enzymes like APX, CAT, and POX in salt tolerant sesame cultivar *Cumhuriyat* as compared to cultivar *Orhangazi*. SOD activity increased by 1.6-fold in a salt tolerant mutant of *Chrysanthemum* compared to a non-tolerant one under NaCl stress (Hossain et al. 2006). An increased activity of SOD enzyme has also been reported under different abiotic stresses in *Catharanthus roseus* (Jaleel et al. 2007), *Pisum sativum* (Ahmad et al. 2008), *M. alba* (Ahmad et al. 2010a), and *Brassica juncea* (Ahmad 2010; Ahmad et al. 2011). SOD activity has also been observed to increase by the application of heavy metals such as cadmium (Shah et al. 2001; John et al. 2009; Ahmad et al. 2011), lead (Verma and Dubey 2003; John et al. 2009), and copper (Lombardi and Sebastiani 2005). Canola overexpressing Mn-SOD confers tolerance to aluminum stress (Basu et al. 2001). Overexpression of Mn-SOD in transgenic *Arabidopsis* showed a twofold increase in Mn-SOD activity and higher tolerance to salt as compared to nontransgenic plants (Wang et al. 2004). Tanaka et al. (1999) demonstrated that expression of yeast mitochondrial Mn-SOD in rice chloroplasts led to a 1.7-fold increase in Mn-SOD as compared to nontransgenic plants. Transgenic *Arabidopsis* with Mn-SOD confers tolerance to heat (Im et al. 2009). Wang et al. (2005) demonstrated that transgenic rice plants expressing Mn-SOD have shown reduced injury and sustained photosynthesis under PEG stress. Overexpression of Cu/Zn-SOD and APX in transgenic tobacco enhanced seed longevity and germination rates after various environmental stresses (Lee et al. 2010). Transgenic tobacco expressing Cu/Zn-SOD have been shown to tolerate chilling and heat stress (Gupta et al. 1993) and enhanced tolerance to salt, water, and PEG stress (Badawi et al. 2004). Prashanth et al. (2008) have also demonstrated that Cu/Zn-SOD confers tolerance to salinity in rice plants.

3.2.2 Catalase

Plant catalases are tetrameric iron porphyrins and play a role in stress tolerance against oxidative stress. Catalases are produced in peroxisomes and glyoxysomes. Catalases are involved in eliminating hydrogen peroxide generated by different environmental stresses (Kim et al. 2008; Ahmad et al. 2010b). Catalases decompose hydrogen peroxide to water and molecular oxygen without consuming reductants and may thus provide plant cells with an energy efficient mechanism to remove hydrogen peroxide (reviewed by Ahmad et al. 2010b). The enzyme is abundant in the glyoxysomes of lipid-storing tissues in germinating barley, where it decomposes H_2O_2 formed during the β-oxidation of fatty acids (Jiang and Zhang 2002) and in the peroxisomes of the leaves of C_3 plants, where it removes H_2O_2 produced during photorespiration by the conversion of glycolate into glyoxylate (Kiani et al. 2008). This is also due to the fact that there is a proliferation of peroxisomes during stress, which might help in scavenging H_2O_2, which can diffuse from the cytosol (Lopez-Huertas et al. 2000; Kusaka et al. 2005).

High temperatures affect the structure of most proteins and thus the activity of many enzymes. Hertwig et al. (1992) have demonstrated that the translation of catalase was hampered at 40°C. Anderson (2002) showed that high temperature is responsible for the decrease in catalase activity in pepper plants. In comparison, the desert plant *Retama raetam* exposed to heat shock temperature showed only a minor inactivation of catalase activity (Streb et al. 1997). Scandalios et al.

(2000) have also observed a reduced catalase activity in maize on exposure to temperatures of 35–40°C.

Sublethal doses of NaCl induce catalase activity in *Nicotiana plumbaginifolia* through activation of *cat2* and *cat3* genes (Savoure et al. 1999). However, catalase activity was found to decrease due to the salt stress because of accumulation of salicylic acid (Shim et al. 2003). Vaidyanathan et al. (2003) have demonstrated that salt tolerant rice cultivars contain higher levels of catalase activity compared to susceptible cultivars. Increase in catalase activity during salt stress has also been shown by other workers in maize (Azevedo-Neto et al. 2006; Arora et al. 2008), in sesame (Koca et al. 2007), and in mulberry (Ahmad et al. 2010a).

Catalase activity has also been found to decrease in presence of heavy metal stress (Mishra et al. 2006; Khan et al. 2007; Mobin and Khan 2007; Ahmad et al. 2011). Verma and Dubey (2003) also demonstrated that the activity of catalase declines in rice plants with increasing concentration of Pb. John et al. (2009) also reported that an increase in Cd and Pb concentrations decreases the catalase activity in mustard. Decrease in catalase may be due to the inhibition of enzyme synthesis or change in assembly of enzyme subunits (Shah et al. 2001).

3.2.3 Ascorbate Peroxidase

APX is an important antioxidant enzyme mainly found in higher plants and algae (Raven 2003). APX helps to detoxify H_2O_2 in the ascorbate-glutathione (= Halliwell-Asada) pathway. APX utilizes ascorbic acid and reduces H_2O_2 to water and monodehydroascorbate (MDA).

$$2\text{AsA} + \text{H}_2\text{O}_2 \rightarrow 2\text{MDA} + 2\text{H}_2\text{O}$$

APX was first isolated from chloroplasts and algae (Shigeoka et al. 1980; Nakano and Asada 1981). Different isoforms of APX which include thylakoid (tAPX), glyoxisomal (gmAPX), stromal (sAPX), and cytosolic (cAPX) have been reported (Shigeoka et al. 2002; Mittler et al. 2004). In comparison to other antioxidants, APX and guaiacol peroxidase (GPX) have a high affinity towards H_2O_2 (Mittler and Poulos 2005). APX isozymes have been found to be most stress responsive among the APX gene family during environmental stress (Mittler and Poulos 2005). APX1 has been found to enhance in response to environmental stress (Mittler 2002; Shigeoka et al. 2002). APX2 is expressed under stressful conditions and its expression is elevated in response to light stress or heat shock (Mullineaux and Karpinski 2002; Panchuk et al. 2002). Cytosolic APX1 has been found to protect *Arabidopsis* plants from a combination of stresses (Koussevitzky et al. 2008). Lu et al. (2007) demonstrated that cAPX improves salt tolerance in transgenic *Arabidopsis*.

Mittler et al. (1999) have demonstrated that suppression of APX1 in tobacco leads to a higher sensitivity of the plant to pathogen attacks. Overexpression of APX1 resulted in enhanced tolerance to oxidative stress in tobacco (Yabuta et al. 2002). Biologists have demonstrated the importance of APX1 by using APX1 knockout mutants. The plants lacking APX1 have showed delayed growth, no response of guard cells towards light, and light stress resulted in an induction of catalase and heat shock proteins (Pnueli et al. 2003). The accumulation of H_2O_2 is responsible for the abnormal closure of stomata in knockout APX1 plants (Pnueli et al. 2003). The induction of heat shock proteins in knockout APX1 plants may be due to an enhanced level of H_2O_2 which is considered as an essential signaling molecule during abiotic stress (Mittler 2002; Neill et al. 2002).

3.2.4 Glutathione Reductase

GR is a flavo-protein oxidoreductase and is found in both prokaryotes and eukaryotes (Romero-Puertas et al. 2006). GR is an important enzyme of the ascorbate–glutathione system and maintains the balance between reduced glutathione (GSH) and the ascorbate pool (reviewed by Ahmad et al. 2010b). Meldrum and Tarr (1935) for the first time reported GR in eukaryotes and yeast, and in 1951 it was also observed in plants (Conn and Vennesland 1951; Mapson and Goddard 1951). Later on GR has been isolated

from different plants and bacteria (Creissen et al. 1991; Creissen and Mullineaux 1995). GR is mainly found in chloroplasts (70–80%), and small amounts have been found in mitochondria, cytosol, and peroxisomes (Edwards et al. 1990; Romero-Puertas et al. 2006). GR catalyzes the reduction of glutathione in the cell. GSH is oxidized to GSSG which should be converted back to GSH in normal cells. Rapid recycling of GSH is more important than the synthesis of GSH. Hence GR and GSH have been found to play a very crucial role in stress tolerance in plants. GR plays an important role in alleviating oxidative stress in plants as evidenced by increased activities of GR during oxidative stress (Contour-Ansel et al. 2006; Khan et al. 2007; Mobin and Khan 2007; Hsu and Kao 2007). Increased activities of GR during drought stress were observed in different plants, e.g. in wheat (Selote and Khanna-Chopra 2006) and in rice (Selote and Khanna-Chopra 2004; Srivalli et al. 2003; Sharma and Dubey 2005). Salt stress also increased the GR activity in rice (Demiral and Turkan 2005; Tsai et al. 2005) and wheat (Sairam et al. 2005). A positive correlation between the increased activity of GR and chilling tolerance has been reported in rice (Guo et al. 2006), in maize (Hodges et al. 1997a, b) and tomato (Walker and McKersie 1993). Heavy metal stress is also responsible for the increase in the activity of GR in plants. Mulberry plants exposed to copper stress exhibit an increased GR activity (Tewari et al. 2006). A positive correlation of increased GR activity in presence of Cd has been reported in potato, radish, soybean, sugarcane, and mustard (Stroinski et al. 1999; Vitoria et al. 2001; Ferreira et al. 2002; Fornazier et al. 2002; Mobin and Khan 2007). Transgenic tobacco plants expressing the *gor2* gene from *E. coli* showed an increase in GR activity (Stevens et al. 2000). Pilon-Smith et al. (2000) observed that cytosol GR increases by 2-fold and chloroplast GR increases by 50-fold in transgenic plants of *B. juncea* expressing the *gor2* gene from *E. coli*. These transgenic plants showed an enhanced tolerance to Cd stress up to 100 μM. Expression of the *gor2* gene from *E. coli* in tobacco (cv. Belw3) showed an increased activity of GR and increases tolerance to paraquat and H_2O_2 stress (Lederer and Böger 2003).

3.3 Nonenzymatic Antioxidants

3.3.1 Ascorbic Acid

Among the small molecular antioxidants in plants, ascorbate is most abundant and is most concentrated in leaves and meristems (reviewed by Ahmad et al. 2010b). It is about five to ten times more concentrated than GSH in leaves (Ishikawa et al. 2006). AsA is present in high concentration in fruits, especially citrus fruits, but the concentration in fruits is not always higher than in leaves (Davey et al. 2000). Some fruits such as blackcurrants and rose hips are famous for their exceptionally high ascorbate content (Ishikawa et al. 2006). AsA occurs in all subcellular compartments, and the concentration varies from 20 mM in the cytosol to 300 mM in chloroplasts (Noctor and Foyer 1998). The synthesis of AsA takes place in mitochondria and is transported to other cell compartments through a proton electrochemical gradient or through facilitated diffusion (Horemans et al. 2000). Franceschi and Tarlyn (2002) reported the presence of ascorbate in the phloem sap of *A. thaliana*. Other species of plants have also been reported to contain ascorbate in the phloem sap, e.g. cucurbita (Hancock et al. 2008). This led to the conclusion that ascorbate is transported from source (leaves) to sink (meristem) (Ishikawa et al. 2006).

Ascorbate plays an important role in plants as an antioxidant and as a cofactor of many enzymes (Ishikawa et al. 2006). As an antioxidant, ascorbate protects plants from oxidative stress. Ascorbate peroxidase utilizes ascorbic acid and reduces H_2O_2 to water, thereby generating monodehydroascorbate (MDA) in the ascorbate–glutathione cycle (Pan et al. 2003). MDA can also be reduced directly to AsA in the presence of the catalytic enzyme MDAR and the electron donor NADPH (Asada 1999). Maddison et al. (2002) have reported that ascorbate plays a role in the defense against ozone. AsA has the capability of donating electrons in various enzymatic

and nonenzymatic reactions and is thus a powerful radical scavenger. It can directly scavenge $^1O_2$, $O_2^{\bullet-}$, and ^{-}OH radicals produced in the cell and can protect membranes against oxidative stress. In plant cells, the most important reducing substrate for H_2O_2 detoxification is ascorbic acid (Turkan et al. 2005). An increase in oxidized ascorbate during Cd stress has been reported by Demirevska-Kepova et al. (2006) in *Hordeum vulgare*. Yang et al. (2008) also reported that drought stress increases the ascorbate content in *Picea asperata*. Water stress results in significant increases in antioxidant AsA concentration in turfgrass (Zhang and Schmidt 2000; Vranova et al. 2002; Jaleel et al. 2007). Ascorbic acid shows a reduction under drought stress in maize and wheat, suggesting its vital involvement in oxidative response (Vertovec et al. 2001; Nayyar and Gupta 2006).

3.3.2 α-Tocopherol

Plants have the capacity to synthesize a lipophylic antioxidant known as α-tocopherol or vitamin E. α-tocopherol scavenges free radicals in combination with other antioxidants (Munne-Bosch and Algere 2003; Massacci et al. 2008). It has also been reported that α-tocopherol protects the structure and function of PSII as it chemically reacts with O_2 in chloroplasts (Lopez-Huertas et al. 2000; Nordberg and Arner 2001). Munne-Bosch and Algere (2003) reported that α-tocopherol helps in membrane stabilization and alleviates the tolerance of plants during oxidative stress. Environmental stresses are responsible for the generation of low molecular mass antioxidants such as α-tocopherol (Lowlor and Cornic 2002; Munne-Bosch and Algere 2003; Mahajan and Tuteja 2005; Martinez et al. 2007).

Falk et al. (2003) reported the upregulation of genes of α-tocopherol synthesis during oxidative stress. Water stress resulted in elevated levels of α-tocopherol in *Vigna* plants (Manivannan et al. 2007) and turfgrass (Zhang and Schmidt 2000).

3.3.3 Reduced Glutathione

Glutathione (L-glutamyl-L-cysteinylglycine, GSH) is a thiol compound composed of L-glutamic acid, L-cysteine, and glycine. GSH is distributed universally in animals, plants, and microorganisms and has an established role as an essential compound of a free radical scavenger (Monneveux et al. 2006). GSH participates in numerous cellular processes and protects cells from the toxic effects of many ROS (Petropoulos et al. 2008). Additionally, GSH is involved in other biological functions, such as regulation of protein and DNA synthesis, protein activities, and maintaining membrane integrity (Cabuslay et al. 2002). Meyer et al. (2005) reported that levels of H_2O_2 are controlled by the action of glutathione. Reduction of glutathione (GSH) and oxidation of glutathione (GSSG) are necessary for controlling H_2O_2 levels in cells and have an important role in redox signaling (Pastori and Foyer 2002. Reduced glutathione is directly involved in the reduction of ROS in plants. Transgenic tobacco expressing glutathione gene withstands oxidative stress (Singh and Verma 2001).

Glutathione is a tripeptide (α-glutamyl cysteinylglycine) and is found in the cytosol, chloroplasts, ER, vacuoles, and mitochondria (Sankar et al. 2007a, b). The nonprotein thiols are nucleophilic in nature and thus are important for the formation of mercaptide bonds with metals and for reacting with selective electrophiles (Rodriguez et al. 2005). In most plants, the major source of these nonprotein thiols is glutathione. Glutathione is considered the most important nonenzymatic antioxidant due to its relative stability and high water solubility (Samarah 2005). It can protect plant cells from environmental stress-induced oxidative stress (Samarah 2005).

4 The Effect of Elevated Atmospheric CO_2 Concentration on Antioxidants and Osmolytes Under Environmental Stress

Elevated atmospheric CO_2 concentration leads to a higher CO_2 concentration gradient between the outside air and the intercellular spaces of the leaves, so that the diffusion of CO_2 into the leaves

and the pCO_2/pO_2 ratio at the sites of photoreduction is increased (Robredo et al. 2007). Therefore, usually photorespiration and the rates of oxygen activation and ROS formation are reduced due to an increased NADPH utilization, whereas the net photosynthetic rate and thus the carbon supply is enhanced, especially in C_3 plants (Polle 1996; Urban 2003; Kirschbaum 2004; Long et al. 2004; Hikosaka et al. 2005; Ignatova et al. 2005). Furthermore, we often find a lower stomatal resistance (Hsiao and Jackson 1999; Li et al. 2003; Marchi et al. 2004; Rogers et al. 2004), which together with the higher net assimilation also leads to a better water use efficiency of photosynthesis (Amthor 1999; Morgan et al. 2001; Urban 2003). As a consequence of these effects, on the one hand there might be less need for antioxidants as elevated CO_2 ameliorates oxidative stress (Schwanz et al. 1996). On the other hand more energy can be provided for energy-dependent stress tolerance mechanisms such as the synthesis of osmolytes and antioxidants. Due to both effects mentioned earlier, elevated CO_2 can increase plant survival under abiotic stress conditions (Ball and Munns 1992; Rozema 1993; Drake et al. 1997; Fangmeier and Jäger 2001; Wullschleger et al. 2002; Urban 2003; Geissler et al. 2010).

Regarding oxidative stress, the antioxidant system can respond differently to elevated CO_2 depending on species or even genotype as well as on treatment duration and growth conditions such as mineral nutrition (Schwanz et al. 1996; Polle et al. 1997; Sanità di Toppi et al. 2002; Pérez-López et al. 2009). Varying responses can be related to a species-specific differential regulation in order to maintain an adequate balance between ROS formation and antioxidant ability under the actual conditions (Pérez-López et al. 2009). However, many studies have reported an increased tolerance to various abiotic stresses under elevated CO_2 due to an alleviation of oxidative stress:

In chestnut trees, photoinhibition due to high irradiance stress was ameliorated, and higher GSH levels were found in juvenile leaves (Carvalho and Amâncio 2002). Sgherri et al. (2000) reported that CO_2 enrichment led to an improved water use efficiency and a decreased photorespiration in *Medicago sativa* under drought stress. As a consequence, the cells showed a higher reducing status, increased ascorbate/dehydroascorbate and GSH/GSSG ratios. There was no demand for a higher GR activity (no CO_2 effect) and less requirement for Ca^{2+} ATPase activity to maintain Ca^{2+} homeostasis under stress conditions. Similarly, in cold stressed maize elevated CO_2 had no effect on SOD, CAT, and APX activities, but the formation of superoxide radicals and membrane injury was reduced (Baczek-Kwinta and Ko cielniak 2003). The alleviation of oxidative stress was probably due to a higher CO_2 assimilation, overcoming the Rubisco limitation under low temperature. In some cases elevated CO_2 even leads to reduced activities of antioxidative enzymes because there is less need for antioxidants: Pérez-López et al. (2009) reported that barley plants exposed to NaCl stress under ambient CO_2 exhibited enhanced activities of SOD, APX, CAT, GR, and dehydroascorbate reductase (DHAR), which was accompanied by ion leakage and lipid peroxidation. Furthermore, the expression ratio of enzyme isoforms changed, e.g. a relatively higher contribution of GR1 relative to GR2 and of Cu/Zn-SOD (which seems to be especially important for salt tolerance in *Hordeum vulgare*) was observed. Elevated CO_2 ameliorated ion leakage and lipid peroxidation, while the plants showed a lower upregulation of the antioxidant enzymes and an even higher relative contribution of GR1 and of Cu/Zn-SOD. The authors explain these results with less ROS generation and a better maintenance of redox homeostasis due to an enhanced photosynthesis and a reduced photorespiration. Similar results were found for *Solanum lycopersicum* by Takagi et al. (2009). NaCl salinity decreased plant biomass, net assimilation, and the transport of assimilates to the sink (stem), while CAT and APX activities increased. Under elevated CO_2 the negative effects of salinity were alleviated, especially when the sink activity was relatively high, and CAT and APX activities decreased compared to ambient CO_2. The improvement of oxidative stress (and of water relations) seemed to cause an activation of sink activity under elevated CO_2.

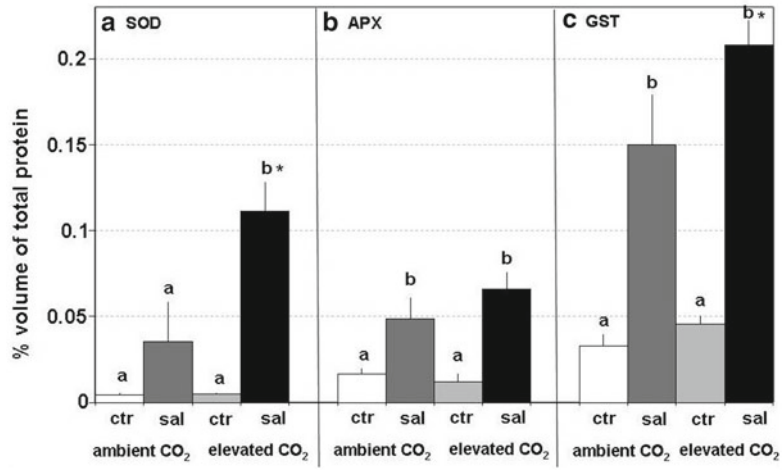

Fig. 1.6 Antioxidant enzyme expressions (relative volume percentages of the spots) in controls and salt treatments (75% seawater salinity) of *Aster tripolium* under ambient and elevated CO_2. (**a**) Superoxide dismutase, (**b**) ascorbate peroxidase, (**c**) glutathione-*S*-transferase. Values represent mean ± SD values of eight gels per treatment. Significant differences ($P \leq 0.05$) between the salinity treatments (within one CO_2 treatment) are indicated by *different letters*, significant differences between the CO_2 treatments (within one salt treatment) are indicated by an *asterisk*. *ctr* control, *sal* salt treatment

In contrast to the studies mentioned earlier, in some cases antioxidant activities are enhanced by elevated CO_2. In ozone stressed *Betula pendula,* elevated CO_2 eliminated the chloroplastic accumulation of H_2O_2, which could be explained by a higher photosynthetic rate, leading to a higher NADPH formation and a more efficient enzymatic detoxification (e.g., via the ascorbate–glutathione cycle; Oksanen et al. 2005). Marabottini et al. (2001) found a higher activity of catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) in drought-stressed *Quercus* under elevated CO_2. Rao et al. (1995) observed a more persistent high activity of glutathione reductase (GR), APX, und SOD in ozone-stressed wheat. Schwanz and Polle (2001) examined the drought tolerant species *Quercus robur* and the sensitive *Pinus pinaster*. They found out that *Q. robur* generally exhibits a higher activity of several antioxidative enzymes; furthermore, elevated CO_2 concentration ameliorated damage caused by drought stress in both species due to a higher stability of antioxidative enzymes and an enhanced SOD activity. Similar results were reported for the facultative halophyte *A. tripolium*. Under ambient CO_2 concentration salt stress led to an overexpression and thus to higher relative activities of the antioxidative enzymes APX, SOD, and glutathione-*S*-transferase (GST), while under elevated CO_2 the expression and activities of these enzymes were further increased (Fig. 1.6; Geissler et al. 2010). Similarly, elevated CO_2 concentration led to a significantly higher content of carotenoids – nonenzymatic antioxidants – in the salt treatments (Geissler et al. 2009b). These results implicate that the enhancement of enzyme expression and activity and the carotenoid content were not high enough to sufficiently eliminate ROS under ambient CO_2 concentration. Under elevated CO_2, however, a higher supply of energy-rich organic substances due to a significantly enhanced net assimilation rate (Geissler et al. 2009a, b) enabled the plants to invest more energy in the energy-dependent synthesis of enzymatic and nonenzymatic antioxidants. Therefore, ROS could be detoxified more effectively, so that salinity tolerance could be improved, manifesting itself in a higher survival rate of the salt-treated plants (Geissler et al. 2009a).

Furthermore, investigations about *A. tripolium* showed that elevated CO_2 concentration does not

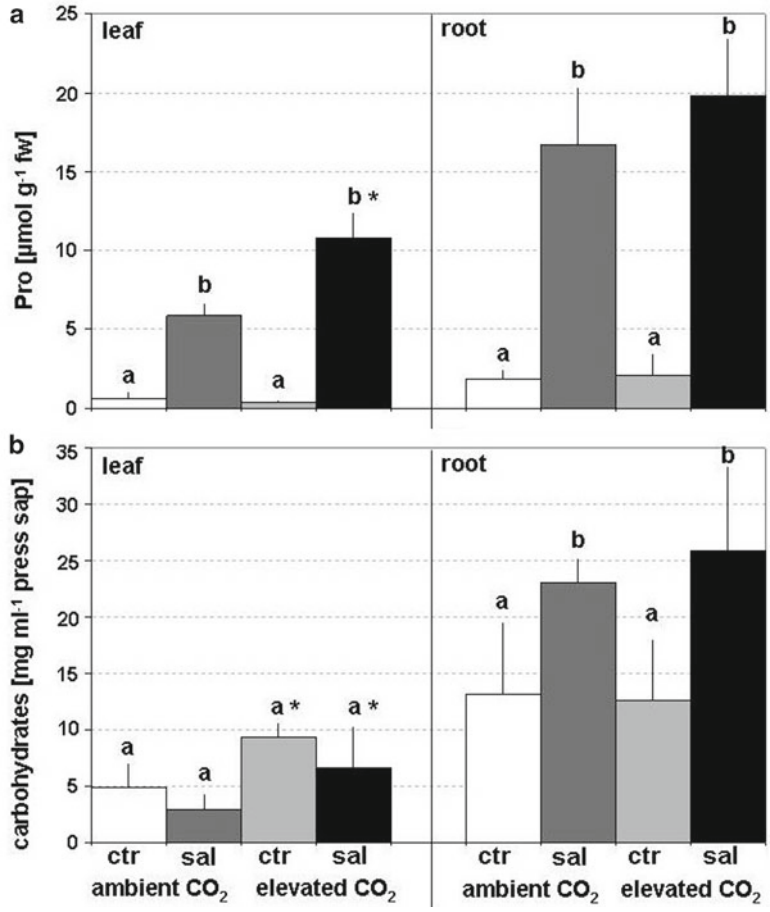

Fig. 1.7 Content of osmolytes in controls and salt treatments (75% seawater salinity) of *Aster tripolium* under ambient and elevated CO_2. (**a**) Proline, (**b**) total soluble carbohydrates. Values represent mean ± SD values of six measurements per treatment. Significant differences ($P \leq 0.05$) between the salinity treatments (within one CO_2 treatment) are indicated by *different letters*, significant differences between the CO_2 treatments (within one salt treatment) are indicated by an *asterisk*. *ctr* control, *sal* salt treatment

only have an effect on antioxidants, but on osmolytes as well. This halophyte employed its additional carbon gain under elevated CO_2 concentration also for a higher synthesis of compatible solutes (Geissler et al. 2009a): Salinity (under ambient CO_2) led to an accumulation of proline in all plant organs and of soluble carbohydrates in the main roots (Fig. 1.7). Under elevated CO_2 concentration, the plants accumulated a higher amount of proline, especially in the leaves which are the primary areas of influence of CO_2. In the main root, there was no necessity of an additional accumulation of proline because this organ is well protected against salt damage due to a high content of compatible solutes even at ambient CO_2 concentration. Furthermore, a higher amount of soluble carbohydrates under elevated CO_2 was found in all plant organs due to the increased photosynthesis and a lower conversion of saccharides to starch. These results are in accordance with the study of Abdel-Nasser and Abdel-Aal (2002) investigating *Carthamus mareoticus* under drought stress: Elevated CO_2 concentration increased the accumulation of total soluble carbohydrates in well watered as well as in stressed plants due to a higher amount of assimilates. The drought-induced inhibition of the sucrose phosphate synthase activity was

annihilated under elevated CO_2, and the drought-induced increase in sucrose content was further enhanced. The content of total amino acids and especially of proline behaved similarly to sucrose, as well as the activities of the proline synthesizing enzymes 1-pyrroline-5-carboxylate reductase (P5CR) and the ornithine aminotransferase (OAT). In contrast, the activity of the proline degrading enzyme proline dehydrogenase (PDH) was reduced by drought stress and further decreased under elevated CO_2.

In contrast to *C. mareoticus*, proline (and other amino acids) do not seem to contribute to salt tolerance in barley, but to reflect a reaction to stress damage, as shown by Pérez-López et al. (2010): Although a better osmotic adjustment (more negative osmotic potential) of salt-stressed plants was recorded under elevated CO_2, the proline content decreased, showing less stress damage. Instead, the accumulation of soluble sugars and other unidentified osmolytes (possibly polyols and/or quaternary nitrogen compounds) was actively enhanced under elevated CO_2, and these substances played an important role in osmotic adjustment and as compatible solutes under saline conditions. Elevated CO_2 provided a higher carbon and ATP supply for salt tolerance mechanisms, enabling the plants to actively increase their compatible solute concentration, which in turn leads to a better water uptake and turgor maintenance for plant growth.

As a summary, it can be concluded that elevated CO_2 concentration can enhance salt and drought tolerance of plants by alleviating oxidative stress, increasing the activity of the antioxidative system, and/or increasing the accumulation of compatible substances, having a positive effect on their suitability as crops on dry and saline soils in future.

5 Conclusion and Future Perspective

Abiotic stresses, especially osmotic and ionic stresses, are responsible for the decrease in yield especially in arid and semiarid regions. It is estimated that 45% of the world's agricultural land experience drought and 19.5% of the irrigated land are affected by salinity. These problems will be further catalyzed by global climate change. Prolonged environmental stresses are responsible for the production of ROS in different cell compartments like chloroplasts, mitochondria, peroxisomes, etc. ROS attack biomolecules, viz., DNA, lipids, proteins, carbohydrates, and disturb the normal functioning of the cell. Under severe stress conditions, ROS ultimately lead to cell death. In order to withstand oxidative stress, plants are equipped with enzymatic and nonenzymatic antioxidants. Many workers have reported the positive effects of SOD, CAT, APX, GR, MDHAR, AsA, glutathione, etc., in combating oxidative damage to the cell. To overcome the deleterious effects of abiotic stresses, plants also accumulate osmolytes and osmoprotectants such as proline and glycine betaine. These compounds are thought to play a role in osmotic adjustment and protect subcellular structures. Elevated atmospheric CO_2 concentration can alleviate oxidative stress, increase the activity of the antioxidative system, and/or increase the accumulation of compatible substances, so it can enhance salt and drought tolerance of plants and their suitability as crops in a future world of climate change.

The biggest challenge to the modern plant scientists is to develop stress-tolerant plants without compromising yield. There can be no doubt that transgenic plants will be invaluable in assessing precisely the role that main antioxidants, ROS, and osmolytes play in the functional network that controls stress tolerance. Researchers should look for defined sets of markers to predict tolerance towards a particular type of stress. While manipulating genes for stress tolerance in important crops, the genes incorporated should contribute to tolerance not only at a certain plant growth stage of interest but also at the whole plant level, because achieving maximum crop yield under saline conditions is the principal objective of all agriculturists. Modern techniques like genomics, proteomics, ionomics, and metabolomics will be helpful to study plant responses to abiotic stresses. Regarding global climate change, it would be desirable to develop model plants not only for understanding stress tolerance mechanisms, but

also their interaction with elevated atmospheric CO_2 concentration in order to assess the suitability of plants as crops in future.

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Prospects of Halophytes in Understanding and Managing Abiotic Stress Tolerance

2

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Abstract

Halophytes are a diverse group of plants with tolerance to high salinity. While most of our crops are glycophytes lacking the genetic makeup for salt tolerance, halophytes are endowed with ability to seize NaCl into their cell vacuoles as an osmoticum. The sensitivity of crops to environmental extremities has become a major limitation to worldwide food production. Study of halophytes can be rewarding as the mechanisms by which halophytes survive and maintain productivity on saline water can be understood to define and manage adaptations in glycophytes. The adaptation mechanisms include ion compartmentalization, osmotic adjustment, succulence, ion transport and uptake, antioxidant systems, maintenance of redox and energetic status, and salt inclusion/excretion. Real benefits can be accrued if sustained efforts are in place to investigate the species-specific regulation during abiotic stresses and extend genetic resource and manipulate stress tolerance mechanisms. Halophytes are also an important plant species with potential for the purposes of desalination and restoration of saline soils, withstand high soil salinity and saline water irrigation, phytoremediation and wetland restoration. It will be invaluable to develop these strategies to ensure sustainability, and future efforts to improve crop performance on marginal and irrigated land.

Keywords

Halophytes • Abiotic stress • Compatible solutes • Antioxidants • Phytoremediation

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1 Introduction

Environment basically consists of balanced interaction between biotic and abiotic factors, and often abrupt perturbations in abiotic factors surrounding the biotic organisms lead to change the homeostasis, consequently creating a stressful condition for the survival of living organisms. Environmental stresses represent the most limiting factors for agricultural productivity. The abiotic stresses such as shade or high light levels, subzero, low or high temperatures, drought, flooding, high salinity, inorganic nutrient imbalance, infection, predation, and natural or man-made toxic compounds and oxidative stress (Bohnert and Sheveleva 1998) cause losses worth hundreds of million dollars each year due to reduction in crop productivity and crop failure (Zhu 2001; Flowers 2004). In view of their sessile nature, plants should have developed some adaptation strategies to manage the changing environmental conditions particularly with the available resources. Therefore, foremost adaptation carried out by terrestrial plants to its surrounding is adjustment in their water potential as low as that of soil in which they are able to grow. In the course of evolution, some plants have evolved and adapted to freshwater habitat for acquiring nutrients from the low concentrations of minerals present in fresh water such as glycophytes, whereas the plants which retained their habitat in nutrient-rich marine environment were found more successful to combat the adverse abiotic stresses and are referred as "halophytes" (Flowers et al. 2010). These plants can be grown using land and water unsuitable for conventional crops and can provide food, fuel, fodder, fiber, resins, essential oils, and pharmaceutical feedstocks (Table 2.1).

2 Halophytes

Soil salinity and irrigated agriculture have coexisted since ancient times, and ever since the problem of salinity in agriculture has become a challenge. Soils are generally classified as saline when the electrical conductivity of the saturated paste extract (ECe) is 4 dS m^{-1} or more (which is equivalent to 40 mM NaCl) and generate an osmotic pressure of approximately −0.2 MPa. Based on this, plants differ greatly in their growth response to saline conditions and therefore classified as "glycophytes" or "halophytes" referring to their capacity to grow on highly saline environments (Munns and Tester 2008). Halophytes are remarkable plants which have the ability to complete their life cycle in a substrate rich in NaCl that normally found toxic to other species and destroy almost 99% of their population (Flowers and Colmer 2008). These are highly evolved and specialized organisms with well-adapted morphological, anatomical, and physiological characteristics allowing them to proliferate in the soils possessing high salt concentrations (Flowers et al. 1977; Flowers and Colmer 2008). Moreover, some halophytes consistently require a particular concentration of NaCl in the growth medium are referred as "obligate halophytes" or "true mangroves" and, apart from their growth in highly saline environment, some halophytes have capacity to grow on the soil devoid of salt are called as "facultative halophytes" or "mangrove associates." This presence or absence of substrate in the form of salt offers advantages for the halophytes in the competition with salt–sensitive plants (glycophytes) for the management of abiotic stress tolerance and utilization of these species for the improvement of crop yield.

In this regard, it is essential to understand the adverse effects of abiotic stresses and tolerance mechanisms developed by the halophytes and exploit such knowledge for the improvement of crop plants which can meet the demand of food, feed, fodder, and industrial raw material. The standard approach to this problem would be to increase the tolerance capacity of conventional crop plants, which otherwise are high yielders. An alternative strategy is to make use of halophytes that already have the required level of stress tolerance and are still productive at high external adverse conditions. Salinity is one of the major abiotic constraints, affecting almost every aspect of plant's physiology at both whole plant and cellular level through osmotic stress in an earlier phase and ionic stress at a later stage of

Table 2.1 List of halophytes used for saline agriculture in Pakistan and other countries (modified from Khan and Qaiser 2006)

Uses	Plant species
Food	*A. hortensis*, *Aizoon canariense*, *Apium graveolens*, *Arundo donax*, *Atriplex halimus*, *Avicennia marina*, *Cocos nucifera*, *Cynamorium coccinium*, *Echinochloa crusgalli*, *Glinus lotoides*, *Glossonema varians*, *H. stocksii*, *Haloxylon griffithii* ssp *griffithii*, *N. schoberi*, *Neurada procumbens*, *Nitraria retusa*, *Ochradenus baccatus*, *Oxystelma esculentum*, *P. sylvestris*, *Pedalium murex*, *Pentatropis nivalis*, *Pheonix dactylifera*, *Pisonia grandis*, *Polypogon monspeliensis*, *Portulaca oleracea*, *Rumex vesicarius*, *S. brachiata*, *S. persica*, *Salicornia bigellovi*, *Salvadora oleoides*, *Sesuvium portulacastrum*, *Solanum incanum*, *Suaeda fruticosa*, *Triglochin maritime*, *Zizyphus nummularia*, *Zygophyllum simplex*
Fodder	*A. griffithii*, *A. halimus*, *A. leucoclada*, *A. tatarica*, *Aegiceras corniculatus*, *Alhaji maurorum*, *Anagallis arvensis*, *Artemisia scoparia*, *Arthrocnemum indicum*, *Atriplex canescens*, *Avicennia marina*, *B. glaucus*, *Beta vulgaris* ssp *maritma*, *Bienertia cycloptera*, *Bolboschoenus affinis*, *Caesalpinea bonduc*, *Camphorosma monspelictum*, *Carex divisa*, *Chloris virgata*, *Cressa cretica*, *Dalbergia sissoo*, *Glinus lotoides*, *Halocnemum strobilaceum*, *Haloxylon stocksii*, *Lobularia maritime*, *Lolium multiflorum*, *Neurada procumbens*, *Orthochloa compressa*, *P. farcta*, *P. juliflora*, *Populus euphratica*, *Prosopis cineraria*, *Raphanus raphanistrum*, *Rhizophora mucronata*, *Salsola tragus*, *Seidlitzia florida*, *Seriphidium quettense*, *Suaeda fruticosa*, *T. repens*, *T. triquetra*, *Trianthema portulacastrum*, *Trifolium fragiferum*, *Vicia sativa*, *Zaleya pentandara*, *Zygophyllum simplex*
Forage	*A. littorali*, *A. macrostachys*, *Aeluropus lagopoide*, *Agrostis stolonifera*, *Aristida adscesho-ines*, *Aristida mutabilis*, *Atriplex dimorphostegia*, *C. ciliaris*, *C. pennesittiformis*, *Cenchrus biflorus*, *Chloris gayana*, *Cynodon dactylon*, *D. aristatum*, *D. scindicum*, *Dactyloctenium aegyptium*, *Desmostachya bipinnata*, *Dichantheum annulatum*, *Diplachne fusca*, *E. crusgalli*, *E. japonica*, *E. superba*, *Echinochloa colona*, *Eleusine indica*, *Eragrostis curvula*, *Festuca rubra*, *Halocharis hispida*, *Halopyrum mucronatum*, *Haloxylon persicum*, *Lasiurus scindicus*, *Nitraria retusa.Oligomeris linifolia*, *P. minor*, *P. pratensis*, *Paspalum pasploides*, *Phalaris arundinacea*, *Poa bulbosa*, *S. helvolus*, *S. ioclados*, *S. kentrophyllus*, *S. tourneuxii*, *S. tremulus*, *S. virginicus*, *Sacchraum bengalense*, *Salvadora persica*, *Sporobolus coroman-delianus*, *Urochondra setulosa*
Ornamental	*Achillea millefolium*, *Alhaji maurorum*, *Ammi visnaga*, *Artemisia scoparia*, *Avicennia marina*, *Caesalpinea bonduc*, *Calotropis procera*, *Camphorosma monspelictum*, *Cassia italic*, *Centella asiatica*, *Ceriops tagal*, *Chenopodium ambrosoides*, *Corchorus depressus*, *Cressa cretica*, *Cynamorium coccinium*, *Erythrina herbacea*, *Evolvulus alsinoides*, *Glinus lotoides*, *Halogeton glomeratus*, *Imperata cylindrical*, *Inula brittanica*, *Ipomoea alba*, *L. gilsei*, *L. sinuatum*, *L. stocksii*, *Leptadenia pyrotechnica*, *Limonium axillare*, *Melhania denhamii*, *Microcephala lamellate*, *Neurada procumbens*, *olanum surrattense*, *Oligomeris linifolia*, *Oxystelma esculentum*, *P. oleracea*, *Pedalium murex*, *Pentatropis nivalis*, *Populus euphratica*, *Portulaca quadrifida*, *Psylliostachys spicata*, *Rumex vesicarius*, *S. quettense*, *Seriphidium brevifolium*, *Solanum incanum*, *Sonneratia caseolaris*, *Thespesia populneoides*, *Trianthema portulacastrum*, *Tribulus terrestris*, *Urginea indica*,*Verbena officinalis*, *Withania sominifera*, *Z. simplex*, *Zaleya pentandara*, *Zygophyllum propinquum*
Chemicals	*Aeluropus lagopoides*, *Ardisia solanacea*, *Calotropis procera*, *Cenchrus ciliaris*, *Clerodendrum inerme*, *Dalbergia sissoo*, *Euphorbia thymifolia*, *Ficus microcarpa*, *Halocnemum strobilaceum*, *Ipomoea pes-caprae*, *K.iranica*, *Knorringia sibirica* subsp. *Kochia indica*, *Mesembryanthemum crystallinum*, *N. schoberi*, *Nitraria retusa*, *Phyla nodiflora*, *Polypogon monspeliensis*, *Raphanus raphanistrum*, *S. taccada*, *Scaevola plumier*, *Sessuvium sessuvioides*, *T. passernioides*, *T. ramosissima*, *T. szovitsiana*, *T. tetragyna*, *Tamarix mascatensis*, *Thomsonii*, *Trianthema portulacastrum*

plant growth (Munns and Tester 2008) and leads to a series of morphological, physiological, biochemical, and molecular changes. In the past 2–3 decades, considerable progress has been made in the evaluation of halophytes to understand their survival mechanisms to be used as crop plants. In the present article, we document different aspects of halophytes, with an emphasis on mechanism of tolerance to salinity, drought and heavy metal tolerance, and their exploitation to manage the problems associated with the abiotic stresses as well as for environmental protection.

Halophytes respond to salt stress at cellular, tissue, and the whole plant level (Epstein 1980). In response to salt stress, the general physiology of halophytes has been reviewed occasionally (Flowers et al. 1977; Epstein 1980; Flowers 1985, 2004) and since then other reviews have examined their eco-physiology (Ball 1988; Rozema 1991; Breckel 2002), photosynthesis (Lovelock and Ball 2002), response to oxidative stress (Jitesh et al. 2006), and flooding tolerance (Colmer and Flowers 2008). Therefore, studies on the halophytes can be instructive from three prospects: first, the mechanism by which halophytes survive and maintain productivity under abiotic constraints can be used to define a minimal set of adaptations required in tolerant germplasm. This knowledge can help to focus the efforts of plant breeders and molecular biologists working with conventional crop plants (Glenn and Brown 1999). Second, halophytes grown in an agronomic setting can be used to evaluate the overall feasibility of high-salinity agriculture, which depends on more than finding a source of tolerant germplasm (Glenn et al. 1997). Third, halophytes may become a potential source of new crops.

3 Halophytes Diversity

Halophytes show immense diversity in habitat and behavior to tolerate the abiotic stress conditions with uneven distribution across the taxa of flowering plants (Flowers et al. 2010). This group of plants has been classified based on their tolerance capacity to salinity stress. Aronson (1989) listed approximately 1,550 species as salt-tolerant based on their capacity to tolerate the salt concentration more than 80 mM NaCl (equivalent to EC 7.8 dS m^{-1}), whereas, plants limiting the growth beyond this concentration were categorized as glycophytes or salt-sensitive. Using similar features, Menzel and Lieth (2003) recorded total 2,600 species as salt-tolerant. However, considering the salt tolerance limit proposed by Aronson (1989) and Menzel and Lieth (2003) which is found to be significantly lower than the salt concentration of seawater (~480 mM Na^+ and 580 mM Cl^-), Flowers and Colmer (2008) defined the halophytes as plants that have evolved and tolerate to complete their life cycle in at least ~200 mM NaCl. Applying the new definition to Aronson's database, Flowers et al. (2010) further classified a total of 350 species as halophytes with major species distributed in 20 orders including 256 families. It has also been suggested that salt tolerance was widely distributed among flowering plant families and had a polyphyletic origin. The authors also stated that distribution and development of evolutionary link of halophytes may account to not more than ~0.25% of the known species of angiosperms.

4 Adaptations to Abiotic Stresses

Halophytes have evolved a number of adaptive traits which allow them to germinate, grow, and achieve their complete life cycle of development under such harsh conditions (Flowers et al. 1977). A variety of studies performed on glycophytes and halophytes subjected to abiotic stresses has demonstrated that impairment in growth under stress condition results from various responses induced through both osmotic effects related to disturbance in plant–water relationships and ionic effects associated with mineral toxicity and deficiency (Lauchli and Epstein 1990). Associated with these primary stresses, higher plants also suffer from secondary stresses provoked by cellular damages especially those induced by oxidative stresses due to imbalance between production and destruction of reduced reactive oxygen species (Zhu 2001).

In order to achieve the tolerance status, three interconnected aspects of plant activity are significant: damage must be prevented, homeostatic condition must be re-established, and growth must resume. At present, there are different mechanisms of abiotic stress tolerance in halophytes that have been proposed which include ion compartmentalization, osmotic adjustment through osmolytes accumulation, succulence, selective transport and uptake of ions, enzymatic and nonenzymatic antioxidant response, maintenance of redox and energetic status, salt inclusion/excretion and genetic control (Flowers and Colmer 2008). Understanding the mechanism of tolerance in halophytes at morphological, anatomical, physiological, biochemical, and molecular levels is crucial to improve the tolerance of the crop plants and their adoption under abiotic stress conditions to exploit such problem soils. A generalized scheme for the plant's response to abiotic stresses and mechanism of stress tolerance is presented (Fig. 2.1).

4.1 Ion Compartmentation

The sensitivity of cytosolic enzymes to salt is similar in both glycophytes and halophytes, indicating that the maintenance of high cytosolic K^+/Na^+ ratio is a key requirement for plant growth under salt conditions (Glenn and Brown 1999). While dealing with Na^+, the cell must also acquire nutrient K^+. The Na^+ ion is the foremost inorganic ion and a cheap source of osmoticum in the halophytes to maintain the osmotic balance under abiotic stresses. Under typical physiological conditions, plant cells require high K^+ (100–200 mM) and lower Na^+ (less than 1 mM) and accordingly the high cytosolic K^+/Na^+ ratio to maintain the osmotic balance (Tester and Davenport 2003) for proper functioning of the cell. Na^+ competes with K^+ for intracellular influx since both these are transported by common channels present on the membranes and, thus, subsequently increase K^+ efflux from intracellular stores as against the higher Na^+ stress built up outside the cell. To maintain a high K^+/Na^+ ratio in the cytosol, plant cell employs primary active transport, mediated by channels and co-transporters for Na^+ extrusion and/or the intracellular compartmentalization of Na^+ into the vacuole (Blumwald 2000). When halophytes are exposed to saline condition, a large increase in extracellular Na^+ level establishes the Na^+ electrochemical potential gradient more than the actual negative electrical membrane potential difference at the plasma membrane (−140 mV) which favor the passive transport of sodium ions from the outer environment inside the cell. Recently, uniporter or ion channel type transporters have been identified for the entry of Na^+ into the cell; these are high-affinity potassium transporter (HKT), low-affinity cation transporter (LCT1), nonselective cation channels (NSCC) like cyclic nucleotide-gated channels (CNGCs) and glutamate-activated channels (GLRs) (Apse and Blumwald 2007). HKTs have been shown to function as Na^+/K^+ symporter and as Na^+ selective uniporters (Horie and Schroeder 2004). In the process of elevated levels of Na^+ outside the cell, the electrochemical gradient makes the sodium uptake passive; however, the efflux of Na^+ outside cell is an active process and requires energy in the form of ATP. In this process, the Na^+/H^+ antiporter (NHX) present on the plasma membrane facilitates the Na^+ efflux. This electroneutral exchange of sodium for protons to facilitate efflux is the only mode of transport that has been measured for efflux under physiological conditions (Apse and Blumwald 2007). Besides the efflux of Na^+, some halophytes have developed mechanism to sequester the Na^+ into the vacuoles as an efficient mechanism to avoid the toxic effects of Na^+ in the cytosol. The transport of Na^+ into the vacuoles is mediated by cation/H^+ antiporters that are driven by the electrochemical gradient of protons generated by the vacuolar H^+ translocating enzymes such as H^+-ATPase and H^+-PPiase (Gaxiola et al. 2007). These transporters play an important role in the sequestration of Na^+ ions into the vacuole or exclusion outside the cell of the halophytes.

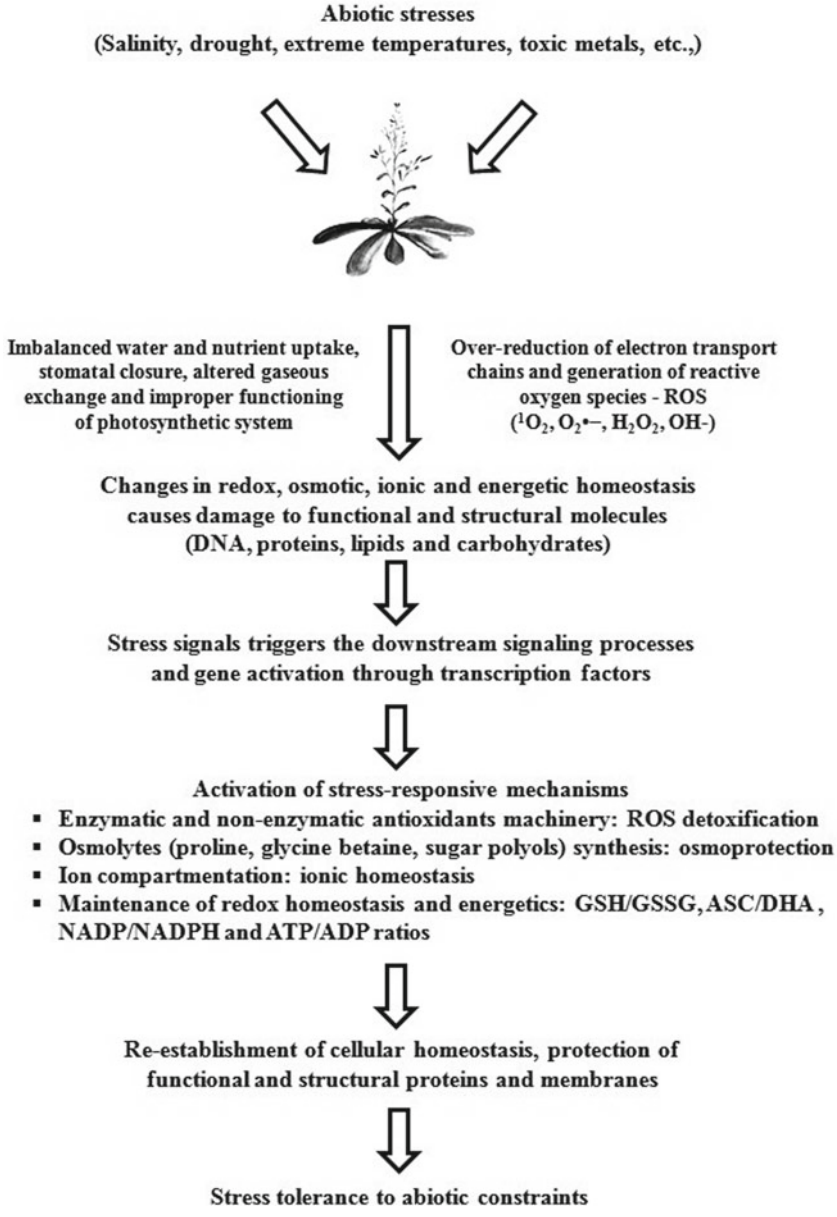

Fig. 2.1 Generalized scheme for plant responses to abiotic stresses and mechanism of stress tolerance. Plants exposed to various abiotic stresses (salinity, drought, extreme temperatures, toxic metals, etc.) initiate the cascade of changes in plants' functioning such as imbalanced water and nutrient uptake, stomatal closure, altered gaseous exchange, improper functioning of photosynthetic systems due to over-reduction of electron transport chains in chloroplast and mitochondria causing generation of reactive oxygen species (ROS). The integrative effect of these factors leads to induce the oxidative damage to functional and structural molecules (DNA, proteins, lipids, and carbohydrates) making the changes in the redox, osmotic, ionic, and energetic homeostasis of the plant. These stress signals triggers the downstream signaling processes and gene activation through transcription factors. Activation mechanisms involve enzymatic and nonenzymatic antioxidants for detoxification of ROS, osmolytes (proline, glycine betaine, sugar polyols) synthesis for osmotic balance and protection to structural molecules, ion compartmentation for ionic homeostasis and maintenance of redox and energetics through the higher ratios of GSH/GSSG, ASC/DHA, NADP/NADPH, and ATP/ADP. The coordinated action leads to re-establish the cellular homeostasis, protection of functional and structural proteins and membranes, and ultimately the tolerance to abiotic stresses

4.2 Succulence

Succulence is commonly called as halosucculence and found to occur within a range of salt concentrations optimal for growth. The sequestration of saline ions into the vacuoles leads to the plant to increase the succulence, one of the common characteristics of the halophytes (Flowers et al. 1977). Succulence minimizes the toxic effects of excessive ion accumulation and has been reported to be associated with accretion of osmotically active solutes for the maintenance of cell turgor pressure. The succulent halophytes unlike glycophytes tend to accumulate sodium in the vacuole to higher levels than in the cytoplasm and as the volume of the vacuole is much greater than that of the cytoplasm in fully expanded cells, the total sodium content of the root will approximate to the sodium content of the vacuole (Yeo and Flowers 1986). Succulent halophytes generally have thick leaves and stems, mainly associated with an increase in the size of their mesophyll cells along with smaller intracellular spaces. It has also been shown that succulent leaves have more and large-sized mitochondria because the succulent halophytes require excess energy for salt compartmentalization and excretion. Whether succulence is a response to salinity or adaptation to salinity is debatable. But as halophytes tend to become succulent in response to salinity (due to physiologically less available water which affects the changes in the integral part of the plant development), the succulence might be the adaptation to salinity stress (Waisel 1972). This adaptive nature of succulence made the halophytes more successful in the course of evolution exposing to various environmental constraints. Most of the halophytes such as *Sesuvium portulacastrum*, *Suaeda* sps., *Lobularia maritime*, *Mesembryanthemum crystallinum*, *Halosarcia pergranulata* subsp. *Pergranulata*, etc. were found more amenable to accumulate the excess Na^+ in their leaves and stems and increase the succulence under optimum NaCl concentrations in the range of 100–400 mM which leads to sequester these saline ions into the vacuole and become more successful for their growth in saline environment (Qi et al. 2009; Lokhande et al. 2010a). Thus it appears that increased succulence could be due to a "diluting" effect on the ion content of cells which might otherwise rise to toxic levels, and sodium acts as a specific stimulant of growth which can be considered as tending to reduce the turgor pressure component of the water potential of the cell (Jennings 1968).

The succulent halophytes are able to balance the growth and ion accumulation through its sequestration into the vacuole; however, some of the halophytes were adapted to saline environment through secretion of salts from salt glands, cuticles or in guttation fluid, re-transported back to the roots and soil via the phloem or become concentrated in salt hairs. Salt glands act as transient cells because they are devoid of vacuole and have a large number of mitochondria and other organelles. The halophytes which secretes the saline ions include *Limonium latifolium*, *Spartina* sps., *Sporobolus spicatus*, *Atriplex* sps., etc. (Ramadan 2000). However, not all halophytes have salt glands; neither do they all discard salt saturated tissue, demonstrating that individual halophytes utilize different salt tolerance traits under different stress periods.

4.3 Osmotic Adjustment

Osmotic adjustment in response to abiotic stresses is an adaptive mechanism in the halophytes in order to maintain their water balance (Flowers and Colmer 2008). Besides the accumulation of inorganic ions and its sequestration in the vacuole, the osmotic balance between vacuole and cytoplasm is also maintained through the synthesis of organic solutes to retain the stability of the proteins in cells in response to drop in the water potential of the environment (Glenn and Brown 1999). Plant cells synthesize a variety of organic solutes such as proline, sucrose, polyols, trehalose and quaternary ammonium compounds (QACs) such as glycine betaine, alaninebetaine, prolinebetaine, choline-*O*-sulfate, hydroxyprolinebetaine, and pipecolatebetaine (Rhodes and Hanson 1993). These are low molecular weight, highly soluble compounds and are nontoxic even at high cellular concentrations (Ashraf and Foolad 2007) without

disturbing intracellular biochemistry and cellular functions (Cushman 2001), protects subcellular structures, mitigate oxidative damage caused by free radicals (Attipali et al. 2004), maintains the enzyme activities under salt stress and protection of cellular components from dehydration injury (Ashraf and Foolad 2007). The osmolytes accumulation is frequently reported in glycophytes and halophytes being continuously exposed to abiotic stresses; however, synthesis of these osmolytes is an energy-dependent process which consumes large number of ATP molecules (Raven 1985), thus affecting the growth. Osmolytes synthesis and their overproduction in transgenic plants has been achieved in transgenic crop plants, however little success has been achieved on the desired protective levels of these osmolytes in plants. In contrast, some plants showed increased tolerance to abiotic stresses after exogenous application of these organic solutes (Ashraf and Foolad 2007). Although increased accumulation of these osmolytes by the plants exposed to abiotic stresses has been reported, not all plant species synthesize the all kinds of osmolytes at a time; some plant species synthesize and accumulate very low quantity of these compounds while some plant species not do so (Ashraf and Foolad 2007).

4.3.1 Proline

Similar to glycophytes, proline accumulation is a common adaptive response to various abiotic stresses. Several studies using transgenic plants or mutants demonstrated that proline metabolism has a complex effect on development and stress responses, and that proline accumulation is important for the tolerance to certain adverse environmental conditions (Hong et al. 2000; Miller et al. 2010). In plants, proline is mainly synthesized from glutamate using two important enzymes such as pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). Proline is synthesized in cytoplasm; however in mitochondria, the catabolism occurs via sequential action of proline dehydrogenase (PDH) producing pyrroline-5-carboxylate (P5C) and its conversion to glutamate using P5C dehydrogenase (P5CDH) (Szabados and Savoure 2009). Halophytes have shown vast diversity for the accumulation of proline in response to abiotic stresses, wherein plants from the Aizoaceae family accumulate large quantities of proline showing its role in osmoprotection (Delauney and Verma 1993). Proline concentrated in the cytosol, chloroplast and vacuoles and compatible with enzyme activity in the cytoplasm showed its significant contribution to osmotic adjustment. Besides being an osmoprotectant, proline also has a role in detoxification of reactive oxygen species and act as an antioxidant, stabilization of proteins and protein complexes and as a signaling/regulatory molecule (Szabados and Savoure 2009). It also function as a protein-compatible hydrotrope (Srinivas and Balasubramanian 1995), alleviating cytoplasmic acidosis, and maintaining appropriate $NADP^+$/NADPH ratios compatible with metabolism (Hare and Cress 1997). Also, rapid breakdown of proline upon relief of stress provides sufficient reducing agents that support mitochondrial oxidative phosphorylation and generation of ATP for recovery from stress and repairing of stress-induced damages (Hare and Cress 1997). In halophytic plant species in response to abiotic stresses, proline accumulation in the cytosol has been shown to contribute substantially to cytoplasmic osmotic adjustment. For example, in cells of *Distichlis spicata* treated with 200 mM NaCl, the cytosolic proline concentration was estimated to be more than 230 mM (Ketchum et al. 1991). In *Sesuvium portulacastrum*, Lokhande et al. (2010a, b, 2011a) found an extensive increase in proline content when the callus and axillary shoot cultures exposed to salt and drought stress alone or under iso-osmotic stress conditions of NaCl and PEG. Higher proline accumulation has also been shown in *S. portulacastrum* plants exposed to various abiotic constraints that include salinity, drought, and heavy metals (Messedi et al. 2004; Slama et al. 2008; Ghnaya et al. 2007; Moseki and Buru 2010; Lokhande et al. 2011b). Such an osmotic adjustment through proline accumulation is also evident in other species like *Plantago crassiflora*, *Salicornia europaea*, *Atriplex halimus*, *A. halimus* subsp. *schweinfurthii*, *Avicennia marina*, *Hordeum maritimum*, *Ipomoea pes-caprae*, *Paspalum vaginatum*, *Phragmites australis*, and *Suaeda* sps.

(Vicente et al. 2004; Reda et al. 2004; Nedjimi and Daoud 2009; Pagter et al. 2009; Lefevre et al. 2009; Sucre and Suarez 2010). Among different halophytic plants, *S. portulacastrum* has been reported as a high proline accumulator, with levels reaching 300 μmol g^{-1} leaf dry matter (Slama et al. 2008). Such a pronounced accumulation of proline and its physiological role in osmotic adjustment may have made the halophytes more successful to grow under adverse environmental stresses.

4.3.2 Glycine Betaine

Among the variety of quaternary ammonium compounds, glycine betaine (GB) is one of the most abundantly occurring and synthesized at higher concentrations in the plants exposing to dehydration stress due to adverse environmental calamities. GB is located in chloroplast where it plays an important role in osmotic adjustment and protection of thylakoid membrane, by maintaining the photosynthetic machinery in active state (Robinson and Jones 1986). GB is synthesized mainly from choline, which is converted to betaine aldehyde and then to GB through sequential enzymatic action of choline monooxyegenase (CMO) and betaine aldehyde dehydrogenase (BADH), respectively. Although other pathways such as direct *N*-methylation of glycine are also known, the pathway from choline to GB has been identified in all GB-accumulating plant species (Ashraf and Foolad 2007). It is widely believed that synthesis and accumulation of GB protects cytoplasm from ion toxicity, dehydration and temperature stress and helps normal functioning of the metabolic machineries in the cell during stressed conditions by stabilizing macromolecule structures, protecting chloroplast and photosynthesis system II (PSII) by stabilizing the association of the extrinsic PSII complex proteins and indirectly interacting with phosphatidylcholine moieties of membranes to alter their thermodynamic properties (Subbarao et al. 2001). It has been shown that tolerant species are more amenable to accumulate higher GB in comparison to sensitive species as a response to abiotic stress imposition. Based on the GB and proline accumulation potential, Tipirdamaz et al. (2006) categorized the halophytes from inland and salt marsh habitats of Turkey. The studies have shown that the species that behaved as GB accumulators appeared poor proline accumulators and vice versa. The GB accumulation reported in the halophytes is generally in the range of 1.5–400 μmol g^{-1} DW and some of the highest GB accumulating halophytes are members of the Chenopodiaceae (*Halocnemum strobilaceum*, *Petrosimonia brachiata*, *Suaeda confuse*), Compositae (*Artemisia santonicum*), and Frankeniaceae (*Frankenia hirsuta*). Increased accumulation of GB has also been demonstrated in other halophytes such as *Beta vulgaris* (Subbarao et al. 2001), *Spartina anglica* (Mulholland and Otte 2002), *Atriplex halimus* (Martinez et al. 2005), *A. Nummularia* (Silveira et al. 2009), and *S. portulacastrum* (Lokhande et al. 2010a, b). Increased GB accumulation has also been correlated with increased betaine aldehyde dehydrogenase gene expression (BADHmRNA) in *Salicornia europaea* and *Suaeda maritima* leaves exposed to salt stress (Moghaieb et al. 2004). Considering the significance of GB in the osmotic balance of the halophytes under stressful environment, different methods can be derived to enhance the concentration of this compound in crop plants to increase their stress tolerance. The approaches can include breeding of sensitive cultivars with their tolerant relatives from halophytes with natural abilities to produce high levels of GB or genetically engineer the sensitive species through transformation of the genes responsible for GB synthesis. Although some progress has been made in introducing the genes for the production of these compounds in naturally accumulating or low-accumulating plant species, levels of these compounds' accumulation in transgenic plant have often been low or insufficient to the plant stress tolerance (Ashraf and Foolad 2007).

4.3.3 Soluble Sugars

In general, modulations in the carbon metabolism and the levels of carbohydrates (sugars) are seen due to changes occurring in the process of photosynthesis and carbon partitioning of the plant at organ level and in whole plants exposing

to abiotic stresses (Gonzalez et al. 2009). Soluble sugars function as metabolic resources and structural constituents of cells, besides acting as signals regulating various processes associated with plant growth and development. Such signaling can modulate stress pathways into a complex network to further orchestrate metabolic plant responses. A variety of sugar compounds such as sucrose, glucose, mannose, maltose, trehalose, and many other sugar alcohols have been studied in response to abiotic stresses (Briens and Larher 1982; Yuanyuan et al. 2009) and the accumulation of soluble sugars has been attributed as an important parameter of osmotic adjustment in the halophytes. Briens and Larher (1982) screened different organs of 16 halophyte species for soluble carbohydrates and other osmolytes and found that all the species accumulated sucrose, fructose and glucose whereas *Plantago maritime*, *Juncus maritimus*, *Phrgamites communis* and *Scripus maritimus* showed the highest accumulation of soluble sugars. The presence of higher amounts of soluble sugars has been reported as main contributors to osmotic adjustment in the *Atriplex halimus* plants exposed to PEG and NaCl stresses and it is correlated with the response of NaCl stress on soluble sugar synthesis (Martinez et al. 2005). The accumulation of total soluble sugars has also been correlated with the variations at genotypic level among two genotypes of *Cakile maritime* namely Jebra and Tabarka which showed differences in the total soluble carbohydrate concentrations. While the content of the sugars was unaffected in the leaves of Jerba plants at moderate salinity, the plants of the salt-sensitive Tabarka showed a slight increase in soluble carbohydrate contents during leaf development. The contribution of this compatible solute group to the "osmotic pool" was found higher in the salt-tolerant Jerba than in the salt-sensitive Tabarka seedlings exposed to 400 mM NaCl stress (Megdichi et al. 2007). Further, *Sesuvium portulacastrum* axillary shoots exposed to salinity stress showed optimum growth at 200 mM NaCl in comparison to control and exhibited increased synthesis of total soluble sugars over proline and glycine betaine (Lokhande et al. 2010b). Salinity-induced soluble sugar accumulation has also been observed in *P. euphratica* (Zhang et al. 2004). Accumulation of soluble sugars has been observed in plants undergoing drought, flooding, and water logging conditions (Chai et al. 2001; Munns 2002; Li and Li 2005). *Chenopodium quinoa* exposed to water deficit and water-logging stresses showed no changes in starch, sucrose, or fructose content but showed increased glucose and total soluble sugar content in stressed plants in comparison to control (Gonzalez et al. 2009). These studies in halophytes demonstrate that soluble sugars play a significant role besides other osmolytes in the osmotic adjustment.

4.4 Antioxidant Systems

The halophytic plants display a cascade of events upon exposure to environmental stresses leading to metabolic disturbance. The cascade of events include physiological water-deficit abscisic acid-regulated stomatal closure in leaves, limited CO_2 availability, over-reduction of electron transport chain in the chloroplast and mitochondria and finally generation of reactive oxygen species (ROS). These ROS are highly toxic and in the absence of protective mechanism in the plant can cause oxidative damage to proteins, DNA, and lipids (Mittler 2002; Miller et al. 2010). Additionally, this may also lead to alteration in the redox state resulting in further damage to the cell (Mittler et al. 2004). To regulate the ROS levels, plant cells are evolved with complex enzymatic and nonenzymatic antioxidant defense mechanisms, which together help to control the cellular redox state under changing environmental conditions. A correlation between enzymatic and nonenzymatic antioxidant capacitance and abiotic stress tolerance has been reported in several plant species such as *Crithmum maritimum*, *C. maritime*, *Plantago* genus, *Sesuvium portulacastrum*, *Mesembryanthemum crystallinum* (Ben Amor et al. 2005; Jitesh et al. 2006; Sekmen, Turkan and Takio 2007; Ashraf 2009; Lokhande et al. 2010a, b, 2011a-c). Superoxide dismutase (SOD) constitutes the first line of defense converting $O_2^{\bullet-}$ to H_2O_2, which is further reduced to

Table 2.2 Examples of halophytic plant species studied for the antioxidant responses in response to abiotic stresses

Plant species	Enzyme/protein/gene studied	References
Avicennia marina	SOD, CAT, POX, APX, MDHAR	Cherian et al. (1999), Jitesh et al. (2006), Kavitha et al. (2008), and Kavitha et al. (2010)
Bruguiera parviflora, *B. gymnorrhiza*	SOD, CAT, APX	Takemura et al. (2000) and Parida et al. (2004)
Beta vulgaris, *B. maritime*	SOD, CAT, APX, GR	Bor et al. (2003)
Crithmum maritimum	SOD, CAT, POX	Ben Amor et al. (2005)
Hordeum vulgare	SOD, POX, CAT, GR	Patra and Panda (1998)
H. maritimum	SOD, CAT, GPX, APX, MDHAR, DHAR, GR	Hafsi et al. (2010)
M. crystallinum	SOD, ferritin, CAT, Mn, Fe, Cu/Zn SOD	Slesak et al. (2002, 2008), Slesak and Miszalski (2003), Hurst et al. (2004), and Parmonova et al. (2004)
Nitraria tangutorum	SOD, CAT, APX	Yang et al. (2010)
Phragmites australis	SOD, CAT, APX, POX, DHAR, GST	Carias et al. (2008)
Phaseolus vulgaris	SOD, APX, CAT, GPX	Jebara et al. (2005)
Sesuvium portulacastrum	SOD, CAT, APX, GR	Lokhande et al. 2010a, b, 2011a-c)
Suaeda nudiflora, *S. salsa*	SOD, CAT, POX	Cherian and Reddy (2003), Wang et al. (2004a), and Fang et al. (2005)
T. halophila	SOD, APX, POX	Taji et al. (2004), Wang et al. (2004a, b), and M'rah et al. (2006)

water and oxygen by ascorbate peroxidase (APX) and catalase (CAT). Monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and glutathione peroxidase (GPX) are an important enzymes involved in regeneration of ascorbate and GSH for the proper functioning of ASC-GSH cycle (Noctor and Foyer 1998; Mittler 2002; Miller et al. 2010).

Halophytes have evolved various mechanisms of adaptations of which increased antioxidant enzyme activities was found one of an important mechanism of stress tolerance. Halophytes have capacity to maintain high metabolic activity even at inhibitory concentrations of intracellular Na^+ due to enhanced antioxidant mechanism (Jitesh et al. 2006). Most of the early studies in halophytes were on photosynthesis and respiration and related to ion compartmentation, osmotic adjustment (Flowers et al. 1977; Fukushima et al. 1997). However, recently, more emphasis has been given on abiotic stresses in relation to antioxidant enzymes in halophytes (Takemura et al. 2000; Cherian and Reddy 2003; Parida et al. 2004; Slesak et al. 2002, 2008; Slesak and Miszalski 2003; Jitesh et al. 2006; Lokhande et al. 2010a–b, 2011a, c). The response of antioxidant enzyme systems in the halophytes exposed to abiotic stresses has been reviewed by Jitesh et al. (2006) and is summarized in Table 2.2. Most of the halophytes have shown increased efficiency of antioxidant enzyme machinery thus removing the ROS levels to a greater extent and maintain the plants survival under stressful conditions.

4.5 Redox and Energetics

The cellular redox state is made tangible in terms of the redox state of the individual redox-active molecules in a cell. For each redox-active molecule, its redox state can be defined as the proportion of reduced molecules relative to the total pool size, or alternatively as the ratio between reduced and oxidized molecules within a pool (Potters et al. 2010). A large number of redox-active compounds such as ascorbate (ASC), glutathione

(GSH), pyridine nucleotides (NADH and NADPH), carotenoids, tocopherols, distinct redox-active phenolics, polyamines and proteins carrying redox-active S-groups are contained in plant cells (Smirnoff 2005; Queval and Noctor 2007). The enzymatic and nonenzymatic antioxidants involved in ROS scavenging significantly contribute to the redox state maintenance of the cellular environment through continuous channeling of these redox-active components which facilitate the proper functioning of the cell under stressful conditions. In general, the maintenance of redox state is correlated with the energy metabolism of the plant cell in terms of ATP/ADP ratio. Under stressful conditions, the ROS produced due to oxidative stress requires more energy in the form of ATP to maintain the cellular homeostasis such as ion compartmentation, osmolytes synthesis, etc. The ATP requirement for these processes is different, such as 3.5, 41 and 50 ATP molecules are required for the synthesis or accumulation of one molecule of Na^+, proline, and glycine betaine, respectively (Raven 1985). Thus, the energetic status of the plant is also dependent on the type of molecule synthesized or accumulated by the plant. It is possible that halophytes evolved to survive under abiotic stress conditions through proper maintenance of a higher redox and energetic status which could have conferred a plasticity to grow under stress. Not much information is available on the redox signaling and energetics in case of the halophytes. Recently, Lokhande et al. (2010c) for the first time demonstrated that maintenance of redox and energy state plays a major role in mediating salinity tolerance and in achieving a balance between tolerance and growth in *Sesuvium portulacastrum.* The plants under optimum levels of NaCl (250 mM) showed retention in the growth whereas significantly toxic levels of NaCl (1,000 mM) disturbed the homeostasis of the plant due to abrupt changes in the ratios of redox-active compounds (ASC/DHA, GSH/GSSG, NADP/NADPH) and energy molecules (ATP/ADP) (Fig. 2.2a–f). Further to stick this work, more efforts could be initiated to unravel the redox and energetic of the halophytes to gain the knowledge of redox control. The concept of a cellular redox state, as proposed by Foyer and Noctor (2005), is very useful in terms of elucidating the importance of redox reactions in gene expression, metabolism control, signal transduction, and cellular defense. The concept must now be developed to identify and quantify those redox-active compounds that are the specific regulators of cellular responses (Potters et al. 2010).

4.6 Genomic Approaches

Plant adaptation to environmental stresses is controlled by a cascade of molecular networks. In this regard, the application of genomic technologies has made more impact on understanding the plant responses to the abiotic stresses (Cushman 2003). The technology has made remarkable success in understanding the abiotic stress tolerance at genome level with potential to modify plants' tolerance for increasing yield under stressful conditions (Bohnert et al. 2006). In contrast to traditional breeding and marker-assisted selection programs, the direct introduction of a small number of genes by genetic engineering has also become tangible and attractive as a rapid approach to improve the plants' stress tolerance (Cushman and Bohnert 2000; Popova et al. 2008) to re-establish homeostasis and to protect and repair damaged proteins and membranes (Wang et al. 2003).

In the course of studies on mechanism of abiotic stress tolerance, *Arabidopsis thaliana* has emerged as an excellent model system (Zhu 2001) because most of the crop plants are glycophytes. However, study of some novel mechanisms unique to halophytes or stress-tolerant plants may be difficult with *Arabidopsis* and this has been made possible by the available genome information on *Mesembryanthemum crystallinum*, which, when compared with the *Arabidopsis* genome, seems to contain a number of transcripts that have no counterparts (Wang et al. 2004b). Thereafter, several halophytes such as *M. crystallinum*, *Suaeda* species, *Atriplex* species have been employed to dissect out the molecular basis of stress tolerance mechanism of the halophytes. Recently, *Thellungiella halophila* (salt cress), a member of the Brassicaceae, has emerged as a

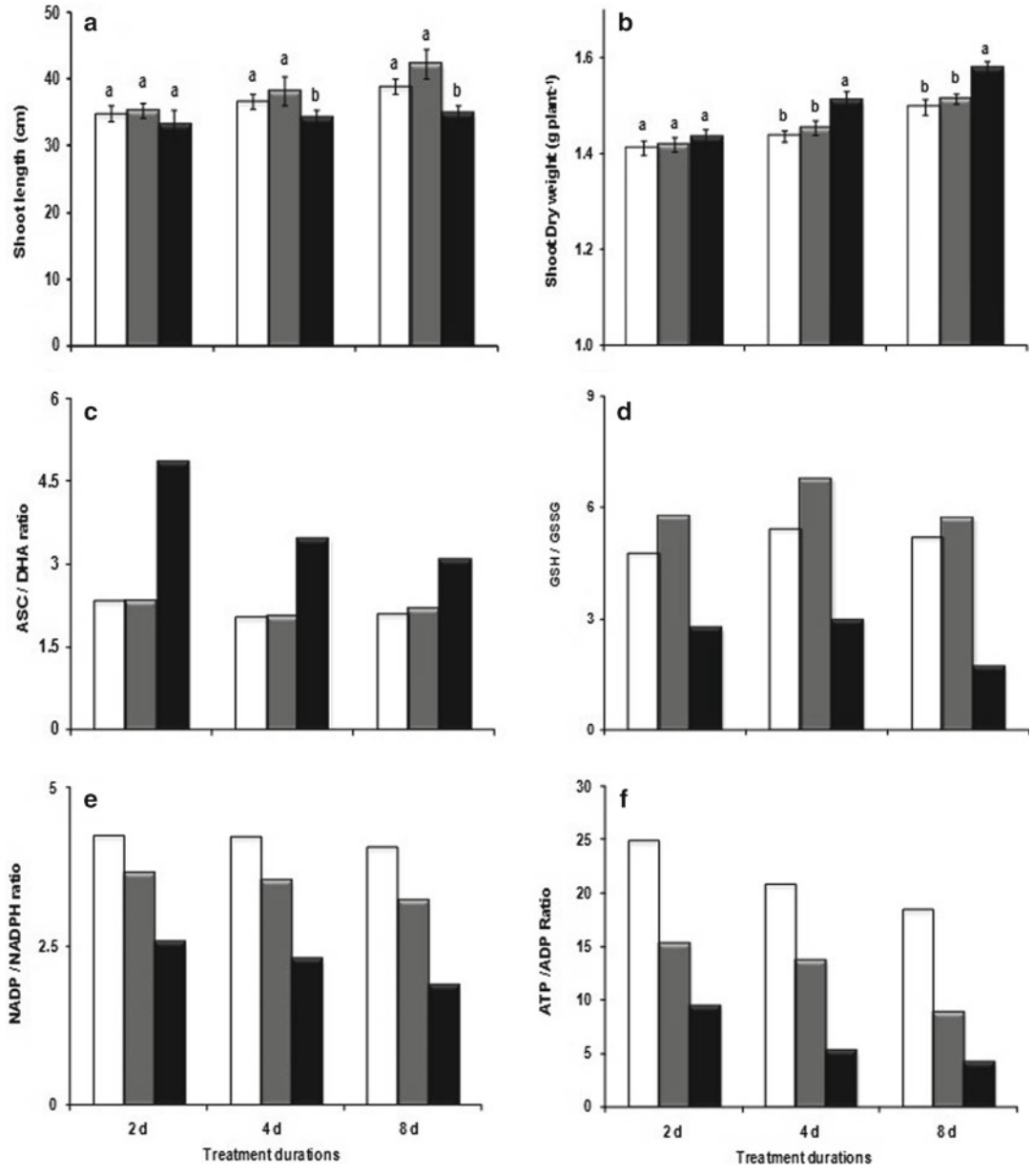

Fig. 2.2 The salinity stress responses (growth, redox, and energy status) of *Sesuvium portulacastrum* exposed to optimum (250 mM) and supra-optimal (1,000 mM) concentrations of NaCl

model for understanding adaptation of the halophytes to abiotic stress tolerance due to its homology with the glycophyte model, *A. thaliana* (Wang et al. 2004b; Amtmann 2009). This halophyte has the ability to grow in high salt concentrations which otherwise become inhibitory for the growth of its salt-sensitive relative *A. thaliana* and other crop plants (Zhu 2001; Nah et al. 2009). The salient features of *T. halophila* such as small diploid genome (240 Mb and $2n = 14$), short and self-fertile life cycle and ease of floral dipping method of transformation have enabled it as a successful candidate for molecular detailing of its response to abiotic stress tolerance and relative

comparison with *A. thaliana.* The comparative genomics of *T. halophila* and *A. thaliana* revealed extensive and novel information on presence of differential genes responsible for abiotic stress tolerance in *T. halophila* in comparison to *A. thaliana* (Nah et al. 2009). Taji et al. (2004) studied the differences in the regulation of salt tolerance between salt cress and *Arabidopsis* by analyzing the gene expression profiles using a full-length *Arabidopsis* cDNA microarray. Only a few genes were induced by 250 mM NaCl in salt cress stress compared to *Arabidopsis*. Even in the absence of stress, a large number of known abiotic- and biotic-stress inducible genes, including Fe-SOD, P5CS, PDF1.2, AtNCED, P-protein, b-glucosidase, and SOS1, were expressed at high levels. The study also found salt cress to be more tolerant to oxidative stress than *Arabidopsis*. The salt tolerance mechanisms between salt-sensitive glycophytes and salt-tolerant halophytes could result from alterations in the regulation of the same basic set of genes involved in salt tolerance among these plants. Kant et al. (2006) used gene-specific primers of *Arabidopsis* that showed similar real-time PCR amplification efficiencies with both *A. thaliana* and *T. halophila* cDNA and concluded that the expression of specific salt tolerance orthologues differs between unstressed and stressed plants of both species.

The development of expressed sequence tags (ESTs) and cDNA libraries using various genomic approaches such as suppressive subtractive hybridization (SSH), differential display reverse transcription-polymerase chain reaction (DDRT-PCR), representational difference analysis (RDA), serial analysis of gene expression (SAGE), and cDNA microarray (Breyne and Zabeau 2001) provided an enormous databases for understanding the genetic network involved in abiotic stress tolerance mechanism of halophytes (Wang et al. 2004b; Kore-eda et al. 2004; Popova et al. 2008). Using this approach, various genes responsible for stress tolerance have been isolated from halophytes and cloned or overexpressed in the bacterial systems as well as sensitive cultivars of glycophytes to enhance the stress tolerance capacity and improve crop yield (Table 2.3).

Transcript-profiling experiments in *Arabidopsis* in response to drought, cold, or salinity stresses using the *Arabidopsis* GeneChip array or full-length cDNA microarrays have shown that extensive changes occur in the transcriptome of *Arabidopsis* (Fowler et al. 1999; Kreps et al. 2002; Seki et al. 2002). It is known that approximately 30% of the transcriptome on the *Arabidopsis* GeneChip 8 K oligoarray changed in stress treatments (Kreps et al. 2002). The expressed sequence tag analyses of *Thellungiella* clones revealed 90–95% identities between *Thellungiella* and *Arabidopsis* cDNA sequences (Wang et al. 2004a, b; Wong et al. 2006). In a comparison of three stresses (cold, low water availability, and saline conditions) as well as recovery from water deficits in *Thellungiella*, Wang et al. (2006) employed an expression profiling strategy to identify stress responses. There was not much degree of overlap among genes responsive to drought, cold, or salinity suggesting relatively few common end responses triggered by these stresses existed in this halophyte. While *Thellungiella* had shown activation of the expression of some well-known stress-responsive genes, it was found to downregulate a large number of biotic stress-related genes under drought and salinity treatments. The study has made a significant step in showing the emergence of *Thellungiella* as a model species for the molecular elucidation of abiotic stress tolerance, and that *Thellungiella* responds precisely to environmental stresses, thereby conserving energy and resources and maximizing its survival potential.

SOS1 (*Salt Overly Sensitive 1*) is known to play key role in the ion homeostasis mechanism movement (Shi et al. 2000). Although *SOS1* has been intensely studied in *Arabidopsis*, its involvement in the salt tolerance of halophytes is not much known. Oh et al. (2009) investigated the role(s) by which *ThSOS1*, the *SOS1* homolog in *Thellungiella*, was involved in modulating the halophytic character using ectopic expression of the gene in yeast and in *Arabidopsis* and *Thellungiella* SOS1-RNA interference (RNAi) lines. The knockdown of SOS1 expression totally altered *Thellungiella* into a salt-sensitive plant like *Arabidopsis*. The authors found that the activity of *ThSOS1* could limit Na^+ accumulation and the distribution of Na^+ ions.

Table 2.3 Source of genes from halophytes for the improvement of abiotic stress tolerance

Source organism	Genes	Trait improved	Target organism	References
Atriplex gmelini	Vacuolar Na^+/K^+ antiporters *AgNHX1*	Eightfold higher activity of the vacuolar-type Na^+/H^+ antiporter	*Oryza sativa*	Ohta et al. (2002)
Medicago sativa	Vacuolar Na^+/K^+ antiporters *MsNHX1*	Increased osmotic adjustment and MDA content	*A. thaliana*	Bao-Yan et al. (2002)
Aeluropus littoralis	Vacuolar Na^+/K^+ antiporters *AlNHX1*	Compartmentalize more Na^+ in roots and keep a relative high K^+/Na^+ ratio in the leaves	*N. tabacum*	Zhang et al. (2008)
Atriplex centralasiatica	Betaine aldehyde dehydrogenase *AcBADH*	Improved synthesis of glycine betaine	*N. tabacum*	Yin et al. (2002)
Suaeda liaotungensis, *Beta vulgaris*, *Atriplex hortensis*, *A. nummularia*	Choline monooxygenase *CMO*	Three- to sixfold increased activity of CMO increases glycine betaine synthesis	*N. tabacum*	Russell et al. (1998), Shen et al. (2001), Tabuchi et al. (2005), and Li et al. (2003, 2007)
Avicennia marina	Monodehydroascorbate reductase (MDHAR)	Ascorbate regeneration and ROS scavenging	*N. tabacum*	Kavitha et al. (2010)
Sesuvium portulacastrum	Fructose-1,6-bisphosphate aldolase *SpFBA*	Strongly expressed in roots than in leaves and stems under abiotic stresses	*Escherichia coli*	Fan et al. (2009)
Mesembryanthemum crystallinum	IMT1, myo-Inositol O-methyl-transferase	Inositol methylation	*E. coli*	Rammesmayer et al. (1995)
Thellungiella halophila	FLC gene	Controls vernalization response pathway	*T. halophila*	Fang et al. (2006)
Suaeda salsa	Peroxiredoxin Q gene *SsPrx Q*	Thioredoxin-dependent peroxidase activity	*E. coli*	Guo et al. (2004)

5 Role of Halophytes in Abiotic Stress Management

5.1 Desalination and Stabilization of Saline Soils

The problem of salinity is widespread covering at least 75 countries (Goudie 1990). Various physical, chemical, and biological approaches have been developed for the reclamation of such saline soils (Shahid 2002). Biological methods include organic manure, crop rotation, salt-tolerant crops (Shahid 2002), as well as vegetative bioreclamation (Qadir and Oster 2004). The reclamation of saline soil using such biological means is also referred as desalinization (Zhao 1991), biodesalination, and desalination of salt-affected soils by halophytes (Rabhi et al. 2009). The potential of plants to accumulate enormous salt quantities depends often on the capacity of their above-ground biomass (hyper-accumulating plants) (Rabhi et al. 2010). This ability could be significant particularly in the arid and semi-arid regions where insufficient precipitations and inappropriate irrigation systems are unable to reduce the salt burden in the rhizosphere of plants and suitable physicochemical methods are too expensive (Shahid 2002). The plant-based method of saline soil stabilization is of importance especially in several developing countries where chemical amendments are getting more and more expensive. In this regard, the use of Na^+ and Cl^- hyper-accumulating plants for soil desalination is often suggested as a strategy (Ravindran et al. 2007). A large number of species has been utilized for soil desalination based on their suitability and capacity to accumulate the salt. Halophytes are one of the important categories of plant species extensively used for this purpose with rice as the only one glycophytic exception (Iwasaki 1987). In order to be useful for desalination purpose, the plant species to be used should have high salt resistance, high biomass production, considerable shoot sodium content, and high degree of economic utilization (such as fodder, fuel, fiber, essential oil, and oil seeds) (Rabhi et al. 2010). Shoot-succulent halophytes such as *Sesuvium portulacastrum* and *Suaeda* sps. meet these criteria since they are able to accumulate enormous Na^+ quantities within their above-ground organs. They can become useful candidates for the desalination of salt-affected soils under nonleaching conditions. Zhao (1991) calculated that *Suaeda salsa* produced about 20 ton DW ha^{-1} and withdraw 3–4 ton NaCl. Ravindran et al. (2007) estimated that *Suaeda*, *Sesuvium*, *Excoecaria*, *Clerodendron*, *Ipomoea*, and *Heliotropium* species could remove 504, 474, 396, 360, 325, and 301 kg NaCl, respectively, from 1 ha land in 4 months.

Selection of suited species is the first step for affordable soil desalination at a wider scale in the arid and semi-arid regions (Rabhi et al. 2009). In a case study, a significant decrease in electrical conductivity of the soil having a 50% saturation percentage was recorded from 33 to 20 dS m^{-1} in the presence of single growth cycle of *J. rigidus* in Egypt (Zahran and Wahid 1982). *Suaeda salsa* showed its potential to reduce the soil Na^+ content at depth 0–10 cm by 2.4 with a density of 15 plants m^{-2} and by 3.8 with a density of 30 plants m^{-2} (Zhao 1991). It has also been demonstrated that the growth of annual glycophytes (*Medicago* ssp.) was much better on the soil previously desalinated with perennial halophytes in saline ecosystems (Abdelly et al. 1995). It was concluded that perennial halophytes desalinize and fertilize the rhizosphere, offering a favorable microhabitat for a better growth of annual glycophytes. Ravindran et al. (2007) evaluated the capacity of six halophytic species (*Suaeda maritima*, *Sesuvium portulacastrum*, *Clerodendron inerme*, *Ipomoea pes-caprae*, *Heliotropium curassavicum* and one tree species *Excoecaria agallocha*) to desalinize the upper 40 cm of soil under field conditions in India. This study demonstrated that after 120 days of cultivation of the halophytes, *Suaeda maritima* and *Sesuvium portulacastrum* showed a decrease in electrical conductivity of saline soil from 4.9 to 1.4 and 2.5 dS m^{-1}, respectively. The potential of native halophytes *Arthrocnemum indicum* and *Suaeda fruticosa* to desalinize saline soils was compared with that of an introduced halophyte, *S. portulacastrum*. In this study, Rabhi et al. (2009) confirmed *S. portulacastrum* as the most suitable

plant with higher accumulation of Na^+ in its shoot parts for desalination purpose in arid and semi-arid regions where precipitation is too low to leach salts from rhizosphere. Similarly, successful germination and growth of *Hordeum vulgare* (barley) was observed on the soil desalinated with salt accumulator halophyte *S. portulacastrum* (Rabhi et al. 2010). Taken together, the reports suggest that salt accumulator halophytes can be exploited as a potential source for desalination of agricultural land in the arid and semi-arid regions as well as for the stabilization of saline lands along the coastal regions of the world.

5.2 Phytoremediation

Contamination of agricultural soil by heavy metals (such as Cu, Cd, Zn, Mn, Fe, Pb, Hg, As, Cr, Se, Ur, etc.) has become a serious environmental concern due to their potential impact on the ecosystems. Such toxic elements are considered as soil and water pollutants due to their widespread occurrence, and their acute and chronic toxic effect on plants grown in such soils as well as on humans living in their surrounding (Yadav 2010). Plants, as sessile organisms have developed diverse detoxification mechanisms against absorbing a diversity of natural and man-made toxic compounds. Pollutant-degrading enzymes in plants are a natural defense system against a variety of allelochemicals released by competing organisms, including microbes, insects and other plants. Therefore, plants act as natural, solar-powered pump-and-treat systems for cleaning up contaminated environments, leading to the concept of phytoremediation (Aken 2008). A variety of plant systems have been studied for phytoremediation practices of contaminated soil; however, each species has limitations to accumulate the toxic metals and detoxify to nontoxic compounds through the enzymatic actions. In the course of evolution from marine to freshwater habitat, halophytes are found most successful group of plants which have shown adaptations to a variety of abiotic stresses, tolerance to heavy metal stress is one of these. In recent years, more emphasis has been placed to remove the toxic metals from contaminated soil and water bodies and reclamation of such lands for sustainable agriculture. In this regard, extensive research is undertaken to exploit the use of metal hyper-accumulating plants and search for a suitable plants that can significantly accumulate heavy metals and metalloids (Zabłudowska et al. 2009). However, phytoremediation constitutes a group of strategies meant not only to reduce the metal load at the contaminated site but also to stabilize the site. These strategies are referred as "phyto-extraction" or "phytostabilization" and the selection of a plant may depend on the level of contamination at the site of concern. Both strategies can be integrated into operation at highly contaminated mine sites with a plant that may not be a hyper-accumulator but can tolerate even very high concentrations of toxic metals (Lokhande et al. 2011b). Various halophytes have evolved distinct morphological specializations for dealing with abiotic stressed environments such as presence of "aerial stilts" in the members of families Rhizophoraceae and "pneumatophores" in the members of Avicennaceae and Sonneratiaceae which enable gaseous exchange and oxygenation for respiration in an anoxic environment (Hutchings and Saenger 1987); however, the members of Myrsinaceae, possess no aerial roots. Table 2.4 presents phytoremediation potential of some halophytes.

Numerous laboratory-based trials suggested that the concentrations of metals required to show significant negative effects on halophytes may be significantly higher when compared to their aquatic and terrestrial floral counterparts (MacFarlane et al. 2007). For example, there were no adverse effects on the growth of *Rhizophora mucronata* and *Avicennia alba* seedlings treated with Zn (10–500 μg ml^{-1}) and Pb (50–250 μg ml^{-1}). In *Kandelia candel* seedlings, only at the highest applied metal concentrations (400 mg kg^{-1} Cu and Zn) inhibition of leaf and root development was observed (Chiu et al. 1995). Similarly, Pb (0–800 μg g^{-1}) had little negative effect on *Avicennia marina* seedlings (MacFarlane and Burchett 2002). Studies have demonstrated the accumulation of metals (Cu, Zn, Pb, Fe, Mn, and Cd) predominantly in root

Table 2.4 Examples of halophytic plant species used for the purpose of phytoremediation

Plant species	Phytostabilization/phytoextraction/ phytoexcretion of heavy metals	References
Sesuvium portulacastrum	Cd, Pb and As	Ghnaya et al. (2007), Nouairi et al. (2006), Zaier et al. (2010a, b), and Lokhande et al. (2011b)
Mesembryanthemum crystallinum	Cd	Ghnaya et al. (2007) and Nouairi et al. (2006)
Halimione portulacoides, *Spartina maritima*	Cd, Cu, Pb, and Zn	Reboreda and Caçador (2007, 2008)
Arthrocnemum macrostachyum, *Spartina argentinensis*	Cd and Cr	Redondo-Gómez et al. (2010a, b)
Triglochin maritima, *Juncus maritimus*, *Sarcocornia perennis*, *Halimione portulacoides*	Hg	Castro et al. (2009)
Atriplex halimus subsp. *Schweinfurthii*	Cd	Nedjimi and Daoud (2009) and Lefevre et al. (2009)
A. halimus	Pb and Cd	Manousaki and Kalogerakis (2009)
Spartina densiflora, *S. maritima*	As, Cu, Fe, Mn, Pb, and Zn	Cambrolle et al. (2008)
Aster tripolium	Cu and Pb	Fitzgerald et al. (2003)
Sarcocornia perennis	Fe, Mn, and Hg	Lilebo et al. (2010)
Halimione portulacoides	Zn, Pb, Co, Cd, Ni, and Cu	Sousa et al. (2008) and Almeida et al. (2009)
Tamarix smyrnensis	Pb and Cd	Kadukova and Kalogerakis (2007), Kadukova et al. (2008), and Manousaki et al. 2008
Juncus maritimus	Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn	Almeida et al. (2006)
Sporobolus virginicus, *Spartina patens*, and *Atriplex nammularia*	Zn, Cu, and Ni	Eid and Eisa (2010)
Salicornia europaea	Cd	Ozawa et al. (2009)

tissue, rather than in foliage, in numerous mangrove species grown in the field conditions, such as *Avicennia* sps., *Rhizophora* sps. and *Kandelia* sps. (Peters et al. 1997). It has also been observed that for some mangroves, concentrations of translocated metals are low, with bio-concentration factors (BCF; ratio of leaf metal to corresponding sediment metal concentration) ranging from <0.01 in *Rhizophora mangle* to 0.06 for other species such as *A. marina* (Lacerda 1997). However, other studies suggest that mangroves may accumulate and translocate some metals with leaf BCFs greater than 1, for example, 1.5–2.4 for *A. marina* (Sadiq and Zaidi 1994), 1.7 for *Aegiceras corniculatum* and 1.2 for *Kandelia candel* (Chen et al. 2003) and behaved as hyper-accumulating species. Lokhande et al. (2011b) recently demonstrated the arsenic (As) accumulation potential of *Sesuvium* exposed to As(V) (100–1,000 μM) for 30 days, wherein the growth of the plant was not affected even after prolonged exposure to arsenic stress with the significant As accumulation (155 μg g^{-1} dry weight) and a bioaccumulation factor of more than ten at each concentration. On the basis of total As accumulation, bioaccumulation factor and known biomass production capacities, the *Sesuvium* like other As hyper-accumulator plants has been suggested to use as potential candidates for application in arsenic removal and land re-vegetation/reclamation projects in the As-contaminated sites of the world.

Heavy metal uptake in halophytes is generally regulated at the root endodermis through modifying uptake from predominantly apoplastic to selective symplastic transport. The contribution of each tissue type is dependent on the molecular properties of the plamsalemma (i.e., specific membrane transport proteins) and on the metal in question (MacFarlane and Burchett 2000). Some halophytic genera such as *Aegiceras* and *Avicennia* secrete excessive Na^+ and K^+ through specialized glands or glandular trichomes on abaxial and adaxial leaf surfaces, while such specialized structures are absent in nonsecretors, for example, *Rhizophora* and *Sonneratia* (MacFarlane and Burchett 1999). Indeed mangroves and a number of other estuarine halophytes with glandular tissue are known to excrete heavy metals concomitantly with other solutes (MacFarlane and Burchett 2000). The variation in morphology/function of nutritive root tissue and glandular tissue to deal with the challenges of excess cations in saline environments could become significant for metal accumulation, transport, partitioning, and excretion among halophytic plant species (MacFarlane et al. 2007).

5.3 Wetland Restoration and Re-vegetation

Mangrove forests are ecologically important coastal ecosystem currently covering 146,530 km of the tropical shorelines of the world (FAO 2003). There has been a steady decline from 198,000 km of mangroves in 1980 and 157,630 km in 1990 (FAO 2003) which represents about 2 and 1% losses per year between 1980–1990 and 1990–2000, respectively (Lewis 2005). Mangroves have provided ecological benefits in terms of shorelines stabilization, reduction in wave and wind energy against shorelines thus protecting inland structures, supporting coastal fisheries for fish and shellfish through direct and indirect food support and provisions for habitat, and support of wildlife. However, increasing human activities and adverse environmental conditions have led to destruction of mangrove forests thus limiting the available resources. Therefore, restoration and re-vegetation of mangrove forest has become important for the development of sustainable agriculture and to avoid the destructive natural calamities. In this context, restoration or rehabilitation of saline lands using potential halophytes will act as an effective strategy. Restoration or rehabilitation is recommended when an ecosystem is altered to such an extent that it can no longer self-correct or self-renew. This could result in total disturbance to ecosystem homeostasis and permanent stopping of the normal processes of secondary succession or natural recovery from damage. Wetlands play an important role in nutrient cycling, sediment accretion, pollution filtration, and erosion control in the world. In addition, they are known for their distinctive flora and rich spectrum of wildlife, especially waterfowl, which makes them more valuable and more prone to human impact than other ecosystems (Mitsch and Gosselink 2007). However, only a small percentage of the original wetlands have remained around the world after over two centuries of intensive development and urbanization. Having lost so many wetlands, it seems that there are many opportunities for wetland restoration along coastal lines, rivers, lakes, etc. The deteriorated saline wetland can be restored using halophytes and salt-tolerant plants. The research on salt-tolerant plants and halophytes has attracted attention of many scientists because these plants could be used for saline agriculture and biomass energy on saline land, and for overcoming the worldwide problem of food shortage and energy crisis. Restoration of areas of damaged or destroyed mangrove forests has been previously discussed by many workers (Brockmeyer et al. 1997; Lewis and Streever 2000; Saenger 2002). Saenger and Siddiqi (1993) described one of the largest mangrove afforestation programs which covered plantings of primarily one species (*Sonneratia apetala*) over 1,600 km^2 on newly accreting mud flats in Bangladesh.

Research in this field of wetland restoration has demonstrated the potential of salt-tolerant plants and halophytes (Wang et al. 2008a, b; Zhang et al. 2008) for restoration and re-vegetation of barren lands and wetlands along the

coastal regions. Use of halophytes from the ancient period for restoration of wetlands is summarized here. *Scirpus* species have been extensively used in constructed wetlands for wastewater treatment as the species has ability to efficiently remove nutrients and pathogens from effluent (Huang et al. 2000; Coleman et al. 2001). Besides *S. robustus* showed efficiency for removal of selenium (Se) from contaminated water demonstrating potential for Se phytoremediation by wetlands (Pilon-Smits et al. 1999). Tissue culture mode of plant regeneration has been suggested as an efficient tool for producing plants required in wetland creation and restoration (Wang et al. 2003). Seliskar and Gallagher (2000) have further proposed that tissue culture-induced somaclonal variation can be advantageous to produce plants with particular characteristics for use in wetland restoration. Further, Wang et al. (2006) evaluated the wetland restoration potentials of selected tissue culture regenerants of ecologically important salt marsh monocots, *Spartina patens*, *S. alterniflora*, *Juncus gerardi*, *J. roemerianus* and *Scirpus robustus* in a simulated marsh fields.

5.4 Saline Agriculture

The rapidly increasing human population in the arid and semi-arid regions of the world has tremendously increased the pressure on the availability of good quality water and land resources for human usage, industry and agriculture. In addition, improper and poor quality irrigation practices have increased the level of under-ground water and large areas have become water logged which eventually results into higher salinities of the soil (Yensen 2006). Salinity affects the growth of the plant to a sever extent thus reducing the crop productivity in the arable lands. It becomes a serious threat of growing any conventional crops which are otherwise sensitive to high salt concentrations and expect the yield at higher level in such saline lands to fulfill the demand of ever-growing human population for food, fodder, shelter and as a raw material for the industrial purposes. The research work on engineering the salt tolerance of the crop plants has not yet produced the successful transgenic plants which could tolerate the excess saline stress and yield more productivity in such conditions. Therefore, extensive efforts should have been undertaken to protect the available resources of freshwater and arable lands in the arid and semi-arid regions of the world through the application of halophytes for the variety of uses as a source of nonconventional cash-crops such as food, fodder, forage, medicinal, ornamental, chemical, timber, and other usage of wood and fibers (Khan and Qaiser 2006). Introduction of these potential halophytes has led to cover the barren saline land along the coastal zones of the world and provided economic benefits to the humans.

A variety of halophytic plant species have been categorized into different groups such as euhalophytes, xerohalophytes, and hydrohalophytes on the basis of their growth performance in variable climatic conditions and salt concentrations in the soil which has been utilized as a source of nonconventional cash-crops (Khan and Qaiser 2006; Khan and Ansari 2008). Hollington et al. (2001) described the successful stories of the utilization of halophytic species for the improvement of sustainable agriculture as well as sources of economy. *Atriplex* species showed the highest productivity and increases water uptake whereas tree species of *Acacia* and *Prosopis* have shown its role in re-vegetation and for biological drainage. Further, the use of raised-bed technology and on-farm seed priming have improved the production and efficiency of a range of halophytic plant species in saline conditions. Zhao et al. (2002) have screened the halophytic species distributed along the coast of China and categorized into different groups and suggested their uses in saline agriculture for the economic purposes. The variety of halophytic species so far used as a nonconventional source for various purposes in Pakistan have been reviewed extensively (Khan and Qaiser 2006; Khan and Ansari 2008) and presented in Table 2.1. Similarly, various strategies have been utilized for managing the saline or alkaline soil for sustainable agricultural production in South Africa (Sharma and Minhas 2005). Besides, Masters et al. (2007) also reviewed the utilization of halophytic grasses and shrubs

(such as *Medicago sativa*, *M. polymorpha*, *Trifolium* sps., *Hordeum vulgare*, *Distichlis spicata*, *Suaeda* sps., *Sporobolus* sps., etc.) in biosaline agriculture for the production of forage and livestock which showed their growth potentials in highly saline soil with salt concentrations >25 dS m^{-1} and produce 0.5–5 ton of edible dry matter $year^{-1}$. The potentials of five halophytic plant species namely *Diplachne fusca*, *Spartina patens*, *Sporobolus virginicus* (Smyrna-smooth), *Sporobolus virginicus* (Dixe-coarse), and *Medicago sativa* have been studied as a source of forage plants in Egypt on the soils irrigated with different concentrations of seawater. The studies showed that *S. virginicus* (Dixe) produced the highest biomass upon irrigated with either 25 or 37.5% sea water, followed by *S. patens* and *D. fusca* whereas *S. virginicus* showed the lowest yield. The studies conducted on cultivation of *Salvadora persica* in semi-arid saline and alkali soils showed its efficiency for growth and as a source of industrial oil on both saline and alkali soils for economic and ecological benefits which is otherwise not suitable for conventional arable farming (Reddy et al. 2008). Therefore, considering the approach of biosaline agriculture more efforts should have been undertaken on the cultivation of nonarable lands with nonconventional plant resources such as halophytes to bring the uncultivable land for the use of economic purposes of the human being. This strategy will help to improve the gross economy of the developing countries.

6 Conclusions and Future Perspectives

Worldwide food production is affected to a large extent by environmental extremities and the sensitivity of crops is a major limitation for achieving higher plant productivity. Plants have evolved adoptive mechanisms which can be understood and exploited as an important resource for development of crops tolerant to extremities. Halophytes show a diversity of growth responses to increasing salinity. Halophytes have evolved to the changing environmental conditions through developing variety of tolerance mechanisms such as halosuculence, ion compartmentation (exclusion/inclusion), osmoregulation, enzymatic and nonenzymatic antioxidants and maintenance of redox and energy status. Research on understanding the abiotic stress tolerance mechanism of halophytes has been on the upfront using wide array of physiological, biochemical, and molecular tools. Some of the halophytes (e.g., *Thellungiella halophila*) that tolerate adverse conditions have become the choice model systems for unraveling the different pathways associated with halophytic behavior. However, research in the context of progress in metabolomics, genomics, and proteomics has to be initiated in diverse halophytic species with the use of advanced techniques to gain detailed knowledge of abiotic stress tolerance.

Halophytes have also been utilized practically for managing the stressful environment and shown to be involved in increasing the economy of developing countries in many parts of the world. Halophytes have shown their role in desalination of saline lands from arid and semi-arid regions as well as the stabilization of saline lands along the coastal sides, phytoremediation of heavy metal contaminated sites and wetland restoration and revegetation through introduction of variety of halophyte species. This has led to developing agriculture on saline lands for supporting the sources of food, forage, fodder, medicine, ornamental and important plant-based chemicals to ever-growing human population. In addition, this may also help in reducing the burden on the crop plants which are facing the productivity problems due to exposure to various abiotic stresses. Considering these applications, constructive strategies have to be developed and implemented for the protection of world's nonrenewable resources, wherein available halophyte diversity can be utilized as an important source. Investigation of the gene regulation and the balance of individual stress tolerance mechanisms will aid in translating the information to other salt-sensitive crops. Such studies will be helpful for ensuring sustainability of future research efforts to improve and manage crop performance on marginal and irrigated land through genetic manipulation.

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UV-B Radiation, Its Effects and Defense Mechanisms in Terrestrial Plants

3

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Abstract

The UV-B is an important component of solar radiation to which all terrestrial and aquatic plants were exposed during the early evolutionary phase of the Earth. Hence the plants, principally terrestrial, have evolved different mechanisms to avoid and repair the UV-B damage; therefore, it is not surprising that photomorphogenic responses to the solar UV-B are erroneously assumed to be adaptations to the harmful UV radiation. The responses to UV-B enhancement include changes in the leaf area, leaf thickness, stomatal density, wax deposition, stem elongation, and branching pattern, as well as in the synthesis of secondary metabolites, alterations in plant–pathogen and plant–predator interactions, and in gene expression. However, under field conditions the ambient solar UV-B provides an important signal for the normal plant development and may be perceived by the plants through nondestructive processes involving both UV-B specific and UV-B nonspecific signaling pathways. The specific signaling pathways include the components UVR8 and COP1 which regulate the expression of a set of genes that are essential for the plants' protection. The nonspecific signaling pathways involve DNA damage, reactive oxygen species (ROS), hormones, and wound/defense signaling molecules. Indeed under the field conditions, the ambient UV-B might more properly be viewed as a photomorphogenic signal than as a stressor. Therefore, it might not be appropriate to evaluate the adaptive roles of plant responses to UV-B cues upon stress tolerance by the simultaneous application of both solar radiation and supplemental UV-B. In this chapter, we analyzed the information regarding

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physiological and morphogenic responses of the terrestrial plants to the UV-B radiation, as well as the events related to UV-B perception, signal transduction, gene expression, and ROS formation from different studies carried out in greenhouses, growth chambers, and field conditions.

Keywords
UV-B radiation • DNA damage • DNA repair • Metabolites • Signaling • Secondary metabolites • Morphogenic responses

1 Introduction: Knowing the Solar UV-B Radiation, a Historical Background

Within the electromagnetic radiation spectrum, the UV radiation describes a spectral range between 200 and 400 nm, which borders on the visible range. The UV radiation is divided into three effective types: UV-A (320–400 nm), UV-B (280–320 nm), and UV-C (200–280 nm). Less than 7% of the sun's radiation reaching the Earth's surface falls approximately in the range between 295 and 400 nm (UV-A and UV-B); the shorter UV wavelengths get filtered out by the stratospheric ozone. Therefore, the lower limit of shorter wavelengths of the solar UV radiation reaching the surface is determined by the stratospheric ozone layer. The stratospheric ozone absorbs virtually all the UV radiation ranging approximately 295 nm and lesser. Although the stratospheric ozone determines the amount of UV-B radiation that reaches the surface of the Earth, its level is significantly affected by variations in latitude and altitude. The level of UV-B radiation over tropical latitudes is higher than in temperate regions due to lesser atmospheric UV-B absorption determined by the solar angle and the ozone layer itself which is thinner in equatorial regions. Thus, the UV-B radiation is relatively high in tropical areas and relatively low in the polar regions. Increases of the UV-B irradiance with increasing elevation above sea level is also known, i.e., measurements of the UV-B irradiance show an average increase between 10 and 19% for every 1,000 m increase in elevation. Besides geographical factors, the atmospheric pollutants (e.g., smoke, aerosols) and especially the weather factors (e.g., clouds, haze) greatly decrease the level of UV-B reaching the Earth's surface. Depending upon the type and height of clouds, liquid water content, and particle distribution, the cover of clouds can attenuate over 70% of the incident UV-B radiation (McKenzie et al. 2007).

Over 35 years ago, it was warned that man-made nitrous oxide and chlorine-containing compounds (e.g., chlorofluorocarbons, CFCs) produce the breakdown of large amounts of ozone in the stratosphere (Crutzen 1972; Molina and Rowland 1974; in Velders et al. 2007). This fact causes the depletion of the stratospheric ozone layer increasing the UV-B radiation at the ground level, especially in Antarctic and Arctic regions as well as in high altitude areas (Ryan and Hunt 2005). By their contributions on chlorine-containing compounds and the depletion of the ozone layer, Crutzen, Molina, and Rowland were awarded with the Nobel Prize for chemistry in 1995. After the Molina and Rowland's work, Farman, Gardiner, and Shanklin, scientists of the British Antarctic Survey, shocked the scientific community during the early middle 1980s by publishing the results of a study showing a springtime ozone *hole* in the Antarctic ozone layer (Farman et al. 1985, in Ryan and Hunt 2005). This fact rang alarm bells worldwide and within the same year, 20 nations including most of the major CFCs producers signed the Vienna Convention, which established a framework for negotiating an international regulation on the ozone-depleting substances. After that, on September 16, 1987 the Montreal Protocol on "Substances that Deplete the Ozone Layer" was opened for nations signature

and entered into force on January 1, 1989 (Velders et al. 2007). At present, after several amendments, all the countries in the United Nations have ratified the Montreal Protocol (http://ozone.unep.org/Meeting_Documents/). However, today the ozone depletion is a global phenomenon and according to the European Ozone Research Coordinating Unit (EORCU) its amount approximately reaches 0.6% per year. The level of UV-B radiation in the biosphere varies, spatially and temporally, quite considerably but the depletion of the stratospheric ozone strongly affects its penetration (Ryan and Hunt 2005). Thus, despite reductions in the production and use of ozone-depleting chemicals, the potential of ozone depletion by anthropogenic emissions or natural causes (e.g., volcanoes) still remains. In this scenario, the level of stratospheric ozone will continue decaying with a severe decline occurring between 2010 and 2019 in the northern hemisphere that may result up to 50–60% increase in the springtime UV-B radiation according to the Global Climate Model (GCM) that is based on the simplified ozone-depletion chemistry (Taalas et al. 2000). Furthermore, the recovery of the stratospheric ozone to early 1980s levels is not predicted until roughly 2050.

2 Solar UV-B Radiation and the Life of Terrestrial Plants

Since the discovery of the ozone layer depletion (~30 years ago) the responses of microorganisms, animals, and terrestrial plants to solar UV-B radiation have been active subjects of many studies (Rozema 2000; Björn et al. 2002; Ryan and Hunt 2005). Prior to building of the atmospheric oxygen and then the stratospheric ozone layer, the UV-C radiation and high levels of both UV-B and UV-A would probably have reached the Earth's surface relatively unattenuated affecting all the living organisms. In this context, the UV radiation seems to be a ubiquitous factor in the course of terrestrial biota? Plant evolution from the early Archean era began as solitary photosynthetic cells (Cockell and Horneck 2001). The UV effects on terrestrial plants, which are principally detrimental, have been demonstrated with some of the most essential components of the biochemical machinery, i.e., DNA molecule and photosystem II (PSII) (Singh et al. 2008). However, when the stratospheric ozone layer developed, the UV-A radiation and a minor portion of the UV-B wavelengths only could reach the Earth's surface due to the atmospheric absorption and scattering of the UV-C radiation. Therefore, how the UV radiation has altered the Earth's environment over geological time periods is essential for understanding the evolutionary history of the earth and also to understand how the UV-B radiation has contributed as selection pressure on the development of terrestrial plants (Björn and McKenzie 2007). In fact, Sagan (Sagan 1973 in Singh et al. 2008) first considered the UV radiation as a selection pressure on the early photosynthetic organisms, when our knowledge on biological effects of the UV radiation on plants was in its infancy. During the evolutionary history of the Earth, the terrestrial plants coevolved under different solar UV-B levels and may have experienced significantly higher UV-B irradiances than the current surface UV-B level (Cockell and Horneck 2001; Rozema et al. 2002). Thereby, the UV-B tolerance acquired earlier probably helps to explain why plants are distributed at lower latitudes or higher elevations, where UV-B irradiances are greater, are less sensitive to high levels of the UV-B radiation than those at higher latitudes and/or lower elevations (Turunen and Latola 2005; Ren et al. 2010). The present rate of atmospheric changes is so rapid that evolution may not keep up with it, particularly in long-living plants like trees. The UV-B environment of terrestrial plants is presently quite variable in both time and space, and thus organisms experience different UV-B doses and adapt to UV-B radiation at different levels (Rozema 2000). In this context it is expected that terrestrial plants respond differentially to increasing solar UV-B. Nevertheless, although studies assessing possible consequences of the ozone depletion have greatly increased our understanding on how living organisms are affected by the UV-B radiation, the focus of these researches may also have distracted the attention from the UV-B radiation as a component of the *ambient light environment* involved in the evolution of life on the Earth's surface (Cockell and Horneck 2001).

3 UV-B Radiation as a Modulator of the Plant Function

Due to their absolute sunlight requirement for survival, the plants are inevitably exposed to solar UV-B radiation. However, from equatorial to the polar regions and from the sea level to high mountains, the terrestrial plants are exposed to greatly different UV-B irradiances, given the geographical differences in UV-B irradiances is much greater than corresponding differences in the total solar radiation (Rozema 2000). The plant chemical photoprocesses respond differently to different UV wavelengths as the biological damage exacerbated as wavelength becomes shorter. Thus, the relative effectiveness of UV-B-ranging wavelengths (effective UV-B irradiance) must be known in order to assess the responses to ozone changes. The effective UV irradiance (E) or dose rate exposure is given by

$$E = \int F(\lambda)W(\lambda)d\lambda,$$

where $W(\lambda)$ is the weighting function (action spectrum) for a specific biological or chemical effect and $F(\lambda)$ is the spectral irradiance, either computed or measured, for a given time (e.g., hour, day, year) and location. As a result, the biological effectiveness of the weighted UV-B irradiances (UV-BBE, biologically effective UV-B radiation) related to different action spectra has different responses to atmospheric ozone changes (Flint and Caldwell 2003). It has been estimated that 1% decrease in the stratospheric ozone concentration would result in nearly 2% increase in the UV-BBE at mid-latitudes. Therefore, the recently projected 15% stratospheric ozone reduction would result in up to 30% increase in the value of UV-BBE in the next three decades (McKenzie et al. 2007).

4 Solar UV-B Radiation: Stress Factor or Beneficial Signal Factor?

From the ozone depletion perspective, the UV-B radiation is considered as an environmental stressor of photosynthetic organisms (Jordan 2002; Rozema et al. 2002; Ballaré 2003; Caldwell et al. 2007). However, from an evolutionary perspective this assumption is questionable. The terrestrial plants have always developed under the solar UV-B and then their genetic machinery coevolved together with the *ambient* UV-B level. Therefore, it can be hypothesized that the metabolic machinery of plants contains all the necessary elements for a *normal coexistence* with the current UV-B level and so the solar UV-B radiation should not be considered as an *environmental stress factor*. In fact, the current level of the ambient UV-B radiation should be considered as a *signal factor* that induces the expression of genes related to the normal plant development (Jenkins 2009). While the UV-B exclusion must be considered as an *anomalous signal factor* that induces the expression and/or repression of another set of genes (Brosché et al. 2002; Stratmann 2003; Hectors et al. 2007). In this context, the solar UV-B radiation appears as a reliable plant effector, but it is not always possible to identify a unique particular reason as explanation of the underlying UV-B effects. In nature, the terrestrial plants are seldom affected by only a single environmental factor; they typically respond to several environmental factors acting in concert (Bruno et al. 2003). Therefore, the influence of changing UV-B in natural ecosystems must be evaluated considering two opposite processes: (a) facilitation; (b) competition. These processes have been recognized as key drivers in a wide range of natural communities and hence, effectiveness of the UV-B radiation will be greatly modified by other environmental factors, in some cases aggravating and in others, ameliorating the overall UV-B effect (Bruno et al. 2003).

5 Responses of Terrestrial Plants to Ambient Solar UV-B

The most extended researches relating to the effects of increasing solar UV-B radiation on terrestrial plants have been performed in both austral and boreal polar regions (Day et al. 2001; Phoenix et al. 2003; Robson et al. 2003; Rozema et al. 2006; Newsham and Robinson 2009). The depletion of the stratospheric ozone is greater in both

Antarctic and Arctic regions than in other nonpolar latitudes where it is less pronounced and subject to other atmospheric factors such as horizontal and vertical ozone transport. In the Antarctic zone, the complete breakdown of the stratospheric ozone occurs only during few springtime days, but the springtime ozone depletion reaches 50–60% on average (Rozema et al. 2005). This event has occurred uninterruptedly for at least 30 years, leading to a marked increase of the solar UV-B irradiance. Since the 1990s frequent occurrence of the springtime ozone hole over the Arctic also occurs resulting in significant ozone depletion and increasing the UV-B irradiance at the ground level (Rex et al. 2004). Similar to the Antarctic area, the ozone loss over the Arctic area is higher in the early springtime than in the growing plant season (late springtime and summer). The Arctic springtime ozone depletion is lower than the Antarctic one and rarely reaches 40–50% on average (Rex et al. 2004). Also, the Arctic ozone loss is extremely sensitive to frequency of sudden stratospheric warming due to the greenhouse effect. Because of the influence of increasing greenhouse gases, the ozone holes may worsen leading to greater ozone depletion over the Arctic and increasing the severity and duration of the Antarctic ozone depletion (Rozema et al. 2005). At present both Antarctic and Arctic polar regions represent one the most extreme UV-B environment and constitute an excellent site to study the responses of terrestrial plants to increased solar UV-B. Although almost all the investigations were carried out in the Antarctic area this ecosystem only has two species of higher plants: *Deschampsia antarctica* and *Colobanthus quitensis* (Convey and Smith 2006). While the terrestrial Arctic ecosystem has more than 160 higher plant species, allowing more species interactions and feedbacks and perhaps providing a more general representative ecosystem response to enhanced UV-B than the more simple two-species Antarctic ecosystem (Rozema et al. 2006). Short- and long-term studies have shown different and controversial effects of both enhanced and excluded solar UV-B radiation on the Antarctic and Arctic flora species (Searles et al. 2002; Phoenix et al. 2003; Robson et al. 2003). However, in three extensive overviews, Dormann and Woodin (2002) and Rozema et al. (2005, 2006) claimed the finding that neither flowering plants nor mosses and lichen species of the polar ecosystems are markedly affected by the enhanced solar UV-B. Almost all the plant parameters related to the growth and photosynthesis were not significantly affected by elevated UV-B simulating 15, 30, or may be higher (e.g., 50%) of the ozone depletion (Rozema et al. 2005). In fact, these overviews contradict many authors who hypothesized that stressful harsh climatic and environmental polar conditions would make the polar plants vulnerable to the enhanced UV-B, and that the repair of UV-B-induced damage could be hampered by the low polar temperatures (Newsham and Robinson 2009; Snell et al. 2009). The absence of significant UV-B effects on polar plants could imply that they are better adapted to high UV-B regimes and capable of preventing and/or effectively repairing the UV-B damage (Rozema et al. 2005). Although this fact may be interpreted that terrestrial plants from polar ecosystems are particularly tolerant to the ozone depletion, in a more generalized way it has been applied to all the plants and ecosystems, especially those located in high UV-B environments (e.g., tropical and subtropical mountain areas). This assumption implies that terrestrial plants occurring naturally in the high UV-B habitats would undoubtedly have evolved specific adaptations that protect them against the deleterious effects of the UV-B radiation. Hence such plants could show a reduced responsiveness mainly due to their reduced sensitivity to UV-B radiation. Similarly, the plants growing in habitats with low UV-B irradiances (e.g., forest underground) could suffer changes even under small variations in the stratospheric ozone layer (Turunen and Latola 2005).

The solar UV-B radiation cannot be regarded as merely an environmental factor causing plant damages because it can also act as an informational signal leading to morphogenic effects on the structure of plants and the overall function of forest ecosystems (Julkunen-Tiitto et al. 2005). For many years both field and laboratory experiments have focused on the UV-B increased scenario, being scarce those on the responses of plants to the current level of solar UV-B (Searles et al. 2001). This lack of information constitutes

an important gap that impedes us to understand the responses of terrestrial plants to solar UV-B changes completely. Many reports consider the solar UV-B as an *environmental stressor* that affects the development of plants (Láposi et al. 2002; Kadur et al. 2007), while others have communicated no detrimental effects of the solar UV-B on the plant growth (Amudha et al. 2010). Some have even reported protective and/or beneficial effects of the solar UV-B radiation (Winter and Rostás 2008). Although there is no conclusive explanation for these contradictory effects, they could obey to variations in the UV-B sensitivity among different species and even among cultivars of the same species (Gilbert et al. 2009; González et al. 2009). In this context, the terrestrial plants have developed different strategies to avoid UV-B radiation reaching the most sensitive cellular targets. A major strategy against penetration of the solar UV-B is based on epidermal screening of the incident radiation (Tattini et al. 2005). The mechanisms that inhibit the penetration of UV-B radiation inside the leaf tissues comprise different leaf structural features such as leaf surface reflectance due to the leaf surface wax and hairs (trichomes) (Liakopoulos et al. 2006; González et al. 2007), epidermal thickness (Hilal et al. 2004), epidermal terpenoids (resin) accumulation (Zavala and Ravetta 2002), and epidermal accumulation of UV-absorbing compounds (Burchard et al. 2000; Agati and Tattini 2010). However, despite largely evolved UV-protection mechanisms, complete UV-B protection is not achieved and a small percentage of the solar UV-B radiation penetrates inside the leaf (Krauss et al. 1997). It is generally accepted that a gradient exists in the ability to screening of UV; the herbaceous plants (being least efficient) towards woody and perennial plants, with the conifers being the most efficient (Krauss et al. 1997). Moreover, the proportion of UV-B radiation reaching the leaf photosynthetic mesophyll is significantly higher in the deciduous broadleaf trees than in the evergreen conifer trees (Julkunen-Tiitto et al. 2005; Turunen and Latola 2005). This indicates a greater susceptibility of the deciduous trees to the enhanced UV-B radiation as well as a greater cost of maintenance (Snell et al. 2009). The reason for the low UV-B transmittance in the conifer needles is that UV-absorbing compounds are located in both vacuoles and epidermal cell walls, whereas in herbaceous plants these are located primarily inside the vacuoles of epidermal cells (Julkunen-Tiitto et al. 2005). Moreover, the soluble flavonoids can be actively and rapidly mediated by the exposure to UV-B radiation whereas the cell-wall bound insoluble phenyl-propanoids represent a more passive UV-screening mechanism (Krauss et al. 1997; Clarke and Robinson 2008). These compounds absorb the UV-B wavelengths effectively, but they also transmit the visible PAR inside the mesophyll cells (Krauss et al. 1997). Interestingly, excess of penetrating UV radiation (UV-A and UV-B) could be converted into visible PAR radiation through both yellow and green fluorescence emission from the epidermal cell-wall bound UV-absorbing compounds (Hoque and Remus 1999). Although the epidermal thickness and concentration of UV-absorbing compounds seems to be the strongest predictors of epidermal transmittance and depth of the UV-B penetration, clear relationships between effectiveness of the accumulation of UV-absorbing compounds and epidermal morphological changes have still not been established, suggesting that other intrinsic plant factors are also important in determining the UV-B screening efficiency. Moreover, the endogenous constitution of plants can affect the chemical composition at both whole and organ level. Even within an individual plant the quality and quantity of secondary metabolites may differ between young and old leaves, as well as between the leaves exposed to the sun and those that remain in the shade (Brenes-Arguedas et al. 2006). Alteration in the accumulation of species-specific UV-absorbing compounds may result in changes in the tissue attractiveness or palatability to insects and herbivores (Izaguirre et al. 2007), pathogen attacks (Stratmann 2003), plant–plant interactions (Sullivan 2005), and changes in litter decomposition processes (Pancotto et al. 2003). Because most of these studies have been conducted on

crop monocultures or isolated pot grown plants, the extrapolation of their responses to natural ecosystems is difficult (Phoenix et al. 2003). One previous ecosystem study found little effect of the ambient solar UV-B on *Sphagnum* bog and *Carex* fen in Tierra del Fuego-Argentina (Searles et al. 2002). Moreover, in related studies the solar UV-B reduced the herbivory, but increased the damage of DNA in the perennial herb *Gunnera magellanica* and reduced both the leaf number and length of the Antarctic species *D. antarctica* and *C. quitensis* (Ballaré et al. 2001). Although overall these studies expand our limited knowledge on how the exposure to natural ambient UV-B can modify the biomass accumulation, population dynamics, and competitive interactions in nonagricultural species and thereby how ecosystems may respond to future UV-B fluctuations. Presently long-time studies are very scarce, only a few studies with more than 4 years under continuous monitoring have been communicated (Robson et al. 2003; Rozema et al. 2006; Trošt-Sedej and Gaberščik 2008). Although the visible radiation can often penetrate dense canopies deeper than the UV-B because of its higher transmittance through leaves, in less dense canopies the situation may be reversed. This implies that the UV-B/PAR ratio should change with the canopy leaf area and leaf architecture (Shulski et al. 2004). In order to understand how the natural ecosystems respond to the ambient solar UV-B radiation, many additional well-designed long-term studies with various plant species are needed in order to understand the different behavior of UV-B and PAR inside the canopy as well as to obtain a complete picture of the gene–environment interactions.

6 Effects of Artificially Enhanced UV-B Radiation

Different to polar studies, earlier researches on nonpolar terrestrial plants were mainly focused on the effects of artificially increased UV-B radiation on crop species rather than ecosystems (Flint et al. 2003). Although, such researches were important to understand the physiological responses and identify possible targets for the UV-B radiation, extensive recent studies have shown that effects of the artificially manipulated UV-B have been often overestimated (Rozema 2000). Moreover, the responses of terrestrial plants to simulated solar UV-B enhancement vary greatly due to artifacts derived from the experimental conditions (Musil et al. 2002a; Flint et al. 2003). A critical point besides the variability of experimental conditions in the evaluation of simulated solar UV-B enhancement is the use of lamps to provide the UV-B radiation. Both UV-fluorescent and broad-spectrum xenon-arc lamps are the most commonly used sources of UV radiation in UV-B enhancement experiments (Flint et al. 2009). In terrestrial studies, the UV-fluorescent lamps are usually used, but in aquatic experiments the xenon-arc lamps are preferred. Although the UV-fluorescent lamps are widely used, they supply more short- than long-wave UV-B radiation compared with the solar spectrum (Musil et al. 2002a). In addition, all the UV lamps emit small but biologically effective UV-C radiation, which is not present in the solar radiation reaching the Earth's surface (Flint et al. 2009). Other debatable question in the studies on UV-B effects is the use of UV filters. The major filters used to exclude either UV-A or UV-B in UV-exclusion studies are: (a) cellulose diacetate, CA, that is commonly used to exclude the UV-C radiation and transmit both UV-A and UV-B; (b) polyester, the generic name for Mylar (trade name of the DuPont Co.) that is used to exclude both UV-C and UV-B and transmit the UV-A only; (c) polychlorotrifluoroethylene, PCTFE (Aclar 22 C) that transmits all the UV radiation (UV-A, UV-B, and UV-C); (d) copolymers of tetrafluoroethylene and hexafluoropropylene, Teflon FEP (trade name of the DuPont Co.) that transmits the radiation at 245 nm and above; (e) polyvinyl fluoride, Tedlar TUT (trade name of the DuPont Co.) that blocks wavelengths in the UV-B region; (f) clear polyethylene, Dura-Film Super 4 (trade name of the AT Plastics Inc.) that blocks the UV radiation up to 380 nm; polymethylmethacrylate, Plexiglas (trade name

of the Arkema): the standard Plexiglas excludes the UV-B wavelengths and a portion of the UV-A region, whereas the UV-T Plexiglas transmits all the wavelengths in both UV-B and UV-A regions (Krizek et al. 2005). Although these filters have been widely used in UV-B studies, their transmittance properties vary leading to erroneous interpretations of the UV effects in long-term experiments (Day et al. 2001). Moreover, CA, the most widely used UV filter produces detrimental effects on plants (Krizek and Mirecki 2004). On the other hand, in greenhouses or growth chambers unrealistic balances frequently occur among the different light spectral regions: UV-B/UV-A/PAR (photosynthetic active radiation (PAR), 400–700 nm) and often levels of PAR lower than in the field conditions are also observed. Low levels of PAR increase the sensitivity of plants to UV-B-induced damages (Pradhan et al. 2006). Additionally, to calculate and compare the doses of UV-B under different spectral regimes the UV-B radiation is weighted (UV-BBE) according to a suitable biological action spectrum or biological weighting function (BWF). To obtain the UV-BBE there are different BWFs available but there is a generalized consensus for the use of the Caldwell's BWF (Flint and Caldwell 2003). Several results, however, have shown that this very steep action spectrum may lead to over- or underestimation of the UV-B effects (Micheletti et al. 2003). Moreover, differences in climatic conditions can also affect the interpretations and comparisons among different studies based on the BWF (Musil et al. 2002b; Flint et al. 2009). Therefore, all the quantitative predictions relating to UV-B enhancement effects could be greatly affected. On the other hand, more recent studies have proposed that increases of the ambient solar UV-B radiation at magnitudes anticipated under the current stratospheric ozone projections will not significantly have large-scale deleterious effects on terrestrial plants even though some species may suffer photosynthesis decreases and growth reductions (Rozema et al. 2006; Xu and Qiu 2007; Newsham and Robinson 2009).

7 Physiological and Morphological Responses to UV-B Enhancement

From indoor and outdoor studies, there is a general consensus that UV-B enhancement produces physiological, biochemical, morphological, and anatomical changes in the plants (Searles et al. 2001). According to the literature, the enhancement of UV-B radiation can affect the terrestrial plants at different functional levels involving conformational changes and damages to different molecules such as DNA, proteins, and lipids (Li et al. 2010). As a result, if damage to macromolecules, that is, DNA is not effectively repaired, the UV-B effect will be translated to the biochemical level with the consequent alteration and/or impairment of the plant functionality (e.g., photosynthetic process, growth, yield). Although it is clear that there is a wide range of both intra- and interspecific sensitivity to UV-B radiation (Gilbert et al. 2009) the terrestrial plants through the evolution have acquired different protective strategies to avoid the adverse effects of UV-B radiation. The two major protective mechanisms are: (a) shielding through the production of soluble phenolics (e.g., flavonoids, anthocyanins, hydroxycinnamic acid derivatives), insoluble polyphenols (e.g., lignin), and cell-wall bound UV-absorbing compounds (Hilal et al. 2004; Clarke and Robinson 2008), as well as by reflection of the UV-B radiation by epicuticular waxes and cuticular structures (Hada et al. 2003; Schmitz-Hoerner and Weissenböck 2003; Agati and Tattini 2010); (b) removal and direct reversion of the DNA lesions induced by UV-B radiation (Tuteja et al. 2001; Britt 2004; Kimura et al. 2004).

7.1 DNA: Damage and Repair

The more important UV-B-induced DNA alterations are the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidine dimers (6–4 photoproducts, 6-4PPs) (Dany et al. 2001). The DNA repair mechanisms operating in

plants include the following processes: (a) direct reversal (DR); (b) photoreactivation that induces photolyases; (c) dark repair (Tuteja et al. 2001; Britt 2004). The DR is a simple mechanism that involves a single-enzyme reaction for the removal of certain types of DNA damage. Alkyltransferases simply extract alkyl groups from the alkylated bases that are transferred to internal cysteine residues and thus inactivate themselves. The best example for DR is the correction of miscoding alkylation lesion O^6-methylguanine, which is generated endogenously in small amounts by the reactive cellular catabolites. This reaction is catalyzed by a specific enzyme, called methylguanine methyltransferase (MGMT), which removes a methyl group from a guanine residue of the DNA molecule and transferring it to one of its own cysteine residues in a rapid and error-free repair process (Tuteja et al. 2001). The photoreactivating enzyme DNA photolyase (PRE) is a DR phenomenon performed by the combined action of one or more photolyases and the visible light (blue, violet, or long-wave UV) (Hidema et al. 2007). Photolyases specifically recognize and bind the pyrimidine dimers to form a complex molecular structure which is stable in absence of the light. After absorbing a blue light photon the pyrimidine dimers are reversed to pyrimidine monomers without excision of the damaged base (Tuteja et al. 2001). The repair reaction is fast and requires about 1 h for completion (Takeuchi et al. 2007). In plants, two specific types of photolyases have been characterized: (a) CPD-photolyase; (b) 6-4PP-photolyase (Tuteja et al. 2001). The dark repair processes include the nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), and other DNA repair pathways. These mechanisms have been observed in several plant species and some of the genes required for the processes were identified (Kimura et al. 2004). The wide class of helix-distorting lesions such as CPDs and 6-4PPs are repaired by the NER process. It is one of the most versatile DNA repair pathway operating in plants. Unlike other DNA repair pathways that are specific repair processes, the NER pathway is capable of removing various DNA damage classes, including those induced by the UV-B radiation (pyrimidine dimers) and chemical agents (bulky DNA adducts) (Kimura et al. 2004). The NER pathway sequentially involves recognition of the DNA damage, incision on the damaged strand, excision of the damage-containing oligonucleotides, and the DNA synthesis and ligation (Liu et al. 2003). Also, the NER pathway is a slow process (about 24 h for completion) and includes several enzymes. There are two subpathways of the NER process that are designated as: (a) global genomic repair (GGR); (b) transcription-coupled repair (TCR). While the GGR pathway repairs the DNA damage over the entire genome, the TCR pathway is selective for the transcribed DNA strand in expressed genes (Kimura et al. 2004). Oxidized or hydrated bases and single-strand breaks are repaired by the BER pathway that is considered an essential process for maintenance of the DNA molecule. The BER mainly removes the DNA damages that are arising spontaneously in the cell from hydrolytic events such as deamination or base loss, fragmented bases resulting from ionizing radiation (e.g., UV-B radiation), and oxidative damage or methylation of the ring nitrogen by endogenous agents. The process that involves the BER mechanism is initiated by DNA glycosylases that release the damaged base by cleavage of the sugarphosphate chain followed by excision of the abasic residue or abasic residue containing oligonucleotides and then the synthesis and ligation of the DNA occurs. The BER pathway involves several enzymatic steps and depends strongly on the presence of nicotinamide adenine dinucleotide (NAD+). Also, the BER process comprises two subpathways that are designated as: (a) BERshort-path; (b) BERlong-path. The BERshort-patch is a DNA polymerase beta-dependent mechanism, while the BERlong-patch is a DNA polymerase delta/epsilon-dependent mechanism (Kimura et al. 2004). The major difference between BER and NER pathways is the way by which the DNA damage is removed. The NER pathway cuts out the damage as a part of an oligonucleotide fragment, while the BER mechanism excises only one nucleotide (Tuteja et al. 2001). The excision repair processes (NER and BER) are very important for maintaining the genome stability

and essential for the survival of plants. The mismatch repair pathway (MMR) is also important in the DNA repair processes when by errors of replication or homologous recombination can be produced mismatched bases. The MMR pathway basically discriminates between correct and incorrect bases and after DNA synthesis the error is corrected (Tuteja et al. 2001). Although most of the present understanding of the eukaryotic MMR has come from studies of the *E. coli* MutS and MutL proteins (Kolodner and Marsischky 1999), recent studies carried out in *Arabidopsis* and rice have reported interesting findings on the MMR pathway operating in plant cells (Tuteja et al. 2001; Kimura et al. 2004). According to the *E. coli* model, the MutS dimer recognizes mispairs and then binds on it followed by the MutL binding, which activates the MutH (endonuclease) that makes a single-strand incision (nick). The MutH incision can be done on either side of the mismatch. Subsequent to incision the excision is initiated and proceeds toward mismatch. To fill the gap (100–1,000 nucleotide gap), the original template strand can then be replicated and finally sealed by ligation. The proteins involved in the last step of eukaryotic MMR are: (a) DNA polymerase δ, RP-A (replication protein); (b) PCNA (proliferating cell nuclear antigen); (c) RFC (replication factor) (Kolodner and Marsischky 1999).

Although the UV-A wavelengths can mediate the photoxidative damage (Turcsányi and Vass 2000) the UV-B radiation is the most important photooxidant agent for terrestrial plants. The DNA damage can also be caused by reactive oxygen species (ROS) and free radicals produced by the UV-B radiation. This damage includes several modifications such as cross-linking, aggregation, denaturation, and degradation (Hidema et al. 2007). The formation of 7,8-dihydro-8-oxoguanine (GO) is a common oxidative DNA lesion generated by a direct modification mediated by ROS. The GO is mutagenic and can mispair with adenine (A) during the DNA replication (Yang et al. 2001). If the resulting A/GO is not repaired before the next round of the DNA replication, a C/G → A/T transversion occurs and the opportunity for repair is lost. The A/GO is repaired via the BER which is initiated by the DNA repair enzyme adenine-DNA glycosylase (Yang et al. 2001). The UV-absorbing compounds (e.g., flavonoids, anthocyanins, hydroxycinnamic acid derivatives, phenolics) accumulating in epidermal and subepidermal cell layers have traditionally been thought to function as UV-B filters, but also play an important role as quenchers of the ROS and free radicals in the amelioration of the UV-B-induced DNA oxidative damage (Agati and Tattini 2010). The UV-absorbing compounds are also effective in reducing the induction of cyclobutane pyrimidine dimers (CPDs) in plants exposed to high UV-B levels (Hidema et al. 2007). The inhibition of CDP formation seems to be high enough to compensate the DNA damage arising even from unusually strong solar irradiations (Tuteja et al. 2001). Other related UV-absorbing compounds, that is, anthocyanins through an anthocyanin-DNA complex could also provide protection against the oxidative damage. Since both anthocyanins and DNA mutually protect each other in vitro, it is likely that such protection mechanism may also operate in vivo (Sarma and Sharma 1999). In the plant cells, anthocyanins are predominantly localized inside the vacuoles and thus their putative role in the protection of DNA should be critically examined. Accepting this fact, it has been demonstrated that the excess accumulation of anthocyanins reduces the amount of blue/UV-A radiation reaching the cell and may sometimes lower the ability to photorepair the damaged DNA. For example, the purple rice is a highly UV-B sensitive species despite possessing an elevated level of anthocyanins in their leaves (Hada et al. 2003). Although significant amounts of flavonoids have been found in the chloroplasts or etioplasts isolated from a wide range of plants growing under both ambient and enhanced UV-B irradiances (Tattini et al. 2005; Agati et al. 2007), it is also likely that some amount of anthocyanins can be present in the nuclei and organelles and then may associate with the DNA molecule, offering to it a certain protection against the oxidative damage (Feucht et al. 2004). In this context, the UV-absorbing compounds seem to have an important protective function against the DNA

damage induced by shorter solar wavelengths (Schmitz-Hoerner and Weissenböck 2003) and so the speculations concerning a great biological risk with regard to increases in solar UV-B radiation after the depletion of the ozone layer are presumably premature.

In the natural populations, both protection and DNA repair are complementary and necessary processes for the plant development. Thereby, it is expected that the plants growing under different UV-B irradiances can exhibit different levels of the DNA protective mechanisms (Turunen and Latola 2005). Under field conditions, the observed DNA damage can often be modified by climatic conditions and then a direct extrapolation of the DNA changes obtained in controlled-environment experiments under artificially enhanced UV-B radiation to plants growing under the ambient solar UV-B is complex and unrealistic. Differences between damage, repair, and defense can be subtle and identification of a particular mechanism does not always occur as the explanation underlying a given phenomenon. For example, the UV-induced degradation of the D1 protein of the PSII can be seen either as damage or as a part of the repair mechanism leading to substitution of the damaged components of the PSII (Turcsányi and Vass 2000). Consequently, understanding these differences and potentially using the DNA repair mechanisms could become very important for producing UV-B-tolerant plants.

7.2 Secondary Metabolites: Flavonoids and Anthocyanins

The increase of secondary metabolites synthesis has been recognized as one of the most frequently observed plant response to UV-B enhancement (Searles et al. 2001; Rozema et al. 2002; Bassman 2004). Considerable attention has been focused, over the past two decades, on the UV-B-induced biosynthesis of phenylpropanoid-derivative compounds, particularly flavonoids and hydroxycinnamic acid derivatives (Jordan 2002; Rozema et al. 2002; Bassman 2004). Although these compounds exhibit important interspecific differences induced by the UV-B radiation, they are often derivatives of the flavonols quercetin and kaempferol (Buer et al. 2010). Quercetin- and kaempferol-derivative flavonoids are usually glycosylated and frequently contain a hydroxycinnamic acid moiety esterified to one of the glycosyl groups (orthodihydroxy B-ring-substituted flavonoids) (Tattini et al. 2004). The flavonoids are ubiquitous molecules occurring in the vacuoles and cell walls of epidermal cells and in nonsecretory and glandular trichomes, and it has been assumed that they primarily have the function of attenuating the shorter solar wavelengths due to their good quantum efficiency (Burchard et al. 2000). In this way, the location of flavonoids in trichomes (Tattini et al. 2004), cuticular wax layers (Fukuda et al. 2008), and epidermal cells (Burchard et al. 2000) may largely prevent that the UV-B radiation reaches sensitive targets within the leaf. However, the flavonoids also have another *protective function* against the shorter solar wavelengths. Considering that the flavonoids with orthodihydroxylated B-ring may efficiently dissipate the excess of energy through tautomeric interconversions (Smith and Markham 1998), scavenge the ROS through the quenching mechanism (Yamasaki et al. 1997; Hilal et al. 2008), and inhibit the formation of free radicals (Neill and Gould 2003; Xu et al. 2008), they can also act as effective antioxidant molecules (Jordan 2002; Tattini et al. 2005; Buer et al. 2010). However, a major criticism regarding functions of the flavonoids is the use of mutants that lack or possess the ability to synthesize flavonoids, which may oversimplify the plant model system for quantifying the UV-B-tolerance/flavonoid-biosynthesis relationships (Bieza and Lois 2001). In this context, contrary to determination of the flavonoid concentration at the whole-leaf level, less attention has been devoted to analyzing the tissue-specific location of individual flavonoids, which may clarify their complex functional roles in both attenuation and antioxidant mechanisms against the high UV-B irradiances (Tattini et al. 2004). Furthermore, the short-term experiments and inappropriate microscopy techniques for visualizing the flavonoids also greatly contributed to this *superficial* conclusion. More recent studies, however, suggest that the biosynthesis of

flavonoids, particularly *internal flavonoid glycosides* may be largely controlled by constitutive morphoanatomical and biochemical features, primarily intended both to prevent the light penetration (Burchard et al. 2000) and to remove the consequent oxidative damage (Apel and Hirt 2004). Agreeing with these findings, Semerdjieva et al. (2003) showed in the *Vaccinium* spp. an inverse relationship between cuticule thickness (primary barrier to UV-B penetration) and the mesophyll accumulation of UV-B-induced flavonoids.

Regarding to ROS scavenging activity of the flavonoids, Yamasaki et al. (1997) proposed a model to address major criticisms on the antioxidant functions of the flavonoids compartmentalized in epidermal vacuoles, and at the same time to explain the preferential UV-B-induced synthesis of flavonoids with effective antioxidant properties in vitro. According to Yamasaki's model, the orthodihydroxy B-ring-substituted flavonoids, not their monohydroxy B-ring-substituted counterparts, are effective substrates for the class III peroxidases, which quench the H_2O_2 freely diffusing from the mesophyll cellular organelles to vacuoles of the epidermal cells. The model was remarkable in calling out the question whether vacuolar flavonoids could be effective in protecting underlying tissues from the damaging shorter solar wavelengths, while not protecting the epidermal cells from the oxidative damage. Epidermal cells and glandular trichomes usually contain much higher concentrations of flavonoids than the mesophyll cells (Burchard et al. 2000), then the H_2O_2 leaked out from the mesophyll cells under high UV-B irradiances can be scavenged by the flavonoid-peroxidase system in the epidermal cells according to the proposal of Yamasaki et al. (1997). Consistent with this idea blackening of the epidermis after a severe light stress is frequently observed in many species under the field conditions. This phenomenon has been ascribed to the polymerization of vacuolar phenolics that result from the penetration of H_2O_2 inside the epidermal cells (Yamasaki et al. 1997). Nevertheless, it cannot be excluded the possibility that other apoplastic flavonoid-depending peroxidases such as guaiacol peroxidase and syringaldazine peroxidase associated with the process of lignification may be involved in the mechanism of ROS scavenging (Hilal et al. 2004). The concept of delocalized scavenging of H_2O_2 by the vacuoles can be applied not only to the organelle–organelle interactions but also to the cell–cell interactions (Yamasaki et al. 1997). It is also acknowledged as a controversy matter whether the ability to accumulate flavonoids, particularly flavonoids with orthodihydroxylated B-rings, and the *tolerance* to UV-B radiation is highly correlated (Dixon et al. 2001; Musil et al. 2002b; Hofmann et al. 2003). The orthodihydroxylated B-ring-derivative flavonoids such as quercetin and luteolin glycosides are accumulated in the vacuoles of the mesophyll cells in *Ligustrum vulgare* leaves exposed to the full sunlight, in presence or absence of the UV-B radiation (Agati and Tattini 2010). This finding, which is consistent with previous reports indicating that the UV-B radiation is not a prerequisite for the synthesis of flavonoids (Tattini et al. 2004, 2005; Jenkins 2009), leads to the conclusion that the light-induced oxidative damage may regulate the biosynthesis of flavonoids, irrespective of the presence of UV-B radiation. The flavonoids and other UV-absorbing phenolics (e.g., hydroxycinnamic acid derivatives) are also synthesized in other abiotic/biotic unfavorable conditions such as drought, salinity, low temperature, heavy metal pollution, pathogen attack, and as feeding deterrent. Besides their antioxidant abilities the flavonoids might exert modulatory effects in the cell through selective actions at different components by cell interactions (Buer et al. 2010). This fact has become increasingly important because attention focuses on the new concept of flavonoids as potential modulators of the intracellular signaling cascades that are vital for the cell functionality (Jenkins 2009).

The anthocyanins, other members of the phenol family, have generally been included into photodamaging-protective compounds (Gould 2004). The anthocyanins show a weak absorption in the shorter UV region (270–290 nm), but their acylated counterparts (hydroxycinnamic acid derivatives) exhibit an increased absorption in the longer UV-B region (310–320 nm) (Neill and

Gould 2003). Because anthocyanins are photoinduced many researchers surmise that they must either have a photoprotective function against the light-induced photooxidation or against the UV-B damage (Hughes et al. 2005). Even without acylation the anthocyanins can significantly attenuate the visible radiation. In fact, the more clear evidences really support the theory of the photooxidative protection, while the role in UV-B protection seems to be much less apparent (Kytridis and Manetas 2006). This assumption, however, contradicts the theory that anthocyanins have a UV-B-filtering role (Neill and Gould 2003). Disagreeing with the last theory the UV-B vulnerability is poorly correlated with the content of anthocyanins. For example, an *Arabidopsis* mutant with enhanced sensitivity to UV-B radiation was found deficient in certain flavonoids, whereas the amount of anthocyanins displayed unchanged. Similarly the responses of a *Brassica rapa* mutant to the supplementary UV-B treatment were mostly independent of the anthocyanin level in leaves (Gould 2004). Agreeing with these findings the anthocyanins often occur in very low concentrations compared to other UV-absorbing compounds, and require a long exposure to the UV-B radiation to be synthesized (Neill and Gould 2003). On the other hand, the red-leafed plants of *Impatiens capensis* and rice displayed significantly worse performances under the UV-B enhancement than their green-leafed counterparts (Dixon et al. 2001). Moreover, it has been communicated that the accumulation of anthocyanins can cause deleterious effects on terrestrial plants after a long-term UV-B exposure (Gould 2004). It has been noted that the DNA damage after a prolonged UV-B treatment was substantially greater in the purple-leafed rice than in the near-isogenic green line. Anthocyanins in the purple rice prevented the photoactivation of photolyases by absorbing some of the incident blue/UV-A light on leaves. Thus, any short-term gain from the absorption of UV-B radiation by anthocyanins would be offset by their property to absorb the visible light and thereby limit the rate of DNA repair (Hada et al. 2003). Furthermore, it has been demonstrated that other abiotic and biotic stresses produce changes in the chemical pattern of anthocyanins (Close and Beadle 2003), then it is obvious that such changes will influence the absorption spectra of anthocyanins under the UV-B enhancement. Also absorptive artifacts due to the dissociation of covalent bonds can occur during the improper isolation of anthocyanins and misread absorption spectra will be generated (Gould 2004).

The accumulation of anthocyanins is usually transient and generally occurs in the vacuoles of peripheral tissues such as palisade and/or spongy mesophyll exposed to high light irradiances, but there are some exceptions (e.g., accumulation in the abaxial leaf tissues and in obligatory shade plants) (Kytridis and Manetas 2006). Perhaps the improved solubility of anthocyanins that in contrast to other flavonoids are nearly always glycosylated allows them to be stored in the vacuole more efficiently than the nonglycosylated flavonoids (Winefield 2002). In fact, the importance of flavonoids should not be overlooked in the discussion of anthocyanin production and UV-B protection. In this context, the flavonoids induced by the UV-B radiation (Agati and Tattini 2010; Buer et al. 2010) are recognized as strong UV-B absorbers, and their UV-B absorption capacity is much stronger than that of anthocyanins (Bieza and Lois 2001). Since the production of anthocyanins represents a conversion of flavonoid precursors that themselves are strong UV-B absorbers, a conundrum appears: if one of the effects of UV-B radiation on plants is to induce the UV-B-protective pigments, why are anthocyanins produced instead of their flavonoid precursors? Characteristics of both flavonoids and anthocyanins absorption spectra must be analyzed to respond this conundrum (Solovchenko and Merzlyak 2008). The flavonoids exhibit two bands in the UV region: (a) short-wave peaking around 280 nm; (b) long-wave situated in the range of 300–360 nm. However, the exact positions of the maxima vary for different flavonoid derivatives. The anthocyanins also have two maxima: one in the UV-B region (270–320 nm) and another in the visible region with a maximum located in the blue-green part of the visible wavelengths (500–540 nm) (Gould 2004). In this way, the UV-B component of the solar spectrum can

be screened by both flavonoids and anthocyanins. However the UV-A radiation, whose proportion in the solar spectrum could be tenfold higher as compared with the UV-B spectrum region also exerts significant effects on plants (Krizek 2004). For example, maximum inhibition of the photosynthesis under natural radiation fluxes is induced by radiation in the UV-A region (Ivanova et al. 2008). Although this fact supports the importance of the UV-protection provided by the flavonoids in the range of 300–360 nm, the high visible fluxes (400–700 nm) also induce a photodamage in plant tissues, especially in the chloroplast (Krizek 2004). The anthocyanins are able to intercept a great proportion of the solar radiation in the range of 500–600 nm, which correspond to the maximum solar energy reaching the Earth's surface (Gould 2004). This finding therefore contributes to support the role that anthocyanins play in the photoprotection of plant tissues (Solovchenko and Merzlyak 2008). In this context, the accumulation of anthocyanins requires visible light and generally coincides with the period of high excitation pressure and the increased potential for the photooxidative damage. The photooxidative damage is produced by an imbalance between the light capture, CO_2 assimilation, and carbohydrate utilization (e.g., greening of developing tissues, senescence, adverse environmental conditions) (Hughes et al. 2005). Thereby, the attenuation of light by anthocyanins may help to reestablish this balance and to reduce the excitation pressure (Kytridis and Manetas 2006). Then, the risk of cellular photooxidative damage is lowered. Also, it would seem that the anthocyanin biosynthesis can enhance under the high light, but it is not usually a prerequisite for the protection against the oxidative stress (Gould 2004).

Like the colorless flavonoids the colored anthocyanins may scavenge the free radicals and ROS (Gould 2004). The anthocyanins diminish the oxidative trend in the leaf simply by filtering out the yellow-green light, because most of the reactive oxygen in plant cells is derived from excitation of the chlorophyll molecule (Neill and Gould 2003). Agreeing with this theory, in juvenile and senescing plants the regulation of photosynthetic apparatus functions is often impaired, making it less efficient in utilization of the absorbed light and therefore prone to the photodamage (Merzlyak et al. 2008a). As a general rule, these situations are accompanied by an increased generation of ROS causing photooxidative damage to the plant and, eventually, its death (Bukhov 2004). Under these conditions, the anthocyanins may afford a detoxifying sink for some ROS when the chloroplast, the first line of the antioxidative defense, is surpassed (Kytridis and Manetas 2006). It is not clear, however, whether the ROS scavenging occurs predominantly through the anthocyanins found inside the vacuole or through their counterparts located in the cytosol. Both anthocyanin forms have impressive antioxidant potentials (Neill and Gould 2003), but due to their proximity to the chloroplastic source of ROS it is more probable that anthocyanins located in the cytosol (mesophyll tissue) than in the vacuole (epidermal tissue) provide the major contribution to antioxidant defense (Kytridis and Manetas 2006). In a similar trend, recent evidences suggest that flavonoids may scavenge the ROS within or near sites of its generation (Schmitz-Hoerner and Weissenböck 2003; Tattini et al. 2005; Agati et al. 2007). Interestingly, equal effectiveness as antioxidant molecules of other colorless phenolics suggests that the putative photooxidative protection afforded by the anthocyanins should be unrelated to their ability to quench oxidants.

Noteworthy the accumulation of anthocyanins in terrestrial plants has always been a contentious issue of the special interest. They often appear in juvenile plants but mature plants usually lack them or display transiently levels under stressful conditions (Merzlyak et al. 2008a). Obviously, upon maturation of the photosynthetic apparatus or its acclimation to stressors the photoprotective *screen* of anthocyanins is no longer required and the juvenile reddish pigmentation disappears. However, unfavorable environmental conditions such as low temperatures, heavy metals, drought, wounding, and pollutants can also predispose the photosynthetic apparatus to photoinhibition and photooxidation, and then the plants may increase, although not necessarily ascribed to, the accumulation of anthocyanins in vegetative organs

(Gould 2004). Accordingly, the production of anthocyanins would fit neatly into the definition of Leshem and Kuiper's (1996) *general adaptation syndrome* (GAS). The GAS indicates that different types of stress evoke similar adaptation responses. In this context, along with compounds such as tocopherols, flavonoids, glutathione, and ascorbate, the anthocyanins may function as general mitigators of the oxidative damage. However, it should be addressed that there is no direct evidence that terrestrial plants benefit from the antioxidant properties of anthocyanins yet (Neill and Gould 2003). Although anthocyanins are of special importance for the photoprotection in senescing leaves, it seems not to be the only function of anthocyanins. It has been suggested, for example, that the red color may also deter aphids from laying their eggs or from feeding on the sugar-rich sap in the phloem. Noteworthy, despite that the autumnal color may be an *extravagancy without a vital function*. This phenomenon that enchants so many tourists each year may hold a vital key to the survival of deciduous trees (Archetti et al. 2009). Also the anthocyanins are involved in the photoprotection of ripening fruits, for example, the chlorophyll in apple fruit peel with high anthocyanin content showed a very high resistance to the photobleaching as compared with the anthocyanin-free zones of the same fruit (Merzlyak et al. 2008b). Despite its function as photoprotective molecules, the anthocyanins may instead serve to decrease the leaf osmotic potential. The resulting depression of leaf water potential could increase the water uptake and/or reduce transpirational losses. This phenomenon may allow to the anthocyanin-containing leaves to tolerate suboptimal water levels. The often transitory nature of the foliar anthocyanin accumulation may allow plants to respond quickly and temporarily to environmental variability rather than through more permanent anatomical or morphological modifications (Chalker-Scott 2002). Interestingly, the anthocyanins also fulfill the less common but important function of avoiding the photodegradation of sensitive molecules. The *Ambrosia chamissonis*, for example, hold strands of laticifers surrounded by an anthocyanin sheath. These laticifers contain thiarubrines, toxic chemicals that are believed to deter herbivory and prevent both fungal and bacterial infections. The thiarubrines are photolabile molecules and are degraded by both visible and UV light giving thiophenes that are less toxic. Page and Towers (2002) have shown that the anthocyanin sheath, by absorbing a proportion of the rays that would otherwise strike the laticifers, protects these light-sensitive defensive chemicals from degradation, and thus provides a mechanism for the antiherbivory under conditions of strong sunlight.

However, although the role of anthocyanins in protecting plant tissues under stress conditions, including the photodamage mediated by both UV-B and visible light, as well as in the pollinator attractiveness and seed dispersion seems to be important, it is clearly evident that the adaptive significance of anthocyanins is still not fully understood (Close and Beadle 2003). Meanwhile two poorly explored areas became interesting: (a) how the increase of anthocyanin production is integrated to tissue responses to UV-B; (b) how the UV-B-induced anthocyanins contribute to the plant survival.

7.3 Morphogenic Responses

Studies carried out in greenhouses or in growth chambers using ultraviolet lamps and filters to simulate different solar UV-B enhancements have been conducted on a variety of terrestrial plants, including economically important crops (Santos et al. 2004) and wild plant species (Zu et al. 2010). Overall these studies showed that the UV-B enhancement besides physiological effects induces a range of morphological changes including: (a) increase/decrease of the leaf area and leaf thickness (González et al. 2002; Hilal et al. 2004); (b) reduction of the plant height (Santos et al. 2004) and increase/decrease of the shoot/root ratio (Furness and Upadhyaya 2002); (c) axillary branching (Kakani et al. 2003); (d) increase of the leaf glandular and uniseriate trichome density (Liakopoulos et al. 2006); (e) deposition of the waxy surface structures (Fukuda et al. 2008); (f) opening of the cotyledon curling (Boccalandro

et al. 2001; Barnes et al. 2005); (g) inhibition of the hypocotyl and stem elongation (Shinkle et al. 2004; Gerhardt et al. 2005); (h) premature leaf senescence (Pradhan et al. 2006). The effects of UV-B also include changes (increase/decrease) in the number and size of flowers as well as in the size of seeds (Kakani et al. 2003; Qaderi and Reid 2005). While some of the UV-B responses constitute a stimulation of the growth (e.g., axillary branching, leaf thickening), others reflect a growth inhibition (e.g., reduced hypocotyl elongation). However, in these experimental setups, frequently unrealistic balances between UV-B/UV-A/PAR are obtained, and in some cases the plants have been exposed to relatively high short-term doses of UV-B, which lack the ecological relevance (Newsham and Robinson 2009). Additionally, the levels of UV-A or PAR as well as other experimental conditions also affect the morphogenic responses, making it difficult to compare the results from different indoor studies. In addition, it is clear that not all the plant species respond in the same way to UV-B exposure (Pliura et al. 2008). In general, the monocots are more morphologically responsive to UV-B than the dicots (Pal et al. 1997). Closely related species or ecotypes, especially when occupy different habitats, also differ with respect to their morphogenic responses (Hofmann et al. 2003). Plant species also differ in the use of PAR and UV-B radiation; while some species use the PAR to trigger responses others use the UV-B radiation. Then the plants responding mainly to PAR radiation will probably be more sensitive to UV-B radiation than the UV-responding ones (Rozema et al. 2005). A critical factor in the UV-B studies is the visible light irradiance, which in growth chambers and greenhouses can be quite different to the natural sunlight (Flint et al. 2009). Indeed, it has been shown that as a result of the insufficient visible wavelengths and, therefore, of unrealistically high UV-B/PAR ratios in indoor studies, the morphogenic effects of the UV-B radiation are magnified (Musil et al. 2002a, b). In fact, even if realistic levels of the UV-B radiation in simulating ozone reductions are used the indoor responses of plants to UV-B radiation may be quite variable and exaggerated in relation to the field. Microclimatic conditions and the interactions of different abiotic and biotic environmental factors additionally contribute to inconsistency between the results obtained in growth chambers or greenhouses with those obtained under the field conditions (Flint et al. 2003; Caldwell et al. 2007). Furthermore, the plant responses to above ambient UV-B radiation (e.g., from stratospheric ozone depletion) have rarely been assessed in the broader context of the possible effects emerging from variations in the UV-B radiation within the *ambient range*. Also there is a significant knowledge gap between field and laboratory studies, which has two major components: (a) the occurrence of certain effects of the UV-B radiation under laboratory conditions has not yet been demonstrated in the field studies; (b) although some indoor responses are known to occur in the field, their functional implications are still unclear. Therefore, the obvious corollary from greenhouses or growth chambers studies is: *study methodologies are as varied as results*. In fact, from the field grown plants, the consensus that effects of artificially changed spectral UV-B irradiances are less pronounced (Searles et al. 2001). While under UV-B enhancement among other changes, leaf thickness, reduced leaf area, decreased plant height, changes in plant architecture, and biomass/yield reduction have been observed (Searles et al. 2001; Flint et al. 2003; Barnes et al. 2005). Nevertheless, the more recent studies have suggested that in the field, primary effects of the most realistic solar UV-B enhancements are subtle morphological and chemical changes with altered carbon partitioning and allocation, but doubt reveals such changes show significant effects on both plant growth and biomass accumulation (Gilbert et al. 2009; González et al. 2009; Morales et al. 2010; Ren et al. 2010; Zu et al. 2010). The morphogenic effects of the realistic UV-B enhancements are not usually considered as primary ecological factors influencing both species abundance and species distribution in relation to other abiotic environmental factors (e.g., drought, temperature, salinity). There are, however, situations where the UV-B-induced morphogenic effects can be ecologically important, giving changes in the

competitive ability with a significant impact on the composition of the plant community (Flint et al. 2003). The UV-B enhancement alters the leaf angle and differential transmission, and absorbance of the UV-B radiation through stands of erectophilous or planophilous plant species may have an important consequence on terrestrial plant responses to the UV-B radiation (Rozema 2000). In a model study, it was predicted that a more planophilous leaf angle in erectophilous species would reduce the UV-B/PAR ratio and therefore the UV-B damage. Of course, this effect may affect the competitive relations among species and also the ecosystem composition (Deckmyn 1996 in Rozema et al. 1997). The morphogenic effects often can be pronounced on different organisms at other trophic levels (Bassman 2004). The UV-B radiation also affects the decomposition of plant materials into ecosystems. Plants grown under the enhanced solar UV-B showed a reduced rate of the litter decomposition when compared to control plants grown under the ambient solar UV-B. The accumulation of UV-B-induced lignin and/or tannin accounts for the reduced litter decomposition rate (Cybulski et al. 2000). Nevertheless, the reduced rate of the litter decomposition can be produced as consequence of detrimental effects of the enhanced UV-B radiation on decomposing fungi and other decomposer organisms (Pancotto et al. 2003). In opposite trend, the plant litter material exposed to the enhanced solar UV-B can be decomposed by photodegradation more rapidly than under the ambient solar UV-B (Gallo et al. 2006). Moreover, it has also been demonstrated that the species growing for several generations under enhanced UV-B radiation show accumulation and exacerbation of the UV-B effects and likelihood they might be heritable (Mpoloka et al. 2007).

Much of the UV-B research on terrestrial plants has concentrated on vegetative plant parts, but fitness of the organisms depend mainly on their successful reproduction. Of particular concern is the detrimental effect of UV-B on the pollen quality observed for some species (Koti et al. 2004; He et al. 2007). This finding suggests that pollination may be an ecologically critical developmental stage vulnerable to the UV-B damage, even in the UV-B-tolerant species. The pollen surface of some species may transmit up to 20% of the incident UV-B radiation (Stadler and Uber 1942 in He et al. 2007), despite the presence of a variety of UV-B-absorbing pigments (Rozema et al. 2001). Thus, the mature pollen grains are potentially susceptible to the UV-B damage during a short period between the dehiscence of anthers and the penetration of the pollen tube into the stigmatic tissue (Koti et al. 2004). This fact may lead to both reduced pollen quality and altered patterns of competition among species affecting the composition of the ecosystem. Furthermore, the UV-B enhancement can alter the production and/or the temporal availability of flowers so as to make the plant a less attractive host for the pollinators and impinge upon competition of plants for the pollinator service, as well as on the reproductive success of the plant/pollinator system (Sampson and Cane 1999).

8 UV-B Radiation and Signaling Pathways

The UV-B radiation triggers diverse responses involving a differential regulation of the genes and participate in several protective pathways including the DNA repair, detoxification of ROS, and production of secondary metabolites as well as in photomorphogenic events (Agrawal et al. 2009). The UV-B responses can be elicited with either high fluence (HF-UV-B, over 15 kJ m^{-2}), intermediate fluence (IF-UV-B, 5–7 kJ m^{-2}), low fluence (LF-UV-B, 1–3 kJ m^{-2}), or very low fluence (VLF-UV-B, less than 1 kJ m^{-2}) (Brosché et al. 2002). However, the exposure of plants to low fluence UV-B promotes the expression of varying genes involved in the UV-B protection, and genes responsible for the production of flavonoids and several phenolic compounds, while as the low fluence photomorphogenic responses seem to be initiated by photoreceptors and no alternative UV-B-absorbing molecules seem to mediate the photomorphogenic UV-B responses (Ulm and Nagy 2005). Also, many components of the protective pathways which lead to the changes of the gene expression in response to

both UV-B radiation and pathogens are similar or identical (upregulation of *PDF1.2*), except the pathways which are distinct (upregulation of *PR-1*), signifies the effects of the high fluence UV-B radiation on the gene expression are unlikely to be due to nonspecific damage and a yet unidentified UV-B photoreceptor (Brosché et al. 2002). The response of plants to UV-B radiation depends upon the adaptation and acclimation to UV-B irradiances, as well as of the interactions with other environmental factors. Moreover, studies carried out with *Arabidopsis* plant suggest that some genes are differentially responsive to UV-B in both 280–290 nm and 300–310 nm ranges, hence could be multiple UV-B photoreception mechanisms (Kalbina et al. 2008). Consequently, the exposure of plants to UV-B radiation can cause multiple responses on the primary and secondary metabolisms as well as different changes on the growth and overall performances. Although other light-dependent photoreceptors (e.g., phytochrome, cryptochrome) have been described (Carvalho et al. 2010), presently a UV-B-specific photoreceptor has still not been described and therefore the basic mechanism of UV-B perception and the signal transduction remain still poorly understood. Nevertheless, the chromophores that could act as photoreceptors to absorb the UV-B radiation exist. Pterins or flavins in their reduced forms are candidates where some experimental work supports the involvement of the perception of UV-B radiation (Galland and Senger 1988a, b in Jenkins 2009). For example, the compounds that antagonize the flavins and pterins impair the UV-B induced anthocyanin synthesis in maize (Jenkins 2009) along with UV-B suppression of the hypocotyl elongation in tomato plants (Ballaré et al. 1995). In addition, other possible chromophore can be a phenolic molecule, with this assumption the *p*-coumaric acid chromophore present in the photoactive yellow protein (PYP), a photoreceptor found in the purple photosynthetic bacteria *Ectothiorhodospira halophila*, enables to absorb in UV-A and blue regions of the solar radiation spectrum (Imamoto and Kataoka 2007). Moreover, an alternative possibility is that the UV-B radiation be sensed through some form of direct activation of a cellular component. Whether or not the UV-B photoreceptor exists the responses to UV-B radiation could be mediated by nonspecific signaling pathways involving the DNA damage, ROS production, hormone synthesis (e.g., salicylic acid, ethylene, jasmonic acid), and wound/defense signaling molecules (e.g., flavonoids, phenolics) (Apel and Hirt 2004; Demkura et al. 2010); or by specific UV-B signaling pathways mediated by the UV-B-specific component UV RESISTANCE LOCUS8 (UVR8) (Cloix and Jenkins 2008.). The UVR8 acts specifically to mediate the UV-B response, together with the expression of genes to establish the UV-B protection (Jenkins 2009). Moreover, the UVR8 also mediates the expression of genes activated at low UV-B fluence level, showing consistency with their involvement in the photomorphogenic UV-B signaling pathway (Brown and Jenkins 2008). No other component is known to act specifically in the photomorphogenic UV-B responses. The transcriptome analysis revealed that a set of approximately 70 indentified genes are stimulated by UV-B under control of the UVR8. Among these several genes are known to have key roles in the UV-B protection mechanism, including those encoding principal enzymes of the flavonoid biosynthetic pathway, as well as DNA photolyases and enzymes involved in amelioration of the photooxidative damage (Jenkins 2009). The findings demonstrate that the *Arabidopsis UVR8* mutant shows severe necrosis under exposure to UV-B levels found in the bright sunlight, whereas it is indistinguishable from the wild type in the absence of UV-B (Brown and Jenkins 2008). The UVR8 regulates the expression of both ELONGATED HYPOCOTYL5 (HY5) and HY5 HOMOLOG (HYH) transcription factors at low UV-B fluence levels. The transcriptome analysis shows approximately the half of genes regulated by the UVR8 is also regulated by the HY5 transcriptor factor, but this is an underestimate and does not take into account the functional redundancy between HY5 and HYH (Brown et al. 2005). Further analysis, however, suggests that the HY5 and HYH transcription factors may regulate all the genes of the UVR8 component, and therefore are pivotal downstream

effectors of the UVR8 signaling pathway. In fact, the HY5 is evidently a very important regulator of the UV-B responses because the *HY5* mutant, similar to the *UVR8* mutant, is very sensitive to UV-B, while the *HYH* mutant is less sensitive indicating that it has a subsidiary role (Brown and Jenkins 2008). These findings demonstrate that the UVR8 is a key regulator of the UV-B protection and therefore helps to promote the survival of terrestrial plants exposed to UV-B radiation (Jenkins 2009).

Another important component of the low UV-B signaling pathway is the CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) (Oravecz et al. 2006). Contrarily to UVR8, the COP1 represses the expression of photomorphogenic genes and the plant development in the darkness. The COP1 acts as an E3 ubiquitin ligase, destroys HY5 and other positive regulators of the expression of photomorphogenic genes (Yi and Deng 2005). Following illumination, the COP1 is inactivated and moves slowly out of the nucleus, enabling the HY5 and other transcription factors to accumulate and promote the photomorphogenesis. In contrast to this function, the COP1 is a positive regulator of the UV-B responses such as the accumulation of flavonoids. Nevertheless, nearly half of genes regulated by the COP1 are controlled by HY5, indicating HY5 a key effector of the COP1 pathway. Both COP1 and HY5 transcriptor factors must act together in the nucleus to evoke the UV-B responses. Furthermore, the positive role of the COP1 seems not to be specific to UV-B because some evidences show a comparable function in several responses to the red light that require the involvement of the phytochrome B. With regard to this theory, the COP1 is required for the nuclear accumulation of the transcriptor factor PHYTOCHROME INTERACTING FACTOR3 (PIF3) in the darkness, although it does not mediate its destruction following the red and far-red illumination (Oravecz et al. 2006). Moreover, a more recent study showed that exposure to supplemental far-red (FR) light compared to red (R) light under UV-B radiation leads to a fast elongation growth and a phenolic accumulation in leaves of the silver birch seedlings (Tegelberg et al. 2004). Whether the COP1 acts positively in other light responses, however, still remains unknown (Jenkins 2009). On the other hand, both UVR8 and COP1 regulate many of the same genes and are required for the low fluence UV-B induction of the HY5 transcriptor factor expression which plays a central role in the regulation of genes involved in the photomorphogenic UV-B responses (Brown and Jenkins 2008). Although both UVR8 and COP1 seem to function in the same pathway, little information is available to explain their functional relationships. Since UVR8 is a UV-B specific component, it may have a direct action of the COP1 in UV-B responses. To explain this fact one possibility is that the UVR8 regulates the nuclear accumulation of the COP1 or vice versa, while another can be that the UVR8 recruits the COP1 into a complex involved in the regulation of the transcription by UV-B. The last hypothesis is supported by a recent study demonstrating that the UVR8 colocalizes with the COP1 and directly interacts with a UV-B-dependent manner (Favory et al. 2009). Besides these, the expression of some genes at low UV-B fluence levels occurs independently of the action of both UVR8 and COP1 components (Jenkins 2009).

According to data of A-H-Mackerness et al. (2001), the expression of genes by intermediate UV-B fluence levels (IF-UV-B) may be regulated partly by the enzymatic ROS formation after the specific UV-B induction, whereas the changes in mRNA levels of the high fluence (HF-UV-B) genes could be due to the formation of ROS as a result of the nonspecific damage to plant cells. However, it seems unlikely that sufficient ROS would be generated by the exposure of plants to the current ambient solar UV-B to cause the activation of the signaling pathway leading to biosynthetic responses; thus, presumably, the activation by ROS would not be UV-B specific (Jenkins 2009). Nevertheless, evidence exists for the involvement of ROS in some morphological changes and gene expression responses initiated by the UV-B radiation. Furthermore, the *Arabidopsis RADICAL-INDUCED CELL DEATH1* (RCD1) transcriptor factor is also involved in the UV-B signaling pathway. Interestingly, the expression of *RCD1* genes is

not significantly changed by the UV-B radiation. Previous study has shown that the SALT TOLERANCE (STO) protein is interacting with RCD1 in vitro being the mRNA level of the *STO* (*SALT TOLERANCE*) gene greatly increased in the *Arabidopsis rcd1-1* mutant after UV-B irradiation. However, the expression of UV-B-induced *HY5* and *CHS* (CHALCONE SYNTHASE) genes is partially inhibited in the *STO* mutant, subsequently, seems to be the RCD1, together with the STO, involved in the *Arabidopsis* UV-B signaling (Jiang et al. 2009).

The cotyledon curling in *Brassica napus* stimulated by both UV-B and H_2O_2 is also inhibited by ascorbate (Gerhardt et al. 2005). In addition, the exposure to relatively high fluence rates of UV-B decreases the abundance of transcripts of the *Arabidopsis LHCB1* gene that encodes the major chlorophyll binding protein of the chloroplast, and this response is inhibited by ascorbate as well as by a scavenger of superoxide radicals (A-H-Mackerness et al. 1999). Interestingly, supplementation of the ambient UV-B under greenhouse conditions increased the formation of CPDs and reduced the leaf area in *G. magellanica*, however did not cause lipid peroxidation being the modulation of the ascorbate content that counters the oxidative stress (Giordano et al. 2004).

The exposure to UV-B stimulates the expression of a set of genes normally induced in response to the pathogen attack, insect predation, and wounding (Stratmann 2003; Ulm and Nagy 2005). It also reduces the level of insect herbivory in a range of species, probably because of increased production of the secondary metabolites, proteinase inhibitors, and other molecules that deter the herbivorous insects (Ibañez et al. 2008). An explanation for the overlap in responses to UV-B, wounding, and pathogens is that the UV-B stimulates the accumulation of ROS and other signaling molecules (e.g., jasmonic acid, ethylene, salicylic acid) that mediate the wounding defense responses (Izaguirre et al. 2007; Demkura et al. 2010). Molecules mediating the responses of some UV-B-regulated genes have been reported (Izaguirre et al. 2003). Alterations in the induction of defense-related genes by the UV-B radiation have been observed in both ethylene *ETR-1* and jasmonic acid *JAR1 Arabidopsis* insensitive mutants (A-H-Mackerness et al. 1999; Jenkins 2009). Also a transgenic *Arabidopsis* plant expressing the salicylate hydroxylase was unable to accumulate salicylic acid and showed a reduced UV-B induction of the several *PR* genes (Surplus et al. 1998). The expression of genes related to the pathogenesis-related proteins (PR) and class I Endo-β-1,3-glucanases (IβGlus I) are also induced by the UV-B radiation (Kucera et al. 2003). The PR genes have been grouped as: (a) intermediate UV-B level genes (PR-5); (b) high UV-B level genes (like PR-1). The IβGlus I are also assigned to PR proteins and constitute the PR-2 family. In addition, it has been demonstrated that the UV-B-induced DNA damage seems to be related to induction of the IβGlus I genes, but not with the synthesis of flavonoids under high levels of UV-B radiation (Kucera et al. 2003). The involvement of ROS in defense signaling is well established and evidences show that superoxide generated by the plasma membrane NADPH oxidase seems to be involved in UV-B-induced regulation of some defense genes, either directly or through the production of H_2O_2 (A-H-Mackerness et al. 2001). In agreement with this assumption, it has been reported that both redox activity of the plasma membrane and cytosolic-free Ca^{2+} homeostasis are involved in the induction of gene expressions by UV-B and blue/UV-A wavelengths in *Arabidopsis* plants (Long and Jenkins 1998). Although the available data supports that ROS might be used by plants to modulate the expression of different genes in response to varying levels of UV-B, little information is available regarding the scope and nature of the ROS production and function in response to the solar UV-B radiation under natural growth conditions. However, the ROS are very dangerous to the cellular integrity and must be eliminated (Agrawal et al. 2009). Most plants scavenge the excessive amounts of ROS using a combination of enzymatic scavengers such as superoxide dismutase, ascorbate peroxidase and glutathione reductase, and nonenzymatic scavengers such as ascorbate, glutathione, carotenoids, tocopherols, and secondary metabolites (mainly flavonoids, hydroxycinnamic acids derivatives, and anthocyanins) (Xu et al. 2008).

Although it is clear that the UV-B radiation stimulates both defense and wound signaling, there is little information on how the UV-B activates components of the signaling pathways. Experiments in tomato indicate that the UV-B radiation initiates similar signaling processes to systemin, an 18-aminoacid peptide that stimulates the wound responses (Ulm and Nagy 2005). Although data suggests that the tomato systemin receptor might also function as a brassinosteroid receptor (Wang and He 2004), more recent evidences indicate otherwise (Holton et al. 2007). In fact, a better knowledge of the mechanisms involved in the signaling pathways can help to understand the functional roles of the solar UV-B radiation in the resistance of plants to environmental factors into the terrestrial ecosystems.

9 Reduced Solar UV-B: A Future Scenario?

Despite the influence that the ambient level of solar UV-B radiation can exert on the plant life, recent findings have shown that the increases of UV-absorbing tropospheric gases (e.g., ozone, SO_2, NO_2) and aerosols can reduce the amount of solar UV-B radiation reaching the Earth's surface (McKenzie et al. 2001). Thus, a further attention is needed to understand how the reduced solar UV-B can have an effect on the dynamics of ecosystems. Similar cues can occur when species originated from highland regions such as mountains (Ren et al. 2010), the Bolivian Altiplano (González et al. 2009) and the Tibet Plateau (Yang et al. 2008) or those from the equatorial regions that naturally receive high UV-B irradiances, are grown in lowland areas and/or high latitudes (Turunen and Latola 2005).

So far the ecological cost of terrestrial plant responses to the solar UV-B radiation has not been studied in detail. Clearly, some morphological and physiological responses must have a cost in terms of resources that in absence of the UV-B could have been allocated elsewhere (Snell et al. 2009). Under this perspective seems to be an interesting point to study the question *how the terrestrial plants respond to the solar UV-B radiation growing under UV-B-reduced irradiances*, because till now data on this topic are very scarce. Like morphological features, emerging findings revealed that the UV-absorbing compounds could be affected by the reduced solar UV-B (Ibañez et al. 2008; González et al. 2009). In this context, the removal of UV-B from the natural solar radiation causes large increases of the growth in *Glycine max* and *Cyamopsis* plants and a marginal increase in the *Vigna radiata* but did not affect the growth of *Vigna mungo* plants (Varalakshmi et al. 2003; Amudha et al. 2010). In addition, it has also been demonstrated that the photomorphogenic regulatory mechanisms, rather than the photosynthesis seems to play key roles in the observed metabolic changes upon exposure to the reduced solar UV-B (Kadur et al. 2007). In agreement with these findings, the prolonged treatment with chronic low doses of UV-B caused changes in the morphology, gene expression, and biomass redistribution without cessation of the growth and in absence of the stress symptoms (Hectors et al. 2007). In fact, the reduced solar UV-B induces probably a plethora of key enzymes into the metabolic pathways transmitting a general plant response, which under a future long-time solar UV-B-reduced scenario will probably affect the plant productivity, species competition, trophic interactions, and ultimately the structure of ecosystems.

10 Conclusion and Future Perspective

The enhanced UV-B radiation produced important physiological and morphological effects on the terrestrial plants, but most of these studies were carried out in greenhouses and growth chambers. Then the extrapolation and quantification of the observed indoor effects to field experiments is very complex, because they can reflect exacerbated responses of the plants and confuse the interpretation of physiological and morphogenic responses, as well as the molecular analysis at both individual species and ecosystem composition. In fact, much remains to be done to define and establish the effects of both increased and

reduced solar UV-B, as well as the signaling pathways to understand how they may be integrated to terrestrial plants growing in the natural environment. *However, although in the solar UV-B alchemy, each successive understanding produces a larger doubt, God does not play dice with the universe! (Einstein).*

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K^+ Nutrition, Uptake, and Its Role in Environmental Stress in Plants

4

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Abstract

The increasing world population makes high yield crop production a necessity in future agriculture. However, the negative effects of the emission of greenhouse gases into Earth's atmosphere and the resulting climate change may impede reaching this goal. New stresses will appear and the existing ones will be exacerbated. Important plant processes such as the acquisition of K^+, which is an essential macronutrient for plants, will be negatively affected. The development of new crop varieties with enhanced capacities in the acquisition of K^+, especially under the future environmental conditions, is an important challenge. One of the first steps may be the identification of the K^+ uptake systems operating in the roots, which may be later improved to enhance K^+ acquisition under stress conditions. Some gene families encoding K^+ transporters, that is, the HAK1-type, and channels, that is, the AKT1-type, key pieces for root K^+ uptake, have been identified. Members of other families of transport systems, such as the cation proton antiporter (CPA) family, or the cyclic nucleotide-gated channel (CNGC) family may also participate in that process. The use of T-DNA insertion lines in the model species *Arabidopsis thaliana* has allowed the demonstration of the role of some of these transport systems and, in some cases, the results obtained in the model plant also apply to crop species. Important points of the regulation of these transport systems are found at the transcriptional and the translational level. Internal K^+ concentrations, plasma membrane potential, reactive oxygen species (ROS), hormones, kinases, and phosphatases are involved in their regulation. The knowledge accumulated to date and that to be obtained in the future could be used in biotechnological approaches to produce more efficient K^+ transporters that endow plants with enhanced performance to face the future environmental challenges. Genetic engineering, natural variability, and the development of -omic technologies are valuable tools for the achievement of these objectives.

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1 Introduction

The increasing world population makes high yield crop production a necessity in future agriculture. One important factor that will affect agriculture worldwide and that prominent climate scientists have been warning of is the dangerous effects of the continual emission of greenhouse gases into Earth's atmosphere. Atmospheric CO_2 concentration, average temperature, and tropospheric ozone concentration will be higher in the near future. Droughts will be more frequent and severe, more intense precipitation events will lead to increased flooding, some soils will degrade, and climatic extremes will be more likely to occur. Existing abiotic stresses such as salinity will be exacerbated. All these changes will produce an environmental stress for plants and will have many important implications for plant physiology and crop yield. Among the processes that will be altered, K^+ nutrition deserves special attention. K^+ is an essential macronutrient that is absorbed from the soil solution by the roots. Environmental stress may reduce K^+ availability and at the same time may produce physiological alterations that impair K^+ acquisition. The development of plant varieties with increased capacity to absorb K^+ may contribute to reducing the negative effects of the abiotic stress conditions derived from climate change. Characterizing the systems involved in the process of K^+ absorption and how they are regulated and the effect that abiotic stresses will have on their functioning will help to obtain the required new plant varieties (Fedoroff et al. 2010).

2 Role of K^+ in Plants

K^+ is an essential macronutrient for plants, composing up to 10% of the total plant dry weight (Taiz and Zeiger 1991) and fulfilling important functions in metabolism, growth, and stress adaptation.

K^+ functions can be classified into those that rely on high and relatively stable concentrations of the nutrient in certain cellular compartments or tissues and those that rely on its movement between different compartments, cells, or tissues (Amtmann et al. 2004). The first class of functions includes enzyme activation, stabilisation of protein synthesis, neutralization of negative charges on proteins, and maintenance of cytoplasmic pH homeostasis (Marschner 1995). Other roles of K^+ are linked to its high mobility. This is particularly evident where K^+ movement is the driving force for osmotic changes – as, for example, in stomatal movement, light-driven and seismonastic movements of organs, and phloem transport. In other cases, K^+ movement provides a charge balancing counter-flux essential for sustaining the movement of other ions. Thus, sugar, amino acid, and NO_3^- transport can be accompanied by a flux of K^+ (Marschner 1995).

The most general phenomenon that requires directed movement of K^+ is growth. Accumulation of K^+ (together with an anion) in plant vacuoles creates the required osmotic potential for cell expansion which relies on its high mobility, and therefore only few other inorganic ions can replace K^+ in this role (Reckmann et al. 1990). Once cell growth has come to a halt, maintenance of osmotic potentials can be carried out by less mobile sugars, and K^+ ions can be partially recovered from vacuoles (Marschner 1995; Poffenroth et al. 1992).

2.1 Range of K^+ Levels Inside the Plant

As for other nutrients, three ranges in internal K^+ concentrations can be distinguished (Marschner 1995): a deficient, an adequate, and a toxic range. K^+ internal levels found in plants may vary depending on the tissue considered and the

nutritional status of the plant, but they are always in the millimolar range. An optimum K^+ concentration of around 50–100 mM in the cytosol is required for the performing of the functions mentioned above (Jones 1983). Many of the K^+-activated enzymes require K^+ at concentrations between 10 and 50 mM to achieve their maximum activity and plant cells keep the cytosolic K^+ concentration around this value (Walker et al. 1996). In contrast, vacuolar concentrations are more flexible and mirror the external supply of K^+. Under K^+ limiting conditions, cytosolic K^+ activity can be efficiently maintained around its optimum value due to the release of vacuolar K^+ ions into the cytosol. Once the electrochemical gradient from the vacuole reaches its limit, cytosolic K^+ activity starts to decrease (Nieves-Cordones et al. 2008; Walker et al. 1996, 1998).

K^+ homeostasis is not necessarily effective in all cell types. In barley epidermal leaf cells, cytoplasmic K^+ levels as low as 15 mM have been reported under saline conditions (Cuin et al. 2003). These cells were still alive but they probably showed very low metabolic activity. Indeed, low epidermal K^+ concentrations reflect selective tissue allocation of K^+ within leaves, which allows the plant to protect metabolically active mesophyll cells against K^+ deficiency.

2.2 Problems Related to K^+ Deficiency

Although current agriculture is based on high levels of fertilization, soils of some intensively cultured lands may became K^+ deficient (Dobermann et al. 1998; Pal et al. 2001; Rengel and Damon 2008; Yang et al. 2004) because of the withdrawal of K^+ during crop harvests (Pal et al. 2001) or due to K^+ losses as a result of lixiviation in sandy soil (Kayser and Isselstein 2005). In other cases, the importance of K^+ has sometimes been overlooked, and financial constraints have forced farmers to prioritize applications of nitrogen (N) over K^+ (Armengaud et al. 2009). As a result, a considerable area of farmland has become K^+ deficient (Andrist-Rangel et al. 2007; Dobermann et al. 1998; Hoa et al. 2006).

K^+ deficiency entails important effects on plant physiology such as limited cellular expansion and reduction of photosynthesis which ultimately results in growth reduction and development impairment. When external K^+ is limiting, its translocation from mature leaves and stem is activated. Under conditions of severe deficiency, these organs become chlorotic and even necrotic. At the cellular and metabolic levels, important negative effects are observed. It has been reported that in barley, K^+-limiting conditions give rise to a decrease in K^+ activity and pH in the cytosol of root cells (Walker et al. 1996). These reductions positively correlated with a decrease in protein synthesis and a subsequent decline in growth (Walker et al. 1998). At the metabolic level, increase in sugars and depletion of pyruvate has been described in roots and shoots (Armengaud et al. 2009). Other changes also take place to (1) maintain carbon flux into amino acids and proteins, (2) decrease negative metabolic charge, and (3) increase the nitrogen–carbon ratio in amino acids. In addition, K^+-deficient plants are more susceptible to abiotic and biotic stresses such as drought, cold, salinity, and fungal attacks (Marschner 1995).

3 K^+ Uptake Systems

Root K^+ uptake occurs through specialized epidermal cells (root hairs) which increase the surface in contact with the soil solution as well as through cortical cells. K^+ must get through root tissues to reach the stele where the xylem vessels will distribute K^+ to the rest of the plant.

There are two possible parallel pathways that K^+ can follow to reach the stele: the apoplastic and the symplastic pathways. The apoplastic pathway is blocked by the Casparian strip that limits K^+ flux into the stele. Therefore, K^+ needs to enter into the symplast to reach the xylem vessels by crossing the plasma membrane of an epidermal or cortical root cell and through K^+ transport systems of different capacities, with the latter constituting crucial pieces in the control of K^+ acquisition.

3.1 High- and Low-Affinity K^+ Uptake

In the 1950s, of last century Epstein reported that in barley roots, K^+ uptake exhibited biphasic kinetics in response to increasing external K^+ concentrations (Epstein and Hagen 1952; Epstein et al. 1963). The first system showed high-affinity for K^+ (K_m for K^+ of 21 μM) and was not inhibited by Na^+. The second system showed low-affinity for K^+ (K_m of 11.4 mM) and was inhibited by Na^+. In maize roots, the low-affinity component was linear (Kochian and Lucas 1982), and traditionally, it has been thought that ion channels are mostly responsible for the low-affinity component (Maathuis and Sanders 1996).

The high-affinity system has been thought to be mediated by transporters because with the reported data on plasma membrane potentials and gradients for K^+ concentrations for barley and *Arabidopsis* (Maathuis and Sanders 1993, 1994; Walker et al. 1996), a channel would not be operative for K^+ influx. A K^+:H^+ symport with a 1:1 stoichiometry has been suggested as the transport mechanism. In *Neurospora crassa*, a fungus which exhibited high-affinity K^+ uptake similar to that present in plants, the high-affinity K^+ uptake was shown to be mediated by a K^+:H^+ symport (Rodriguez-Navarro et al. 1986). In plant cells, as in fungal cells, the membrane potential is sustained by the H^+-ATPase, supporting the idea of the plant K^+:H^+ symport. Other features observed in the high-affinity K^+ uptake in plants are the upregulation by K^+ starvation, the lack of discrimination between K^+ and Rb^+ and the inhibition by NH_4^+ (Kochian and Lucas 1988; Maathuis and Sanders 1996; Rodríguez-Navarro 2000; Rodríguez-Navarro and Rubio 2006).

3.2 Molecular Entities Mediating Low- and High-Affinity K^+ Uptake

3.2.1 Shaker Channels

The identification of the genes involved in K^+ uptake began in the 1990s by using the functional complementation of *Saccharomyces cerevisiae* mutants defective in the endogenous K^+ uptake systems *TRK1* and *TRK2*. In 1992, the Arabidopsis *Shaker* K^+ channels *AKT1* and *KAT1* were isolated (Schachtman 1992; Sentenac et al. 1992). Both AKT1 and KAT1 did not exhibit inactivation through time which suggested that they mediated long-term K^+ supply (Gaymard et al. 1996; Schachtman 1992). *AtAKT1* expression was preferentially localized in the peripheral cell layers of the root mature regions, which was consistent with a role of AtAKT1 in root K^+ uptake (Lagarde et al. 1996). However, *AtKAT1* is mainly expressed in leaves discarding its participation in this process (Szyroki et al. 2001).

After the cloning of *AtAKT1*, several cDNAs encoding K^+ channels with homology to AKT1 were obtained from other species. For instance, SKT1 from potato (Zimmermann et al. 1998), LKT1 from tomato (Hartje et al. 2000), TaAKT1 from wheat (Buschmann et al. 2000), OsAKT1 from rice (Golldack et al. 2003), DKT1 from carrot (Formentin et al. 2004), CaAKT1 from pepper (Martínez-Cordero et al. 2005), NKT1 from tobacco (Sano et al. 2007), and VvK1.1 from grapevine (Cuellar et al. 2010).

Intriguingly, another cDNA from Arabidopsis, *AtKC1*, encoding a protein with high homology to K^+ channels was cloned, but it never exhibited ion currents when expressed alone in heterologous expression systems (Reintanz et al. 2002). Later, it was shown that AtKC1 together with the syntaxin SYP121, regulated AtAKT1 function by forming a ternary complex (Honsbein et al. 2009) (see below). Homologues of AtKC1 have also been found in other plant species (Downey et al. 2000; Wang et al. 2002).

3.2.2 HAK/KT/KUP Transporters

High-affinity K^+ uptake in plants resembles that mediated by SoHAK1, a high-affinity K^+ transporter from the yeast *Schwanniomyces occidentalis*. By using an RT-PCR approach, a plant homolog, HvHAK1, was isolated from barley (Santa-María et al. 1997). Yeast expression of HvHAK1 demonstrated that it was a high-affinity K^+ transporter that did not discriminate between K^+ and Rb^+ and was inhibited by Na^+ and NH_4^+, in agreement with the characteristics of high-affinity

K^+ uptake previously described in barley roots. Homologous transporters were later isolated in Arabidopsis (Fu and Luan 1998; Kim et al. 1998; Quintero and Blatt 1997). One of them, AtHAK5, showed a high homology to HvHAK1 and no discrimination between K^+ and Rb^+ (Rubio et al. 2000). Phylogenetic analyses show that the HAK family (also named KT or KUP family) of transporters is composed of four clusters and that all high-affinity K^+ transporters from this family characterized so far belong to Cluster I. These Cluster I transporters are encoded by genes that are rapidly upregulated in root cells when the supply of the external K^+ is removed (Rodríguez-Navarro and Rubio 2006). When expressed in yeast they mediate K^+ uptake of similar characteristics to plant high-affinity K^+ uptake such as low K_m values for K^+ or sensitivity to NH_4^+, suggesting that they are major components of this system (Rodríguez-Navarro and Rubio 2006).

After *AtHAK5* cloning, numerous genes encoding K^+ transporters of the HAK family were identified in other plant species: 17 genes in rice (Bañuelos et al. 2002), *GsKT1* in cotton (Ruan et al. 2001), *LeHAK5* in tomato (Wang et al. 2001, 2002), *CnHAK1* and *CnHAK2* in *Cymodocea nodosa* (Garciadeblas et al. 2002), four genes in *Mesembrianthemum cristallinum* (Su et al. 2002), *LjKUP* in *Lotus japonica* (Desbrosses et al. 2004), *VvKUP1* and *VvKUP2* in *Vitis vinifera* (Davies et al. 2006), *AlHAK* in *Aeluropus littoralis* (Su et al. 2007), five genes in *Phragmites australis* (Takahashi et al. 2007b, c), *CaHAK1* in pepper (Martínez-Cordero et al. 2004), and *ThHAK5* in *Thellungiella halophila* (Alemán et al. 2009b).

3.2.3 HKT Transporters

In 1994, a wheat cDNA, *TaHKT1*, was isolated, and it encoded a protein with homology to the TRK transporters from yeast (Schachtman and Schroeder 1994). Its heterologous expression showed that TaHKT1-mediated high-affinity K^+ uptake coupled with Na^+ symport in addition to low-affinity Na^+ uptake (Rubio et al. 1995). Because Na^+-activated high-affinity K^+ uptake has never been demonstrated in higher plants, the involvement of this system in high-affinity K^+ uptake seems unlikely. Later studies have involved HKT transporters from different plant species to Na^+ uptake from the external solution and Na^+ movements within the plant through loading and unloading at the xylem and the phloem (Berthomieu et al. 2003; Garciadeblas et al. 2003; Golldack et al. 2002; Haro et al. 2005; Horie et al. 2007; Laurie et al. 2002; Rus et al. 2001, 2004; Uozumi et al. 2000). This has focused the research on the physiological roles of plant HKT towards adaptation to saline environments rather than K^+ nutrition.

3.2.4 Cation Proton Antiporters

This superfamily of antiporters is composed of three families: the monovalent cation:proton antiporter-1 (CPA1) family (eight members), the monovalent cation:proton antiporter-2 (CPA2) family also referred to as the CHX family (28 members), and the NhaD family (two members) (Maser et al. 2001; Saier et al. 1999). In plants, Na^+/H^+ and K^+/H^+ antiporters belonging to the first family (CPA1) are likely critical determinants of salt tolerance and K^+ homeostasis either by compartmentalization in cellular organelles, for instance NHX transporters, or by extrusion from the cell, for example SOS1 transporters (Apse et al. 1999; Leidi et al. 2010; Pardo et al. 2006; Rodriguez-Rosales et al. 2008; Shi et al. 2003). On the other hand, some members of the CPA2 family have been related to K^+ acquisition. KEA5, AtCHX17, and AtCHX13 are upregulated in K^+-starved roots (Cellier et al. 2004; Shin and Schachtman 2004; Zhao et al. 2008). AtCHX13 was indeed shown to complement a K^+ uptake deficient yeast mutant showing a K_m $K^+(Rb^+)$ of 136 μM (Zhao et al. 2008), although its expression in the root tip suggested a role in sensing external K^+ rather than mediating K^+ uptake.

3.2.5 Cyclic Nucleotide-Gated Channels

Cyclic nucleotide-gated channels (CNGCs) share structural homology with *Shaker* channels, although they lack a GYGD motif (Szczerba et al. 2009; Talke et al. 2003). Published data has shown that their activation is cGMP- and/or

cAMP-dependent and that most CNGCs do not discriminate among monovalent cations, have a limited Ca^{2+} permeability, and are blocked by NH_4^+ and external Mg^{2+} (Balague et al. 2003; Demidchik and Maathuis 2007; Leng et al. 2002). A notable exception is AtCNGC2 that exhibited a high degree of K^+ selectivity as opposed to Na^+ (Hua et al. 2003), a feature that is unknown in animal CNGCs. In particular, some CNGCs have shown features related to K^+ uptake. For instance, AtCNGC10 was shown to rescue K^+ channel mutants of *Escherichia coli* (LB650), yeast (CY162), and *atakt1* Arabidopsis plants (Kaplan et al. 2007). In heterologous systems, AtCNGC3 can function as a Na^+ and K^+ uptake pathways (Gobert et al. 2006). Moreover, promoter-driven GUS activity data has shown that, in seedlings, AtCNGC3 is mainly expressed in epidermal and cortical root tissues, something consistent with a role in K^+ uptake.

3.3 Advances in the Characterization of Entities Mediating Root K^+ Uptake

Although the characterization in heterologous systems together with gene expression patterns strongly suggested that many of the aforementioned entities participated in root K^+ uptake, a clear demonstration was still pending. It was necessary to demonstrate the transport activity of such systems in vivo, in their native environment. Furthermore, a subcellular localization compatible with K^+ uptake (plasma membrane localization) was also required.

The first successful study on this topic demonstrated that an Arabidopsis T-DNA knock-out mutant in AKT1, *atakt1-1*, had reduced plant growth in the presence of 2 mM NH_4^+ when external K^+ was below 1 mM (Hirsch et al. 1998). These results suggested that K^+ channels could also mediate K^+ uptake in the high-affinity range of concentrations. Further studies showed the presence of two components of K^+ uptake: an NH_4^+-insensitive AKT1-mediated component and an NH_4^+-sensitive component (Spalding et al. 1999). A later study showed that AKT1 fused to GFP localized to the plasma membrane in tobacco mesophyll protoplasts (Hosy et al. 2005). AtAKT1 function is regulated by the AtKC1 subunit, whose gene is strongly expressed in roots (Reintanz et al. 2002) and upregulated by salt stress in the shoot (Pilot et al. 2003). When AtKC1 is coexpressed together with AtAKT1 in heterologous systems, a shift in the activation threshold toward more negative values is observed (Duby et al. 2008; Geiger et al. 2009), probably preventing AKT1 from mediating K^+ efflux. Recently, it has been shown that AtKC1 forms a ternary complex with AtAKT1 and a syntaxin (SYP121) that mediates K^+ uptake in Arabidopsis roots (Honsbein et al. 2009). When expressed in Xenopus oocytes, the characteristics observed in this ternary complex are more similar to those observed in native Arabidopsis roots than those recorded without the syntaxin (Honsbein et al. 2009), indicating that *in planta*, the functional unit is the ternary complex.

As for HAK transporters, experiments performed with T-DNA insertion mutants in Arabidopsis demonstrated that root high-affinity K^+ uptake was impaired in the *athak5-1, athak5-2*, and *athak5-3* mutants which lacked a functional AtHAK5, (Gierth et al. 2005; Rubio et al. 2008). Moreover, in the *athak5* plants, NH_4^+ did not inhibit Rb^+ uptake, while this cation did so in WT plants, indicating that AtHAK5 mediates the NH_4^+-sensitive high-affinity K^+ uptake in plants, in agreement with the sensitivity to NH_4^+ that AtHAK5 shows when expressed in yeast (Rubio et al. 2000). Recently, it has been shown that adult *athak5-3* plants display lower plant biomass due to reduced K^+ uptake when they are grown for several weeks at 10 μM K^+ (Nieves-Cordones et al. 2010), evidencing that this transporter supports growth at very low-external K^+ concentrations. These results are in agreement with another study performed in agarose plates in which AtHAK5 mutants seedlings exhibited reduced root growth when grown below 10 μM K^+ (Qi et al. 2008). Moreover, in this study, it was also reported that AtHAK5 fused to an epitope of the human influenza virus hemaglutinin protein (HA epitope) localizes to the plasma membrane.

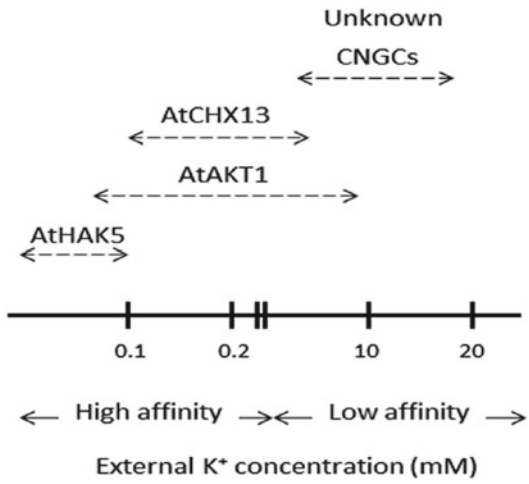

Fig. 4.1 Molecular entities participating in K^+ uptake in Arabidopsis roots. The graphic shows the external K^+ concentrations at which the high- and the low-affinity K^+ uptake systems operate. In the high-affinity range, AtHAK5 is the only system mediating K^+ uptake when the external concentration is below 10 μM. At higher concentrations, AtHAK5, AtAKT1, and AtCHX13 may be the main systems participating in this process. In the low-affinity range of K^+ concentrations, AtAKT1, AtCHX13, unidentified members of the CNGC or unknown systems could contribute to K^+ uptake. *Dashed horizontal lines* depict the range of external K^+ concentrations in which the system just above plays its important role in root K^+ uptake

As stated above, AtAKT1 also contributes to K^+ uptake in the high-affinity range of concentrations. Recent studies with single *athak5*, *atakt1* and double *athak5, atakt1* mutants in combination with NH_4^+ and Ba^{2+} that inhibit AtHAK5 and AtAKT1, respectively, have allowed the establishment of the relative contributions of each of these two systems to K^+ uptake from diluted solutions (Rubio et al. 2010). AtHAK5 is the only system-mediating K^+ uptake at concentrations below 10 μM. Between 10 and 50 μM K^+, AtHAK5 and AtAKT1 have been demonstrated to be the only systems contributing to K^+ uptake. Above 50 μM K^+, both systems are thought to act, and at concentrations higher than 200 μM, the contribution AtHAK5 decreases and the only system operating is probably AtAKT1, although the contribution of other unknown systems cannot be ruled out (Fig. 4.1).

Recent research has demonstrated that members of the HKT transporter/channel family mediate important Na^+-tolerance mechanisms in plants mainly by improving K^+/Na^+ homeostasis. Previous work based on the identification of a quantitative trait locus (QTL) determining salt tolerance showed that Kna1 in *Triticum aestivum* controls the selectivity of Na^+ and K^+ transport to shoots, resulting in a high K^+:Na^+ ratio in leaves (Dubcovsky et al. 1996; Gorham et al. 1987, 1990; Luo et al. 1996). Other loci identified by QTL analyses in durum wheat, Nax1 and Nax2, also contributed to salt tolerance (Lindsay et al. 2004; Munns et al. 2003). Furthermore, the presence of Nax1 and Nax2 was shown to enhance K^+ accumulation in leaf blades and sheaths, leading to the model that Nax1 and Nax2 mediate K^+-loading into the xylem (James et al. 2006). These QTLs are attributable to polymorphisms in copies of wheat HKT genes, TmHKT1;4-A1 and TmHKT1;4-A2 (also named TmHKT7-A1 and -A2) for Nax1 and TmHKT1;5 and TaHKT1;5 for Nax2 and Kna1 (Byrt et al. 2007; Huang et al. 2006). Independent analysis of another QTL in rice, named SKC1 (shoot K^+ content), resulted in an identical model for the function of the rice gene OsHKT1;5 (Ren 2005). The SKC1 QTL was due to point mutations in OsHKT1;5 that replace several amino acid residues in the salt-tolerant cultivar NonaBokra (Ren 2005).

Studies performed in different null mutant types of the class I HKT transporter AtHKT1 has shed light into the mechanisms by which this entity controls Na^+/K^+ homeostasis under salt stress. According to these studies, AtHKT1 may be involved in Na^+ recirculation in plants by operating in Na^+ loading in the phloem (Berthomieu et al. 2003) or removal of Na^+ from the xylem preventing the accumulation of Na^+ in the leaves (Davenport et al. 2007; Horie et al. 2006; Sunarpi et al. 2005). In agreement with this model, AtHKT1 overexpression in the root pericycle improves salt tolerance (Moller et al. 2009).

Concerning K^+ homeostasis, mutations in OsHKT1;5 and AtHKT1 have also been found to reduce K^+ and enhance Na^+ accumulation in shoots during salt exposure, contributing to enhanced salinity stress (Ren 2005; Sunarpi et al. 2005). Interestingly, the disruption of AtHKT1 in mutants of the salt overly sensitive (SOS) pathway

prevented the K^+-deficiency symptoms observed in *sos* mutants when grown at low K^+ concentrations and improved cellular K^+/Na^+ ratios when compared with single *sos* mutants under saline conditions (Rus et al. 2001, 2004).

All the results described above refer to HKT transporters belonging to the class I of this family. They have been shown to mediate Na^+ transport and the effect upon K^+ nutrition may be indirect. On the other hand, some members of the Class II of HKT transporters can operate as Na^+–K^+ symporters under some conditions (Haro et al. 2005; Jabnoune et al. 2009; Rubio et al. 1995), and a contribution to K^+ uptake may be expected. However, mutations in the OsHKT2;1 gene do not have a strong impact on high-affinity K^+ (Rb^+) uptake into intact rice roots (Horie et al. 2007). Many of the presently characterized HKT class two genes are also induced by K^+ starvation, including those in wheat, barley, and rice (Garciadeblas et al. 2003; Horie et al. 2001; Wang et al. 1998). Therefore, in addition to mediating K^+ uptake, this system mediates Na^+ uptake, allowing Na^+ to act as a substitute nutrient for K^+ in K^+-starved rice plants under moderate Na^+ concentrations (Horie et al. 2007), supporting the long-standing hypothesis that Na^+ may substitute for K^+ when this nutrient is scarce (Flowers et al. 1983; Mengel and Kirkby 1982).

Regarding CHX transporters, it has been shown that disruption of AtCHX17 led to lower root K^+ concentrations under saline and K^+-deprivation conditions, although a subcellular localization and a functional characterization for AtCHX17 remain to be assessed (Cellier et al. 2004). On the other hand, AtCHX13 was localized to the plasma membrane and AtCHX13 knock-out and over-expressing mutant plants showed impairment and enhancement of K^+ uptake, respectively (Zhao et al. 2008). All these results suggested that this transporter may be involved in K^+ uptake *in planta*.

With respect to CNGCs, AtCNGC10 is targeted to the plasma membrane, transports both K^+ and Na^+ and partially rescues Arabidopsis *akt1* mutant plants (Kaplan et al. 2007). Results obtained in antisense lines of this channel, which displayed lower expression than WT plants, indicated a role in Na^+/K^+ homeostasis in roots by providing a pathway for Na^+ influx and K^+ efflux at the root/soil interface (Guo et al. 2008). Similar results were obtained for AtCNGC3. A null mutation in this channel altered both short-term Na^+ influx and K^+ uptake at high-external K^+ conditions, suggesting an alternate role in nonselective monovalent cation uptake at the plasma membrane level (Gobert et al. 2006).

4 Regulation of the Transport Systems

4.1 Transcriptional Regulation

In general terms, genes encoding AKT1-like K^+ channels do not show great differences in expression levels in response to the external supply of K^+ as it has been observed for AtAKT1 and CaAKT1 (Lagarde et al. 1996; Martínez-Cordero et al. 2005; Pilot et al. 2003). However, K^+ withdrawal from the growth solution increased *TaAKT1* transcript levels (Buschmann et al. 2000), and NaCl treatments or hormonal addition to the external medium produce changes in *AtAKT1* expression (Kaddour et al. 2009; Pilot et al. 2003).

Substantial differences are found in the expression pattern of the genes encoding HAK1-type K^+ transporters in comparison to that observed in the aforementioned genes for K^+ channels. It seems that an important point in the regulation of this type of transporters resides in the control of gene expression.

K^+-starvation is a common inducer in the gene expression of the HAK1-type K^+ transporters. This induction has been observed in *HvHAK1* (Santa-María et al. 1997), *AtHAK5* (Ahn et al. 2004; Armengaud et al. 2004; Gierth et al. 2005; Qi et al. 2008; Shin and Schachtman 2004), *OsHAK1* (Bañuelos et al. 2002), *LeHAK5* (Nieves-Cordones et al. 2007; Wang et al. 2002), *CaHAK1* (Martínez-Cordero et al. 2004), and *ThHAK5* (Alemán et al. 2009b). The reduction in root K^+ concentration below a threshold level has been proposed as the stimulus that could trigger the increase in the transcription of these genes (Martínez-Cordero et al. 2005).

Some other factors have been shown to considerably modify gene transcription of the HAK1-type genes. *CaHAK1* expression in K^+-starved plants was reduced, if half of the nitrogen of the growth solution was supplied as NH_4^+ (Martínez-Cordero et al. 2005). Similarly, *AtHAK5* promoter activity was diminished in K^+-starved plants after exposure to NH_4^+ (Qi et al. 2008). Conversely, *HvHAK1* transcript levels were increased by the presence of NH_4^+ in the growth solution in K^+-sufficient plants (Fulgenzi et al. 2008). Intriguingly, *LeHAK5* was downregulated when NO_3^- was supplied again after a withdrawal period (Wang et al. 2001). Another case in the regulation of HAK transporters was the increase in *AtHAK5* mRNA levels after exposing plants to sucrose in the absence of light (Lejay et al. 2008). Similar results were found previously in Arabidopsis when plants were grown in the sucrose-containing media MS (Rubio et al. 2000): It was observed that in K^+-sufficient plants *AtHAK5* expression was high; in this medium, K^+ starvation did not further increase the expression levels, probably because K^+ was substituted for NH_4^+ in these experiments.

Another important factor affecting the expression of these genes seems to be the presence of salinity. This was illustrated, for example, in the decrease of *LeHAK5* (Nieves-Cordones et al. 2007), *AtHAK5*, and *ThHAK5* (Alemán et al. 2009b) transcripts when plants were starved of K^+ in the presence of Na^+. Importantly, salinity decreased, to a lesser extent, the levels of *ThHAK5* mRNA in *T. halophila* than those of *AtHAK5* transcripts in Arabidopsis.

There is not much information about the signal transduction elements involved in the induction of HAK-type K^+ transporters expression. In 2004, the important role of reactive oxygen species (ROS) in the signaling events after removing K^+ from the growth solution was described for Arabidopsis (Shin and Schachtman 2004). Recently, it has been proposed that ethylene signaling acts upstream the increase of ROS during K^+ deprivation (Jung et al. 2009).

In tomato plants, *LeHAK5* expression levels correlated with steady plasma membrane potentials registered in root cells (Nieves-Cordones et al. 2008) and high-affinity K^+ uptake (Nieves-Cordones et al. 2007). Changes in plasma membrane potentials are one of the first signals that root cells sense after a stress is applied (Wang and Wu 2010). In tomato roots, the recorded plasma membrane potentials were importantly affected by long-term changes in the composition of the growth solution. For instance, the presence of NH_4^+ and K^+ starvation hyperpolarized and Na^+ depolarized plasma membrane potentials, which produced an increase and a decrease in the *LeHAK5* mRNA levels, respectively. Short-term exposure of depolarizing agents such as CCCP or Vanadate to K^+-starved roots also decreased *LeHAK5* expression. These changes in *LeHAK5* expression at both long- and short-term denoted a tight regulation at the transcription level and they also indicated that LeHAK5 contribution to K^+ uptake could be limited to some specific conditions even if K^+ deficiency was still present (Nieves-Cordones et al. 2008).

4.2 Post-translational Regulation

Recent studies have gained insights into AtAKT1 regulation. By analyzing mutants sensitive to low K^+ stress, a CBL-interacting protein kinase, CIPK23, turned out to be essential in the activation of AtAKT1, therefore permitting Arabidopsis plants to grow under low K^+ conditions (Xu et al. 2006). Two positive regulators of CIPK23, CBL1, and CBL9, which are two Calcineurin B-like proteins, were also found. Both CBL's could phosphorylate CIPK23 which became active after this phosphorylation (Fig. 4.2). Activation of the CBL's depended on Ca^{2+} as demonstrated by patch-clamp recordings (Li et al. 2006). Later on, it was shown that the network was more complex, being CIPK6 and CIPK16, in addition to CIPK23, able to interact with CBL1, CBL2 and CBL3 and CBL9 (Lee et al. 2007). Furthermore, a PP2C phosphatase (AIP1) inactivated AKT1 by dephosphorylating the latter. AIP1 bound AKT1 through AKT1's ankyrin domain. In addition, formation of heterotetramers of AKT1 subunits with AtKC1 also serves as another mode of regulation in which such heterotetramers display different

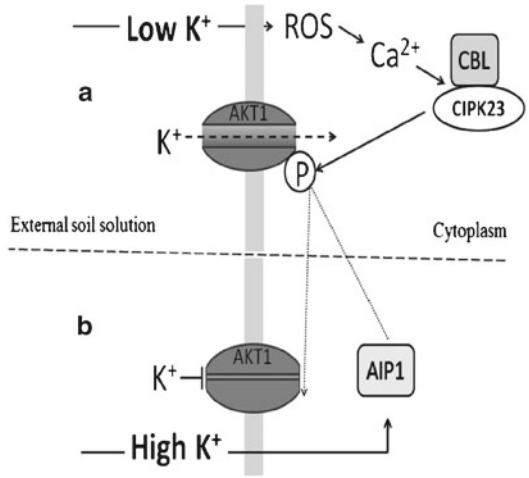

Fig. 4.2 Regulation of the activity of AtAKT1 by the CBL–CIPK complex and by the phosphatase AIP1. (**a**) Low K^+ stress provokes an increase in ROS levels that can possibly be translated into a cytosolic Ca^{2+} wave. This wave mainly activates CBL1, CBL9, and CIPK23. The activated CBL/CIPK complex phosphorylates the AtAKT1 channel, resulting in its activation (channel open). (**b**) At high-external K^+, the AIP1 phosphatase is thought to dephosphorylate AtAKT1, resulting in the inactivation of the channel (channel closed)

voltage dependence and sensitivity to external K^+ (Duby et al. 2008; Geiger et al. 2009).

The only report in which the regulation of HAK1-like K^+ transporters at the protein level was discussed revealed that HvHAK1-mediated Rb^+ uptake in yeast cells was modulated by the HAL4/5 kinases and the PPZ1 phosphatase (Fulgenzi et al. 2008). Both types of enzymes seemed to negatively regulate HvHAK1 activity in K^+-starved yeast cells.

4.3 Signaling Molecules: ROS and Hormones

ROS and hormones represent two of the most important signaling mechanisms in plants. They are produced when stimuli act upon certain types of cells and they constitute an effective means of communication between cells, adjusting plant to the new environmental conditions. In recent years, several hormonal activities and ROS have been related to responses that take place after changes in the K^+ status or to direct/indirect regulation of K^+ uptake, suggesting a role in this process. In some cases, significant advances in the mechanisms by which some of these molecules regulate K^+ nutrition have been reported (Fig. 4.3).

4.3.1 ROS and Ethylene

ROS have been shown to accumulate in a discrete region of roots active in K^+ uptake and they are translocated during low K^+ stress (Shin and Schachtman 2004). Knock-out of an NADPH oxidase (*rhd2* plants) prevented upregulation of genes that are normally induced by K^+ starvation, such as *AtHAK5*, but the high-affinity K^+ uptake remained unchanged. Application of H_2O_2 restored the expression of those genes induced by K^+ deficiency in *rhd2* plants. Both ethylene production and the transcription of genes involved in ethylene biosynthesis also increased when plants were deprived of K^+ (Jung et al. 2009; Shin and Schachtman 2004). However, in the ethylene insensitive2-1 (*ein2-1*) mutant, the ethylene-mediated low K^+ responses were not completely eliminated, suggesting that some K^+ deprivation-induced responses are either ethylene independent or EIN2 independent. Ethylene signaling stimulated the production of ROS and thereby it seems to constitute an earlier step in low K^+ response. Nevertheless, elements acting upstream ethylene signaling in the onset of low K^+ responses are still unknown. These results suggested that K^+-dependent regulation of *AtHAK5* mRNA levels relied, at least to some extent, on this ethylene-ROS pathway.

4.3.2 Abscissic Acid

Abscissic acid (ABA)-mediated control of ROS levels has been invoked to explain its function in protecting plants against oxidative conditions caused by many stress conditions, including nutritional deficiencies (Rubio et al. 2009). For instance, K^+ deprivation increases ABA levels both in the root and in the shoot (Kim et al. 2009) that would produce, among the responses of this hormone, a decrease in plant transpiration. Moreover, exposure of Arabidopsis roots to ABA evoked a dramatic reduction in the transcript levels of SKOR, the

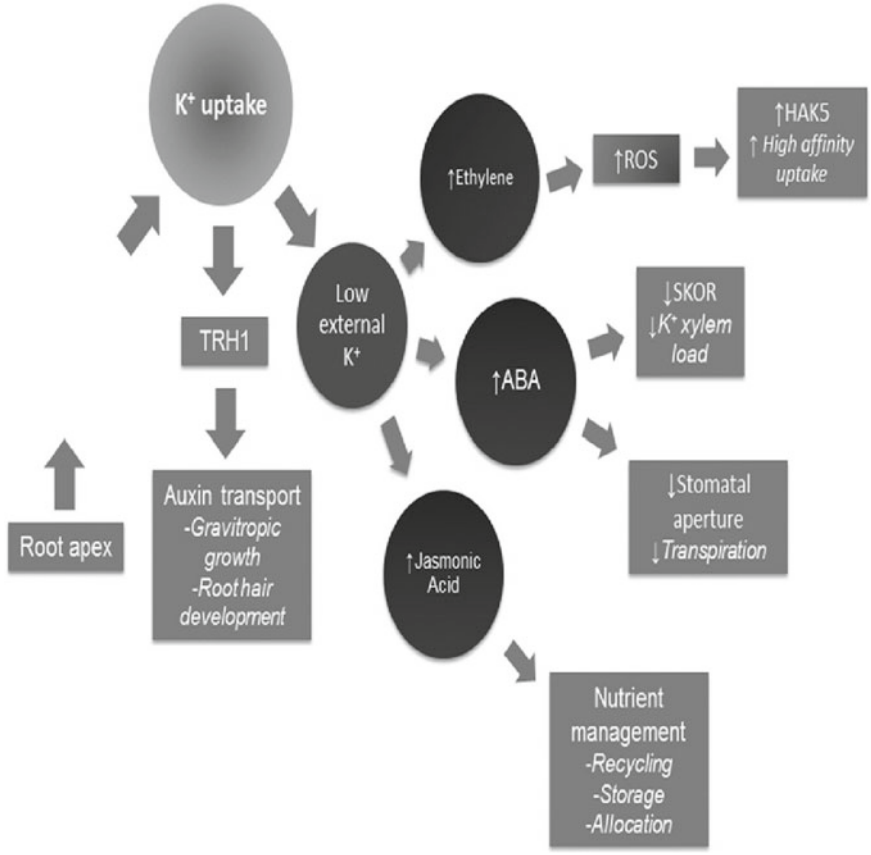

Fig. 4.3 Hormonal responses in relation to K^+ uptake. Root apex is an important source of cytokinins and when it is removed, rapid efflux of K^+ is observed. Such efflux can be reversed by adding cytokinins. K^+ transport through TRH1 greatly contributes to auxin gradients in the root and therefore supporting auxin responses such as gravitropic growth and root hair development. Low-external K^+ concentrations trigger stress-related pathways by increasing ethylene, abscisic acid (ABA), and Jasmonic acid levels. Ethylene activates reactive oxygen species (ROS) production, which mediates AtHAK5 upregulation and, in turn, high-affinity K^+ uptake. The ABA increase reduces stomatal aperture in leaves and in roots, it induces SKOR downregulation and thereby decreases K^+ xylem load. Jasmonic acid production activates several pathways which aim at enhancing nutrient recycling, storage, and allocation processes

outward-rectifier K^+ channel involved in K^+ release in the xylem (Gaymard et al. 1998). Interestingly, important reductions in SKOR mRNA levels in the roots were also observed due to K^+-starvation (Pilot et al. 2003), suggesting that this reduction could be related to the ABA increase under low K^+ growth conditions.

4.3.3 Jasmonic Acid

Upregulation of the genes controlling the first three steps of jasmonic acid (JA) biosynthesis has been observed in K^+-deprived plants (Armengaud et al. 2004). In contrast, K^+ resupply to these plants downregulated the expression of 19 JA biosynthesis-related genes. Interestingly, these transcriptional responses were observed mainly in the shoot, but not in the root, indicating that there is organ specificity in the K^+-mediated control of JA accumulation. This specificity could be related to the increased nutrient recycling observed in senescent leaves of K^+-deprived plants, a process that is triggered by JA (He et al. 2002). If we consider the exact position of ethylene and jasmonate signals within the K^+ starvation response, there is still a gap to be filled.

4.3.4 Auxins

Both physiological and molecular evidences show a relation between K^+ transport and polar auxin transport. For example, it was observed that Arabidopsis mutant plants that lack activity of the K^+ transporter TRH1 (AtKUP4/AtKT3) displayed agravitropic root growth and impaired root hair development and that these defects correlated with altered auxin transport or perception, as shown by the fact that addition of auxins rescued these *trh1* phenotypes (Vicente-Agullo et al. 2004). K^+ status may also affect auxin accumulation, as observed by reduced expression of genes controlling auxin biosynthesis, such as CYP79B2 and CYP79B3, when K^+-deprived Arabidopsis plants were resupplied with this nutrient (Armengaud et al. 2004). Conversely, a role for auxins in the control of K^+ homeostasis is supported by the fact that treatment of maize plants with auxins increased expression of the gene encoding the K^+ transporter ZMK1 (Philippar et al. 1999). Such transcriptional induction is in agreement with the increase in K^+ transport observed in maize coleoptiles protoplasts upon auxin application. These results contrast with those found in Arabidopsis in which exposure to the synthetic auxin 2,4 D drastically reduced AKT1, AtKC1, and SKOR root mRNA levels (Pilot et al. 2003).

4.3.5 Cytokinins

Cytokinins have long been implicated in the regulation of K^+ transport in plants (Abutalybov et al. 1980; Abutalybov and Akhundova 1982; Alizade et al. 1988; Green and Muir 1979; Shabala et al. 2009). It has been described that K^+ release into the xylem (as measured by $^{86}Rb^+$ release into root exudates) is inhibited by micromolar concentrations of kinetin (Collins and Kerrigan 1974; Hong and Sucoff 1976; Rains 1969). These observations are in agreement with the important decrease in the SKOR root transcript levels after exposure to benzyladenine (Pilot et al. 2003). In another study (Albacete et al. 2009), it was shown that xylem K^+ (but not Na^+) concentration was strongly correlated with leaf size and maintenance of the photosynthetic apparatus in tomato under salt stress, as was leaf xylem zeatin concentration, highlighting another interesting cytokinin–K^+ interaction. At the root level, cytokinins seemed to play a role in the regulation of K^+ fluxes in root epidermis (Shabala et al. 2009). Removal of the root apex, an important source of cytokinins, evoked significant K^+ efflux from root segments that was rapidly reversed by the addition of exogenous kinetin. Regarding regulation of K^+ transport systems, exposure of roots to the synthetic cytokinin, benzyladenine, produced a rapid decrease in the mRNA levels of the Shaker subunits AKT1 and AtKC1 in addition to the one previously mentioned of SKOR (Pilot et al. 2003). This effect of benzyladenine mirrors that observed after the addition of exogenous auxins. Nonetheless, studies revealing the underlying mechanisms of this interaction cytokinins–K^+ are lacking.

5 The Arabidopsis Model

5.1 Integration of Uptake Systems

Presently, root K^+ uptake systems from Arabidopsis are the best characterized and it is possible to integrate all the information available to generate a comprehensive model. Plants show a high concentrative capacity for K^+ (Martínez-Cordero et al. 2005; Nieves-Cordones et al. 2007), mediated by an active transport mechanism (Maathuis and Sanders 1994) through HAK transporters. As described in a previous section, the disruption of *AtHAK5* led to lower K^+ uptake rates and growth impairment at 10 μM K^+ and lower K^+ concentrations (Nieves-Cordones et al. 2010). Together with the micromolar K_m obtained when AtHAK5 was expressed in yeast (Rubio et al. 2000), this suggested that at the lowest K^+ concentrations, AtHAK5 is the only system mediating root K^+ uptake, in agreement with the higher K_m values in *athak5* plants (Gierth et al. 2005). AtHAK5-mediated uptake probably occurs via H^+–K^+ symport, as membrane potentials registered in root cells were not negative enough to drive passive flux of K^+ into root cells. Currently, the mechanism of transport through AtHAK5 remains elusive.

When external K^+ concentrations are above 10 μM K^+, AtAKT1 becomes an active player. In such conditions, the electrochemical gradient is

compatible with K^+ fluxes through channels and therefore AtAKT1 could mediate K^+ uptake. Indeed, this idea is further supported by the fact that *athak5* mutants did not display a defective phenotype at 20 μM K^+ (Pyo et al. 2010), exhibited Rb^+ uptake above that K^+ concentration (Rubio et al. 2008) and only double mutants *atakt1,athak5* lacked any of these features (Pyo et al. 2010; Rubio et al. 2010). These results strongly suggested that the channel was mediating K^+/Rb^+ uptake in this context. It is worth noting that in this range of K^+ concentrations, there is an overlap in the K^+ uptake capacities between AtHAK5 and AtAKT1 which made the phenotypic study of their corresponding single mutants difficult. AtAKT1 contribution to K^+ uptake starts in the low micromolar range, but the upper limit is not well defined. At 1.4 mM K^+, *atakt1* plants exhibited lower plant biomass and lower tissue K^+ concentrations, including an upregulation of *AtHAK5* that reflects clear symptoms of K^+ deficiency stress (Rubio et al. 2008). Therefore, with growth solutions containing around 1 mM K^+, the AtAKT1 function was not compensated by other systems such as AtHAK5, and thus AtAKT1-mediated uptake seems to be unique. At higher concentrations, for example, 10 mM K^+, *atakt1* mutant plants exhibited normal growth and tissue K^+ concentrations, denoting that other systems were indeed able to support growth under these conditions (Nieves-Cordones et al. 2010; Rubio et al. 2010). Similar results were obtained in the *athak5,atakt1* double mutants showing that AtHAK5 was not important in this range of concentrations. Interestingly, a complete inhibition of K^+ uptake and plant growth were not observed in the aforementioned double mutant at 0.5 mM K^+ (Rubio et al. 2010), suggesting that unknown systems are mediating K^+ nutrition in this *athak5,atakt1* line. Candidate systems thought to mediate K^+ uptake in this mutant line are AtCHX13 (Zhao et al. 2008) or non-selective channels regulated by cyclic nucleotides (CNGC's) (Demidchik and Maathuis 2007; Li et al. 2005).

5.2 Usefulness of This Model to Understand Other Species

Arabidopsis has been proven to be a useful plant model for the understanding of physiological processes in other plant species such as tomato, pepper, and *T. halophila* (Alemán et al. 2009b; Martínez-Cordero et al. 2005; Nieves-Cordones et al. 2007; Rubio et al. 2008). With this in mind, it has been shown that lower upregulation of HAK transporters due to salt stress when plants are deprived of K^+ is a common feature observed in barley, tomato, *T. halophila*, and *A. thaliana*. In contrast, regulation by NH_4^+ of HAK transcript levels was found to be different in Arabidopsis with respect to tomato but similar to barley and pepper. Moreover, CIPK–CBL pathways from Arabidopsis (like that regulating K^+ uptake through AKT1) are conserved among species and can interact with other *Shaker* channels from other species such as VvK1.1 from grapevine and ZmK1 from maize (Cuellar et al. 2010; Geiger et al. 2009). This interaction has allowed the production and characterization of functional channels in Xenopus oocytes.

On the other hand, comparing Arabidopsis with monocots species becomes more difficult. For instance, genome complexity in monocots implies that some K^+ transport systems are found as different isoforms (e.g., HvHAK1A B) that could exhibit differential regulation and contribution, something not yet observed in Arabidopsis. Moreover, HKT transporters belonging to subfamily II are present in monocots and absent in dicots such as Arabidopsis. Therefore, although some general assumptions regarding K^+ uptake could be valid for monocots, for example, occurrence of high- and low-affinity components with similar properties or regulation of HAK transporters by K^+, NH_4^+, and Na^+, in-depth studies addressing the contribution of the different transport systems deserves a monocot model.

6 Future Environmental Stresses Affecting K^+ Acquisition

Current agriculture faces many environmental abiotic stresses such as drought, salinity, or high temperatures. In the future, climate change will worsen the effects of these stresses. In addition, the continuous over-fertilization of crops to maintain production and the irrigation with low-quality water will also produce negative effects. All these abiotic stress conditions will have an important impact on K^+ acquisition by the plant (Fig. 4.4).

6.1 High CO_2

Stomatal movements and, in turn, plant transpiration are regulated by CO_2 levels in the atmosphere. These elevated atmospheric CO_2 concentrations could induce stomatal closure and thereby reduce plant transpiration. Implications of low transpiration rates in K^+ uptake were studied in short-term experiments (hours) and it was found that the accumulation of K^+ by the cells of the root was unaffected by water flux, whereas the passage through the root to the shoot via the vessels was linearly related to it (Bowling and Weatherley 1964). Similarly, K^+ transport to the shoot was shown to be increasingly reduced at low transpiration rates (high relative humidity) in comparison to normal conditions, whereas K^+ absorption by the root was less affected by such changes in transpiration (Hooymans 1969).

A long-term study concluded that, in response to sustained exposure to elevated CO_2 concentration, biomass is enhanced by 47% in C3 plants, 21% in CAM plants, and 11% in C4 plants (Poorter and Navas 2003). Generally, elevated CO_2 alters root architecture and fine-root turnover (Tingey et al. 2000) and increases the proportions of fine roots and secondary roots, implying an expansion of the rhizosphere (Curtis et al. 1994; Norby 1994; Pregitzer et al. 1995). Several studies have demonstrated that elevated CO_2 increases the root to shoot ratio (Norby 1994; Rogers et al. 1996; Rogers et al. 1994; Stulen and den Hertog 1993), thereby improving the capacity of the root system to acquire nutrients from the soil. In fact, it was described that after 2 years of exposure to elevated concentrations of CO_2 (around 700 ppm), an increase in the K^+, Pi, and N accumulation was observed in every organ in *Larix kaempferi*, together with an increase in dry matter (Shinano et al. 2007). The enhanced

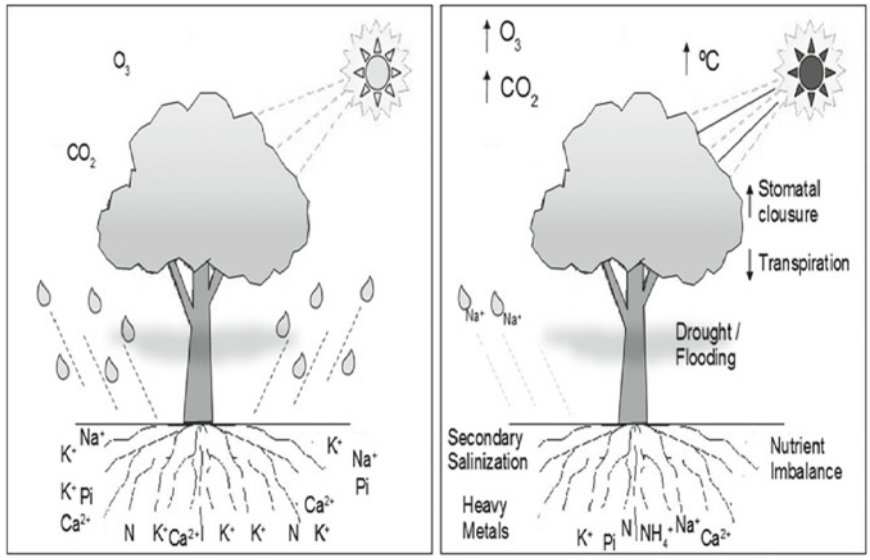

Fig. 4.4 Predicted effects of global warming and consequences over K^+ nutrition. An increase in CO_2, O_3, and temperature is expected, enhancing stomatal closure, and reducing transpiration. Irrigation with low-quality water may cause secondary salinization. Drought and flooding periods may also alter soil moisture triggering nutrient imbalance

uptake of nutrients in plants exposed to elevated CO_2 resulted from increased root biomass rather than increased root activity.

Although, as stated above, elevated CO_2 may have a positive effect on biomass production, important adverse effects also take place. Some plant species could respond to high CO_2 by a downregulation of net photosynthesis rate, which may be attributed to an end-product inhibition by elevated carbohydrate concentration in the leaf, combined N and P deficiency. In addition, photoinhibition, possibly due to the damage of PS core complexes, may also occur (Reddy 2010).

6.2 High Temperature

High temperatures may be the main factor in global change that could decrease crop yield (Battisti and Naylor 2009). In general, increased soil temperature appears to have a more consistent effect on root nutrient uptake kinetics than elevated CO_2 (Bassirirad 2000). A number of studies have demonstrated that changes in soil temperature can directly affect root transport properties for K^+ (Siddiqi et al. 1984), NH_4^+ (Chapin et al. 1986), NO_3^- (Bassirirad et al. 1991, 1993; Cumbus and Nye 1982), and PO_4^{3-} (Chapin 1974, 1977; Cumbus and Nye 1985). Temperature sensitive processes, such as root respiration, which affects ion movement across the root, could partially explain the observed changes in nutrient uptake with increasing soil temperature. However, in maize roots, it was observed that the absorption rates for K^+ increased with temperature but they reached a maximum between 25 and 35°C, whereas root respiration did not reach its optimum even at 40°C (Bravo and Uribe 1981). Interestingly, the enhanced uptake with temperature was observed in both high- and low-affinity components of K^+ uptake, revealing a general improvement in K^+ uptake capacity by the root.

6.3 High and Low Soil Moisture

Variations in moisture levels are mainly driven by precipitation, and are also affected by temperature and other factors that determine evapotranspiration rates. As for water supply by rain, climate change may lead to lower rainfall values in some lands, which would ultimately produce drought and episodes of water logging and floods in other areas. These opposite water disorders evoke a series of stress responses in plants. First, drought leads plants to a stand-by state in which general physiological processes such as transpiration and growth are greatly diminished to prevent further water loss. K^+ homeostasis can also be affected, as K^+ deficiency symptoms often appear on drought stressed maize, grain sorghum, and soybeans (Wiebold and Scharf 2003). These symptoms may occur even if the soil tests high for K^+. If root growth is inhibited by dry soil, K^+ uptake decreases and deficiency symptoms are induced. There are additional problems derived from low water potentials in the soil, like salinity. Water loss from the soil increases salt concentrations (for instance, sodium salts) leading to toxic levels of these salts in the plant and further impairing nutrient homeostasis.

Conversely, soil flooding causes oxygen deficiency (hypoxia), leading to a reduction in adequate soil conditions for plant growth (Ponnamperuma 1972), and adversely affecting nutrient uptake in plants (Pezeshki et al. 1999). The mineral nutrition of plants in response to flooding depends on plant species and soil type (Kozlowski 1984; Pezeshki 2001). In flood-intolerant species, the concentrations of N, P, and K^+ in foliages are often, but not always reduced by flooding (Kozlowski 1984; Pezeshki 1995).

Furthermore, flooding and drought together with extreme temperatures could modify chemical and physical properties of the soil, which can lead to reduced nutrients mobility and absorbance or leaching of some individual nutrients.

The final outcome resulting from the interaction of these climate changes described above on K^+ uptake and nutrition requires further investigation. Nevertheless, it clearly indicates that K^+ uptake is going to be negatively affected and special attention is needed in this issue to ensure plant performance in the field.

6.4 Salinity

Most crops are glycophytes (Greenway and Munns 1980) and have been grown in soils with

low salinity. Thus, the mechanisms developed to absorb, transport, and manage nutrients may not operate as efficiently under salinity as they would under non-saline conditions. Salinity negatively affects plant development and growth and, therefore, reduces crop yield and quality (Laüchli and Epstein 1990).

Over 800 million hectares of land worldwide are affected by salinity (Munns 2005), comprising nearly 7% of the world's total land area and approximately 5% of cultivated land (Amtmann et al. 2004). The expected drought periods that global warming is predicted to cause will entail more water used by farmers. As water resources are becoming more scarce, secondary salinization will be increased as the water quality is likely to be reduced.

There are two types of effects of salt stress on plants: osmotic effect and the specific effect. The latter includes the toxic effect, the nutritional imbalance and the oxidative stress. The osmotic effect results from the reduction of the soil water potential due to salt accumulation. Plant cells respond with osmotic adjustment by synthesizing compatible organic solutes and by accumulating ions from the external environment (Niu et al. 1995). By doing this, plants can revert water flow and permit growth (Kurt et al. 1986). The specific effect depends on the salt species present. The most abundant salt under salinity conditions is NaCl and therefore, the specific effect is mainly derived from the high Na^+ and Cl^- concentrations. Na^+ specifically affects K^+ nutrition because Na^+ and K^+ share physicochemical properties and Na^+ competes for the K^+ binding sites that are essential for the cellular function. More than 50 enzymes are activated by K^+ and Na^+ cannot replace its function (Bhandal and Malik 1988). Therefore, K^+ nutrition under salt stress is greatly impaired.

6.4.1 Salt Stress Affects K^+ Nutrition

One of the key physiological processes disrupted by high Na^+ concentrations is the maintenance of cellular and whole-plant K^+ homeostasis (Cakmak 2005; Flowers et al. 1983; Gaxiola et al. 1992; Kader and Lindberg 2008; Kronzucker et al. 2006; Peng et al. 2004; Rains and Epstein 1967; Santa-María and Epstein 2001; Takahashi et al. 2007a; Zhu et al. 1998). Reductions in K^+ tissue concentrations can be a consequence of the inhibition of K^+ uptake by Na^+ (Alemán et al. 2009a; Kochian et al. 1985; Kronzucker et al. 2006), stimulation of root K^+ efflux (Lynch and Lauchli 1984; Nieves-Cordones et al. 2010; Shabala et al. 2006) (Cramer et al. 1985; Nassery and Baker 1975), and differential allocation between organs (Graifenberg et al. 1995; Naidoo and Rughunanan 1990). Inhibition of K^+ uptake by high-external Na^+ concentrations is a consequence of the competition between K^+ and Na^+ for the K^+ uptake systems. The characteristics of such an inhibition may depend on the conditions and species. Thus, the addition of 3 mM Na^+ to the growth media resulted in a 50% suppression of K^+ influx in maize plants (Kochian et al. 1985), and this effect was limited to the low-affinity transport range for K^+. In barley, high-external Na^+ was shown to inhibit both high- and low-affinity K^+ uptake (Kronzucker et al. 2006, 2008) and the same has been observed in many other studies (Botella et al. 1997; Fu and Luan 1998; Martínez-Cordero et al. 2005; Rains and Epstein 1967; Rubio et al. 2000; Santa-María et al. 1997). Differences in Na^+ effects may originate from differences in experimental conditions, but in some cases they are due to differences between species. For example, the inhibition of K^+ uptake by Na^+ has been observed in Arabidopsis and in its salt-tolerant relative *T. halophila* when grown under several saline conditions. However, the inhibition was less intense in *T. halophila* in comparison to Arabidopsis (Alemán et al. 2009a). The lower reduction of K^+ uptake in *T. halophila* was concomitant to lower Na^+ absorption rates, whereas the contrary was observed in Arabidopsis. It could be concluded that plant species that differ in salt tolerance may have different K^+ uptake systems that results in different sensitivities to high-external Na^+ which may constitute important tools for improving plant K^+ nutrition efficiency under salinity conditions.

K^+ efflux from salinity-induced plants is another process that contributes to the reduction of tissue K^+ concentration. The strong membrane depolarization caused by high Na^+ uptake favors K^+ efflux via depolarization-activated outward-rectifying K^+ channels (Shabala et al. 2006). This is a Na^+-specific effect because isotonic mannitol solution causes significant membrane

hyperpolarization, resulting in increased K^+ uptake (Chen et al. 2005; Cuin and Shabala 2007; Shabala et al. 2000; Shabala and Lew 2002). In agreement with this, net K^+ balances in Arabidopsis roots grown for 14 days in the presence of 30 mM NaCl and low-micromolar K^+ concentrations reflected a prominent K^+ loss (Nieves-Cordones et al. 2010), although other studies have shown that under steady-state conditions high-external Na^+ reduced K^+ efflux (Kronzucker et al. 2008).

Salinity also interferes with K^+ sensing because high Na^+ suppresses the induction of high-affinity K^+ uptake by low K^+ (Alemán et al. 2009b; Nieves-Cordones et al. 2007, 2010) leading to lower K^+ uptake capacity under these conditions.

The reduction in tissue K^+ concentrations due to salinity also leads to a redistribution of this macronutrient to maintain its concentration buffered in metabolic active cells. For example, it has been observed that in leaves of plants grown at 50 mM external NaCl, K^+ concentrations decreased preferentially in mesophyll cells, whereas at higher salt levels, K^+ concentrations decreased only in epidermal cells (Fricke et al. 1996). Since K^+ is more compatible with cellular (i.e., cytoplasmic) processes than Na^+, K^+ is preferentially retained in metabolically active (i.e., mesophyll) compared with metabolically less active (i.e., epidermis) tissues or cell compartments (Cuin et al. 2003).

6.5 Effect of Other Nutrients

The increasing world population makes high yield crop production a necessity in agriculture. The use of fertilizers has raised crop yield considerably (Stewart et al. 2005). The expansion of agriculture has lead to an important increase in global K^+ consumption [4.4% per year between 1999 and 2005] and K^+ fertilization to maintain crop production is a regular cultural practice. Global K^+ consumption reached 33.9 Mt K_2O in 2008.

In some cases, over-fertilization occurs, which implies a financial and an environmental cost. Moreover, the high input of fertilizers to crops may lead to the inhibition of K^+ acquisition because of the presence of high concentrations of other nutrients. It is, therefore, important to optimize the efficiency of fertilizer usage. Cultivating crops that acquire and/or utilize K^+ more efficiently can reduce the use of K^+-fertilizers which would be environment and economic friendly (White and Brown 2010). Efforts to minimize fertilizer input and to develop nutrient-efficient, high-quality crops rely on detailed understanding of the exact interaction among the nutrients for uptake and the possible deficiency effects of a given nutrient caused by the supply of other nutrients (Amtmann and Armengaud 2009).

6.5.1 NH_4^+

Interaction between NH_4^+ and K^+ has been studied since long. Several studies have shown that external NH_4^+ decreases K^+ uptake (Deanedrummond and Glass 1983; Kirkby 1968; Martínez-Cordero et al. 2004; Pyo et al. 2010; Rubio et al. 2010), which is probably due to the fact that NH_4^+ and K^+ share some features such as charge value, hydrated ion diameter, and their effect on membrane electric potentials (Wang et al. 1994). In agreement with this, amelioration of NH_4^+ toxicity by K^+ supply has been shown (Cao et al. 1993).

Since the description showing that NH_4^+ specifically inhibits the non-AKT1 pathway of root K^+ transport (Santa-María et al. 1997; Spalding et al. 1999), some advances have been made in the understanding of the NH_4^+–K^+ interaction in K^+ uptake. The use of Arabidopsis mutants lead to the demonstration that the non-AKT1, NH_4^+-sensitive component of high-affinity was exclusively mediated by AtHAK5 (Pyo et al. 2010; Rubio et al. 2010).

6.5.2 Cross-Talk in the Responses to K^+, NH_4^+, NO_3^-, and Pi

The development of molecular biology techniques and the availability of plant genome sequences have accelerated the identification of transport systems for all plant nutrients. Now the research focus is moving towards the understanding of the regulation of these systems and cross-talk between them (Ohkama-Ohtsu and Wasaki 2010).

A relationship between K^+ supply and regulation of NO_3^- and NH_4^+ transporters seems to exist. NO_3^- transporters are downregulated in K^+-starved plants (Armengaud et al. 2004) and NH_4^+

transporters are upregulated by K^+ deprivation (Maathuis et al. 2003). Short-term (6 h) K^+ deprivation in maize plants leads to the rapid upregulation of a maize NO_3^- transporter NRT2 homolog (Schachtman and Shin 2007), suggesting that factors other than changes in NO_3^- metabolism may act on NO_3^--sensing mechanisms. Moreover, it has been recently described that K^+ and NO_3^- sensing share a protein kinase in the signaling pathway. As mentioned previously CIPK23 is known to be involved in K^+ signaling through phosphorylation of the AKT1 channel (Krouk et al. 2010), and CIPK23 is also an NO_3^--inducible gene which is downregulated in *chl1* (*nrt1.1*) mutants.

Regulation of Pi and K^+ transporters may also share signaling elements. Sucrose supply induce the Pi transporters, PHT1;4 and PHT3;1, as well as the K^+ transporter HAK5 upstream of hexokinase (HXK) sugar sensing pathways (Lejay et al. 2008). Other NO_3^-, SO_4^{2-}, and K^+ transporters are also upregulated by sucrose but downstream of the HXK sugar sensing pathway. On the other hand, genes encoding an MAP kinases, transcription factors, and nutrient transporters are induced by K^+ and Pi deprivation (Wang et al. 2002).

ROS may be a common signaling element of the plant response to the deficiency of several nutrients. ROS in roots is a common feature in response to NO_3^-, Pi, K^+, and SO_4^{2-} deprivation. Although this response occurs because of the deprivation of several macronutrients, it appears that there are some differences in localization as well as differences in the molecules that produce the ROS (Schachtman and Shin 2007), thus allowing some specificity.

7 Biotechnological Perspectives

To improve crop productivity, it is necessary to understand the mechanisms of plant responses to environmental changes with the ultimate goal being the increase of food availability. There are concerns about our ability to increase, or even maintain, crop yield and production in the context of global environmental change and its associated abiotic stresses (Tester and Langridge 2010). Furthermore, the current increment in biofuels production adds more doubts about our capacity to produce enough food.

The correlation between the increased frequency of extreme environmental events and global warming, underlie an urgent need for protective measures including the development and introduction of new crop cultivars with enhanced tolerance to environmental stresses (Etterson and Shaw 2001; Mittler 2006). Different strategies could be carried out for this purpose, and each of them will present certain advantages and inconveniences that need to be taken into consideration. Two of these strategies, natural variability exploitation and genetic engineering, that could be used for the improvement of the K^+ uptake systems in plant roots, will be discussed below.

7.1 Natural Variability and QTL Mapping

Natural variability offers a large resource of polymorphism, which is often explored to identify traits with environmental adaptive value or quality properties. Responses to environmental conditions depend on numerous genes and are typically controlled by QTLs. Genomic mapping of such QTLs may lead to the identification and cloning of important regulatory genes or allelic variants. It could also provide genetic markers for molecular breeding and/or cloned genes for genetic engineering for the improvement of stress tolerance in plants (Papdi et al. 2009).

Natural variability is often based on minor genetic changes, generating small quantitative alterations in responses to environmental conditions. One single nucleotide change, so-called single nucleotide polymorphism (SNP), could lead to different plant yield (Fleury et al. 2010; Papdi et al. 2009; Rafalski 2002; Xing and Zhang 2010).

The identification of genetic variability which affects stress responses requires phenotypic screenings capable of distinguishing between plants with different stress tolerance. Although previously tedious and time-consuming, next-generation sequencing of natural accessions can reveal sequence variability at the genome scale and will facilitate the large-scale identification of SNPs in different ecotypes and varieties. This is one of the goals of the "1001 Genomes Project," spearheaded by Magnus Nordborg, Joe Ecker,

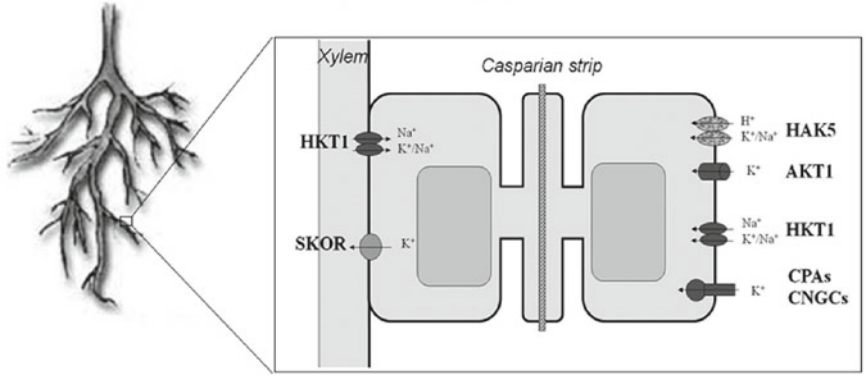

Fig. 4.5 Proposed points of genetic engineering for obtaining plants with improved K^+ nutrition: Upregulation of known root K^+ uptake transporters (HAK5) and channels (AKT1); selective upregulation of Na^+/K^+ transporters (HKT); upregulation of unknown K^+ transporters (CPAs, CNGCs). In addition, protein activity and selectivity may be also modified to enhance K^+ uptake

and Detlef Weigel among others, http://1001genomes.org/where 617 accessions have been committed as of 2010-6-2.

There are many examples of successful uses of genetic markers and QTL mapping. ERECTA was the first Arabidopsis gene that was mapped as a main QTL, and it regulates transpiration efficiency by controlling leaf photosynthesis efficiency and stomatal conductance (Masle et al. 2005). Freezing tolerance is controlled by seven QTLs in Arabidopsis. QTL mapping revealed that the C-repeat binding factor (CBF) locus is the most important component in cold acclimation (Alonso-Blanco et al. 2005).

As mentioned above, the identification of QTLs for determining salt tolerance or K^+ accumulation have highlighted the importance of HKT transporters in these processes. High-throughput ionomic coupled with genomic analysis allowed the identification of the genetic alteration that drives the natural variation in shoot Na^+ accumulation in Arabidopsis populations (Rus et al. 2006). Polymorphism of the *AtHKT1* gene and sensitive wild populations of Arabidopsis illustrate the importance of this transporter in salt tolerance. Other examples are the already mentioned SKC1, a rice HKT-type Na^+-selective transporter involved in unloading Na^+ from the xylem, characterized as a QTL for salt tolerance (Ren 2005); or the durum wheat Nax1 and Nax2 loci, linked to Na^+ exclusion which correspond to the Na^+ transporters HKT1;4 (HKT7) and HKT1;5 (HKT8), respectively (Byrt et al. 2007; Huang et al. 2006). Recently, RAS1 (Response to ABA and Salt 1) were found by using QTL mapping of a recombinant inbred population derived from Landsberg erecta (Ler; salt and ABA sensitive) × Shakdara (Sha; salt and ABA resistant). This transcription factor have been shown to play an important role in salt tolerance and ABA sensitivity (Ren et al. 2010).

As we can see, the analysis of natural variation in crop plants and Arabidopsis has provided an unprecedented amount of information on the genetic and molecular mechanisms that determine intraspecific variation and adaptation. It can be anticipated that this trend will continue in the next decade, especially with the broad implementation of "-omics" technologies for the precise analysis of natural variation at different levels (Alonso-Blanco et al. 2009).

7.2 Genetic Engineering

Two main strategies can be developed by researchers to attain biotechnologically improved plants: Modifying the protein quantity of interest or modifying the protein quality to make it more selective or active. These two strategies may be achieved with modifications at the nucleic acid level that will encode the protein. These strategies may be applied to K^+ uptake systems to improve K^+ nutrition (Fig. 4.5).

7.2.1 Modification of Gene Expression

Although all cells in an organism contain essentially the same DNA, cell types and cell functions vary because of qualitative and quantitative differences in their gene expression. Overexpression and downregulation of key genes will provide plants with the necessary resources to grow properly in the global warming challenge. The nutrition of K^+, one of the most important macronutrients presently found in most commercial fertilizers, could be improved in this way. Thus, enhanced expression of K^+ transporters and channels could result in an increase in plant K^+ uptake which could lead to high-yield production. At the same time, improved K^+ nutrition could lead to enhanced salt tolerance, because of the aforementioned competition between K^+ and Na^+, or to alleviation of drought stress as K^+ is a known osmolite (Amtmann et al. 2004; Römheld and Kirkby 2010).

To our knowledge, only two plasma membrane K^+ transporters or channels have been overexpressed. Overexpression of the barley high-affinity K^+ transporter HvHAK1 in Arabidopsis transgenic plants has been characterized (Fulgenzi et al. 2008). They showed increased K^+ uptake when plants were deprived of K^+ but not in K^+-sufficient conditions. Also a correlation between HvHAK1 transcript level and K^+ uptake was absent. The cation:proton antiporter AtCHX13 has also been overexpressed (Zhao et al. 2008). Driven by the 35S promoter, AtCHX13 increases K^+ (Rb^+) uptake in intact plants with a K_m of 196 μM, although it is not clear whether AtCHX13 localizes to the plasma membrane of the root cortex in native tissue. More K^+ transporters and/or channels have to be upregulated to unravel their biotechnological potential. Biotechnological success has been recently reported by cell-specific overexpression of an HKT transporter. Specific expression of *AtHKT1* in the mature root stele leads to a reduction of root-to-shoot transfer of Na^+ and increased salinity tolerance (Moller et al. 2009). Interestingly, not only Arabidopsis but also rice plants exhibit salinity tolerance with the specific expression of *AtHKT1* in root cortex cells (Plett et al. 2010).

7.2.2 Modification of Protein Activity

Future modification of K^+ transporters and channels may contribute to K^+ nutrition under adverse conditions such as high-external concentrations of K^+ uptake inhibitors. The finding of transporters and channels with higher affinity for K^+ (lower K_m) and/or higher transport capacity (higher V_{max}) could lead to obtaining plants better suited to cope with the problems derived from climate change. Some advances have been made for this purpose and several random and site-directed mutagenesis have been developed. *S. cerevisiae*, as a screening system model, has allowed the identification of modifications in the gene sequence encoding the transporters that confer salt tolerance or enhanced K^+ uptake (Garciadeblas et al. 2007; Mangano et al. 2008; Rubio et al. 1995, 1999). None of these studies have shown whether the enhanced yeast growth was due to a higher V_{max} or to an increase of transporter quantity at the plasma membrane. Only Garciadeblas and colleagues (2007) have reported on a mutated transporter of the moss *Physcomitrela patens* (PpHAK1), with a lower K_m for K^+, but still high for high-affinity K^+ uptake (200 μM).

Although good candidates for increasing K^+ uptake in salt environments have been described for some K^+ transporters, no biotechnological advances in plants have been made to date (or to our knowledge). Promising candidates would finally confirm *in planta* the hypothesis that the K^+/Na^+ ratio and not absolute Na^+ concentration in the cytoplasm is critical for stress tolerance (Amtmann et al. 2004; Maathuis and Amtmann 1999) and that by modifying root K^+ uptake systems salt tolerance can be increased. In addition, the possibility that improved K^+ uptake ameliorates the plant response to other stresses such as drought and cold should be also considered.

8 Conclusion and Future Perspective

The development of new crop cultivars with enhanced tolerance to environmental stresses such as salinity, heat, etc., is a necessity for modern

agriculture, as these stresses will be exacerbated as a consequence of future climate change. One of the targets for improvement is the plant's capacity to maintain/control its mineral nutrition. As regards to the acquisition of K^+, the research developed in the past decades has allowed the identification of key pieces to this process, namely the identification of genes encoding the transport systems, the regulation of these genes and the elements involved in modulating the activities of these transport systems. Relevant transcription factors and promoter regions will hopefully be identified in the near future, which in turn will constitute objectives and targets of further research. Another set of targets in need of study are composed by the regulatory proteins that have been shown to interact with structural proteins involved in K^+ acquisition. In this sense, elements of Ca^{2+} signaling pathways such as kinases, phosphatases, and Ca^{2+} binding proteins are of special importance. In addition, key players involved in hormone and ROS homeostasis should be also considered. The development of genetic engineering tools, the -omics approaches and the exploitation of natural variability should produce advances in the understanding of the process of K^+ acquisition that could lead to the development of improved crop varieties better suited for facing future challenges.

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Temperature Stress and Responses of Plants

5

Anna Źróbek-Sokolnik

Abstract

Among the abiotic environmental factors temperature is the most important factor which significantly affects life processes of all organisms. Temperature stresses experienced by plants are usually classified into three types: (a) chilling stress (occurring at temperatures below freezing), (b) freezing stress (occurring at low temperatures above freezing), and (c) high temperature stress. This chapter shows the influence of low and high temperature to physiological and metabolic processes in plants. The consequences of chilling and freezing or heat stresses are presented as well as mechanisms of plant resistance to low or high temperature and adaptation or/and acclimatization possibilities is reported in this chapter.

Keywords

Temperature • Vernalization • Stratification • Metabolism • Freezing • Acclimatization • Adaptation

1 Introduction

Temperature is an abiotic environmental factor that significantly affects life processes in all organisms by modifying membrane properties, enzyme activity levels, the rate of chemical reactions and diffusion, viscosity of vacuole solution and the cytoplasm, phloem, and xylem solutions in plants (Sung et al. 2003). Living organisms can be classified into three groups, subject to the preferred temperature of growth (Fig. 5.1). This chapter analyzes the impact of temperature on plant growth with emphasis on plant response to temperature stress.

It is believed that land plants evolved in a tropical climate. This evolution process was spurred not so much by a warm climate, but by the stability of ambient temperature. Plants gradually migrated into temperate regions both north and south of the equator as they developed mechanisms that allowed them to accommodate

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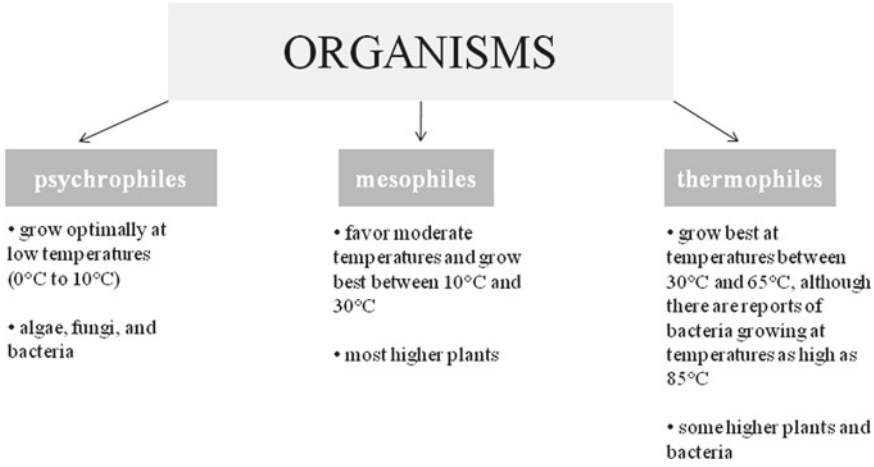

Fig. 5.1 Classification of the living organisms, subject to their preferred temperature of growth

wider variations in temperature on both a daily and a seasonal basis (Fitter and Hay 2002). The growth and development of plants involves a countless number of biochemical reactions that are sensitive to temperature. Plant life is generally limited by the freezing point of water at the low end of the temperature scale and the irreversible denaturation of proteins at the high end. Temperature is a critical factor in the plant environment, and it may play a significant role in growth and development. Growth is defined as an increase in dry weight, while development is the increase in the number and/or dimension of organs by cell division and/or expansion: leaves, branches, spikelets, florets, root apices, etc., including those present in seed embryos. It also seems that the rate of plant development tends to be controlled primarily by temperature, and it is less sensitive to other environmental factors. The development of vegetation is determined by a broad variety of environmental factors that exert combined effects. Plant organisms are rarely affected by individual factors, and temperature stress is usually accompanied by water stress and, in consequence, oxidative stress (Fitter and Hay 2002). Temperature can also play a part in controlling the pattern and timing of plant development, and this accounts for the below phenomena.

1.1 Vernalization

In some plant species, a period of low temperatures is required to induce flowering, while in other plants, low temperatures only accelerate flowering or have no effect at all. Plants with a vernalization requirement experience a period of low temperatures in late fall and/or winter at the stage of seed imbibition or young seedlings (annual winter crops) or upon reaching vegetative maturity (biennial and perennial plants) (Kim et al. 2009). Flowering is induced in the temperature range of 0 to +10°C. The duration of the vernalization period, that is, the required number of days with low temperatures, varies subject to species, and it usually reaches from 2 weeks to several weeks (Dennis et al. 1996; Amasino 2006). In seeds, temperature stimuli are perceived by the embryo, while in seedlings and matured plants, this signal is sensed by apical meristems. A vernalized meristem retains competence following the reception of the inductive signal. When the signal is absent for a longer period of time, the plant is de-vernalized, and a similar effect can be achieved by exposing the plant to higher temperatures (around 40°C for 1–2 days) (Tretyn et al. 2003). The mechanisms underlying vernalization have not yet been fully explained. It is believed that low temperatures lead to changes in the permeability

of cell membranes and/or the level of expression of "vernalization" genes. Phytohormones, in particular gibberellin, significantly contribute to this process (Sheldon et al. 2000; Amasino 2005).

1.2 Stratification

Stratification is a popular method of breaking seed dormancy that has been used for centuries. This technique involves the storage of seeds in a moist and well-ventilated environment at relatively low temperatures in the range of 1–10°C. Stratification is generally defined as the process of subjecting seeds to cold or warm and cold conditions in a moist and ventilated environment to break the dormancy stage. Low temperature, high moisture content, and oxygen supply during the treatment induce deep physiological and biochemical changes in seeds. Stratification leads to the decomposition of germination inhibitors in seeds, and it induces the production of growth stimulators: cytokinin, gibberellin, and auxin. At various stages of the dormancy breaking period, changes are noted in the quantitative ratio of various stimulators which modify the seeds' sensitivity to light and temperature and support dormancy breaking in various dormancy mechanisms (e.g., Baskin and Baskin 1998; Opik and Rolfe 2005; Wróbel et al. 2005).

1.3 The Effect of Temperature on Membranes, Enzymes, and Metabolic Processes

An increase or a decrease in temperature changes the kinetic energy of particles, accelerating their motion and weakening hydrogen bonds in macromolecules. All of the reactions contributing to growth are catalyzed by enzymes whose activity depends on their precise, three-dimensional, tertiary structures, to which the reacting molecules must bind exactly for each reaction to proceed. As the temperature rises, tertiary structures are damaged, reducing enzyme activity and reaction rates (Price and Stevens 1999). The asymmetry of response curves, such as Fig. 5.2a, b, is the net result of an exponential increase in the reaction rate, caused by increased collision frequency, and increasingly modified by the thermal denaturation of macromolecules (Fitter and Hay 2002).

The effect of temperature on enzyme activity is not a simple correlation. Activity levels rise with an increase in temperature, but only within a temperature range that guarantees the enzyme's stability (Cornish-Bowden 2004). When the critical temperature is exceeded, enzymes undergo thermal denaturation, and their activity drops rapidly. The average rate of enzymatic reactions increases twofold with every 10°C increase in temperature within the range that does not cause enzyme denaturation (Fig. 5.3). The correlation between temperature and the increase in enzymatic activity is described by temperature coefficient Q_{10} which illustrates changes in reaction rate when the temperature increases by 10°C:

$$Q_{10} = \frac{v_{(t+10)}}{v_t}.$$

Parameter Q_{10} applies only in a nondenaturing range of temperatures, it is enzyme specific and determined by the activation energy of the catalyzed reaction. Enzyme activity reaches the highest level at optimal temperature. The representative values of temperature coefficients (Q_{10}) for selected plant processes measured at varying intervals within the range 0–30°C are determined at 1–2.3 (e.g., light reactions of photosynthesis ~1; diffusion of small molecules in water: 1.2–1.5; water flow through seed coat: 1.3–1.6; water flow into germinating seeds: 1.5–1.8; hydrolysis reactions catalyzed by enzymes: 1.5–2.3; root axis extension: 2.3). Coefficient value reaches 2–3 for dark reactions of photosynthesis, 0.8–3 for phosphate ion uptake into storage tissue, and 2–5 for potassium ion uptake into seedlings. Grass leaf extension is characterized by Q_{10} of 3.2, and the relative growth rate is marked by coefficient value of 7.2 (Fitter and Hay 2002). The observed optimal temperature is the product of two processes: an increase in the reaction rate related to an increase in kinetic energy and an increase in the rate of thermal denaturation of an enzyme above a critical temperature point. When the

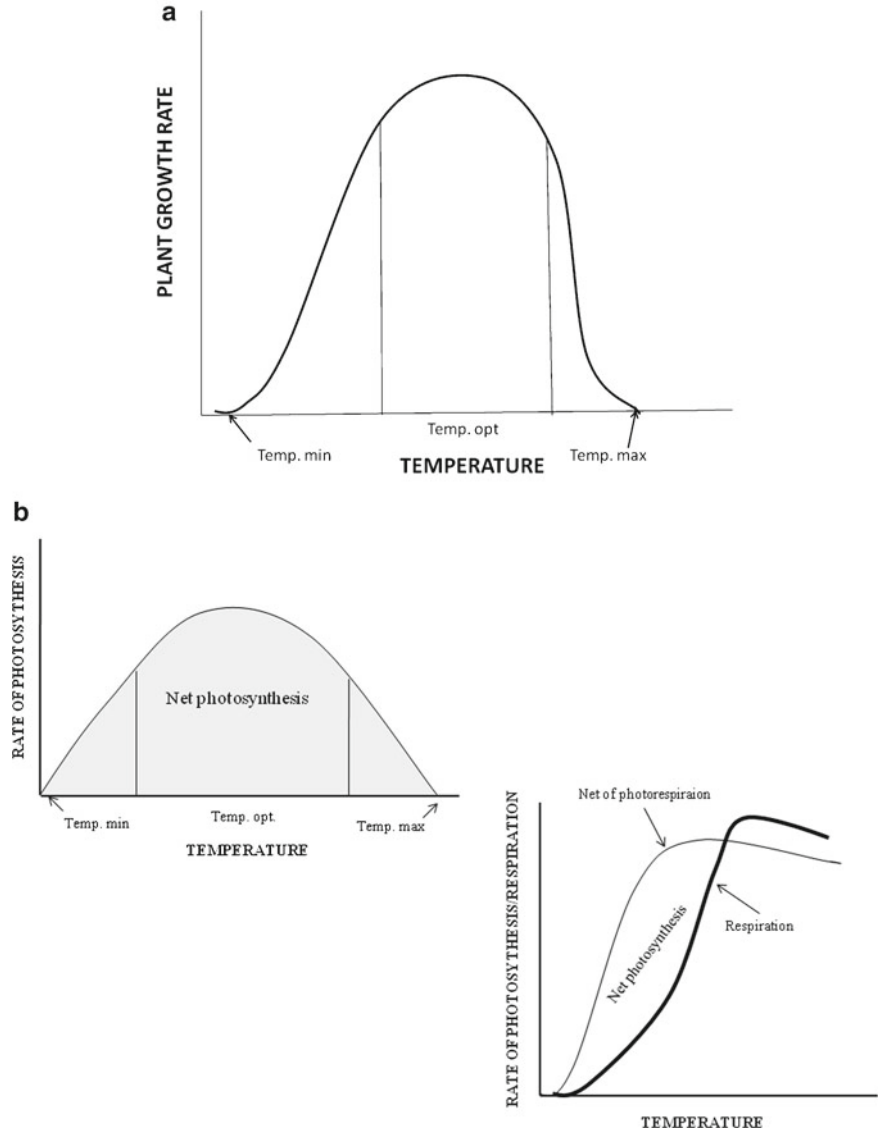

Fig. 5.2 Schematic illustrations of plant responses to temperature (adapted from Fitter and Hay 2002). (**a**) The response of plant growth rate; (**b**) the influence of temperature on the rate of photosynthesis and respiration

second parameter is higher, a drop in activity levels is noted. For most enzymes, the optimal temperature falls within the range of 30–45°C. Enzymes are irreversibly denatured and inactivated at temperatures higher than 60°C. The enzymes of thermophilous organisms (such as thermal spring bacteria) remain active and attain maximum reaction rates at higher temperatures. The highest temperature at which an enzyme is not thermally inactivated under given conditions determines the enzyme's thermal stability.

An alternative approach involves the application of the Arrhenius equation (from chemical kinetics) to plant processes:

$$k = A \exp(-E_a / RT),$$

where k is the rate constant, E_a is the activation energy for the process, A is the constant, R is the gas constant, and T is the temperature expressed on the absolute temperature scale.

Arrhenius constants (E_a/R for the process) can be useful in biochemical comparisons between

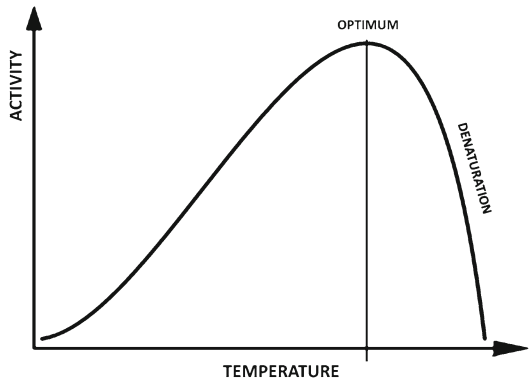

Fig. 5.3 The influence of temperature on enzyme activity

species (Criddle et al. 1994; Levine 2005) and in analyses of plant membrane changes during cooling and freezing.

Higher temperatures increase the liquidity of membrane lipid layers. A temperature drop has the opposite effect: biological membranes become more rigid and the activation energy of membrane enzymes increases. The above phenomenon is as the result of thermotropic changes in the lipid phase. Temperature modifies the organization of fatty acid residues in phospholipids and galactolipids, the components of various membranes. The configuration of polyunsaturated fatty acid residues is more difficult to reorganize at lower temperatures than that of saturated fatty acids, but polyunsaturated fatty acids residues "melt" more easily at higher temperatures. Temperature-induced changes in the liquidity of the cell membrane or its selected domains that modify the structure and function of membrane proteins. Cell membrane's response to temperature variations may also be determined by its sterol content or interactions with other nonlipid organic compounds (Sung et al. 2003; Alberts et al. 2004). Temperature-induced changes in membrane properties also significantly affect water regulation in cells, and secondary water stress may occur when the rate of water uptake by the roots is slower than leaf transpiration. At temperatures below 0°C, liquid water changes into solid ice. Ice crystals are formed inside the protoplast which could lead to structural damage. Extracellular formation of ice may cause cell dehydration. The component processes of plant growth do not all respond to temperature in the same way. For example, in most crop species, gross photosynthesis ceases at temperatures just below 0°C (minimum) and above 40°C (maximum), with the highest rates being achieved in the range of 20–35°C. In contrast, rates of respiration tend to be low below 20°C but, owing to the thermal disruption of metabolic controls and compartmentation at higher temperatures, they rise sharply up to the compensation temperature, at which the rate of respiration equals the rate of gross photosynthesis, and there can be no net photosynthesis (Wilkinson 2000; Fitter and Hay 2002; Jenks and Hasegawa 2005; Wahid et al. 2007).

Temperature stress in plants has been broadly researched, and the problem has been widely addressed by review articles (Wang et al. 2003; Wahid et al. 2007; Jan et al. 2009), books discussing various types of stress (Wilkinson 2000; Fitter and Hay 2002; Jenks and Hasegawa 2005), studies investigating the negative effects of extreme temperatures (Iba 2002; Sung et al. 2003), etc. It should be noted that unlike homeothermic animals, plants are unable to maintain their cells and tissues at a constant optimum temperature, therefore, their metabolism, growth, and development are profoundly affected by changes in environmental temperature. This suggests that as sessile organisms, plants must be able to sense transient fluctuations as well as seasonal changes in temperature and respond to these changes by actively adjusting their biology to fit the subsequent temperature regime. Temperature is a major environmental factor that changes from season to season and undergoes daily fluctuations and short, erratic lows and highs. For this reason, the stress-inducing role of temperature is difficult to define unambiguously since the response to various temperatures is determined by the plants' ability to adapt to different climate regimes. Vegetation occurs in climate zones characterized by extreme temperatures of −50 to +50°C, that is, within a range of 100°C. The margin of thermal tolerance that conditions the stability of life processes in most plants is relatively wide, ranging from several degrees above zero to around 35°C, and it is genetically determined. Many genotypes specific

Table 5.1 Factors which determinate temperature of above- or under-ground organs (adapted from Fitter and Hay 2002)

Leaf/above-ground organs	– The amount of solar radiation intercepted	
	– The potential for energy exchange with the environment	
	– Time of day (regular diurnal variation of solar elevation)	
	– Month (typical seasonal variation)	
	– Cloud cover	
	– Wind force and origin of air mass (irregular, short-term variation)	
	– Position in the canopy (e.g., "sun" or "shade" leaf)	
	– Altitude above soil surface	
	– Canopy characteristics, including leaf shape, dimensions and surface properties	
Roots/under-ground organs	– Seasonal and diurnal variations in energy exchange – The interception of solar radiation by the canopy – The account of the depth below the soil surface	Determine how much energy reaches the soil surface
	– Soil properties which influence the energy balance at the soil surface, and the transfer of heat through the soil (e.g., moisture content, bulk density, color/albedo, and the vegetative or litter cover)	

to extreme climate conditions, from arctic to tropical, have a much wider tolerance margin. In principle, plants in the dormant state (dry germs and seed embryos, dehydrated dormant organs) are far less sensitive to temperature change, and they are able to survive through periods of extreme temperature unharmed. Metabolically active tissues have thermal activity limits which, when exceeded, lead to a reversible drop in the rate of life processes to a minimum level. Further temperature change (referred to as critical or lethal temperature) causes permanent damage to cell structures, it affects cell metabolism, impairs vital life processes, and kills the protoplasm. During evaluations of plant response to extreme temperatures, special attention should be paid to the temperature of the plant which often differs from ambient temperature. In the summer, leaf temperature often exceeds ambient temperature by up to several degrees. Higher differences are noted in plants whose leaves are positioned horizontally, such as apple trees. In the spring and autumn, the night temperature of leaves, in particular when the sky is clear, may be even several degrees lower than ambient temperature (Wilkinson 2000; Fitter and Hay 2002; Jenks and Hasegawa 2005). At a given moment, leaf temperature is determined by several factors (Table 5.1). Roots demonstrate a stronger growth response to extreme temperatures than the above-ground parts of plants, and the above applies to both extreme cold and extreme heat (Fig. 5.4). During the evolution process, roots became adapted to more stable temperatures. Nonetheless, the temperature of both the roots and other under-ground organs is also determined by factors presented in Table 5.1.

Plants can adapt to changes in the temperature regime through the evolution of genotypes with more appropriate morphologies, life histories, physiological and biochemical characteristics, or by plasticity. Plants also adapt to changing temperatures during the growing season by plastic responses.

2 Low Temperature

Periodic temperature drops below zero degrees are reported on around 64% of the Earth's surface. The lowest temperatures are noted in Antarctica, reaching around −50°C in coastal areas and up to −90°C in the interior. The minimum temperature at which a given species can survive is one of the main criteria determining plant distribution on our planet. In a temperate climate, low-temperature stress eliminates or inhibits the growth and yield of valuable plants and crops (Xin and Browse

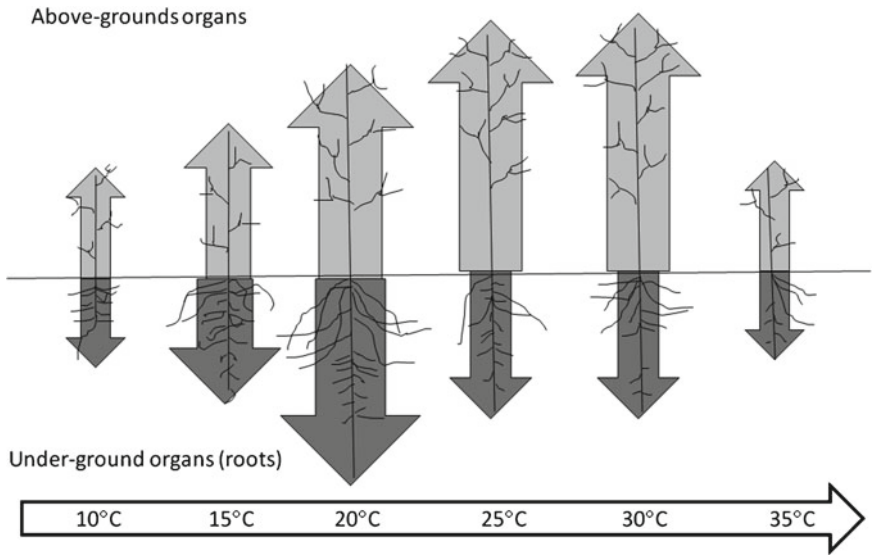

Fig. 5.4 Schematic illustration of the influence on growth and morphology of roots and above-ground organs of potato seedlings (adapted from Marschner 1995)

2000; Jan et al. 2009). Plants indigenous to colder regions are usually well adapted to chilling temperatures and are, therefore, not significantly impaired by cold periods, apart from a general slowing down of the metabolic rate and growth. In a temperate climate, plants respond differently to freezing temperatures and the winter environment than other factors that occur irregularly. In the winter, chilling temperatures do not come as a surprise for plants that have adapted to the periodic, adverse vegetation factors in the course of evolution. Low temperatures are accompanied by short daytime and low radiation intensity. The adaptation to growth inhibiting factors is characteristic of the dormant state (Jan et al. 2009).

There are two types of injuries a plant can sustain through exposure to low temperatures (Fig. 5.5). On the other hand, many plants that are native to cold climates can survive extremely low temperatures without injury (Levitt 1980).

An analysis of freezing winter temperatures as an environmental stressor should also account for the impact of other adverse factors such as low light intensity and short daytime. The above conditions arrest the growth and development of vegetation (Hopkins 2006).

The plants' ability to survive freezing and other adverse temperature changes differs from the remaining stressors. Levitt's stress avoidance theory (1980) does not apply in this case. Plants are unable to avoid freezing temperatures, and they can only protect themselves from the negative consequences of cold by increasing their tolerance to chilling. Many plants enter the dormant state to survive harsh winter weather. This is a typical feature of adaptation to freezing which is a genetically inherited trait.

Plants can be classified into three categories based on the range of lethal temperatures and the characteristics of mechanisms conditioning their resistance to low temperatures (Fig. 5.6).

2.1 Consequences of Chilling and Freezing Stress

There are two theories explaining the plants' primary response to temperature stress. The first concept, formulated by Lyons (1973), states that low temperatures induce the phase transition of cell membranes where a liquid-crystal structure is transformed into a crystal (gel) phase.

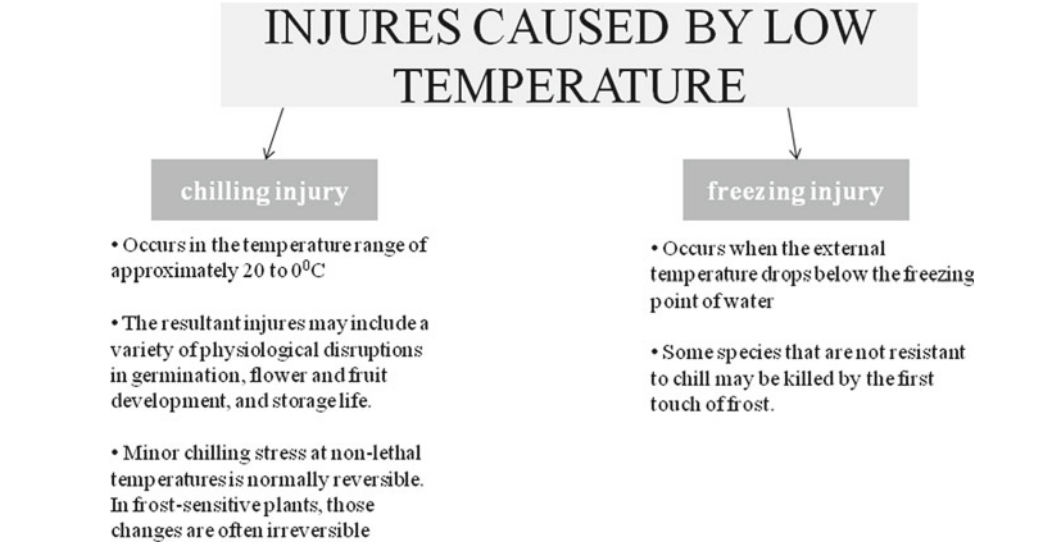

Fig. 5.5 Two types of injuries a plant can sustain through exposure to low temperatures (adapted from Stushnoff et al. 1984)

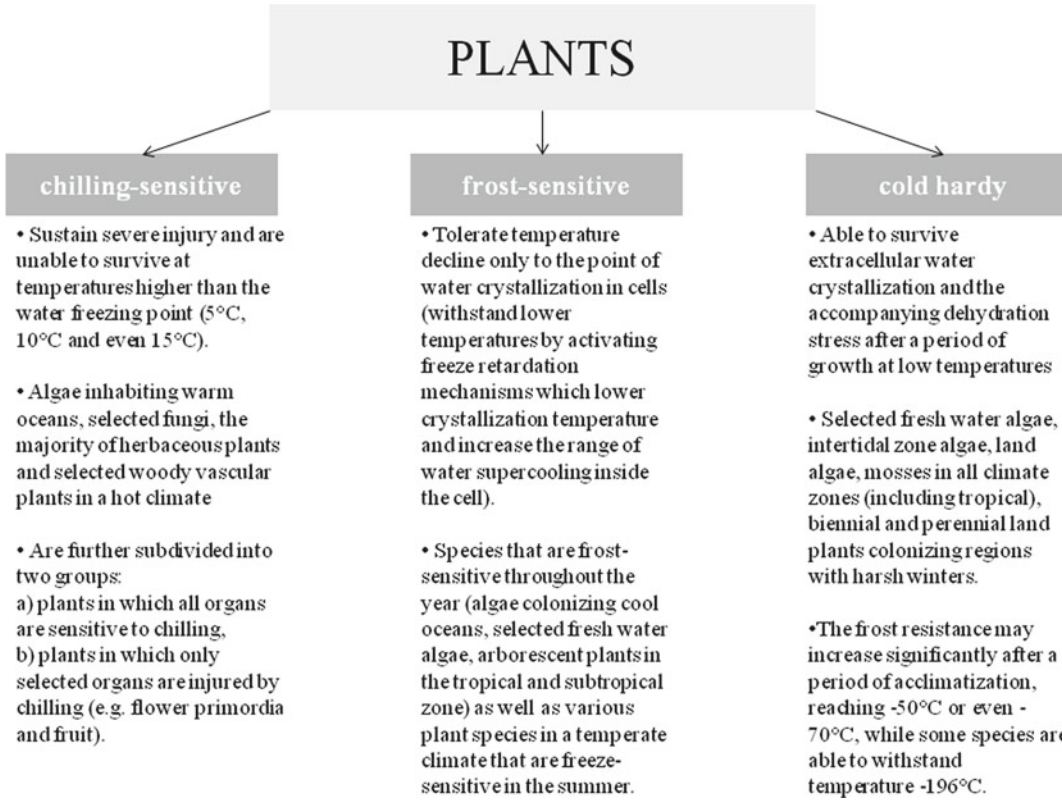

Fig. 5.6 Classification of the plants, subject to their range of lethal temperatures and the characteristics of mechanisms conditioning their resistance to low temperatures (adapted from Stushnoff et al. 1984)

Thermotropic phase changes are the primary cause of membrane dysfunctions that lead to irreversible damage and cell death. The above may produce reactive oxygen species and the accompanying oxidative stress. According to recent research, the phospholipid which initiates the phase transition of the cell membrane is phosphatidylglycerol (PG). If a PG molecule contains fatty acids with a high melting point, that is, saturated fatty acids, then the phase transition of this lipid takes place relatively easily at low temperatures and this, in turn, induces the transformation of other phospholipids and galactolipids adjacent to PG (Los and Murata 2004; Wang et al. 2006). According to the second chilling injury theory, the primary cause of damage is the sudden increase in the concentration of free calcium ions in the cytosol (Minorsky 1989). Calcium ion concentrations increase as calcium channels in the plasmalemma become opened due to sudden depolarization (Lecourieux et al. 2006). In chilling-sensitive plants, calcium opens the stomata, and transpiration significantly exceeds water uptake by the roots (Liang et al. 2009). In many sensitive species, the first indication of cold stress is striking wilting of the leaves, despite optimal water supply in the soil (Mahajan and Tuteja 2005; Solanke and Sharma 2008). The release of calcium ions into the cytosol has many secondary effects, including induced gene expression which could result from changes in the content or distribution of cell hormones, mainly abscisic acid (ABA). This phenomenon is in particularly related to the acidification of the cytoplasm at low temperatures (and the corresponding alkalization of the vacuoles) which, at least in part, is actively controlled by H^+-transport from the cytoplasm to the vacuole catalyzed by H^+-ATPase located on the vacuolar membrane. The inactivation of this enzyme has been reported to occur much earlier than other symptoms of cell injury (Yoshida et al. 1999; Lindberg et al. 2005). Chilling affects the entire internal environment of each cell and each molecule within the cells (Kartsch and Wise 2000). The rate and extent of injury is determined by temperature, its duration as well as the chilling rate. Sudden temperature drops (thermal shock) have particularly damaging consequences. The lower the temperature and the longer its effect, the greater the extent of the sustained injury (Mahajan and Tuteja 2005; Solanke and Sharma 2008). Plant structures and physiological cell processes have varied sensitivity to chilling temperatures (Fig. 5.7). Most injuries are sustained in the cell membrane which may represent a potential site of perception and/or injury (Lindberg et al. 2005). There are changes in the viscosity and liquidity of the membrane, leading to an increase in diffusion resistance and, in many cases, enzyme inactivation. The reversibility of those effects is determined by the severity of damage. Changes in chemical composition may be observed as the result of lipid degradation, the release of fatty acids and changes in the activity of metabolizing enzymes, peroxidation, disintegration of lipid–protein bonds, and higher membrane permeability. The chemical composition of the cytoplasm and differences in lipid quality in various chilling-sensitive species determine the phase transition point, that is, the point at which the membrane is transformed from a liquid-crystal state into a gel state (Solanke and Sharma 2008; Jan et al. 2009). This change in the membrane's physical state impairs its normal functioning. In most chilling-sensitive plants, the phase transition point is around 10°C. Chilling sensitivity is mostly related to a higher content of saturated fatty acid residues in lipids, while the cold-hardiness mechanism is explained by the desaturation of fatty acids which enables the plant to quickly acclimatize to low temperatures. The above is only one of the factors explaining variations in the plants' response to temperature stress (Lindberg et al. 2005; Zhang and Tian 2010). Interactions between membrane components, including lipid–lipid and lipid–protein, are also believed to play an important role. Higher sterol concentrations increase membrane rigidity. The role of membrane proteins during chilling is also a source of controversy, but there is general agreement that conformational changes in protein–lipid systems may lead to membrane disintegration and dysfunction (Los and Murata 2004; Lindberg et al. 2005). Frost-induced

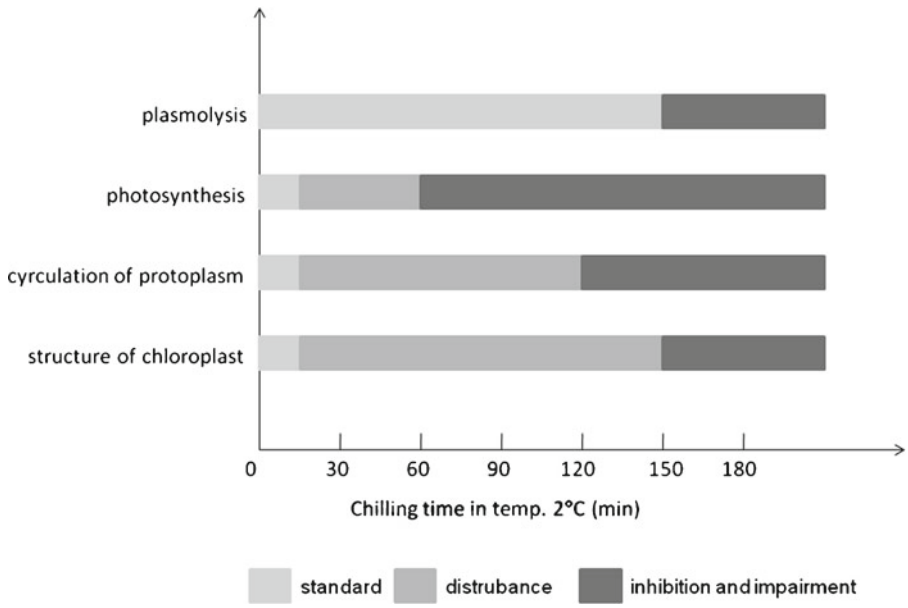

Fig. 5.7 Functional disturbance occurred in chilling-sensitive plants, subjected to stress duration (adapted from Kacperska 1998)

changes may lead to inhibited protoplast movement, excessive protoplast vacuolization, damage to the endoplasmic reticulum, drop in turgidity, and higher membrane permeability. Cytoplasmic streaming and photosynthesis, including thylakoid functioning in chloroplasts (as demonstrated by enhanced in vivo chlorophyll fluorescence), are most susceptible to reversible disruptions. Irreversible damage, including injuries caused by stressors other than temperature, is also most likely to affect thylakoid membranes, mostly photosystem II. Chloroplast lipids undergo various metabolic changes in both chilling-sensitive and cold-hardy plants. Higher levels of galactolipase activity and, consequently, higher free fatty acid concentrations are noted in the chloroplasts of chilling-sensitive species (faba beans, beans, tomatoes, maize) than in cold-hardy plants (spinach, pea). Lower temperatures disrupt the maintenance of the proton gradient in thylakoid membranes conditioning ATP synthesis. Powerful radiation during or directly after chilling intensifies the relevant injuries and retards, or even disables, damage repair in both chilling-sensitive and cold-hardy plants. Long-term frost inhibits the synthesis of chlorophyll and starch (Muller et al. 2005; Liang et al. 2009; Sun et al. 2010). Other membranes (plasmalemma and tonoplast) are damaged after relatively longer exposure, as demonstrated by membrane cells' ability to plasmolyze and vital staining. Those injuries are irreversible. Other metabolic functions are marked by varied sensitivity to low temperatures which cause metabolic disorders and lead to toxin accumulation, for example, respiration efficiency may be higher or lower subject to environmental factors that accompany freezing temperatures. Chilling may also inhibit the activity of many oxidoreductive enzymes, such as catalase, leading to the accumulation of hydrogen peroxide and the production of free radicals (Suzuki and Mittler 2006; Liang et al. 2009; Sun et al. 2010). In sublethal cold stress, fruit ripening and seed germination are most severely inhibited (Kumar and Bhatla 2006).

Frost leads to the appearance of stress which is linked not directly to low temperature, but to freezing (crystallization) of water in the plant (Mahajan and Tuteja 2005). Intracellular and extracellular crystallization produces different effects. Ice crystals are formed readily in those parts of the plant where temperature drops most

rapidly and where water freezes most easily (due to high water potential), mostly vascular bundles and intracellular spaces in above-ground parts where water vapor undergoes condensation. Ice crystals spread quickly via vessels and other tissues with uniform structure. The presence of air-filled intercellular spaces as well as tissues with lignified or cutinized walls slows down crystallization. Ice formation is accelerated by ice-nucleation active bacteria of the genera *Ervinia* and *Pseudomonas.* The proteins formed on the outer bacterial cell wall react with water particles and facilitate the formation of ice crystals at temperatures just below 0°C. In the absence of ice-nucleation active bacteria on the surface of tissues and on the walls of intracellular spaces, ice formation would begin at temperatures several degrees lower due to the supercooling of water solutions.

If tissue is supercooled rapidly (e.g., faster than 5 $K\,min^{-1}$) and the cells have high water potential, or if cell water had been first deeply supercooled, ice may be formed in the protoplast. The above invariably leads to cytoplasm destruction and cell death (Fitter and Hay 2002; Rajashekar 2000; Jan et al. 2009; Janska et al. 2010). Water freezing in intracellular spaces is a less dangerous phenomenon. In nature, where temperature decline is generally slow (1–5 $K\,min^{-1}$), crystallization usually takes place outside the protoplast in intracellular spaces and between the cell wall and the protoplast (partly due to the extracellular fluid having a higher freezing point, i.e., lower solute concentration, than intracellular fluid). The above leads to extracellular crystallization. Vapor pressure decreases in the spaces above ice, and a water potential gradient is created between the unfrozen interior of the cell and the extracellular environment. Water moves along this gradient into extracellular spaces where it is crystallized (Fitter and Hay 2002; Jan et al. 2009; Janska et al. 2010). Cells are dehydrated (secondary stress) and they contract due to desiccation. The lower the surrounding temperature, the longer it takes for an equilibrium to be reached between the water potential above ice and inside cells, and the greater the effect of cell dehydration (Solanke and Sharma 2008).

Multiple forms of membrane damage can occur as a consequence of freeze-induced cellular dehydration including expansion-induced-lysis, lamellar-to-hexagonal-II chase transitions, and fracture jump lesions. The above leads to cell contraction and the associated changes in reactions between the plasmalemma and the cell wall, partial loss of plasmalemma due to exocytosis and endocytosis, changes in the structure of the plasmalemma and other cell membranes, and the creation of protein-deprived lipid areas in the membrane. The greatest damage is done to the plasmalemma. Dehydration also increases the concentration of solutions in the cytosol and the cell sap, leading to higher salinity (Mahajan and Tuteja 2005; Solanke and Sharma 2008; Jan et al. 2009). Conformational changes in proteins found in the plasmalemma and other membranes lead to changes in the activity of various membrane enzymes, including ATPases responsible for the movement of protons and other ions through membranes (Lindberg et al. 2005). Some ions, accumulated in cells by ion transporters (e.g., potassium ions), are diffused after thawing into intracellular spaces together with water, for example, in leaf tissue. Certain proteins, such as the thylakoid coupling factor, become dissociated in the process. The effect of chill injury on life processes is often visible when plants resume their normal growth after freezing temperatures subside. Even partial degradation of thylakoid membranes inhibits photosynthesis, and the process may be reversible. PS II activity may be partially or completely inhibited, and the balance between the light-dependent phase and CO_2 assimilation may be upset. There is a rise in photorespiration intensity (Alam et al. 2005). Changes in the mitochondria and the respiration process are not as profound. In strongly dehydrated cells, the membrane undergoes lyotropic phase transitions, and hexagonal arrangements are formed in lipid bilayers of a single membrane or two layers of two adjoining membranes (e.g., plasmalemma and endoplasmic reticulum). The membranes' primary structure is not always restored after thawing, and water is diffused into the extracellular environment together with ions through membrane

channels. Cell dehydration caused by extracellular crystallization increases the concentrations of salt and organic acids in the protoplast which, in turn, may lead to protein denaturation and enzyme inactivation (Mahajan and Tuteja 2005; Solanke and Sharma 2008). Few enzymes remain active at below zero temperatures, but some of them are activated, such as phospholipase D which catalyzes the hydrolysis of phospholipids (Ruelland et al. 2002). The degradation of membrane lipids begins during freezing and after thawing, releasing unsaturated fatty acids which are peroxidized. Chlorophyll may be also be photooxidized in green tissues exposed to light (Sung et al. 2003). Chill injuries may occur not only during freezing, but also during the thawing of tissue. Plant survival is also determined by post-thawing environmental factors – rapid temperature growth and high light intensity may disturb metabolic pathways in cells and cause additional damage. During rapid melting of ice, the cell is rehydrated, and it quickly increases its volume. The above leads to tension and cracks in cell structures, mostly in the cytoplasm which is the site of primary cell injury. The above changes have less damaging consequences for dormant plants. In a temperate climate, winter frost is not a typical stressor for plants, but freezing temperatures could be a source of stress if they occurred in the spring or summer (Muller et al. 2005).

2.2 Resistance to low Temperature, Acclimatization

Resistance is related to frost tolerance, that is, the ability of the organism to survive low temperatures without damage. In regions characterized by seasonal climate change, plants' resistance to freezing fluctuates periodically – it is the lowest during intensive elongation growth in the spring, and it rises significantly in the fall when growth is arrested by the direct effect of low temperature or the combined effect of shorter daytime and temperature drop (Li et al. 2005a, b). Frost resistance is usually achieved by preventing ice formation in the symplast. An important mechanism preventing or delaying symplastic ice formation is frost plasmolysis. Poorly hydrated plants which are acclimatized to water stress usually show increased cold resistance, for example, plants which are extremely tolerant to drying out, for example, embryos of ripened seeds, can be conserved alive at −200°C without damage (Jan et al. 2009). Species-specific cold resistance is a genetically programmed trait that can be modified by both endogenous and exogenous factors. For a vast number of species, frost tolerance is not a static feature, but it is closely correlated with season, it fluctuates in various growing periods, and it is not identical for all organs (Rorat et al. 2006; Hekneby et al. 2006). The above-ground parts of wheat seedlings were acclimatized even to −20°C, but the roots' sensitivity to frost did not change. Acclimatization can be accelerated by hardening the plants, that is, exposing them to increasingly lower temperatures on successive days, initially above zero, followed by insignificantly below zero (Li et al. 2005a; Zhang and Tian 2010). This process is continued for several weeks. Plants are characterized by the greatest frost resistance 1–3 weeks from the beginning of exposure to freezing temperatures. The period of deacclimatization, that is, dehardening, is much shorter, and it usually lasts several days. The higher the ambient temperature, the faster the deacclimatization process. After dehardening, repeated exposure to frost can severely damage many plants (Li et al. 2004; Burbulis et al. 2008).

Plant organs are also marked by varied sensitivity to frost (Li et al. 2005a; Rorat et al. 2006). Roots are most susceptible to the damaging effects of freezing temperatures, shoots are less sensitive, while tree trunks and older branches are characterized by the highest frost resistance (Muller et al. 2005; Kato-Noguchi 2007). Snow cover minimizes the temperature drop in the soil, and it protects crops from freezing. The cold sensitivity of flowers is determined by the given species' phenological growth stages (Thakur et al. 2009; Ohnishi et al. 2010).

ABA stimulates and speeds-up plant hardening. According to Weiser (1970), acclimatization, and perhaps also hardening, is determined by modifications in gene expression. In this case,

ABA can enhance cold resistance if it is able to induce the expression of the respective genes (Gusta et al. 2005). Gibberellins and auxins deliver an opposite effect. Substances that retard gibberellin synthesis accelerate hardening. Intensive nitrogen fertilization generally delays dormancy and increases susceptibility to freezing. Heavy potassium fertilization has the opposite effect by increasing the frost resistance of both herbaceous and arborescent plants. The concentrations of sugar and other osmoprotectors that protect the cell from dehydration increases in the cytosol and vacuoles (Liang et al. 2009).

Fluid supercooling inside the cell is yet another factor that increases the plants' cold resistance by delaying crystallization in the cell. The presence of substances dissolved in the vacuolar sap lowers crystallization temperature. In small, weakly vacuolated cells, water may undergo deep supercooling. In large and hydrated parenchymal cells and xylem vessels, the supercooled state is very unstable, and it rarely lasts longer than several hours. Supercooling provides temporary protection against freezing caused by, for example, strong ground frost. In tissues comprising small, densely packed and weakly vacuolated cells whose walls prevent ice crystals from spreading, a supercooled state may persist until the temperature drops below a threshold value. The accumulation of nonpolar lipids on the surface of the plasmalemma also prevents ice penetration from the apoplast to the cell interior. In herbaceous plants, the supercooling of water is observed at −1 to −15°C, and in arborescent plants at −30°C, and even −50°C. Such a high degree of supercooling is observed only in some living tissues, such as core parenchymal cells, meristematic tissue, leaf bud scales, and flower buds. When ambient temperature drops below the critical supercooling point, this meta-stable state is rapidly disrupted, and ice is formed inside the cells, ultimately leading to their death. In some extremely frost-resistant tree species, the protoplasm is able to vitrify. Vitrification is stimulated by a high concentration of sucrose and other sugars. In this relatively stable condition, it is possible to cool cells almost to absolute zero without destruction (Rajashekar 2000; Hopkins 2006; Jan et al. 2009).

Membranes are restructured under exposure to cold before the temperature drops below zero. In these conditions, the water potential is gradually lowered with a simultaneous drop in the osmotic potential due to the accumulation of carbohydrates in vacuoles. ABA is accumulated, and it induces the synthesis of specific proteins. The next stage brings intensified changes in the cell membrane – degradation of phosphatidylcholine and phosphoinositol, accompanied by a continued increase in ABA levels and protein synthesis modifications (Gusta et al. 2005; Lindberg et al. 2005). Cryoprotectants, substances that directly protect the membrane from damage, are also synthesized at this stage. Rigid membranes are less likely to be deformed during frost-induced dehydration, and they protect cells against freezing more effectively. This parameter is largely dependent on the sterol content of cells (Hopkins 2006; Janska et al. 2010).

In addition to membrane unsaturation, it appears that lipid asymmetry in the membrane also contributes to the physical structure of the membrane at low temperature (Gomès et al. 2000).

The mechanism protecting chloroplast membranes enables the plant to begin photosynthesis as soon as ambient temperature increases.

The cold resistance of plants is also determined by the following mechanisms:

1. Thermal insulation which delays and minimizes heat loss, for example, shoot apices are often covered with dense foliage (rosette plant habit) or they winter under a layer of leaves or litter (geophytes). Frost tender organs are often rejected before the onset of very low temperatures (deciduous plants shed leaves in the fall). In high mountainous regions of tropical zones, the leaves of large rosette plants close above the tip at night to protect the interior from freezing (Hopkins 2006).
2. Water freezing in intertissue spaces, for example, between the seed coat and the embryo or between bud scales, where extensive areas are covered with ice.

3. Cell structures are protected against excessive dehydration with an accompanying increase in the effectiveness of barriers that prevent ice crystals from propagating from the apoplast inside the cell. The following mechanisms are involved:
   (a) Osmotic pressure increases to keep water inside the cell, and the water potential decreases due to the accumulation of osmotically active compounds (simple sugars and oligosaccharides, polyols, low-molecular-weight nitrogen compounds, such as selected amino acids) in vacuoles and hydrophilic proteins in the cytoplasm (Rorat 2006; Liang et al. 2009). The share of highly polar lipids in the membrane structure increases, such as phosphatidylcholine and phosphatidylethanolamine in the plasmalemma and cytoplasmic membranes or digalactosyldiacylglycerol in chloroplast membranes, which increases matrix interactions inside the cell.
   (b) The membrane is enriched with more stable lipids containing polyunsaturated fatty acid residues, selected sterols, and cryoprotectants are accumulated in the cytoplasm to protect cell structures against strong dehydration (Lindberg et al. 2005; Zhang and Tian 2010). These substances stabilize membrane structure and prevent conformational protein changes. They counteract the accumulation of salt ions and selected organic acids in the cell, and they protect proteins against denaturation. Small proteins, whose synthesis is enhanced or induced under exposure to low temperatures, play a protective role. Some of them show significant homology to proteins synthesized in response to water stress, for example, to dehydrin (Rorat et al. 2006). The cell wall plays an important role in protecting the cell against the adverse consequences of dehydration, and it is the main barrier to ice penetration.

In addition to mechanisms responsible for resistance to the primary consequences of frost, cold-resistant plants develop acclimatization mechanisms that enable them to avoid secondary thermal stress at below zero temperatures, such as photoinhibition, draught, oxygen deficiency (under ice cover), or mechanical effects of ice load (Alcázar et al. 2011).

3 High Temperature

Heat stress occurs when a rise in temperature has negative consequences for a plant. It is a complex function of intensity (temperature in degrees), duration and the rate of temperature increase. For plants inhabiting very cold climates such as the Arctic, temperatures in the region of 15°C can already be a source of heat stress. In a temperate climate, heat stress takes place in the temperature range of 35–40°C. In scientific literature, heat stress denotes temperatures that exceed the optimum values by around 10–15°C (Larkindale et al. 2005). Plants can be divided into three groups, subject to their sensitivity to high temperature (Fig. 5.8). In geographic zones with a hot climate, in habitats marked by high fluctuations in daily temperature (soil surface, littoral zone, shallow waters) or seasonal fluctuations and in volcanic areas, temperature levels can be lethal for vascular land plants. High absorption of solar energy during windless weather can increase the temperature inside plant tissues in excess of the ambient temperature. Creeping grass shoots, the runners and tillers of young plants can also be subjected to heat stress. The lethal temperature range (thermal death point) is determined by the duration of tissue exposure to high temperature (Table 5.2). Only single-celled organisms can complete their life cycle during continued exposure to temperatures higher than 50°C, while only prokaryotic organisms can survive in temperatures higher than 60°C.

3.1 Consequences of Heat Exposure

At very high temperatures, severe cellular injury and even death may occur within minutes or even seconds (due to denaturation and/or aggregation of proteins), while at moderately high temperatures,

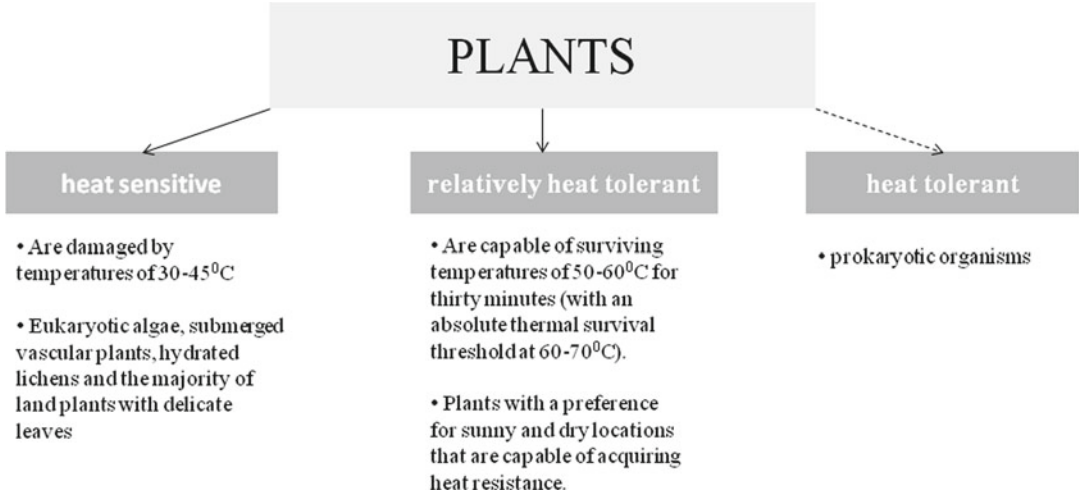

Fig. 5.8 Classification of the plants, subject to their sensitivity to high temperature (adapted from Stushnoff et al. 1984)

Table 5.2 The lethal temperature range (thermal death point) characteristic for varied types of plants

Type of plants	Thermal death point
Aquatic plants and plants growing in shaded habitats	38–42°C following several hours of exposure
Temperate plants with hydrated and metabolically active organs	45–55°C following several hours of exposure
Desert plants	Higher than 60–65°C for more than several hours per day

injuries or death may occur only after long-term exposure (due to disruptions in basic metabolic processes). The adverse effects of overheating are directly noticeable. The morphological symptoms of heat stress include scorching of leaves and twigs, sunburns on leaves, branches and stems, leaf senescence and abscission, shoot and root growth inhibition, fruit discoloration and damage, and reduced yield. Cell size reduction, closure of stomata, and curtailed water loss is observed at the tissue and cellular level. At the subcellular level, major modifications occur in chloroplasts (changing the structural organization of thylakoids, loss of grana stacking or its swelling) (Wahid et al. 2007; Mitra and Bhatia 2008). In vascular land plants, the negative consequences of elevated temperature are often related to secondary stress, namely, a negative water balance (leading to the perturbation of many physiological processes) due to intensive leaf transpiration during daytime. Under field conditions, high temperature stress is frequently associated with reduced water availability (higher during daytime than at night). Heat stress may secondarily induce oxidative stress via the generation and the reactions of activated oxygen species (Xu et al. 2006; Almeselmani et al. 2006).

Metabolic pathways and processes show varied sensitivity to temperature which may result in a deficit or an excess of selected metabolites. It is generally believed that the processes taking place in membranes are most sensitive to temperature change. A heat-induced increase in membrane liquidity (either by denaturation of proteins or an increase in unsaturated fatty acids) and changes in reactions between lipid and protein components impair membrane functions (Savchenko et al. 2002; Wahid et al. 2007), including the functioning of ion and water channels, ion transporters, metabolite transport, energy generation, and other processes. Ion leakage

from the cell is observed; photosynthesis and respiration are also impaired (Wahid et al. 2007; Wang et al. 2009). It has also been suggested that changing membrane fluidity plays a central role in sensing (plant thermometer) and influencing gene expression both under high and low temperatures (Plieth 1999). Photochemical reactions in thylakoid lamellae and carbon metabolism in the stroma of chloroplast have been suggested as the primary sites of injury at high temperatures (Yang et al. 2006a; Wang et al. 2009). Thylakoid membranes are particularly sensitive to high temperature, and this especially applies to photosystem II whose activity is greatly reduced or even partially stopped under high temperatures (Salvucci and Crafts-Brandner 2004; Camejo et al. 2005; Marchand et al. 2005). High temperature has a greater influence on the photosynthetic capacity of C3 plants than C4 plants (Crafts-Brandner and Salvucci 2002). Heat shock reduces the amount of photosynthetic pigments (Wang et al. 2009), soluble proteins, rubisco binding proteins (RBP), large-subunits (LS), and small-subunits (SS) of rubisco in darkness but increases them in light (Kepova et al. 2005). Moreover, heat stress greatly affects starch and sucrose synthesis, as demonstrated by the reduced activity of sucrose phosphate synthase, ADP-glucose pyrophosphorylase, and invertase (Wahid et al. 2007; Sumesh et al. 2008). In any plant species, the ability to sustain leaf gas exchange under heat stress is directly correlated with heat tolerance. During the vegetative stage, high daytime temperature can cause damage to compensated leaf photosynthesis, reducing CO_2 assimilation rates (Crafts-Brander and Salvucci 2002; Morales et al. 2003). Photosynthesis is more sensitive to heat than dark respiration which could have additional consequences under prolonged stress, including the depletion of carbohydrate reserves and plant starvation (Sumesh et al. 2008). Heat stress rapidly increases selected phytohormone levels, including ABA, ethylene, and salicylic acid (SA), and it decreases cytokinin and gibberellin concentrations (Dat et al. 2000; Talanova et al. 2003; Larkindale and Huang 2004). The overlapping effects of the above changes in hormone levels speed-up plant aging.

3.2 Mechanism of Plant Resistance to High Temperature

Plants rely on two adaptation mechanisms to survive high temperatures: the ability to prevent excessive temperature growth in tissues or alleviate its effects and the heat tolerance of the protoplasm.

Survival in hot, dry environments can be achieved in a variety of ways, by combinations of adaptations (Fitter and Hay 2002). Plants growing in a hot climate avoid heat stress by reducing the absorption of solar radiation. This ability is supported by the presence of small hairs (tomentose) that form a thick coat on the surface of the leaf as well as cuticles, protective waxy covering. In such plants, leaf blades often turn away from light and orient themselves parallel to sun rays (paraheliotropism). Solar radiation may also be reduced by rolling leaf blades. Plants with small leaves are also more likely to avoid heat stress: they evacuate heat to ambient more quickly due to smaller resistance of the air boundary layer in comparison with large leaves. Plants rely on the same anatomical and physiological adaptive mechanisms that are deployed in a water deficit to limit transpiration. In well-hydrated plants, intensive transpiration prevents leaves from heat stress, and leaf temperature may be 6 K or even 10–15 K lower than ambient temperature. Many species have evolved life histories which permit them to avoid the hottest period of the year. This can be achieved by leaf abscission, leaving heat-resistant buds, or in desert annuals, by completing the entire reproductive cycle during the cooler months (Fitter and Hay 2002). Such morphological and phonological adaptations are commonly associated with biochemical adaptations favoring net photosynthesis at high temperatures (in particular C_4 and CAM photosynthetic pathways), although C_3 plants are common in desert floras (Fitter and Hay 2002).

Heat tolerance is generally defined as the ability of the plant to grow and produce economic yield under high temperatures. This is a highly specific trait, and closely related species, even different organs and tissues of the same plant, may vary significantly in this respect. The above

is affected by climate conditions and the species' geographic origin. Plants native to cold regions (tundra, high mountain ranges) are much more sensitive to heat than temperate flora. The latter, in turn, are more susceptible to high temperatures than desert and tropical plants. The highest heat tolerance is demonstrated by selected sedge and grass species, mainly C_4 plants. Heat tolerance is associated with greater enzyme thermostability and a higher share of saturated fatty acids in membrane lipids which increases the lipid phase transition (melting) temperature and prevents a heat-induced increase in the membrane's liquidity. It is believed that PG is the phospholipid initiating phase transitions in thylakoid membranes. Heat tolerance leads to a rapid genome reaction even during short-term overheating. The biosynthesis of heat stress proteins (HSP) which prevent macroparticle denaturation is induced (Kotak et al. 2007; Al-Whaibi 2010). During exposure to high temperature, plants synthesize two groups of HSP: four high-molecular weight HSPs (HSP 100, HSP 90, HSP 70, HSP 60) and several low-molecular weight HSPs (smHSPs). Those proteins remain stable over a certain period of time, and they are probably the main factor enabling plants to survive a temperature increase. HSPs are found in the cytoplasm and organelles such as the nucleus, mitochondria, chloroplasts, and endoplasmic reticulum. The tolerance conferred by HSPs results in improved physiological phenomena such as photosynthesis, assimilate partitioning, water and nutrient-use efficiency, and membrane stability. Those improvements make plant growth and development possible under heat stress (Wang et al. 2004). The HSPs/chaperones may be involved in stress signal transduction, gene activation, and the regulation of the cellular redox state. They also interact with other stress-response mechanisms such as the production of osmolytes and antioxidants (Kotak et al. 2007; Wahid et al. 2007; Al-Whaibi 2010). In heat-stressed plants, the induction of HSP synthesis inhibits the biosynthesis of other proteins. A plant's resistance to heat is determined by protein synthesis in cells that are lost with age. For this reason, aging organs (and organisms) have impaired ability to acclimatize to high temperature. Smaller quantities of HSPs are also determined at optimal temperature, but in this environment, they play a different role than during and after stress. Under optimal conditions, HSPs regulate the formation of protein structures from newly emerged polypeptide strings to protect the cell from proteins that are nonfunctional due to synthesis "errors." At excessively high temperatures, HSPs minimize cell injuries by protecting cell proteins from denaturation and creating chelate bonds with ions leaking from the vacuoles into the cytosol (Kotak et al. 2007; Wahid et al. 2007; Al-Whaibi 2010). An increased content of ABA mediates the acclimation/adaptation of plants to desiccation by modulating the up- or downregulation of numerous genes (Talanova et al. 2003; Wahid et al. 2007). It is suggested that the induction of several HSPs (e.g., HSP70) is regulated by ABA (Snyman and Cronje 2008). Increased ethylene secretion at high temperatures leads to the abscission of reproductive organs; this is accompanied by both reduced levels and transport capacity of auxins to reproductive organs (Wahid et al. 2007). Among other hormones, SA has been suggested to be an important component of signaling pathways in response to systemic acquired resistance (SAR) and the hypersensitive response (HR) during heat stress (Kawano et al. 1998; Wang and Li 2006). Gibberellins and cytokinins have an opposite effect on high temperature tolerance than ABA. The potential roles of other phytohormones in plant thermotolerance are yet unknown (Wahid et al. 2007). Under stress, different plant species may accumulate a variety of osmolytes such as sugars and sugar alcohols (polyols), proline, tertiary and quaternary ammonium compounds, and tertiary sulphonium compounds (Singh and Grover 2008). The accumulation of such solutes may contribute to enhanced stress tolerance of plants, for example, proline and glycinebetaine may buffer the cellular redox potential under heat and other environmental stresses (Wahid and Close 2007); gama-4-aminobutyric acid (GABA) has a physiological role in the mitigation of stress effects (Kinnersley and Turano 2000). High-temperature stress induces the production of phenolic compounds such as flavonoids and

phenylpropanoids. The heat-induced increase in the activity of phenylalanine ammonia-lyase (PAL) is considered to be the cell's main acclamatory response to heat stress (Wahid and Ghazanfar 2006; Wahid 2007). Carotenoids of the xanthophylls family and selected terpenoids, such as isoprene or tocopherol, stabilize and photoprotect the lipid phase of thylakoid membranes during exposure to strong light and/or elevated temperatures (Wahid and Ghazanfar 2006; Wahid 2007). The expression of stress proteins is an important adaptive mechanism for environmental stress tolerance. Most stress proteins are soluble in water and, therefore, they contribute to stress tolerance, presumably by hydrating cellular structures (Wahid and Close 2007). Heat stress also induces the synthesis of other plant proteins, including ubiquitin (Sun and Callis 1997), cytosolic and chloroplasts Cu/Zn-SOD (Tang et al. 2006) and Mn-POD (Brown et al. 1993), cytosolic (Iba 2002) and chloroplasts APX (Tang et al. 2006), and other antioxidant enzymes (Sairam et al. 2000; Almeselmani et al. 2006), proteins of late embryogenesis abundant (LEA) (Goyal et al. 2005), and dehydrins. Their main function is to protect cellular and sub-cellular structures against oxidative damage and dehydrative forces.

3.3 Adaptation to High Temperature

Plants adapt to heat already after several hours of exposure to a temperature that evokes a stress response, but remains below the lethal temperature level (Xu et al. 2006). For most land plants, heat stress is triggered at temperatures slightly above 35°C, and in grasses at 38–40°C. The loss of resistance (dehardening) is a slower process that lasts several days in optimal growth conditions (Sung et al. 2003; Burke and Chen 2006). During acclimatization, the structure of the cell membrane changes by increasing the share of saturated fatty acids in the lipid layer. More unsaturated acyl residues are removed from the *sn*-2 position in a glycerolipid molecule by the respective hydrolases. They are replaced with saturated fatty acid residues (mostly 18-carbon chains) with the involvement of the respective acetyltransferases and lipid transport proteins. At the current state of knowledge, it remains unknown whether a higher or a lower degree of membrane lipid saturation is beneficial for high-temperature tolerance (Klueva et al. 2001; Rahman et al. 2004). It is believed that the synthesis of HSP is also an effective mechanism protecting the plant from high temperature and other HSP synthesis-inducing stressors. In many plants, heat tolerance varies on a seasonal basis in view of their growth cycle and changes in seasonal temperature (Froux et al. 2004). During active growth, all plants are highly sensitive to temperature stress. Selected species of land plants increase their resistance to heat only in the summer, while others demonstrate the highest level of tolerance during winter dormancy. Dormant plants become resistant to stress upon reaching a developmental stage induced by factors other than high environmental temperature. In many land plant species, noticeable changes in heat tolerance are not observed. Due to the close correlation between drought and high temperature, the effects of each stressor on field-grown plants can be difficult to distinguish, and adaptations to arid environments can be effective only if they lead to avoidance or tolerance of both stresses (Fitter and Hay 2002).

4 Conclusion and Future Perspective

Temperature is a major environmental factor that changes from season to season and undergoes daily fluctuations. For this reason, the stress-inducing role of temperature is difficult to define unambiguously since the response to various temperatures is determined by the plants' ability to adapt to different climate regimes. Plants exhibit a variety of responses to different (high and low) temperatures, which are depicted by symptomatic and quantitative changes in growth and morphology. However, it must be remembered the ability of the plant to cope with or adjust to the temperature stress varies across and within species as well as at different developmental stages. Generally heat or low temperature

stress induces structural changes in tissues and cell organelles, disorganization of cell membranes, disturbance of leaf water relations, and impedance of photosynthesis by effects on photochemical and biochemical reactions and photosynthetic membranes. In response to temperature stress, plants manifest numerous adaptive changes. Metabolic pathways and processes show varied sensitivity to temperature which may result in a deficit or an excess of selected metabolites (such as HSPS, osmoprotectants, antioxidative enzymes, etc.). According to this, it is important to discover the induction of signaling cascades leading to profound changes in specific gene expression is also considered an important temperature-stress adaptation. Molecular knowledge of response and tolerance mechanisms will pave the way for engineering plants that can tolerate high or low temperatures and could be the basis for production of crops which can produce economic yield under temperature-stress conditions (Iba 2002; Wahid et al. 2007).

Although physiological mechanisms of heat and low temperature tolerance are relatively well understood, further studies are essential to determine physiological basis of assimilate partitioning from source to sink, plant phenotypic flexibility which leads to tolerance, and factors that modulate plant temperature-stress response. It is known that complex traits of abiotic stress phenomena in plants make genetic modification for efficient stress tolerance difficult to achieve. However, the modification of a single trait resulted in several cases in significant improvements in stress tolerance. By now, little is also known about the molecular mechanisms underlying signaling components during stress response and adaptation. It must be also remembered, alteration of further upstream molecules in the pathway often activates a much wider network of genes, other than stress-specific ones. The discovery and use of new stress-tolerance-associated genes, as well as heterologous genes, to confer plant stress tolerance (including those unique to extreme growth-environment organisms, e.g., halophytes, thermophilic organisms), has been the subject of ongoing efforts to obtain tolerant plants. It must be also remembered, plants receive various stresses from their surrounding environment, which affect them in a complex manner (plant is usually subjected to many abiotic and biotic stresses at the same time). That means, it is necessary to identify the key strategies that plants use to deal with complex stresses of both biotic and abiotic origin (Iba 2002; Wahid et al. 2007).

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Responses and Management of Heat Stress in Plants

6

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Abstract

On the eve of global climate change, temperature increase, is the most evident phenomenon. This temperature increase is posing severe threat for sustainable crop production in many countries across the globe in the form of heat stress. Plants respond in many ways to the prevailing high temperature environment, and several inter- and intraspecific differences are reported. Heat stress produces quite tangible changes at cell, tissue, and organ levels. Photosynthetic acclimation to heat stress, synthesis and accumulation of primary and secondary metabolites, induction of stress proteins are among the major adaptive responses to heat stress. The important genes expressed in response to heat stress include heat shock protein (*hsp*) genes, dehydrins (*dhn*), senescence-associated (*sag*) genes, stay-green (*sgr*) genes. As mechanisms of heat stress tolerance, plants display the maintenance of membrane stability, scavenging of ROS, production of enzymatic and nonenzymatic antioxidants and adjustment of compatible solutes. Plant thermotolerance can be improved by various means; major being the mass screening and morphological and biochemical markers-assisted selection, identification, and mapping of QTLs conferring heat resistance, conventional and molecular breeding, and exogenous use of

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osmoprotectants and stress-signaling agents. Although pretty well understood, more research efforts are required to understand novel aspects of heat tolerance including molecular cloning and characterization of genes/proteins and understanding the basis of growth improvements with seed pretreatments and plant acclimations. In this chapter, we discuss the plant responses to high temperature stress and integrated approaches, such as genetics, breeding and management options to improve the resistance in plants against heat stress.

Keywords
Heat stress • Metabolites • Molecular breeding • Osmolytes • Pretreatment strategies

1 Introduction

Plant growth and development throughout the globe is controlled, in one way or the other, by the prevailing environmental conditions. Abiotic stresses, including temperature extremes, salinity and drought, are serious intimidation to the sustainability and productivity of economic plants. Current climatic model predicts that global air temperature may increase by 1.1–6.4°C with doubling of atmospheric CO_2 (Kim et al. 2007; Lobell and Field 2007). According to Intergovernmental Panel on Climatic Change (IPCC), there was an increase of 0.5°C during the past 100 years in ambient temperature, which is expected to rise by 0.2°C per decade for the next two decades and 1–3.4°C per decade warmer in the year 2100 (IPCC 2007). Such a rise in the global temperatures greatly influences the agricultural production, specifically in terms of aggravating the associated effects of salinity, drought, mineral toxicity stresses or as the case may be.

Global warming is negatively affecting the agricultural activities. On exposure to high temperature, crop yield is decreased because of shortened life cycle and accelerated senescence in different agro-climatic zones (Porter 2005). Higher temperatures, either of days and nights or of soil and air, hamper plant growth or cause considerable pre- and postharvest losses (Hall 2001). Injuries occur due to short- and long-term exposure to high temperature. Severe heat stress for short-terms can permanently damage to cells and tissues due to calamitous collapse of cellular organization (Schöffl et al. 1998). Long-term effects may be reduction in the size of tissues and organs and hampered morphological development (Gilani 2007; Rasheed 2009).

Heat stress substantially affects yields of many economically important cereals such as wheat, rice, maize, etc., and effects are quite often registered at the reproductive stages. Photosynthesis is the fundamental basis for carbon accumulation, growth and biomass yield in plants. Photosynthetic response of terrestrial plants can potentially change ecosystem carbon balance and cycling under global warming (Gunderson et al. 2000). Increased ambient temperature affects plant productivity by damaging photosynthesis (Al-Khatib and Paulsen 1990). According to Berry and Bjorkman (1980), at moderately higher temperatures suppression of photosynthetic rate is reversible; nonetheless upon exposure to extremely high temperature, the whole system of photosynthesis may be permanently damaged. It may also decrease chlorophyll content, net photosynthetic rate, and stomatal conductance (Morales et al. 2003). Likewise, upon exposure to severe heat stress, net photosynthesis is substantially inhibited due to impaired supply of Rubulose-1, 5-biphosphate (RUBP) (Law and Crafts-Brandner 1999).

Sudden heat stress may either denature the membrane proteins or increase the unsaturated fatty acids, leading to increased ion-leakage and thus the loss of cellular functions (Savchenko et al. 2002).

Such damages occur due to production of activated (AOS) and reactive oxygen species (ROS) and dehydration-induced changes in phase transitions due to high temperature (Nishida and Murata 1996; Liu and Huang 2000). Both the AOS and ROS react with pigments, membranes, enzymes, and nucleic acids, and change their structure and functions (Smirnoff 1993; Scandalios 1993; Sairam et al. 2000). Electrolyte leakage, a measure of stress injury to membranes, varies in relation to membrane abilities to take up and retain solutes and reflect stress-induced changes in their potential. Studies indicated that finding genotypic variability for heat tolerance based on leaf electrolyte leakage might be more effective to screen plants for relatively hot areas (Li et al. 1991; Rahman et al. 2004; Wahid and Shabbir 2005). Heat stress decreases the activities of antioxidant enzymes leading to increase in injury to cell membranes by lipid peroxidation and leaf senescence (Liu and Huang 2000).

Heat stress tolerance in plants is an intricate phenomenon involving a great variety of response, mechanisms, and management practices. Determination of responses and possible strategies to improve heat stress tolerance is important to grow crop plants in the heat-stressed areas of the world. In this perspective, this chapter presents explicit responses and some pragmatic management options to avert the high temperature stress effects in plants.

2 High Temperature Stress: Responses

Plants subjected to heat stress show a range of responses and manifest mechanisms to cope with its adversaries. These changes can be discerned at whole plant to subcellular and molecular levels. An individualistic account of all these responses is briefly described below.

2.1 Growth and Phenology

High temperature is a major determinant of agricultural production throughout the world and its effects are evident at all critical growth stages starting from seed germination to final yield harvested. An account of changes in the phenology of plants has been described in the following lines.

Seeds put to germinate at supraoptimal temperatures show reduced or even inhibited germination. In soybean, heat stress changed the protein expression profiles and reduced the seed germination and seedling vigor, which appeared to determine the seed quality attributes (Egli et al. 2005; Ren et al. 2009). Seed germination, seedling emergence and its establishment is prone to increased temperature in most of the plant species (Grass and Burris 1995; Burke 2001; Ashraf and Hafeez 2004; Wahid et al. 2007). Columbo and Timmer (1992) demonstrated that black spruce plant seedlings are more susceptible to high temperature stress than adult plants. Maize shows optimal germination and growth at 20–30°C and 28–31°C, respectively (Hughes 1979; Medany et al. 2007; Farooq et al. 2008a, b, 2009).

There are conflicting reports about the post-emergence seedling growth in maize under heat stress. For instance, some studies show that maize coleoptile was more heat tolerant at all stages of seedling development (Venter et al. 1997; Momcilovic and Ristic 2007), while in some other studies on maize, upon exposure to 40°C, there was a substantial reduction in coleoptile growth and at 45°C growth was completely stopped (Weaich et al. 1996, Akman 2009). Heat stress lowered the activity of specific enzymes and thus reduced the protein synthesis in germinating maize embryos (Riley 1981). Likewise, seedling growth and development of cotton (*Gossypium hirsutum* L.) was also reduced under heat stress (Mahan and Mauget 2005).

High temperature is a major environmental factor that determines the sustainability of crop growth and yield in some regions (Blum 1988; Al-Khatib and Paulsen 1999). Plants grown in warmer environments have much lower biomass than those grown at optimum or lower temperature (Kim et al. 2007). High temperature reduced the plant growth by affecting different mechanisms (Sibley et al. 1999; Wollenweber et al. 2003). For example, it decreased the dry weight,

Table 6.1 Effect of heat stress on yield and yield components of the bread and durum wheat genotypes

Genotype	Control	Hat stress	Genotype × temperature
Grains per spike			
Bread wheat mean	70 ± 0.85	70 ± 0.88	ns
Golia 69	69 ± 1.31 a	70 ± 1.43 a	ns
Sever	71 ± 1.10 a	71 ± 1.05 a	ns
Durum wheat mean	63 ± 0.75	64 ± 0.74	ns
Acalou	66 ± 0.98 a	65 ± 0.95 a	ns
TE 9306	60 ± 0.99 b	63 ± 1.15 a	ns
Individual grain weight (mg)			
Bread wheat mean	56.54 ± 1.08	48.73 ± 0.90	***
Golia 69	47.11 ± 0.84 a	43.75 ± 0.90 a	**
Sever	64.67 ± 1.03 b	53.53 ± 1.23 b	***
Durum wheat mean	72.06 ± 0.60	59.97 ± 0.61	***
Acalou	72.42 ± 0.70 a	57.69 ± 0.86 a	***
TE 9306	71.74 ± 0.95 a	62.55 ± 0.70 b	***
Yield per spike (g)			
Bread wheat mean	3.97 ± 0.09	3.43 ± 0.07	**
Golia 69	3.26 ± 0.08 a	3.06 ± 0.09 a	ns
Sever	4.58 ± 0.11 b	3.78 ± 0.09 b	***
Durum wheat mean	4.52 ± 0.06	3.84 ± 0.06	***
Acalou	4.76 ± 0.08 a	3.77 ± 0.08 a	***
TE 9306	4.29 ± 0.09 b	3.92 ± 0.08 a	**

ns nonsignificant, significant at the **0.01, and ***0.001 levels of probability, respectively; for each *Triticum* species, different letters in the same column refer to significant differences between genotypes. *Source*: Dias and Lidon (2009)

growth and net assimilation rates of shoot (Wahid 2007). Likewise, heat shock affected the meristematic activity and reduced the growth of various parts mainly the leaves (Salah and Tardieu 1996). Applied heat stress arrested the cell wall elongation and altered cell differentiation (Potters et al. 2007).

Reproductive growth is more critically affected by the prevailing high temperature stress during anthesis and seed growth. Pollination is especially sensitive to heat stress. The mature pollens are more sensitive, and quite often fail to fertilize (Dupuis and Dumas 1990). Heat stress interferes with the development of pollen mother cell and microspore and causes male sterility (Sakata et al. 2000; Sato et al. 2006; Abiko et al. 2005). In the event of successful pollination, heat stress affected the kernel development in maize (Monjardino et al. 2005) and reduced the kernel density and reproductive growth in maize, wheat and *Suneca* during kernel development (Wilhelm et al. 1999; Maestri et al. 2002). Dias and Lidon (2009) did not find any effect of heat stress on number of grains per spike in both durum and bread wheat; nonetheless upon exposure to heat stress during grain growth, grain size was substantially reduced in both bread and durum wheats (Table 6.1). Likewise, heat stress also reduced the grain yield in both wheats (Table 6.1). However, different genotypes responded variably in terms of grain size and grain yield and a strong relationship between genotypes and temperature has been observed (Table 6.1).

High temperature also results in the boll and flower bud abortion in cotton, pea, and brassica, possibly owing to limited water supply and nutrients during reproductive development (Hall 1992; Guilioni et al. 1997; Young et al. 2004). During seed development, heat stress was found to affect seed storage process and kernel quality like starch and protein metabolism during grain filling in maize (Wilhelm et al. 1999; Maestri et al. 2002).

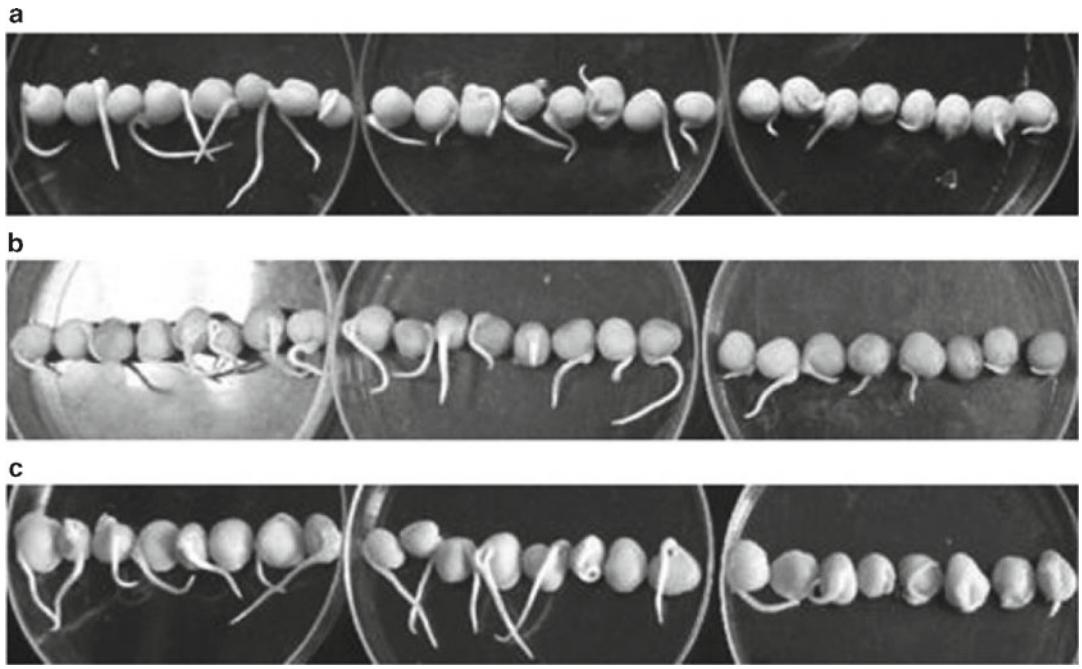

Fig. 6.1 Hypocotyl elongation phenotype of different pea varieties for 36 h in the dark at 22°C after heat acclimation and stress [(**a**) local variety, (**b**) Shandong variety, (**c**) Taiwan variety]. *Left* is expressed as control (22°C); *middle* is expressed as the germinated seeds were acclimated at 37°C for 1 h, followed at 22°C for 1 h, and then stressed at 48°C for 2 h. *Right* is expressed as the seeds were stressed at 48°C for 2 h. After Tian et al. (2009) with permission

2.2 Anatomical and Developmental Responses

Like other abiotic stresses, heat stress brings about quite a few morphogenetic and histological modifications. At whole plant level, generally cell size is reduced (Santarius 1973; Berry and Bjorkman 1980). There may be several morpho-anatomical modifications in cells and tissues such as increased densities of stomata and trichomes and greater xylem vessels area of shoot and root in *Lotus creticus* seedlings (Banon et al. 2004). On exposure of grapes to heat stress, cell membrane permeability was substantially increased and mesophyll cells were severely damaged (Zhang et al. 2005). High temperature also causes various changes at subcellular level. For instance, in chloroplast, it changed the thylakoids structure in maize (Karim et al. 1997) and resulted in loss of swelling and stacking of grana (Gounaris et al. 1984). In grapes, heat stress damaged the mesophyll cells, which showed round-shaped chloroplasts, swollen stroma lamellae, badly affected the antenna complex of photosystem (PS) II (Carpentier 1999), clump formation of vacuolar contents, disrupted cristae and deformed mitochondria (Zhang et al. 2005).

Heat stress restricted the emergence and elongation of hypocotyls in three pea (*Pisum sativum*) varieties (Tian et al. 2009). Nonetheless, heat acclimation for 1 h at 37°C improved the germination and hypocotyl development (Fig. 6.1). In the sprouting buds of sugarcane, heat stress badly affected the differentiation of various cells and tissues (Rasheed 2009). Here the major changes were noted on mesophyll cell expansion and development of vascular connections (Fig. 6.2).

2.3 Physiological and Metabolic Responses

In hot environments, plants exhibit various physiological and metabolic responses. The most

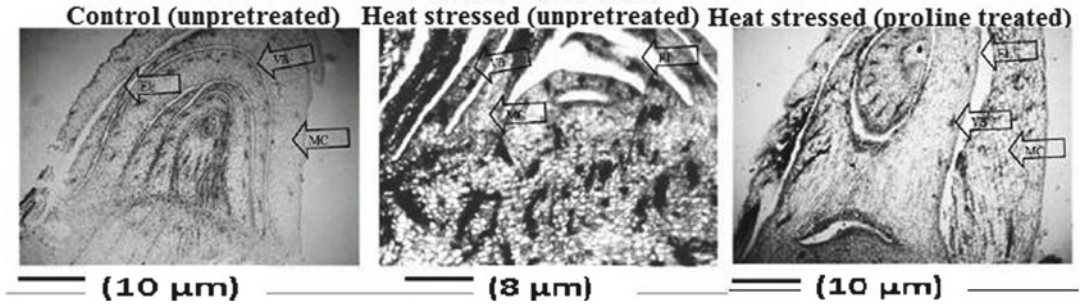

Fig. 6.2 Sprouting bud of sugarcane under control condition (*left*). Effect of heat stress on the histological changes in sprouting buds of sugarcane after 36 h of exposure (*middle*) and role of proline in mitigating heat stress effect (*right*). *Source*: Rasheed (2009)

important of those may be the changes in carbon fixation, oxidative stress; tissue water status and metabolites accumulation. These responses are briefly discussed below.

2.3.1 Photosynthesis

Heat stress causes photosynthetic acclimation and alters the physiological processes directly and changes the developmental patterns indirectly (Downton and Slatyer 1972). All the steps, processes, and aspects of photosynthesis are prone to increased ambient temperature (Al-Khatib and Paulsen 1990). The photosynthesis in C_3 plants is more affected by high temperature than C_4 plants (Wahid and Rasul 2005). The maize seedling grown at 25°C and transferred to 35°C for 20 min led to 50% inhibition in photosynthesis (Sinsawat et al. 2004). Maize showed maximum net photosynthesis near 31°C, decreased at temperature above 37°C and was completely inhibited near 45°C (Crafts-brandner and Salvucci 2002). Heat stress diminished the net photosynthetic (Pn) and stomatal conductance substantially in many plant species (Ranney and Peet 1994; Crafts-Brandner and Salvucci 2002; Morales et al. 2003); in this regard, Pn in developed leaves was more sensitive than mature leaves (Karim et al. 1997, 1999).

Photosynthetic apparatus are highly sensitive to heat temperature and inhibited when leaf temperature exceed 38°C in most plants (Edwards and Walker 1983). PS-II, water splitting and oxygen evolving complex (OEC) in photosynthesis are more heat-sensitive components of photosynthesis (Havaux 1993; Pastenes and Horton 1996a; Heckathorn et al. 1998a). Extensive studies show that both PS-I and PS-II are damaged by increased temperature. In barley and potato, heat stress damaged PS-I and PS-II and affected electron transport (Havaux 1998; Szilvia et al. 2005). Heat stress damaged the antenna complex of PS-II and reduced photosynthetic behavior (Carpentier 1999; Rokka et al. 2000; Zhang et al. 2005). High temperature during greening led to the inactivation of PS-I and PS-II (Sasmita and Narendranath 2002). High temperature increased chlorophyll a:b ratio and decreased chlorophyll:carotenoid ratio in sugarcane (Wahid 2007).

High temperature alters the energy sharing by changing the action of Calvin cycle and other metabolic processes such as photorespiration, synthesis and stability of the Rubisco enzyme (Holaday et al. 1992; Pastenes and Horton 1996b), disruption of electron transport activity and bound RUBP supply by heat stress (Ferrar et al. 1989). Extreme temperature reduced the activation state of Rubisco enzyme in the exposed leaf tissue and increased the RUBP (Feller et al. 1998; Crafts-Brandner and Law 2000), which inhibited photosynthesis as compared to control plants (Sharkey et al. 2001). High temperature enhances chlorophyllase activity and decreases the quantities of photosynthetic pigments (Todorov et al. 2003). The loss of chlorophyll is good indicator of heat tolerance in wheat (Ristic et al. 2007, 2008). High temperature modifies the activities of carbon metabolism enzymes,

especially the Rubisco (Ferrar et al. 1989; Holaday et al. 1992; Pastenes and Horton 1996a, b). Moreover, activities of starch and sucrose synthesis enzymes are greatly influenced (Chaitanya et al. 2001; Vu et al. 2001).

2.3.2 Reactive Oxygen Species and Oxidative Damage

Like other abiotic stresses, heat stress evokes the ROS generation including hydrogen peroxide (H_2O_2), superoxide radical (O^{2-}), singlet oxygen ($^1O_2$) and hydroxyl radical (OH^-), and induces oxidative stress (Mittler 2002; Taiz and Zeiger 2006; Potters et al. 2007). Chloroplast and mitochondria are the major sites where superoxide radicals are regularly produced, whereas some quantities are also produced in microbodies. Principally, ROS causes peroxidation of membrane lipids, destruction of pigments, and modification of membrane functions (Xu et al. 2006). The OH^- appears to be more damaging than other ROS, which is formed with the combination of O_2^- and H_2O_2 in the presence of Fe^{2+} and Fe^{3+} in trace amounts in Haber–Weiss reaction (Apel and Hirt 2004). The OH^- is greatly damaging to chlorophyll, proteins, lipids, DNA, and other important macromolecules (Sairam and Tyagi 2004).

Tolerant plants have the tendency to protect themselves from the damaging effects of ROS with the synthesis of various antioxidant systems (Apel and Hirt 2004). This protection starts with the conversion of O^{2-} by superoxide dismutase (SOD) into H_2O_2 with the help of ascorbate peroxidase (APX) or catalase (CAT). A number of physiological processes are affected by the overexpression of SOD in plants, including removal of H_2O_2, toxic reductants, biosynthesis and degradation of lignin in cell walls, auxin catabolism, etc. (Scandalios 1993). Activation of APX is due to the physiological injuries occurring in plants under heat stress (Mazorra et al. 2002).

Increased levels of ROS under high temperature cause cellular injury due to reduced antioxidant activity in the stressed tissues (Fadzillah et al. 1996; Mittler et al. 2004). In order to increase the heat tolerance, the levels and activities of antioxidants must be increased to protect against high temperature-induced oxidative stress. Studies conducted in this regard revealed that heat acclimated turf grass showed reduced ROS production owing to enhanced ascorbate and glutathione synthesis (Xu et al. 2006). It is suggested that antioxidant capacity of cells can be increased by some signaling molecules (Gong et al. 1997; Dat et al. 1998). Nonetheless, research is imperative to add to the list of potential signaling molecules, which may enhance the antioxidant production in cells exposed to heat temperature stress (Wahid et al. 2007).

2.3.3 Water Relations

Heat stress drives the rapid loss of water from the plant surface, causes tissue and organ dehydration, and restricts growth in plant species, for example, sorghum (Machado and Paulsen 2001), tomato (Mazorra et al. 2002), and sugarcane (Wahid and Close 2007). Heat stress produces osmotic strain on the growing tissues due to diminished root hydraulic conductance and tissue water status (Jiang and Huang 2001; Morales et al. 2003). Likewise, it may result in substantial reduction in sorghum (*Sorghum bicolor*) leaf growth and leaf water content and water potential in wheat (Shah and Paulsen 2003).

Heat stress also disrupts the uptake and translocation of water, ions, and organic solutes across the plant membranes, interferes with photosynthesis and respiration, increases evapo-transpiration rate, reduces the leaf osmotic potential and increases the chlorophyll fluorescence (Tsukaguchi et al. 2003; Huve et al. 2005; Taiz and Zeiger 2006). It results in stomatal closure and reduces the tissue water contents (Berry and Bjorkman 1980; Wahid et al. 2007). Heat stress-induced water stress thus is closely associated with reduction of soil water contents (Talwar et al. 1999).

2.3.4 Osmolytes Accumulation

Accumulation of certain low molecular mass organic compounds, generally called compatible solutes or osmoprotectants, is an important adaptive mechanism in plants subjected to abiotic stresses including temperature extremes (Hare et al. 1998; Sakamoto et al. 1998). Several osmolytes, including sugars and sugar alcohols (polyols), proline, tertiary, and quaternary ammonium

compounds and tertiary sulphonium compounds, are reported to accumulate in different plant species exposed to stress conditions (Sairam and Tyagi 2004; Wahid et al. 2007).

Among different compatible solutes, enhanced synthesis of soluble sugars, free proline and glycinebetaine (GB) has been more frequently studied for their osmoregulatory and protective roles (Matysik et al. 2002; Bohnert et al. 2006; Wahid 2007; Wahid et al. 2008; Farooq et al. 2008a). GB plays a great role as osmoprotectant in plants under a range of abiotic stresses including high temperature (Sakamoto and Murata 2002). However, the ability of plants to synthesize GB under stressful conditions varies among species (Ashraf and Foolad 2007). For example, sugarcane under heat stress (Wahid and Close 2007) and maize under drought (Quan et al. 2004) and chilling (Farooq et al. 2008c) and rice under drought (Farooq et al. 2008a) are reported to accumulate large amounts of GB. Like GB, increased free proline accumulation in higher plants in response to abiotic stresses has also been reported (Kavi Kishore et al. 2005). Biosynthesis of GB or proline may buffer the cellular redox potential under heat and other abiotic stresses, suggesting their functional significance (Rontein et al. 2002). The accumulation of soluble sugars was greatly implicated for improved heat tolerance of sugarcane (Wahid and Close 2007). In view of the importance of osmoprotectants accumulation, more concerted efforts on engineering pathways for enhanced biosynthesis of osmolytes may be fruitful (Ashraf and Foolad 2007).

2.3.5 Metabolite Synthesis

Heat stress leads to the accumulation of a range of primary and secondary metabolites. Primary metabolites are either direct products of carbon fixation (e.g., sugars, organic acids) or are synthesized after preliminary transformations of primary metabolites (e.g., amino acids, betaines alcohol sugars). Like other abiotic stresses, the accumulation of primary metabolites under heat stress has also been well documented (Iba 2002; Zhu 2003). Important primary metabolites showing accumulation under heat stress include free proline, GB, soluble sugars, etc., (Wahid 2007; Wahid and Close 2007; Wahid et al. 2008). In a recent study, using cluster and principal component analyses, it was revealed that out of 122 primary and secondary metabolites determined using advanced techniques like GC-MS and amino acids analyzer, only sucrose, quinate, trans-aconitate, guanine, γ-amino butyric acid (GABA), and ethanolamine held relationships with the high temperature tolerance of sugarcane bud chips (Rasheed 2009).

On the contrary, the synthesis and accumulation of secondary metabolites are less well understood under high temperature stress. Secondary metabolites are biosynthesized in plants from the intermediates of primary carbon metabolism via phenylpropanoic acid, shikmic acid, mevalonic acid, and methyl erythritol phosphate pathways (Taiz and Zeiger 2006). Recently, it is reported that heat stress induces production of secondary metabolites including phenolics, flavonoids, phenyl propanoids, and plant steroids (Bharti and Khurana 1997; Wahid 2007; Wahid et al. 2008). Carotenoids show a role in protecting cellular structures in various plant species under different stress types (Havaux 1998). Studies show that lipid layer of the thylakoid membranes are stabilized and photoprotected by various carotenoids and some terpenoids such as isoprene and α-tocopherol. Exposure of plants to strong light and high temperatures caused the partitioning of xanthophylls (violaxanthin, anthraxanthin, zeaxanthins, etc.) between the light-harvesting complexes and lipid phase of thylakoid membranes and increases membrane thermostability (Havaux 1993, 1998).

Isoprenoids are low molecular weight volatile compounds, synthesized via mevalonic acid pathway (Taiz and Zeiger 2006); their emission from leaves confers their role in heat tolerance (Loreto et al. 1998; Sharkey 2005). Although their synthesis is cost intensive, they show compensatory benefits in terms of heat resistance (Funk et al. 2004). Plants, capable of emitting higher amounts of isoprene, photosynthesize better under heat stress, which indicates a relationship between isoprene emission and heat tolerance (Velikova et al. 2004). Isoprene emission protects PSII under high temperature (Sharkey 2005),

whereas the endogenous production of isoprene protects the biological membranes by directly binding with singlet oxygen ($^1O_2$) by virtue of isoprene-conjugate double bond (Velikova et al. 2004).

Phenolics are the largest class of secondary metabolites and include flavonoids, lignin, anthocyanin, etc. Accumulation of soluble phenolics under heat stress is accompanied with increased activity of phenyl ammonia lyase (PAL) but decreased activity of peroxidase polyphenyl lyase (Taiz and Zeiger 2006). Acclimation to heat stress is triggered by the biosynthesis of phenolic compounds induced by high temperature (Rivero et al. 2001). They act as efficient antioxidants in plant tissues under stressful conditions (Dixon and Paiva 1995; Sgherri et al. 2004). Levels of flavonoid (e.g., anthocyanins) are greatly altered in plant tissues under heat stress (Oren-Shamir and Nissim-Levi 1999; Sachray et al. 2002; Wahid et al. 2008).

Plant steroids, a class of secondary metabolites, also influence a variety of functions under stressful conditions. Brassinosteroids (BRs) and ginsenosides are important plant steroids whose physiological importance to high temperature tolerance in plants has been explored. Studies confirm that BRs confer tolerance to high temperature stress in brassica and tomato seedlings, by inducing the biosynthesis of major heat shock proteins (Dhaubhadel et al. 1999). Production of ginsenosides, another important plant steroid, has been reported in all organs of *Panax quinquefolius*. It is recently reported that growing season had a great effect on the ginsenosides biosynthesis *P. quinquefolius* plants grown at high temperatures had 49% higher concentrations of storage root ginsenosides than respective control plants (Jochum et al. 2007; Wahid and Tariq 2008).

2.4 Molecular Responses

Transcritional regulation plays an important role in plant defense from heat stress (Singh et al. 2002). Heat stress induces numerous genes encoding transcriptional factors, which are involved in heat stress response and tolerance (Chen and Zhu 2004; Kotak et al. 2007). Different studies revealed that several genes are up- and downregulated by abiotic stresses (Kawasaki et al. 2001; Provart et al. 2003; Nogueira et al. 2003). Elevated temperature affects the gene expression in storage protein synthesis and starch metabolism during grain filling stage in rice (Yamakawa et al. 2007). Heat stress changes the pattern of gene expression, which is important for thermotolerance (Yang et al. 2006). An account of various genes and proteins showing expression under heat stress is given below.

2.4.1 Heat Shock Genes and Proteins

Several transcriptome studies have identified many stress-responsive genes and encoding transcriptional factors during environmental stresses. Recent transgenic approaches suggest that heat tolerance is a multigenic character. Heat shock induces many genes, which are attributed to heat shock elements (HSE).These HSE are situated in the promoter region of *hsp* genes (Hubel and Schöffl 1994). Transgenic approach confirmed that Heat-shock transcription factor (HSF) binding to pentameric nucleotides (5′-nGAAn-3′) of HSE sequences (Perisic et al. 1989; Sung et al. 2003). This HSF–HSE interaction and transcriptional activation is quite conserved in nature. This multigenic phenomenon modifying the expression pattern of transcription factors motivate a series of genes (Dong et al. 2003). Studies show that *Hot1-Hot4* genes of *Arabidopsis* may function to improve heat tolerance; Hot1 is identified as *Hsp101* in *Arabidopsis thaliana* (Hong and Vierling 2000). *HsfA1* acts as master regulator of thermotolerance in tomato by reducing the expression of heat shock genes in co-suppression lines (Mishra et al. 2002).

Hsfs are essential for gene expression in response to high temperature (Nover et al. 2001). Various studies show that distinctive *hsp* genes are not expressed in germinating pollen. Only *hsp18* and *hsp70* genes are transcribed in response to heat stress (Wahid et al. 2007). A defective heat shock response of mature maize pollen was due to inefficient induction of heat shock gene transcription (Hopf et al. 1992). Enhanced expression of HSP70 assisted in translocation,

proteolysis, protein translation, protein folding, aggregation, and refolding of denatured proteins (Zhang et al. 2005; Iba 2002; Weggle et al. 2004; Gorantla et al. 2007). Recent studies revealed that α-amylase genes in seeds of rice reduced the seed weight and chalkiness during ripening under heat stress (Asatsuma et al. 2006; Yamakawa et al. 2007).

The synthesis and accumulation of heat shock proteins (HSPs) through heat shock factors (HSFs) network play great role in plant responses to heat stress (Wang et al. 2004; Kotak et al. 2007). Amounts of specific mRNA synthesis, mRNA stability, translation efficiency and alteration in protein activity increase in plants as a result of gene expression (Sullivan and Green 1993). All organisms synthesize HSPs upon exposure to high temperature. Heat stress altered gene expression in reproductive organ of plant (Dupuis and Dumas 1990; Oshino et al. 2007). Abortion of development and demarcation of pollen mother cell due to heat shock is due to tissue specific alterations in gene expression (Sakata et al. 2000; Abiko et al. 2005). In plants, a heat shock of 8–10°C above ambient temperatures induces the synthesis of both high (60–110 kDa) and low (15–30 kDa) molecular weight HSPs (Vierling 1991; Waters et al. 1996; Sun et al. 2002). These HSPs were induced either to protect the plant from injury or to help repair the injury caused by the heat stress (Leshem and Kuiper 1996). The synthesis of HSPs occurred in different plant species when they were exposed 10–15°C above growing temperatures (Dubey 1999). Their synthesis is extremely fast, diverse, and intensive in a variety of organisms (Parsell and Lindquist 1993; Wahid et al. 2007).

Both cytosolic and organelle synthesis of HSPs has been well studied. Some HSPs that accumulate in the cytosol at 27°C and in the chloroplast at 43°C and 37°C respectively, appeared to play a role in photosynthesis and thermotolerance (Heckathorn et al. 1998b). In maize, high temperature induced the synthesis and accumulation of chloroplast protein elongation factor *EF-TU*, which defended the chloroplasts proteins from heat-induced damage (Ristic et al. 2004; Momcilovic and Ristic 2007). Maize *EF-TU* is a 45–46 kDa HSP confined to chloroplast stroma is involved in development at heat tolerance in maize (Ristic and Cass 1992; Bhadula et al. 2001; Moriarty et al. 2002). In maize, heat shock of 40°C induces the synthesis of HSPs18 (Nieto-Sotelo et al. 2002). Interaction of HSPs 22 kDa with the *Chenopodium album* and common bean chloroplast membranes affects the composition of membrane and decreases its fluidity; thus increasing the efficiency of ATP transport (Barua et al. 2003; Simões-Araújo et al. 2003).

Mitochondrial HSPs have been isolated from pumpkin (*Cucurbita pepo*) cotyledons under high temperature stress (Tsugeki et al. 1992; Kuzmin et al. 2004). They act as molecular chaperones in vitro (Schöffl et al. 1998; Guo et al. 2001; Kim and Schöffl 2002), prevent aggregation of denatured proteins (Sheffield et al. 1990), aid in folding of nascent polypeptides and refolding of denatured proteins (Lee et al. 1994; Goloubinoff et al. 1999). They also resolubilize the denatured aggregated proteins (Parsell et al. 1994). HSP68 synthesis was restricted to mitochondria as a precursor protein, but its synthesis increased during heat shock in cell (Neumann et al. 1993). When wheat, maize, and rye seedling were exposed at 42°C, five mitochondrial LMW HSPs (19, 20, 22, 23, and 28 kDa) were induced in maize and only one (20 kDa) in rye and wheat mitochondria each; the tolerance of maize was higher than wheat and rye (Korotaeva et al. 2001). The specific nucleus-encoded HSPs have been identified in potato, maize, soybean, barley, and tomato (Neumann et al. 1993; Nautiyal and Shono 2010), peas (Ko et al. 1992; Watts et al. 1992) under heat stress.

Although with less certainty, some putative functions have been assigned to HSPs when produced under normal or high temperature conditions. The rapid accumulation of HSPs may play a significant role in the safety of metabolic apparatus of the cell. Some HSPs are produced in some developing cells under control condition (Hopf et al. 1992) during embryogenesis, germination,

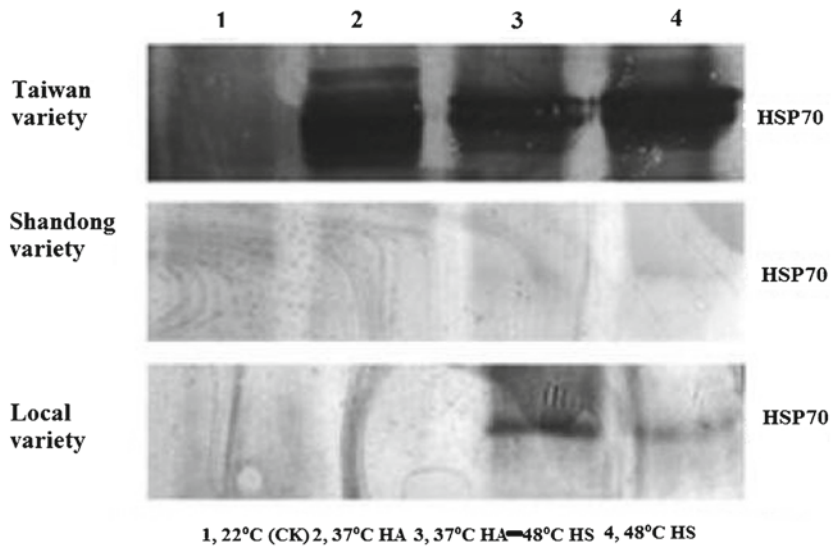

Fig. 6.3 Effect of heat acclimation and stress on the expression of HSP70 in hypocotyls of different pea varieties. After Tian et al. (2009) with permission

pollen formation, fruit set and its maturation (Vierling 1991; Sun et al. 2002 Prasinos et al. 2005; Wahid et al. 2007). For instance, HSPs were produced in greater amounts in etiolated maize seedling after 5-h exposure to high temperature stress (Lund et al. 1998). Acquired thermotolerance depends upon the synthesis of HSPs and their cellular localization (Heckathorn et al. 1999; Korotaeva et al. 2001). In arid and semi arid areas, plants may accumulate significant amount of HSPs in response to high leaf temperatures. In 2-day-old soybean seedlings, HSPs appeared to maintain the conformation of other proteins, as an aid for the acquired thermotolerance (Jinn et al. 1997). The wide diversity and abundance of HSPs is important for altering the plant response to high temperature stress (Waters et al. 1996). The mature pollen was susceptible to high temperature and pollen viability was extremely reduced due to nonproduction of HSPs. A distinct set of HSPs was induced in male tissues of maize under heat stress (Dupuis and Dumas 1990). HSPs (64 and 72 kDa) were induced in germinating pollens under heat stress (Frova et al. 1989). In a recent study, Tian et al. (2009) reported the improved heat tolerance of young pea seedlings due to enhanced synthesis of HSP70 (Fig. 6.3).

2.4.2 Dehydrins

Dehydrins (DHNs), belonging to subclass of LEA group II (Dure et al. 1989), are produced at the later stages of seed development in various plant species under drought, salinity, low temperature, heat stress, nutrients deficiency, and ABA application (Close 1996; Campbell and Close 1997; Svensson et al. 2002; Wahid and Close 2007; Pulla et al. 2007; Rurek 2010). D-11 from cotton (Baker et al. 1988), RAB16 (responsive to ABA) in rice (Mundy and Chua 1988) and RAB17 in maize (Vilardell et al. 1990) were cloned and characterized as DHN genes (Campbell and Close 1997; Ismail and Hall 1999 Koag et al. 2003). Immunological evidence indicated that DHNs are expressed in cyanobacteria (Close and Lammers 1993), brown algae (Li et al. 1997), liverworts (Hellwege et al. 1994), ferns (Reynolds and Bewley 1993), ginkgo (Close and Lammers 1993), and conifers (Jarvis et al. 1996). Using immunological studies, DHNs were detected in the nucleus, cytoplasm, mitochondria, chloroplasts, and vacuole (Close 1996; Campbell

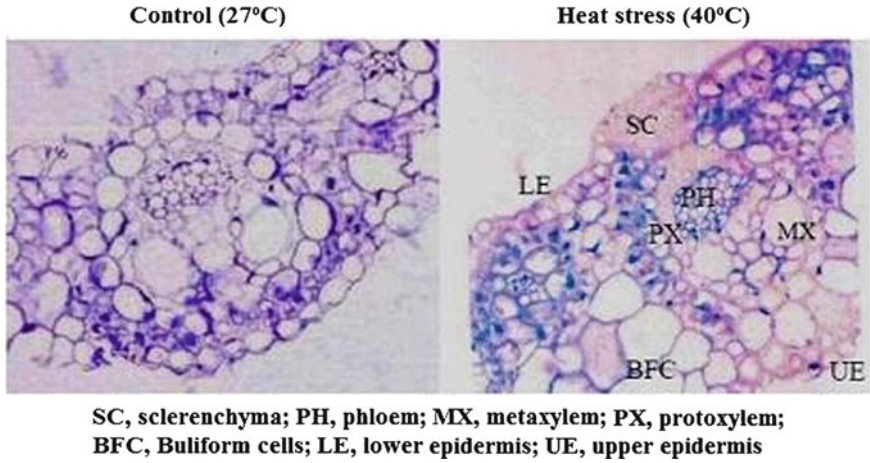

Fig. 6.4 Immunohistochemical expression of dehydrins in the leaf of sugarcane clone HSF-240 under control and heat stress. The dehydrins were found to associate to stele, lower and upper epidermis, and buliform cells during heat stress, as evident from golden brown color in staining. *Source*: Gilani (2007)

and Close 1997; Wahid et al. 2007) and found to be associated with cytoplasmic membranes system under abiotic stresses (Koag et al. 2003). Immuno-histolocalization studies revealed that the DHNs are associated with the mesophyll, vascular, and dermal tissues of heat-stressed sugarcane (Gilani 2007, Fig. 6.4). In maize, all parts of mature embryos show dehydrin accumulation (Godoy et al. 1994). In recent studies, three low molecular weight dehydrins were reported to be expressed in sugarcane leaves in response to heat stress (Wahid and Close 2007).

2.4.3 Senescence-Associated Genes

Temperature, pathogenic infection, drought, and nutrient deficiency; wounding and shading may increase leaf senescence (He et al. 2001). Thus about 183 senescence-associated genes (SAGs) are involved in energy metabolism, gene expression regulations, protein biosynthesis regulations, pathogenicity, stress and flower development (Liu et al. 2008). QTLs for some senescence-related traits have been mapped on chromosome 2A, 3A, 3B, 6A, 6B, and 7A in winter wheat subjected to heat stress (Vijayalakshmi et al. 2010). A number of encoding SAGs for proteinases such as serine proteinase in parsley (Jiang et al. 1999), cysteine proteinase in *Arabidopsis* (Lohman et al. 1994) and aspartic proteinase in *Brassica* (Buchanan-Wollaston and Ainsworth 1997) are associated with leaf senescence. A large number of SAGs and defense genes has been reported to express during leaf senescence in maize (Smart et al. 1995), barley (Kleber-Janke and Krupinska 1997), rice (Lee et al. 2001), *A. thaliana* (Lohman et al. 1994; Oh et al. 1996; Gepstein et al. 2003), tomato (John et al. 1997; Drake et al. 1996), radish (Azumi and Watanabe 1991), and *Brassica napus* (Buchanan-Wollaston and Ainsworth 1997).

Heat stress accelerates the senescence and results in decreased assimilation partitioning to grains (Spano et al. 2003). For instance, high temperature induced the expression of dehydration responsive genes (ERD1), which is known as SAG15. This gene also protects the cells from injury (Weaver et al. 1999). A combine effect of heat-shock and drought induced a senescence-associated gene (SAG12), at least in *Nicotiana tabacum*, which improved the stress tolerance in plants (Rizhsky et al. 2002). Heat shock (40°C) induced *tmr* genes in *Agrobacterium*, which delays the senescence. This was achieved by an inducible promoter such as HS6871 from soybean (Smart et al. 1991). Chen et al. (2002) identified 18 transcription factors such as WRKY

genes and its protein in response to senescence and environmental stresses, including heat stress, which improved the agronomic characters of crop plants.

2.4.4 Stay-Green Gene

Photosynthetic responses of annual plants can be improved by extending duration of vegetative growth and delaying leaf senescence (Thomas and Howarth 2000). Stay-green (Sgr) proteins are responsible for the green-flesh and retention of chlorophyll during senescence (Park et al. 2007; Barry et al. 2008). The trait stay-green is divided into five types such as type A, B, C, D, and E on the basis of its chlorophyll retention during leaf senescence (Thomas and Howarth 2000). Overexpression of *Sgr* gene reduces the loss of chlorophyll and delays early senescence of developing leaves (Park et al. 2007). Sgr synthesis has been reported in many plants such as sorghum (Tao et al. 2000), maize (Rajcan and Tollenaar 1999), rice (Cha et al. 2002; Park et al. 2007), durum wheat (Spano et al. 2003), tomato (Akhtar et al. 1999; Barry et al. 2008), pea (Sato et al. 2007) *A. thaliana* (Oh et al. 2000; Ren et al. 2007), oat (Helsel and Frey 1978), and *Festuca pratensis* (Armstead et al. 2006).

A stay-green protein potentially downregulates the chlorophyll degradation at transcriptional level and delays senescence (Nam 1997; Park et al. 2007). Delaying leaf senescence resulted in about 11% increase in carbon fixation in *Lolium temulentum* (Thomas and Howarth 2000). Tollenaar and Daynard (1978) demonstrated that some maize varieties such as L087602 shows stay-green phenotype, which increases the water, carbohydrates, and protein contents in the husks, cobs, and seeds. Nguyen (1999) demonstrated that stay-green genes delay leaf senescence in sorghum and reduce lodging in heat-stressed and low moisture areas. In fact stay-green is used as a selection criterion in warm areas (Acevedo et al. 1991; Kohli et al. 1991). For instance, most lines of wheat are sensitive to heat stress while some lines are heat tolerant due to stay-green character (Rehman et al. 2009).

3 High Temperature Stress: Management

Despite the fact that heat stress affects all the aspects of growth and development in plants, its effects may be mitigated by adopting various approaches. Some of the pragmatic strategies in this regard are detailed below.

3.1 Exploitation of Genetic Variability

As mentioned above, heat tolerance is a multigenic trait, which offers the opportunity of improving plants against heat stress. Temperate genotypes show less dry matter production and reduced yield due to high temperature stress as compared to tropical ones (Giaveno and Ferrero 2003). Attempts have been made to find the genetic differences in plants based on morphological and physiological criteria (Wahid et al. 2007; Khan et al. 2008). For instance, high temperature stress during grain filling can reduce setting and filling of seed by accelerating senescence thereby reducing crop yields (Harding et al. 1990; Siddique et al. 1999). This is because, resources required are utilized by plants for heat stress tolerance and limited amount is available for reproductive growth (Hall 1992). Search for genotypic variation in heat resistance on the basis of leaf electrolyte leakage is important for improving heat tolerance (Li et al. 1991). It is known that membrane stability is positively associated with crop yield under heat stress (Rahman et al. 2004). Such association was important for survival of wheat when exposed to high temperature at anthesis stage (Saadalla et al. 1990).

3.2 Conventional Breeding and Molecular Strategies

Conventional and modern breeding methods have been practiced for improving plant stress tolerance for the last many decades. For conventional

breeding, major challenges include germplasm mass screening, selection criteria, and identification of heat tolerant materials (Wahid et al. 2007). Among the several screening and selection methods, heat tolerance index (HTI) based on recovery of plants from heat stress has been proposed in case of sorghum (Young et al. 2001). In tomato, for example, under stress, there was a positive relationship between fruit set and yield. In this regard, many studies suggested that heat stress is not the only reason for impaired seed setting, nonetheless heat stress driven reduction in pollen tube growth and fertilization are rather more important (Wahid et al. 2007).

Present-day molecular and biotechnological tools have contributed enormously to understand the complexity and cellular pathways of stress responses and signal transduction mechanisms under abiotic stress responses (Sreenivasulu et al. 2006). Molecular studies involving cDNA arrays have revealed several genes, which are upregulated by several biotic and abiotic stresses (Kawasaki et al. 2001; Provart et al. 2003; Nogueira et al. 2003). Many of these genes are involved in signaling pathways via encoding proteins particularly mitogen-activated protein kinase (MAPK), histidine kinase, Ca^{2+}-dependent protein kinase (CDPK), SOS3 Ca^{2+} sensor family and numerous transcription factors.

Marker-assisted selection (MAS) and genetic transformation are two main approaches to improve heat stress tolerance in plants, which proved worthwhile in improving our understanding of stress tolerance mechanisms at molecular level (Foolad 2005). The use of MAS approach requires identifying genetic markers associated with genes or QTLs contributing to whole or individual stress tolerance. A number of research efforts have identified genetic markers related to different environmental stresses at various growth stages of plants to facilitate understanding of genetic relationships for stresses tolerance (Foolad 2005). However, limited studies have identified genetic markers related to high temperature tolerance in plant. Available studies show that in a number of *Arabidopsis* mutants, four QTLs were found to be involved in acquiring thermotolerance in heat-sensitive mutants (Hong and Vierling 2000). Use of restriction fragment length polymorphism (RFLP) mapped 11 QTLs for pollen germination and pollen tube growth in heat-stressed maize plants (Frova and Sari-Gorla 1994; Wahid et al. 2007).

3.3 Seed Treatments and Plant Acclimation

In addition to the above, seed treatment and planting materials and foliar spray of with various organic and inorganic agents has proven their worth in enhancing heat tolerance in a number of plant species. For instance, presowing GB treatment in barley (Wahid and Shabbir 2005), H_2O_2 treatment in maize (Wahid et al. 2008), and presprouting soaking of sugarcane bud chips with GB and proline (Rasheed 2009) have been implicated with great success in improving high temperature tolerance at germination and subsequent growth stages (Fig. 6.2). Epibrassinolide, a subclass of brassinostreoids, treatment modulated the translational machinery, resulting in higher HSPs synthesis and rapid resumption of protein synthesis during and after the application of high temperature stress (Dhaubhadel et al. 1999; Kagale et al. 2007). More so, precondition of tomato plants (Morales et al. 2003) and acclimation of turfgrass (Xu et al. 2006) to heat stress resulted in better growth of plants in heat-stressed environments.

4 Conclusion and Future Perspective

Responses of plants to heat stress may be symptomatic to quantitative. Despite the fact that heat stress responses are well evident at all growth stages, reproductive growth stages are more prone to heat episodes. Other heat stress effects may entail structural changes in tissues and cell organelles, disturbance of leaf water relations, and decline in the rate of photosynthesis, production of ROS and lipid peroxidation, changes in enzymatic and nonenzymatic antioxidants, and synthesis of secondary metabolites are also important.

The adaptive mechanisms of plants in response to heat stress include the induction of signaling cascades resulting in expression of specific gene. Synthesis of HSPs is a universal heat stress response in plants. Recently, evidence is pouring in on the synthesis and accumulation of some other stress-related proteins. All such proteins function as molecular chaperones and maintain three-dimensional structure of membrane proteins for sustained cell metabolism and plant survival under heat stress. Use of classical and modern breeding protocols, hunting genetic diversity for high temperature tolerance, use of presowing seed treatments and planting materials, and preconditioning/hardening of plants for high temperature tolerance has been beneficial. Although a considerable progress has been achieved in understanding the heat responses of plants, yet there is need for further understanding the biochemical and molecular basis of heat tolerance for improvement of yield benefits from hot environments. Recent molecular biology and gene transfer protocols can play important roles in this regard. Since benefits of seed treatments have been accrued at advanced growth stages (Wahid and Shabbir 2005), the basis of such changes needs to be explored.

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Understanding Chilling Tolerance Traits Using *Arabidopsis* Chilling-Sensitive Mutants

7

Dana Zoldan, Reza Shekaste Band, Charles L. Guy, and Ron Porat

Abstract

Many plants of tropical and subtropical origin are severely damaged when exposed to chilling temperatures between 2 and 15°C. In contrast, the cruciferous plant *Arabidopsis thaliana* is chilling tolerant and, therefore provides an alternative model plant system for the identification of chilling tolerance traits. In this chapter, we describe physiological, biochemical, and molecular responses of *Arabidopsis* class 1 chilling-sensitive (*chs*) mutants to low temperatures. These mutants, including *chs1, chs2* and *chs3*, are extremely chilling-sensitive and wilt and turn yellow in just a few days after transfer to low temperatures of 4–13°C. Overall, following exposure to chilling, class 1 *chs* mutants suffer from: (1) loss of chlorophyll and decrease in photosynthetic efficacy resulting in lack of starch accumulation, (2) damage to cellular membranes resulting in increased electrolyte leakage, and (3) accumulation of the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2). At the molecular level, transcriptome analysis studies following exposure to 10°C for 48 h using the Affymetrix ATH1 genome array reveal remarkable changes in expression patterns of between 1,500 and 3,000 genes, which are significantly differentially expressed ($p \leq 0.05$ and up- or down-regulated by a factor of at least 4) in *chs1, chs2*, and *chs3* mutants compared to wild-type (WT) plants. The main functional categories of up-regulated genes by chilling include "stress," "protein," and "signaling," whereas the main categories down-regulated by chilling were "photosynthesis," "tetrapyrrole synthesis," "carbohydrate metabolism," "cell wall," and "lipid metabolism". Overall, these and other studies using *Arabidopsis* chilling-sensitive mutants allow the recognition of major genetic traits crucial for plant survival under chilling conditions.

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1 Introduction

Low temperature is an important environmental factor that greatly influences the growth, development, survival, and geographical distribution of plants (Levitt 1980). Whereas most plant species from temperate regions can acclimatize to cold and can survive exposures to deep freezing temperatures, plants of tropical and subtropical origin are severely injured when exposed to low nonfreezing temperatures between 0 and 15°C (Lyons 1973; Lynch 1990; Wang 1990). Symptoms of chilling injury often include cessation of growth, wilting, chlorosis, necrosis, and eventually plant death (Lyons 1973; Graham and Patterson 1982; Maruyama et al. 1990; Allen and Ort 2001). In addition to its adverse effects on plant growth and development, chilling sensitivity also imposes major limitations on the postharvest storage and handling of many fruits and vegetables, because it necessitates storage at relatively high temperatures that do little to delay deterioration and spoilage (Paull 1990).

In contrast to our knowledge of plant responses to other abiotic stresses, such as freezing, drought, salinity, and heat, little is known regarding the molecular basis in regulating chilling tolerance or about the signal transduction networks involved in its acquisition. In previous reviews, the occurrence of chilling damage was attributed mainly to the general disruption or dysfunction of cellular metabolic processes (Lyons 1973; Graham and Patterson 1982; Markhart 1986). In this respect, it has been suggested that an important primary event in the occurrence of chilling injury is an alteration in the physical state of the cellular membranes, which leads to dysfunctional selective permeability and increases in solute leakage from the cells (Lyons 1973; Markhart 1986; Nishida and Murata 1996). Another factor that affects susceptibility to chilling is the status of the cellular antioxidant defensive system required to avoid accumulation of toxic reactive oxygen species (ROS). Indeed, overexpression of antioxidant defensive genes, such as ascorbate peroxidase, superoxide dismutase, and catalase (CAT) enhanced chilling tolerance (Van Breusegem et al. 1999; Payton et al. 2001), whereas repression of catalase gene expression reduced chilling tolerance (Kerdnaimongkol and Woodson 1999). Finally, several reports have suggested that various stress genes, usually related to other types of stress responses, may also contribute to the acquisition of chilling tolerance. For instance, it has been suggested that heat shock proteins (HSPs) (Sabehat et al. 1998) and dehydrin genes (Ismail et al. 1999) may also be factors in chilling tolerance in plants.

In this chapter, we will summarize data obtained from studies on *Arabidopsis* chilling-sensitive (*chs*) mutants, and will suggest how the adoption of *Arabidopsis* as a plant model system could improve our basic understanding regarding the molecular and biochemical nature of chilling tolerance. Overall, *Arabidopsis* has many advantages as a plant model system and its chilling tolerance makes it an excellent study subject for the identification of major genetic traits important for low temperature survival of plants.

2 Using *Arabidopsis* as a Genetic Resource for Identification of Chilling Tolerance Traits

It is believed that chilling-sensitive species evolved in warm tropical regions where there was no selection pressure favoring growth at low temperatures. In contrary, the dispersal of plants to cooler climates necessitated the acquisition of chilling tolerance and other low temperature

tolerance traits. Indeed, trees and shrubs in temperate regions are able to grow at low chilling temperatures, and become dormant during the coldest portion of the winter when temperatures are below 0°C (Levitt 1980). Many herbaceous annual species such as *Arabidopsis* normally overwinter in their vegetative state, survive both chilling and freezing conditions, and switch to a reproductive stage during the spring, when temperatures rise. Thus, over-wintering herbaceous annual species can be very resistant to chilling, and continue their growth and development, albeit more slowly, at low temperatures. Indeed, it has been shown that *Arabidopsis* can complete its entire life cycle and produce fertile seeds when sown and grown under continuous chilling temperatures of 4–6°C (Hasdai et al. 2006). Therefore, naturally chilling-resistant plants, such as *Arabidopsis*, may provide valuable genetic resources for the identification of chilling tolerance traits which, in the future, may be incorporated into horticultural important chilling-sensitive crops (Tokuhisa and Browse 1999; Porat and Guy 2007).

Arabidopsis mutants and forward genetic approaches have become useful tools to study the molecular and physiological responses and traits of plants to low temperature stress. Because *Arabidopsis* is freezing tolerant and can modulate its freezing tolerance by cold acclimation, and is easily genetically modified, creating and characterization of mutants deficient in freezing tolerance has been a productive strategy to uncover major determinants of signaling pathways and regulators of gene expression at low temperatures. Forward genetic analysis has identified a number of transcription factors like *HOS9*, *HOS10* and other regulators of freezing tolerance like ESKIMO1 (ESK1). *HOS9* and *HOS10* encode a homeodomain and MYB transcription factors, respectively, (Zhu et al. 2007). Loss-of-function mutations in *HOS9* and *HOS10* cause significant decreases in basal and acquired freezing tolerance. Mutations in ESK1 (Xin and Browse 1998) result in constitutive freezing tolerance. Also, mutations in a transcriptional adaptor protein ADA2b causes constitutive freezing tolerance (Vlachonasios et al. 2003). Another example of forward genetics includes the *chy1–10* mutant that is freezing-sensitive after cold acclimation. The *chy1* mutant accumulates ROS. Map-based cloning of *CHY1* revealed that the mutant encodes a peroxisomal β-hydroxyisobutyryl–CoA hydrolase needed for valine catabolism and fatty acid β-oxidation, and suggests a role in cold stress signaling, and freezing tolerance for peroxisomal metabolism (Dong et al. 2009).

Arabidopsis mutants have also helped to bring light to the relationships of lipid metabolism and low-temperature exposure particularly as the isolation of mutants with altered lipid compositions has assisted in biochemical and molecular approaches to understanding lipid metabolism and membrane function (Wallis and Browse 2002). Further, the availability of a variety of plant lines with specific changes in membrane lipids has afforded valuable resources to study the structural and adaptive roles of lipids. Presently, there are at least five types of *Arabidopsis* fatty acid metabolism mutants that grow well at 22°C, but are injured at low temperatures (2–6°C). The mutant lines include *fab1* (Wu et al. 1997), *fad2* (Miquel et al. 1993), *fad5*, *fad6* (Hugly and Somerville 1992), and the *fad3-2 fad7-2 fad8* triple mutant (Routaboul et al. 2000). Some examples that could be equated to chilling injury include the *fab*1 mutant of *Arabidopsis thaliana* that was derived from the Columbia ecotype following mutagenesis with ethane methyl sulfonate (EMS). The mutant contains increased levels of saturated fatty acids, particularly increased proportions of 16:0 in all of the major membrane lipids of the leaf tissue (Lightner et al. 1994). When subjected to 2°C, after 14 days, major changes in chloroplast ultrastructure were observed (Wu et al. 1997). The mutant chloroplasts were irregular in shape with poorly defined and broken envelopes. By 21 days at a low temperature, chloroplasts had largely disappeared from the mutant, but some chloroplast remnants remained visible in most mesophyll cells. The loss of chloroplast ultrastructure correlated with a major loss of photosynthetic function as indicated by Chlorophyll *a* fluorescence data. Nevertheless, the *fab1* plants were able to largely recover upon return to 22°C. Given that

fab1 plants are unable to maintain photosynthetic function at 2°C and will die after 5–7 weeks, the sensitivity of *fab1* plants to 2°C may result from increased levels of phosphatidylglycerol (PG) having high-melting-point molecular species (containing only 16:0, 18:0, and 16:1, Δ^3-trans fatty acids) (Kim et al. 2010). A *fab1* suppressor line, S7, had reduced levels of 16:3 fatty acid in leaf galactolipids compared to WT and was identified by map-based cloning as a hypomorphic allele of *lysophosphatidic acid acyltransferase1* (*lpat1*), *lpat1-3*. The *lpat1-3* mutation was found to strongly affect fatty acid composition of PG, with the proportion of high-melting-point molecular species in PG being reduced from 48.2% in *fab1* to 10.7% in *fab1 lpat1-3* (S7), a value that was close to the 7.6% found in wild type.

The ability to modulate membrane fatty acid unsaturation has long been thought to be critical to membrane function and cell viability for poikilothermic organisms like plants. The *Arabidopsis fad3-2, fad7-2, fad8* triple fatty acid desaturase mutant was found to be only subtly impacted upon short-term exposure to low temperatures, with small decreases in photosynthetic quantum yield, ΦII, were observed in the mutant (Routaboul et al. 2000). However, long-term exposure to 4°C resulted in lower fluorescence parameters, chlorophyll content, photosynthetic processes, and thylakoid membranes in the triple-mutant. When taken together, the examples outlined above demonstrate the value in forward genetic approaches to understanding plant low-temperature responses.

Overall, in contrast to nearly all chilling-sensitive species, with *Arabidopsis* it is relatively easy to screen large mutagenized populations for individuals sensitive to chilling phenotype resulting from a loss-of-function mutation in an important gene. Furthermore, there are *Arabidopsis* populations with insertional mutations, in which the compromised genetic locus can be readily obtained, and the mutated gene can be isolated and sequenced. Additional follow-up complementation studies can confirm the linkage of a mutant gene with the ability of the plant to tolerate chilling.

3 *Arabidopsis* Chilling-Sensitive Mutants

In order to study the molecular basis of chilling tolerance in *Arabidopsis*, EMS-mutagenized M_2 populations of *Arabidopsis* were grown at 22°C for 2 weeks after which they were transferred to 10 or 15°C and were screened for the appearance of chilling-sensitive mutants (Schneider et al. 1995a). Of about 20,000 M_2 plants examined, 21 mutants were identified that appeared normal at 22°C, but developed chlorosis or necrosis when shifted to lower temperatures (Schneider et al. 1995a). The chilling-sensitive mutants were categorized into four different phenotypic classes according to the severity of their chilling damage symptoms: class 1 mutants (*chs1–3*) turned yellow, wilted and died, in class 2 mutants (*chs4*) only the mature leaves became necrotic, and class 3 mutants (*chs5–6*) developed yellow chlorotic patches, but continued to grow and develop at low temperatures; and in class 4 (*chs7–15*) only part of the leaf near the rosette turned yellow (Schneider et al. 1995a). Crosses among mutants in different phenotypic classes showed that those in the first three classes were found only in a small number of loci (Schneider et al. 1995a).

Detailed characterization of the *chs1* mutant revealed that it was sensitive to temperatures below 18°C, and that exposure to low temperatures resulted in defects in chloroplast maintenance and integrity, including disruption of chloroplast protein accumulation and altered steryl-ester metabolism (Hugly et al. 1990; Patterson et al. 1993; Schneider et al. 1995b). Furthermore, it was found that only the leaf tissues of *chs1* plants were injured at low temperatures whereas germination, root and callus growth were unaffected by chilling. All these findings suggest that the function of the *chs1* gene product may be required to maintain chloroplast function at low temperatures. Overall, the *chs1* mutant was found to be extremely sensitive to low temperatures, and after 3 days at 13°C, as the plants become irreversibly injured and could not be rescued upon returning them to normal temperatures (Schneider et al. 1995b). Interestingly, it was also

noted that the *chs1* mutants were much more sensitive in terms of leaf yellowing and time till the initiation of wilting to exposure to 15°C than to 5°C (Schneider et al. 1995b).

Transcriptome profiling studies among approximately 8,000 *Arabidopsis* genes that compared gene expression patterns in wild-type (WT) plants with those in 12 chilling-sensitive mutants showed that the expression of more than 1,000 genes in normal plants was unaffected by chilling at 13°C but was affected by at least two-fold in class 1 *chs* mutants (Provart et al. 2003; Zhu and Provart 2003). In the light of these microarray expression data, it was suggested that the normal functions of the mutated *chs1*, *chs2*, and *chs3* genes might be to prevent widespread chilling damage effects on transcriptional regulation (Provart et al. 2003). It was further observed that the profiles of gene expression of the various class 1 *chs* mutants (*chs1*, *chs2*, and *chs3*) at 13°C were very similar to each other, which supports the idea that the products of these genes might perform related biological functions. In the future, identification of the gene products of class 1 *chs* mutants by means of mapping and chromosome walking technologies will certainly provide important insights into the molecular basis of at least some major factors that are crucial for plant survival at chilling temperatures.

A recent report on the *chs3* mutant has demonstrated the expected arrested growth and chlorosis phenotype when grown at 16°C or when shifted from 22 to 4°C. *chs3* plants exhibited chlorotic and spontaneous lesion phenotypes when grown at 16°C as older leaves turned yellow and died, and emerging leaves became water-soaked (Yang et al. 2010). Evidence presented indicated that *chs3* plants exhibit an activated defense response at 16°C, which was suppressed to WT levels at 22°C. Map-based cloning of *chs3* gene revealed an unusual disease resistance protein belonging to the TIR-NB-LRR class and also having a zinc-binding LIM domain at the carboxyl terminus. The mutation of a G-to-A substitution at the ninth intron–exon junction appears to lead to abnormal splicing and the formation of a truncated protein. Thus, *chs3* seems to be a gain-of-function mutation as the mutation led to the constitutive activation of the TIR-NB-LRR domain. An activated defense response was supported by hydrogen peroxide (H_2O_2) analyses with 3,3′-diaminobenzidine (DAB) staining. Strong staining was observed in *chs3* plants grown at 16°C, but not in WT plants under the same conditions indicating H_2O_2 levels were high in the mutant. The *chs3* growth and defense phenotypes could be suppressed by *eds1*, *sgt1b* and *rar1*, and partially suppressed by *pad4* and *nahG*, but not by *npr1* and *ndr1*. These findings reveal an unexpected linkage between defense responses and cold stress, and points to a mutual interaction between cold signaling and defense responses (Yang et al. 2010).

Beside *chs3*, the only other gene among the 21 *chs* mutants identified by Schneider et al. (1995a) that has been cloned so far is *chs5*; it belongs to class 3 of *chs* mutants, which become chlorotic at low temperatures but otherwise continue to grow and develop normally. Genetic and sequence analysis demonstrated that the *chs5* mutation occurred in the coding region of 1-deoxy-D-xylulose 5-phosphate synthase (DXS), an enzyme belonging to the nonmevalonate pathway localized in the chloroplast (Araki et al. 2000). DXS functions in the synthesis of isoprenoid compounds like carotenoids, xanthophylls, sterols, and isopentynyl chains of cytokinins and chlorophylls. Once again, as in the case of the *chs1* mutation, it seems that among the diverse components of cellular machinery that chloroplast is especially vulnerable to low chilling temperatures.

In another independent study, Tokuhisa et al. (1997) identified additional *Arabidopsis* EMS and T-DNA insertion chilling-sensitive mutants that were indistinguishable from WT plants when grown at 22°C, but exhibited visible chilling symptoms after 42 days of growth at 5°C. Under these conditions, the chilling symptoms that were identified included chlorosis, reduced and impaired growth (small stature, reduced leaf growth, high anthocyanin content, and distorted leaf morphology), necrosis, and death. Thus, two independent populations of chilling-sensitive mutants have been identified so far in *Arabidopsis*: class 1 *chs* mutants described by Somerville and coworkers (Schneider et al. 1995a) which showed

apparent chilling damage symptoms after a short exposure (3–7 days) to mild temperatures (10–15°C), and the mutants described by Browse and coworkers (Tokuhisa et al. 1997) which showed apparent chilling symptoms only after a much longer period of 6 weeks at a very low temperature of 5°C (Schneider et al. 1995a; Tokuhisa et al. 1997). The first type of mutants, especially class 1 *chs* mutants, most likely encode proteins crucial for basic aspects of cell survival at chilling temperatures, whereas the second type of mutants probably involves governing particular mechanisms of adaptation to low temperatures of older cells and organs or are characteristic of older cells. One of the latter T-DNA-tagged mutants, *paleface1* (*pfc1*), that becomes chlorotic during growth at 5°C, was cloned and encodes a specific 16 S rRNA methylase, which is required for maintenance of a particular step in pre-RNA processing in the chloroplasts, and which is apparently sensitive to low temperatures (Tokuhisa et al. 1998).

Further cloning and isolation of additional *Arabidopsis* chilling-sensitive genes will greatly improve our understanding regarding the molecular basis of chilling tolerance (Tokuhisa et al. 1998; Araki et al. 2000). In the following sections, we will analyze in more details the physiological, biochemical and molecular responses of class 1 chilling-sensitive mutants, including *chs1*, *chs2*, and *chs3*, to low temperatures, and based on that will aim to identify crucial traits required for survival of *Arabidopsis* plants under chilling conditions.

4 Physiological and Biochemical Responses of *chs1*, *chs2*, and *chs3* Mutants to Chilling

Class 1 chilling-sensitive mutants include *chs1*, *chs2*, and *chs3*. In addition to previous observations regarding the responsiveness of *chs1* plants to chilling (Hugly et al. 1990; Patterson et al. 1993; Schneider et al. 1995b), we hereby show that upon transfer of 2-week-old plants from a normal temperature of 22°C to a moderate chilling temperature of 10°C all class 1 *chs* mutants, including *chs1*, *chs2*, and *chs3*, turned yellow, wilted and eventually died (Fig. 7.1). The yellowing and growth arrest phenotypes become visible in 7 days after transfer to chilling temperatures, and these phenotypes become more severe as the time of exposure to chilling increased up to 3 weeks (Fig. 7.1). Furthermore, we found that in addition to what has been reported previously for *chs1* (Hugly et al. 1990), *chs3* mutants are also extremely sensitive to low temperatures, as they showed a severe dwarfism phenotype when grown at a moderate chilling temperature of 18°C (Fig. 7.2). Overall, all class 1 chilling-sensitive mutants are very sensitive to chilling, and turn yellow and wilt in just a few days after exposure to low temperatures.

To further evaluate the effects of chilling on *chs1*, *chs2*, and *chs3* plants, we examined their chlorophyll content, photosynthesis efficacy and electrolyte leakage rates at various periods after exposure to 10°C. It can be seen that the earliest event that was observed within 4 days of exposure to chilling was a sharp decline in chlorophyll content (Fig. 7.3a), and this was followed by a gradual decrease in photosynthetic efficacy (determined by measuring chlorophyll fluorescence; Fv/Fm ratio) which continued up to 21 days of exposure to 10°C (Fig. 7.3b). An increase in electrolyte leakage rates was evident after 7 days at 10°C and gradually became more severe as the plants were kept for longer periods at 10°C (Fig. 7.3c).

In accordance with the observed decreases in chlorophyll contents and photosynthetic efficacy, we found that *chs1*, *chs2*, and *chs3* plants were unable and did not accumulate starch in their leaves as observed in WT plants (Fig. 7.4). Following exposure to low temperatures, WT plants continued to produce assimilates by photosynthesis, but since growth was slowed down they accumulated starch. In contrast, the *chs1*, *chs2*, and *chs3* mutants were defected in their photosynthetic machinery (as observed by the decrease in chlorophyll content and photosynthesis efficacy) and, therefore, did not produce sugars and did not accumulate starch following exposure to low temperatures (Fig. 7.4).

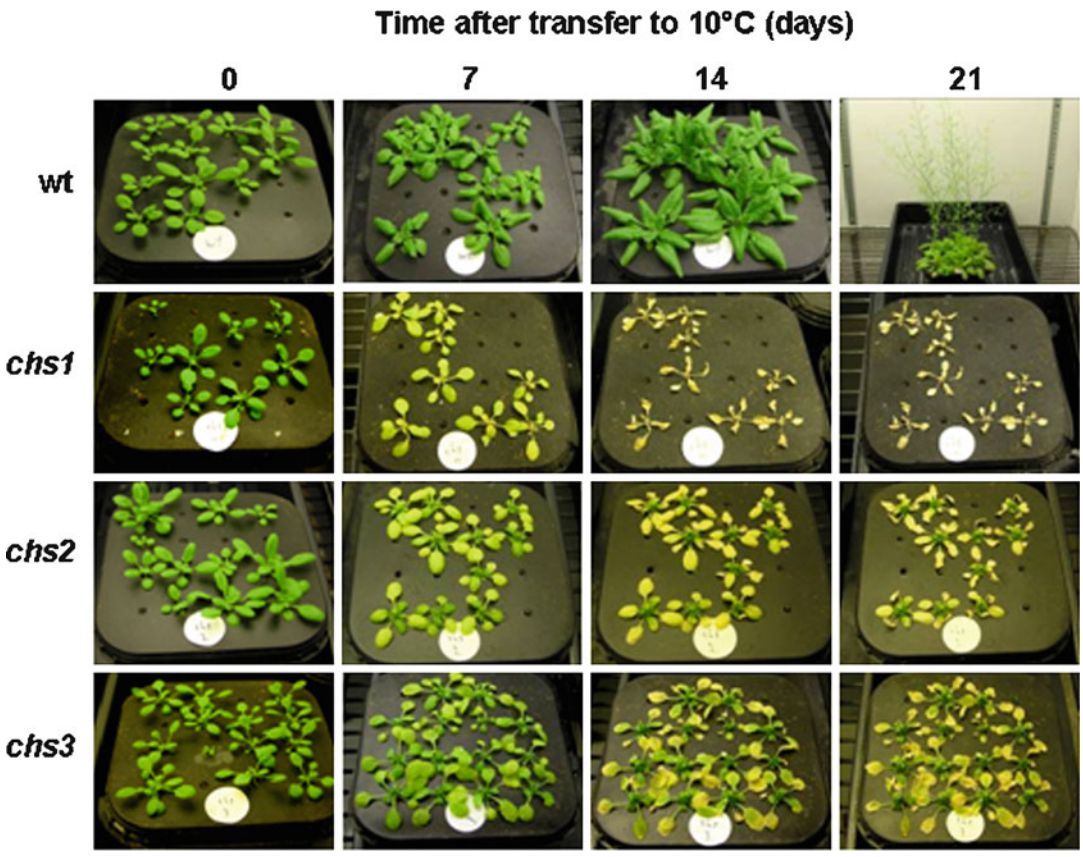

Fig. 7.1 Phenotypes of wild-type and *chs1*, *chs2*, and *chs3* mutants following transfer to chilling conditions. Plants were grown for 2 weeks at 22°C and afterwards transferred to a chilling temperature of 10°C

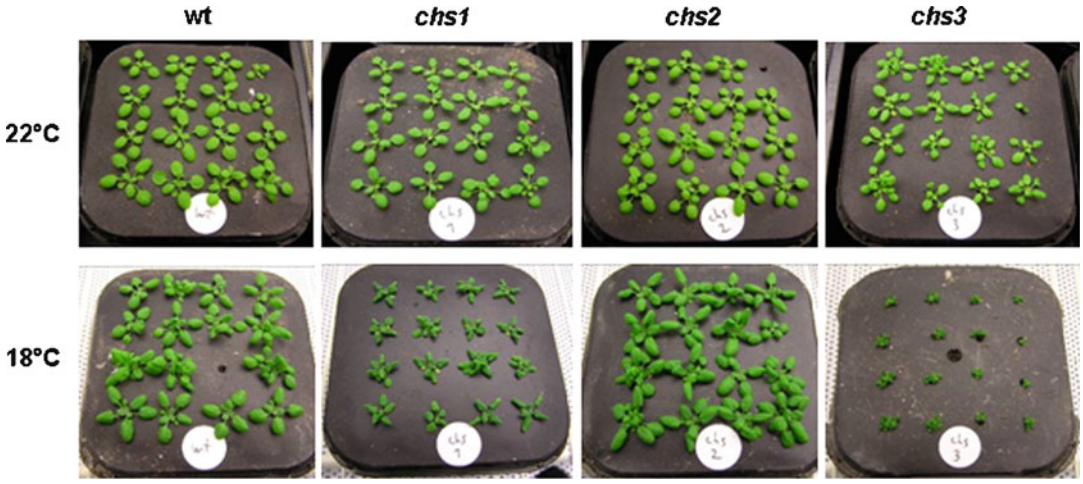

Fig. 7.2 Effects of a moderate growth temperature of 18°C on the phenotypes of wild-type and *chs1*, *chs2*, and *chs3* mutants. Plants were grown from sowing at 22 or 18°C, and photographs were taken after 3 weeks of growth at each temperature

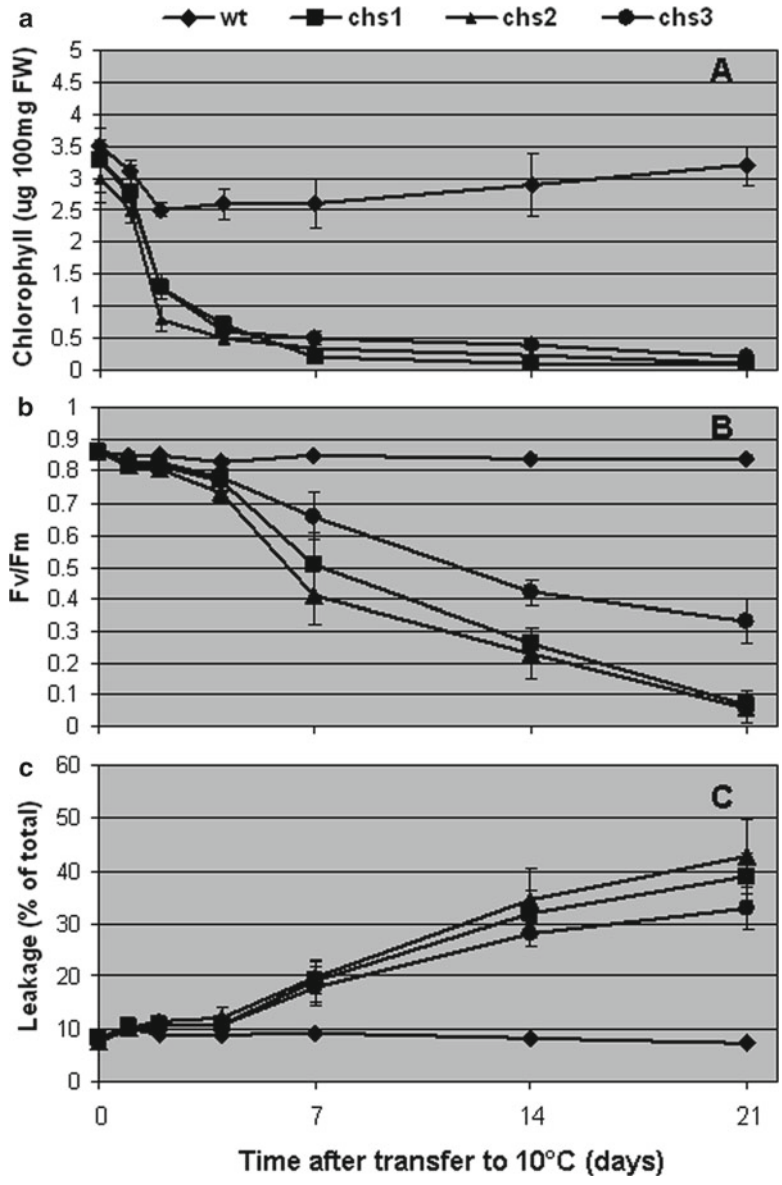

Fig. 7.3 Effects of chilling on chlorophyll content, photosynthesis efficacy, and electrolyte leakage rates of wild-type and *chs1*, *chs2*, and *chs3* mutants. Plants were grown for 2 weeks at 22°C and afterwards transferred to a chilling temperature of 10°C. (**a**) Chlorophyll content, (**b**) photosynthesis efficacy, and (**c**), electrolyte leakage. Data are means ± S.E. of five replications

Another response of *chs1*, *chs2*, and *chs3* mutants to chilling was enhanced accumulation of ROS in general, and H_2O_2 particularly, as observed by DAB staining experiments (Fig. 7.5). It can be seen that WT plants remained clear and did not accumulate H_2O_2 following exposure to chilling, whereas leaves of *chs1*, *chs2*, and *chs3* mutants were stained by brown spots indicating presence of H_2O_2 (Fig. 7.5). The accumulation of H_2O_2 was somewhat more pronounced in *chs2* and *chs3* plants as compared with *chs1*, and after exposure to the lower temperature of 4°C as compared with 13°C (Fig. 7.5).

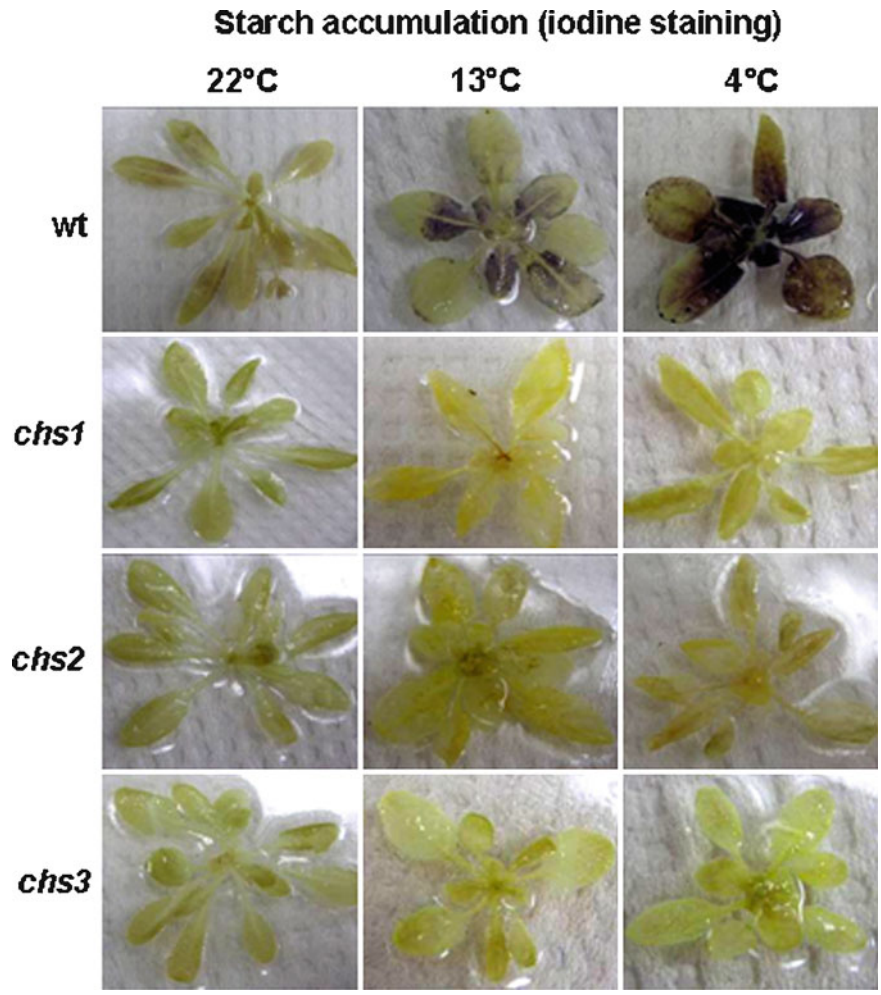

Fig. 7.4 Effects of chilling on starch accumulation in wild-type and *chs1*, *chs2*, and *chs3* mutants. Plants were grown for 2 weeks at 22°C and afterwards transferred to chilling temperatures of 13 and 4°C. Starch accumulation was indicated by iodine staining after 7 days at chilling conditions

5 Mapping of the *chs1*, *chs2*, and *chs3* Genes

Identification of the gene products of class 1 *chs* mutants by means of mapping and chromosome walking technologies will provide important insights into the molecular basis of chilling tolerance traits crucial for plant survival at low temperatures. To facilitate the mapping of *chs1*, *chs2*, and *chs3* genes, we crossed the mutants present in Colombia (Col) background with Lansdberg erecta (Ler) WT plants, and scanned populations of F_2 plants for chilling sensitivity phenotypes upon transfer to low temperatures. Afterwards, we extracted DNA from chilling-resistant plants and searched for possible linkage of PCR markers with the chilling tolerance phenotype. Accordingly, based on polymorphism between Col and Ler ecotypes, we mapped the *chs1*, *chs2*, and *chs3* genes to the following chromosomal locations (Fig. 7.6):

(1) *chs1* – was mapped to chromosome 1 in the region between 5.9 and 7.0 Mb.

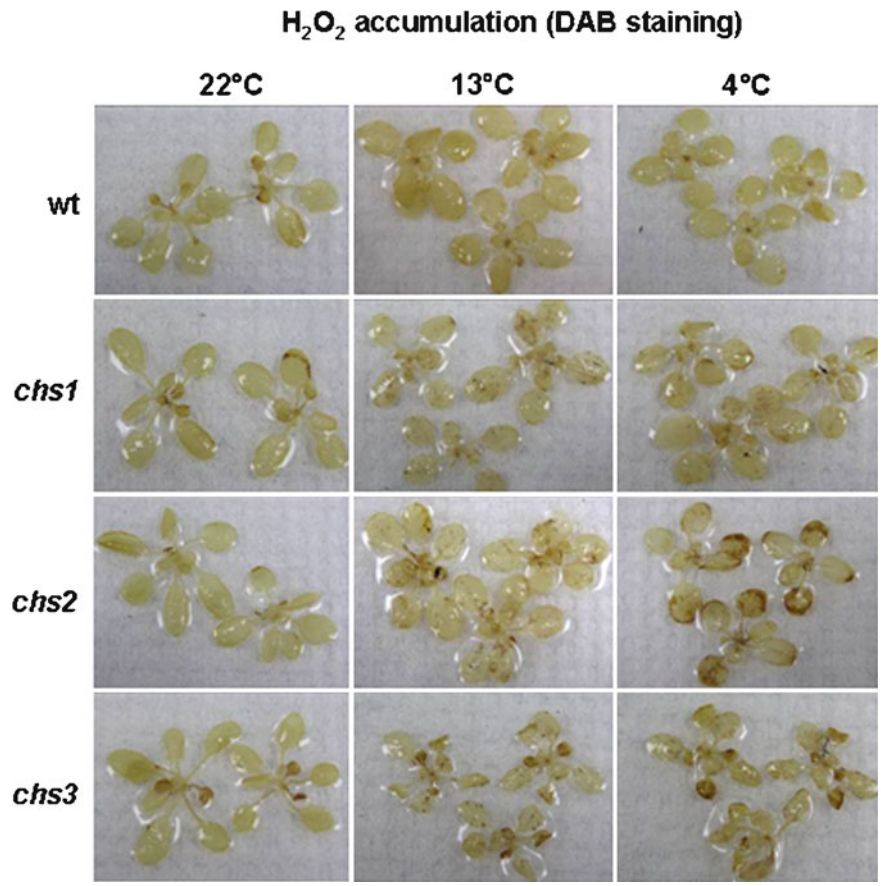

Fig. 7.5 Effects of chilling on H_2O_2 accumulation in wild-type and *chs1*, *chs2*, and *chs3* mutants. Plants were grown for 2 weeks at 22°C and afterwards transferred to chilling temperatures of 13 and 4°C. Accumulation of hydrogen peroxide was indicated by DAB staining after 7 days at chilling conditions

(2) *chs2* – was mapped to chromosome 4 in the region between 9.02 and 9.95 Mb.
(3) *chs3* – was mapped to chromosome 5 in the region between 5.41 and 7.37 Mb.

This initial mapping of the *chs1*, *chs2*, and *chs3* genes to a final resolution of approximately 1 Mb will facilitate in the future the final cloning and isolation of these important genes that are crucial for survival of *Arabidopsis* plant at low chilling temperatures. As indicated in Sect. 3, the *chs3* gene was recently mapped to the top of chromosome 5 and its sequence was identified (Yang et al. 2010).

6 Effects of Chilling on the Transcriptome of *chs1*, *chs2*, and *chs3* Mutants

To identify transcripts that exhibited significant changes in their abundance after exposure to chilling, we performed pair-wise comparisons, and selected transcripts that had ANOVA values of $p \leq 0.05$ and that were induced or repressed by a factor of at least 4 after exposure of *chs1*, *chs2*, and *chs3* mutants to 10°C for 48 h, as compared with their corresponding expression levels in WT

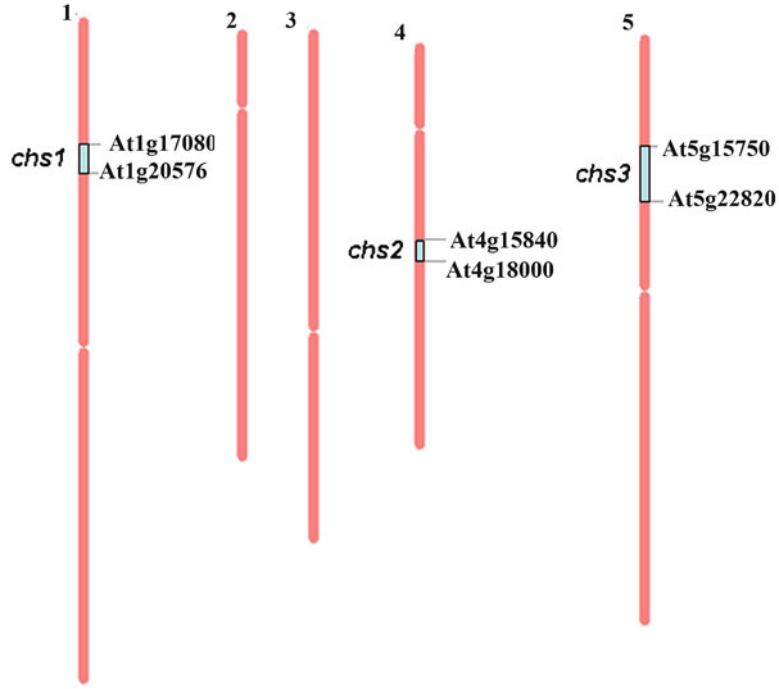

Fig. 7.6 Chromosomal locations of the *chs1*, *chs2*, and *chs3* loci. The *chs1* gene was mapped to chromosome 1 in the region between 5.9 and 7.0 Mb, *chs2* was mapped to chromosome 4 in the region between 9.02 and 9.95 Mb, and *chs3* was mapped to chromosome 5 in the region between 5.41 and 7.37 Mb

Table 7.1 Effects of chilling (48 h at 10°C) on the transcriptome of *chs1*, *chs2*, and *chs3* mutants

	Probe sets differentially expressed at $p \leq 0.05$ and *induced or repressed by a factor of at least 4*		
Pair-wise comparison	Up-regulated	Down-regulated	Total
chs1/wild type	913	591	1,504
chs2/wild type	1,523	1,717	3,240
chs3/wild type	1,293	1,426	2,719

Data include numbers of probe sets on the Affymetrix ATH1 Genome Array differentially expressed at $p \leq 0.05$ and induced or repressed by a factor of at least 4

plants. Doing so, led to the identification of 1,504, 3,240, and 2,719 probe sets whose expression significantly changed after exposure to chilling in *chs1*, *chs2*, and *chs3* mutants, respectively (Table 7.1).

To define the degree of similarity and differences between gene expression patterns in the various class 1 *chs* mutants in response to chilling, we conducted a Venn diagram comparison (Fig. 7.7). It was found that the expression of a large group of 1,426 probe sets was similarly affected by chilling in all class 1 *chs* mutants, and that of 1,207 additionally genes were similarly affected in both *chs2* and *chs3* mutants (Fig. 7.7). Thus, *chs1*, *chs2*, and *chs3* mutants endure similar molecular responses following exposure to chilling stress.

In order to assign the chilling-induced differentially expressed genes in *chs1*, *chs2*, and *chs3* mutants into corresponding molecular functions, we performed functional categorization analysis using the MapMan software (Thimm et al. 2004). Doing so revealed that the main functional categories up-regulated by chilling in the mutants were "stress," "protein," and "signaling," whereas the main categories down-regulated by chilling

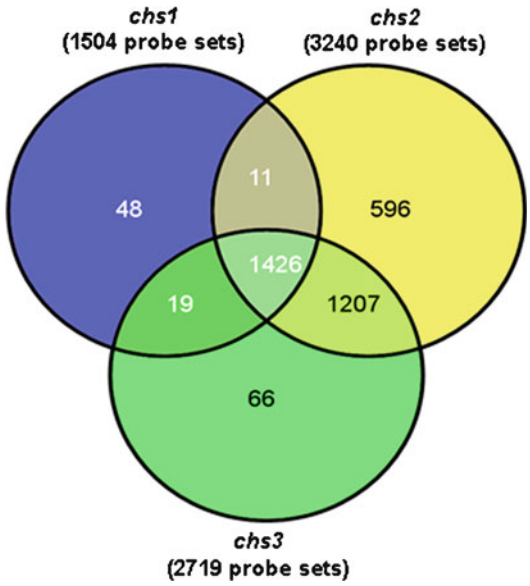

Fig. 7.7 Venn diagram illustrating the overlapping and differences in gene expression patterns among the various *chs1*, *chs2*, and *chs3* chilling-responsive regulons. The numbers on the diagram indicate the amount of overlapped probe sets

were "photosynthesis" and "tetrapyrrole synthesis," "major carbohydrate metabolism," "cell wall," and "lipid metabolism" (Table 7.2). A more or less similar response consisting differential expression of stress, photosynthesis, and protein, carbohydrate and lipid metabolism genes, following exposure to chilling were reported also in several other chilling-sensitive commodities, such as rice, sunflower and potato as well as chilling-tolerant poplar trees (Yan et al. 2006; Fernandez et al. 2008; Oufir et al. 2008; Maestrini et al. 2009). The up-regulated category of "protein" included massive up-regulation of genes involved in protein degradation, and particularly transcripts encoding members of RING finger and F-BOX proteins belonging to the ubiquitin protein degradation pathway. In addition, the "protein" category further included massive down-regulation of genes involved in protein synthesis. The up-regulated category of "signaling" included mainly up-regulation of receptor kinase genes and calcium signaling genes, as previously reported (Bhattacharjee 2009). The observed down-regulation in "lipid metabolism" genes included mainly suppression of transcripts involved in fatty acid synthesis and desaturation; the latter is known to be a crucial factor required for adaptation to chilling (Murata et al. 1992; Nishida and Murata 1996).

The overall meanings of these findings are that under chilling conditions class 1 *chs* mutants are defective in normal gene expression related to the photosynthesis machinery and carbohydrate metabolism, and cell wall and lipid metabolism. In addition, the *chs* mutants were in severe stress as indicated by massive up-regulation of stress genes, and suffered from imbalanced protein metabolism (suppression of protein synthesis and induction of protein degradation).

7 Conclusion and Future Perspective

From the current evaluations of the physiological, biochemical and molecular responses of class 1 *chs* mutants to chilling, we have arrived at the following main conclusions:

(1) Under chilling conditions, class 1 *chs* mutants are defective in normal gene expression related to photosynthesis, chlorophyll synthesis and major carbohydrate metabolism (Table 7.2). Furthermore, these molecular observations were confirmed by biochemical measurements indicating a rapid loss of chlorophyll content and decrease in photosynthetic efficacy (Fig. 7.3), leaf yellowing (Fig. 7.1), and inability to accumulate starch as observed in WT plants (Fig. 7.4).

(2) Under chilling conditions, class 1 *chs* mutants are defective in normal gene expression related to lipid metabolism including down-regulation of fatty acid synthesis and desaturation (Table 7.2). Furthermore, these molecular observations were supported by conductivity measurements indicating a continuous increase in electrolyte leakage rates upon transfer of the *chs* mutants to chilling, thus indicating accumulated damage to cellular membranes (Fig. 7.3).

(3) Under chilling conditions, class 1 *chs* mutants activated a battery of stress-related genes,

Table 7.2 Functional categorization of *chs1*, *chs2*, and *chs3* regulon genes

	chs1 regulon		*chs2* regulon		*chs3* regulon	
Functional categorization	Up	Down	Up	Down	Up	Down
Photosynthesis	1	16	2	48	2	38
Major CHO metabolism	2	12	4	26	2	21
Minor CHO metabolism	9	3	15	16	12	12
Glycolysis	2	3	4	5	4	3
Fermentation	0	0	2	1	1	0
Gluconeogenesis	1	0	2	0	1	0
OPP	0	1	2	3	2	2
TCA/transformation	0	0	0	5	0	5
Electron transport/ATP	4	2	5	3	4	3
Cell wall	15	35	27	83	22	77
Lipid metabolism	9	31	24	57	18	49
N-metabolism	1	0	3	3	2	3
Amino acid metabolism	10	9	31	36	19	24
S-assimilation	0	3	0	4	0	4
Metal handling	4	3	6	8	5	7
Secondary metabolism	19	17	33	46	39	27
Hormone metabolism	27	25	47	46	41	42
Tetrapyrrole synthesis	0	11	0	21	0	17
Stress	73	15	99	47	81	44
Redox regulation	12	7	21	17	19	12
Polyamine metabolism	0	1	1	1	1	1
Nucleotide metabolism	6	2	9	19	7	11
Misc	59	48	91	113	83	101
RNA and transcription	64	48	120	133	105	113
DNA	4	6	9	18	7	13
Protein	115	32	211	140	177	105
Signaling	103	21	141	72	120	66
Cell	18	7	28	44	27	35
"Micro RNA"	0	0	0	0	0	0
Development	11	10	19	23	15	18
Transport	43	32	68	61	54	56
Not assigned	296	223	496	579	433	495

Functional categorization was performed according to MapMan software (http://gabi.rzpd.de/projects/MapMan/). The functional groups of "Photosynthesis," "Major CHO metabolism," "Cell wall," "Lipid metabolism," and "Tetrapyrrole synthesis" down-regulated in *chs* mutants are marked by light shading, while the groups of "Stress," "Protein," and "Signaling" up-regulated in *chs* mutants are marked by dark shading

indicating that the plants were under severe stress conditions (Table 7.2). This observation was further supported by our findings regarding enhanced accumulation of ROS, as observed by DAB staining for detection of H_2O_2 levels (Fig. 7.5).

(4) Following exposure to chilling, class 1 *chs* mutants suffered from imbalanced protein metabolism, demonstrated by suppression of transcripts involved in protein synthesis and massive induction of transcripts belonging to the ubiquitin protein degradation pathway. These processes obviously lead to progressive destruction of normal cellular activity.

Overall, based on our studies with *Arabidopsis* chilling-sensitive mutants, we conclude that several biochemical and molecular traits are apparently crucial for plant survival under chilling

temperatures; these include maintenance of photosynthetic activity and carbohydrate metabolism, maintenance of normal lipid metabolism, maintenance of stress tolerance and capability to detoxify accumulation of ROS, and maintenance of normal cellular function including normal protein turnover and cellular signaling processes.

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Root Form and Function in Plant as an Adaptation to Changing Climate

8

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Abstract

Climate variables including temperature, atmosphere CO2, and precipitation are expected to change during this century. As consequence, in the short- and long-term, the increase of soil temperature, salinity, drought, and water-logging stresses could be the more exceeding problems for agricultural productivity and the functioning of the natural ecosystems. Root system represents the first and more sensitive target of the climate change, being seriously damaged in its form and function, and consequently strongly contributes to limit plant growth, development and crop productivity. This review focuses on changes of root morphology, architecture, distribution and dynamics, and on essential root physiological processes, such as water and nutrient uptake in response to soil warming, salinity, drought, and waterlogging. The literature appear sometimes to be controversial due to the complexity of root system characterized by different root types, genetically, developmentally, and functionally distinct, and by diverse root morphological parameters such as total root length, biomass, specific root length (SRL), and tissue density and fineness differently involved on root stress responses. For example, the change on total root length and dry weight, the lateral root formation, the depth of rooting and the root dynamics represent the preferential strategy for plant species in water-limited environments. Whereas, the development of aerenchyma, tissue containing enlarged gas spaces with a low-resistance pathway to oxygen, often accompanied by the aerotropic and extensive lateral roots formation, the herringbone-type root architecture, the emergence of adventitious roots and the presence of anatomical barriers are expressed in flooded root. Salinity reduces plant growth and yield by two mechanisms, osmotic stress and ion cytotoxicity. It is difficult to separate the osmotic effect

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from specific ion effects that overlap during the development of salinity stress, thereby some uncertainty exists regarding the relative importance of both mechanisms. The responses of root cells are finalized to maintain their own correct functionality, despite the condition of elevated Na+ concentration. The genetic diversity of the root system in the plant response to climate change was also reported.

Behind the root "form" changes, many metabolic and physiological pathways are involved in the plant adaptation to climate change. The maintenance of lower respiration rate, carbohydrate metabolism and cell expansion and elongation, often mediated by hormones, are expressed in roots grown in dry soil. At molecular level, the deposition of proline, metabolite responsible of the total osmotic adjustments, the higher expansin and xyloglucan endotransglycolase/hydrolase (XTH) activities, enzymes of the cell wall extension, represent the physiological mechanisms implemented by plants for improving their drought tolerance. In waterlogging soils and also in presence of salinity, the adaptation of physiological mechanisms are addressed to improve the cellular energy status and reduce the accumulation of toxic end products that acidify the cytosol or damage membrane integrity. Remarks on proteomic and molecular aspects which represent a future approach to individuate the plant strategies for their adaptation to the climate change are also included.

Keywords

Root system • Drought • Precipitation • Salinity • Temperature • Waterlogging

1 Introduction

Climate change variables such as temperature (warming), precipitation (drought and flooding), and atmospheric CO_2 concentrations (CO_2 fertilization) have greater impact on agricultural productivity, ecosystem structure and function. Plant has a strong photohydraulic system involved in water and nutrient uptake, a sink for the photoassimilates in which the root system plays a key role in determining plant responses to climate change. Further, the root system, by its respiration, turnover, exudation processes, and interactions with the soil biota, plays a critical role in controlling the soil C storage and cycling and ultimately in the feedbacks of terrestrial C cycling to climate change. Hence, the root system could be considered as sink and source of carbon dioxide, the main driver of climate change, and a better understanding of its responses could provide useful information in the plant adaptation to the future atmospheric composition. In the present chapter, we will mainly focus on how direct (temperature, drought, flooding) and indirect (salinity) components of climate changes influence the root form and function in higher plants.

Let us consider the root form as a "photographical description" of the root system at the tri-, bi-, and one-dimensional levels, as determined by biometric parameters. The root form includes the root morphology, architecture, distribution, and dynamics. Root morphology means "superficial features of the whole and single root axis" (Lynch and Nielsen 1996) such as the length, mass, surface area, volume, and diameter. Further morphological parameters derived from the formers and having a functional significance (Ryser 1998) are the root length ratio (RLR) (root length per unit of the plant's dry mass), root mass

ratio (RMR) (root mass per unit of the plant's dry mass), specific root length (SRL) (root length per unit of root dry weight), root fineness (RF) (root length per unit root volume) and root tissue density (RTD) (root dry mass per unit root volume). The root architecture is defined as the spatial configuration of the root system (Lynch and Nielsen 1996) and is generally estimated in terms of topology (Robinson et al. 2003). Root topology, which refers to the distribution of the branches within the system, can lie within two extreme types: the "herringbone" type in which branching is confined to the main axis and the "dichotomous" type exhibiting a more random branching (Fitter and Stickland 1991). The root distribution, which refers to the deployment of the root axis in terms of biomass or length along the soil profile is described by root mass density (RMD) (root mass per unit soil volume) and root length density (RLD) (root length per unit soil volume). Finally, the root dynamics includes the root production, mortality, and turnover (ratio of root number present at a time point to the number of roots produced up to that time) and life span.

The root system as defined by Robinson et al. (1991) "…is the result of an evolution strategy to solve the problems of soil resources acquisition…." Hence, the main function of the roots is the capture of belowground resources, such as water and nutrients, from that "…heterogeneous and porous system …" (Robinson et al. 1991) which is the soil environment. The climate change has impact on the plant C allocation and respiration, water and nutrients uptake which are important physiological processes in terms of C (Hinsinger 1998, 2001; Hinsinger et al. 2003). Finally, we will discuss the impact of climate changes on the root's "secondary functions" such as storage of carbon and nutrients and supply of energy to belowground food web and microorganisms (Hinsinger et al. 2005, 2006).

2 Roots and High Temperature

The Fourth Assessment Report (AR4) of the Inter governmental Panel on Climate Change (IPCC) of United Nations predicted an approximately 1.8–4°C increase in global mean air temperature during this century (IPCC 2007). Soil temperatures are also expected to increase reflecting the future atmospheric temperature trend (Pollack et al. 1998). The plant root system will be affected by soil temperatures which will have a significant impact on its form and function and therefore on plant development and productivity. Lots of study have been done on the effects of temperature on the root system (Cooper 1973; Voorhes et al. 1981; Kaspar and Bland 1992; McMichael and Burke 2002), relatively few information have focused on integrated root development, growth, metabolic responses to soil warming and how roots preserve its form and function under warming soil conditions is not completely clarified.

Considerable evidence indicates that the root growth increased in response to increased soil temperature up to an optimum threshold, typical for each species and depending partly on their native temperature regime (McMichael and Burke 1998), beyond which root growth decreased. Faster elongation rates of whole root system were observed in the temperature range of 5–23°C for *Eucalyptus* species (Misra 1999), 10–30°C for sunflower (Seiler 1998), 10–15°C for winter wheat (Gavito et al. 2001) and bog and fen plant communities (Weltzin et al. 2000). Supraoptimal soil temperatures, on the other hand, reduced the root growth in many species such as *Agrostis stolonifera* (>35°C, Huang et al. 1998), *Lactuca sativa* (>35°C, Qin et al. 2007; He et al. 2009) and wheat (>38°C, Tahir et al. 2008). McMichael and Burke (2002) grouped the different taxa in relation to optimum of temperature for the root growth, pointing out the influence (incidence) of diverse genetic background on temperature-dependent root growth pattern among the plant species due to their different acclimation strategies. For example, a diverse response and adaptation, in terms of both root length and mass, was evident among genetically diverse sunflower (Seiler 1998) wheat genotypes (Tahir et al. 2008) and in two *Agrostis* species; where the root system of *A. scabra* was more thermotolerant growing up to 45°C (Terceck et al. 2003) than that of *A. stolonifera* which grew upto 23°C only (Pote et al. 2006). These observations suggested that genetic diversity in root growth contributes to the survival of

plant species and to improve their productivity under high soil temperature conditions and deserves further studies.

To better understand the temperature-induced root responses between and within plant species is needed to consider that the root systems comprise of different root types which are distinct genetically, developmentally, and functionally and differently respond to soil environmental stresses (Waisel and Eshel 2002). Several examples can be mentioned in this regard: the first root axes of pearl millet showed a higher elongation rate in response to the increase of temperature (from 20 to 32°C) with respect to the second one (Gregory 1986), the primary root of sunflower was less inhibited at temperature above 35°C than lateral roots (Seiler 1998), the fine roots were more sensitive to soil warming (Pregitzer et al. 2000), the SRL (root length/root mass) and specific root area (root area/root mass) increased in warmer soil in the root finest fraction (<0.5 mm) only (Bjork et al. 2007). However, more studies are needed for gaining a better knowledge on how the different root types/orders respond to high soil temperatures.

The morphological responses and the acclimation of the root to higher temperatures involve the integration of many metabolic and physiological pathways. It is well-known that the root growth depends on the supply of carbohydrates which are sharply consumed by higher root maintenance respiration in warmer soil conditions. Therefore, the maintenance of lower respiration rate may represent an important basis for the thermotolerance of the root systems and, ultimately, for the plant adaptation to the higher soil temperatures. Indeed, the lower energy required for root maintenance permitted to *Agrostis scabra* to grow up to 45°C while the root growth of *Agrostis stolonifera*, heat-sensitive species was inhibited above 27°C (Rachmilevitch et al. 2008). Other plant species such as *Citrus volkameriana* (Bouma et al. 1997), *Bellis perennis*, and *Poa annua* (Gunn and Farrar 1999), adapted to warmer soil exhibited a lower maintenance respiration rate of their root systems.

However, several authors pointed out that the temperature-induced root growth patterns also depend on radiation flux which influencing the photosynthesis determines a variation of the carbohydrate supply to root system. Indeed, the tap and lateral root elongation rate of sunflower were weakly correlated with soil temperature and sharply dependent on the amount of radiation intercepted (Aguirrezabal et al. 1994). Further, the root biomass and length of temperate northern grassland species dominated by *Holcus lanatus* were strongly affected by incident radiation and not by soil temperature (Edwards et al. 2004). Consequently, in order to understand root responses to warming soil, it is necessary to separate the effects of photosynthetically active radiation by soil temperature.

Root growth is not only associated with the carbohydrate metabolism but is also correlated with other cellular processes, such as cell expansion and elongation. By analyzing the spatial distribution of expansion growth along the primary root axis of *Zea mays*, Walter et al. (2002) observed a greater extension accompanied by a maximal expansion activity of the growing zone with the rising temperature (from 21 to 26°C). Furthermore, several lines of evidence suggested that the root morphological changes to high temperatures might be mediated by hormones. Qin et al. (2007) demonstrated that the application of the ethylene precursor (1-aminocyclopropane-1-carboxylic acid, ACC), in lettuce seedlings, mimicked the high temperature-induced root morphological changes (inhibition and increase of the root length and diameter, respectively) and the addition of ethylene biosynthesis inhibitors such as aminooxyacetic acid (AOA) or aminoisobutyric acid (AIB) relieved these effects.

The soil temperatures fluctuated over a wide range of temporal (diurnal and seasonal) and spatial (depth) scales (Kaspar and Bland 1992) determining soil-zone temperatures. This soil gradient affects the orientation of the root axis, mainly nodal and seminal roots which observed a plagiotropic growth (growth at angles from the vertical). For example, the seminal roots of *Zea mays* which at 17°C grew horizontally, above and below this temperature showed a more vertical growth (Onderdonk and Ketcheson 1973). The result of the diverse root orientation was a different root distribution in terms of length and mass within the

soil profile. Root mass of a community constituted by *Cardamine hirsuta*, *Poa annua*, *Senecio vulgaris*, and *Spergula arvensis* was reduced at the surface soil layers by elevated temperatures (Kandeler et al. 1998). Similar results were pointed out by Soussana et al. (1996) in the root of perennial ryegrass (*Lolium perenne*).

Different studies have been focused on the influence of the soil temperature on the root dynamics although no consistent patterns have been observed. The root production and mortality, especially of the fine-roots, determining the soil C inputs and soil microbial activity, play a critical role in regulating agro- and ecosystem C balance and, ultimately, in the below-ground CO_2 efflux. An increase of root turnover in response to soil warming were observed in maple tree seedlings (Wan et al. 2004), Norway spruce stand (Majdi and Ohrvik 2004) and *Eriophoretum vaginatum* (Sullivan and Welker 2005) unlike of temperate steppe perennial species (Bai et al. 2010), Douglas-fir (Johnson et al. 2006), maple (Côté et al. 1998) and oak trees (Joslin et al. 2001) whose root dynamics were not affected by high soil temperatures.

Warming is one of the main factors of climate change along with altered precipitation, elevate atmospheric CO_2 and N deposition and each of these may be expected to vary independently as well as to be interdependent. For example, elevated CO_2 reducing the evapotranspiration increased the soil moisture (Nelson et al. 2004), the increase of the soil temperature stimulated the soil microorganism activity causing a higher N availability for the plant (Rustad et al. 2001) and finally soil warming interacted with the concomitant drought stress also (Kramer and Boyer 1995). In this respect, combinations of different climate change factors are possible and it would be interesting to know the root responses to the interactive effects of these factors. For example, Tierney et al. (2003) reported a strong relationship between root production and soil temperature in a hardwood forest which contrasted with the results of Joslin et al. (2001). Tierney et al. (2003) justified this discrepancy by difference in water availability between their site with that of Joslin et al. (2001) pointing out the water availability and soil temperature interactions in the root growth responses. Bai et al. (2010) revealed that the temperature-induced inhibition on the root production and mortality of temperate perennial steppe species was observed with increased precipitation while the root dynamics was improved under ambient precipitation. Further, the effects of the increase of temperature (from 10 to 15°C) and N supply on the total root length of winter wheat were highly significant and additive (Gavito et al. 2001), Majdi and Ohrvik (2004) observed that the addition of N reduces the risk of root mortality in Norway spruce contrasting the effect of soil warming. The reduction of the root biomass of a mixing plant species (*Cardamine hirsuta*, *Poa annua*, *Senecio vulgaris,* and *Spergula arvensis*) at 0–10 cm of soil layers (Kandeler et al. 1998) and the greater induction of the root production and mortality in Acer spp. (Wan et al. 2004) by higher soil temperatures were observed at elevated but not at ambient CO_2. Soil warming influences several root functions such as nutrient and water captures (St.Clair and Lynch 2010) which are fundamental physiological processes of the plant development and productivity and the functioning of the terrestrial ecosystems. In general, high temperatures increased the nutrient acquisition up to a peak of a maximum activity and then decline. Over the range of 14–34°C two cultivars of red maple increased the net nitrate uptake reaching a maximum absorption at 24°C (Adam et al. 2003). Roots of *Eucalyptus nitens* treated with 20°C showed a greater nitrate and ammonium uptake rates than that exposed to 10°C (Garnett and Smethurst 1999). The K (Ching and Barber 1979) and the P uptake (Mackay and Barber 1984) were increased in response to the moderate increase of temperature up to 29°C. However, Gavito et al. (2001) pointed out that the specific absorption rates are temperature dependent for each nutrient, varying from 10 to 15°C. During the vegetative growth, winter wheat reduced both the root and shoot N concentrations leaving unchanged the P concentration. Further, *E. nitens* showed the different Q_{10} values of the NO_3^- and NH_4^+ uptake rates estimated as 1.88 and 1.31 between 10 and 20°C, respectively (Garnett and Smethurst 1999).

Moreover, nutrient uptake appeared to be controlled by warmer temperatures through a direct and an indirect effect, that is, via root system changes and/or via plant–soil interactions, respectively. Direct changes in root morphology could explain the temperature-induced effects on K uptake (Ching and Barber 1979) and P uptake (Mackay and Barber 1984), as well as changes of the fluidity of fatty acids and thermostability of plasma membrane (Clarkson et al. 1988; Sibley et al. 1999), the uptake kinetics (BassiriRad et al. 1993, 1996; BassiriRad 2000; Adam et al. 2003) the root cell energy by respiration process (Atkin et al. 2000), plant nutrient demand (Lainè et al. 1993; Gavito et al. 2001). Thus, suggesting that temperature directly effects on root physiology of nutrient uptake. On the other hand, the temperature-induced indirect effects on nutrient uptake were mainly correlated with the capacity of the soil warming to influence the nutrient availability through changes on the biogeochemical, nodulation, and mycorhizzation processes and/or nutrient transport, at the rhizosphere level. Mineral weathering, decomposition of organic matter and exchange reactions between soil solid-solution phases, the biogeochemical processes involved in the nutrient availability, occurred at accelerated rates in warmer soils (Pregitzer and King 2005). For example, an increase of N mineralization was observed in a litter and a sandy mineral soil from forest of *Pinus sylvestris* (Ross et al. 1999) and in a boreal Norway spruce stand (Stromgren and Linder 2002). The nutrient movement toward the root system was improved at moderate supraoptimal soil temperatures which increased the ion diffusion and transpiration-driven mass flow. Therefore, the nutrient uptake was enhanced by the higher nutrient concentration around the root axis in warming soil. In this respect, Ching and Barber (1979) observed that temperature changes (from 15 to 29°C) determined a sharp improvement of the K uptake due to increase of the diffusion flux (+160%) rather than the enlargement of the root surface area (+70%).

An important agricultural and ecological function of the root system is the biological nitrogen fixation of the legume species by bacterial infection considering that half of the 320 Tg of N input to terrestrial ecosystems annually comes from the biological fixation of N_2 (Paul and Clark 1996). Symbiotic nitrogen fixation was positively influenced by increased soil temperature which raised nodule mass and activity. However, although Rhizobium can tolerate high soil temperature (30–35°C), the extreme temperatures (>40°C) strongly affected the bacterial infection and N_2 fixation, at different degree among the host plant species (Zaharan 1999). The total N_2 fixation of *Trifolium repens* was enhanced by an increase of the temperature in the 7–13°C range while it was not influenced by temperatures above 13°C (MacDuff and Dhanoa 1990). Warmer soil also affected other main symbiotic relationship of the higher plants, that is, the plant host-mycorrhizal fungi interactions. Increasing the soil temperature, root length colonization (LRC) was improved (Heinemeyer and Fitter 2004) and an increase in development of arbuscular mycorrhizal hyphae was observed which consequently helped in higher P uptake by roots of pea plants (Gavito et al. 2003). However, Olsrud et al. (2004) pointed out that the positive relationship between the mycorrhizal development and warmer soil was an indirect effect due to an increased C allocation toward the roots in response of the concomitant low soil moisture content rather than a direct temperature effect on the root system (Olsrud et al. 2004).

3 Roots and Altered Precipitation

The climate change models of the Intergovernmental Panel on Climate Change predicted that, as a consequence of temperature increases, the precipitation pattern will vary (IPCC 2007). At high latitudes, it should increase in winter and decrease in summer, and in various regions of Central and South Europe will also increase the frequency and duration of summer precipitation. Thus, the climate change will be responsible for determining an increased risk of both soil drought and waterlogging.

Generally, water is believed to be available for plant uptake at soil water potential greater than

wilting point, −1.6 MPa, which is considered the limiting value for the growth and development of many mesophytic plants. Suboptimal water availability, that is, drought soil condition is in fact considered a major constraint limiting the crop productivity. Soil drought more severely affect shoots than roots (Spollen et al. 1993) which have the ability to grow under mild stress condition. This differential sensitivity represents an important advantage to plants and allows them a greater exploration of soil for ensuring a supply of water and increasing their probability to survive at dry condition.

Drying soils produce a range of effects on the plant root system and varies from species to species. The total root length and dry weight, the lateral root production, the depth of rooting and the root dynamics represent the major traits influenced by water deficit. Several evidences indicated that the primary root length of maize was inhibited at −0.5 MPa water potential after 24 h (Fan and Neumann 2004). The reduction in the primary root elongation of *Arabidopsis thaliana* was observed at moderate water stress (−0.2 MPa) (van der Weele et al. 2000), similar results were observed in *Pinus pinaster* whose root system was increased at −0.15 MPa and reduced at −0.66 MPa) (Triboulot et al. 1995). In White oak (less sensitive) whose root elongation rate was reduced in the range of −0.4 and −0.8 MPa and completely ceased at −1.2 MPa soil water potential (Kuhns et al. 1985). The rapid soil drying conditions determined an intense reduction of the elongation rate of the *Opuntia ficus-indica* roots after 3 days of water deficit while the gradual drought stress caused the root inhibition after 9 days of water stress (Dubrovsky et al. 1998). Additionally, as response to soil drying, lateral roots varied in length in a specie-specific manner. The length of the first-order lateral roots was more reduced in maize than in wheat making this species less sensitive to water deficit (Ito et al. 2006).

What physiological mechanisms are involved for maintaining the root elongation at low water potential? Over the last 20 years, Sharp and coworkers (Sharp et al. 2004; Ober and Sharp 2007) pointed out detailed results on the regulation of maize primary root growth under soil drought conditions. In particular, they observed that elongation rate of the apical region (<3 mm, region 1) of the water-stressed roots (−1.6 MPa) was maintained same as well-watered roots but was reduced and completely inhibited in the basal root regions (3–7 mm region 2; and >7 mm, region 3, respectively). The mechanisms involved in the maintenance of the elongation rate in the region 1 may be due to the following reasons: (1) accumulation of the plant hormone abscissic acid (ABA), (2) the osmotic adjustments, and (3) the cell wall extension properties. Saab et al. (1990) and Sharp et al. (1994) showed that endogenous ABA accumulated in the apical region of water-stressed maize roots causing prevention of excess ethylene production and playing a regulatory role in the ion homeostasis (Ober and Sharp 2003). Accumulation of proline was responsible for as much as 45% of the total osmotic adjustments in the apical root region of maize seedlings under water stress (Voetberg and Sharp 1991). Further, Wu et al. (1996) reported differential responses of cell wall extension properties in maize seedlings. The expansion and xyloglucan endotransglycolase/hydrolase (XTH) activities were higher in the apical region of water-stressed than well-watered roots. It is interesting to note that ABA accumulation was involved in both processes increasing the proline transport and the XTH activity in the apical region of water-stressed roots underlying its regulative role in drought stress (Ober and Sharp 1994; Wu et al. 2001). In addition, Fan and Neumann (2004) found that the water stress induced an acidification in the apical region of maize roots which maintained the growth in presence of drought stress. Consistent with these results, transcriptomic and proteomic analyses of root growth of maize seedlings in responses to soil drought revealed that the water stress induced gene expression and the cell wall protein abundance were largely region specific. The apical region exhibited a greater gene and protein numbers involved for the root adaptation to drought (Yamaguchi and Sharp 2010).

Under water stress conditions, beyond the root length, other root morphological parameters were modified such as root diameter or SRL. Water-stressed roots became thinner than that of

well-watered plant such as, *A. thaliana* (van der Weele et al. 2000), rice (Trillana et al. 2001), maize ((Liang et al. 1997; Hund et al. 2009) and rootstocks of *Vitis vinifera* (Baurle et al. 2008). Root thinning as adaptive traits in dry environments could imply: (1) a lower construction cost for unit of root length (Sharp et al. 1988; Ho et al. 2005), (2) a faster elongation rate of root axis (Thaler and Pages 1996; van der Weele et al. 2000), (3) a greater root proliferation in moisture-disturbed soils (Eissenstat 1991), and (4) a limitation on radial expansion to conserve water (van der Weele et al. 2000). However, since the thicker roots were positively correlated with an increase of water transport (Passioura 1988; Doussan et al. 1998) and a greater RLD in deep soil layers (Azhiri-Sigari et al. 2000; Kato et al. 2006). The reduction of the root diameter as plant adaptive traits in drying soils was still questioned.

Generally, the soil dried gradually from the upper layers producing a sharply vertical soil-moisture gradient. Padilla and Pugnaire (2007) observed that in a semi-natural field during the summer, the soil moisture was <8% and >20% at 5 and below 30 cm of soil depths, respectively, and this difference disappeared in the spring season. This vertical soil-moisture gradient in the rainfed or non-irrigated conditions was also observed in field experiments by Songsri et al. (2008), Bucci et al. (2009), and Cheng et al. (2009). As the soil environment became drier, root system changed its distribution becoming deeper since deep-rooted plants had an improved ability to extract water from well-watered deep soil layers compared to shallow-rooted. This shift on root distribution in response to water stress has been confirmed by Schenk and Jackson (2005) who collected data of >1,300 records of root distribution of individual plants from deserts, scrublands, grassland, and savannas along the soil depths, showed that the "absolute" rooting depth was more strongly correlated with the mean annual precipitation in all plant growth except shrubs and trees. However, Bucci et al. (2009) observed that shallow-rooted shrub species of *Patagonia steppe* were correlated with leaf negative water potential than deeply rooted ones. Padilla and Pugnaire (2007) observed that species characterized by deep roots (*Salsola oppositifolia* and *Retama sphaerocarpa*) had consistent access to deep water content and pointed out a successful survival compared with shallow-rooted plants (*Ephedra fragilis*, *Olea europaea*, and *Pinus halepensis*) which died as drought progressed. Further, root depth was found to be the preferential strategy for plant species in dry ecosystems (Canadell et al. 1996), long dry periods (Paz 2003), dry sandy soils (Yamada et al. 2005), and seedling of dry forest (Markesteijn and Poorter 2009). The root depth as drought-adaptive trait was exploited in crop breeding programs for improving the yield in water-limited environments. Two drought-tolerant maize hybrids exhibited around three times more axile roots in the deeper soil layers compared drought-sensitive ones (Wan et al. 2000). Drought-tolerant genotypes of sorghum were characterized by deeper roots (Ludlow et al. 1990) and the ability to produce roots in deeper soil layers could markedly improve the drought tolerance of wheat cultivars (Manschadi et al. 2006). Change in root distribution under water stress was reported among genotypes in cowpea (Matsui and Singh 2003), white clover (Annicchiarico and Piano 2004), and chickpea (Kashiwagi et al. 2006). Beside the deep rooting, the higher RLD at lower soil depths has been identified as a drought adaptative trait that permits to stabilize the pod yield and the harvest index in drought-avoiding peanut genotypes (Songsri et al. 2008) and the ability to produce roots in deeper soil layers could markedly improve the drought tolerance of wheat cultivars (Manschadi et al. 2006). However, the direct relationship between the deeply rooted system and increased water absorption from deeper soil layers has not been clearly demonstrated and it was generally based on indirect evidences with the above-ground biomass and/or yield parameters. This consideration pointed out the following question: did root form changes root functional modification? Hund et al. (2009) demonstrated that the root system of CML444, drought-tolerant maize germoplasm, compared with that of SC-Malawy, moderate drought-tolerant, exhibited a deeper roots accompanied by higher ability to absorb water from deep layers. Further, the

deep-rooting is an important root architecture strategy for efficient water uptake in dry environments. Indeed, Manschadi et al. (2008) observed that the narrower angle of the seminal roots causing a deeply rooted architecture was a trait to exploit in breeding for improved wheat cultivars for water-stressed environments. Further, the investment in terms of carbon allocation toward specific root types, such as tap root in bean, determining a deeply rooted architecture improved the water acquisition efficiency (Ho et al. 2005).

In the agro-ecosystems, plants usually faced with several different environmental stresses acting in combination or in sequence and then it must optimize their resource allocation for the construction and maintenance of root systems. For example, the reduction of the soil water status caused the co-presence of both water stress and mechanical impedance to root growth (Whitmore and Whalley 2009). Ho et al. (2005) demonstrated that the "dimorphic" root system exhibited the best performance in environments characterized by multiple stresses. The bean genotype BAT477, exhibiting root architecture with both shallow and deep root localization, was well adapted in the presence of suboptimal water and phosphorus availability.

Besides the root morphological and architectural traits, the regulation of root water flow represented a physiological adaptive mechanism to soil water limitation. Root hydraulic conductance (Kr) was generally reduced when soil dried (North and Nobel 1991, 1992; Nardini et al. 2002). However, it has been observed that, when seedlings were exposed to moderate water stress, drought-sensitive plants increased the Kr (Nardini et al. 1998) and drought-tolerant plants decreased it (Lo Gullo et al. 1998). The capacity and the time-course necessary to recover Kr was a key factor of plant adaptation to seasonality in water availability under water stress, especially in Mediterranean species. The timing of recovery seemed to be correlated with the recovery of growth of root tip pre-existing and the new lateral root formation (Lo Gullo et al. 1998; Dubrovsky et al. 1998). Other than apoplastic pathway, the root hydraulic conductance was also related with water movement occurred along the cell-to-cell path by the aquaporins, water channel proteins (Javot and Maurel 2002; Tyerman et al. 2002; Maurel 2007). Several studies suggested that aquaporin-mediated transport was important in the regulation of root water flow under drought stress (Siemens and Zwiazek 2004), although their actual function was still unclear. Indeed roots of sunflower exposed to water limitation exhibited an up- and downregulation of different aquaporin genes (Sarda et al. 1999) and transgenic plants of *Arabidopsis* and tobacco, that constitutively over-expressed PIP1b, PIP1;4 or PIP2;5, had adverse effects on plant growth under drought stress (Aharon et al. 2003; Jang et al. 2007).

The drought stress altered the root functionality such as the nutrient uptake. Substantially, drought reduced the uptake of phosphorous (P) in barley (Shone and Flood 1983) and rye grass (Jupp and Newman 1987) and nitrogen (N) in maize (Buljovicic and Engels 2001), *Pseudoregneria spicata* (BassiriRad and Caldwell 1992a), and *Artemisia tridentata* (BassiriRad and Caldwell 1992b). Conversely, N uptake was not affected by drought stress in *Agropyron desertorum* (BassiriRad and Caldwell 1992a) while P uptake was increased in *Artemisia tridentata* (Matzner and Richards 1996). These controversial results could be due to the wide influence of drought stress on all other factors/components involved in actual rate of nutrient uptake from soil, such as (1) soil processes that provide the nutrient availability at root surface; (2) anatomical (endo- and exodermis), morphological (length and surface area), and architectural (shallow and deep rooting) root characteristics; and (3) physiological (nutrient transporters) root characteristics.

4 Root and Excess Water

Excess water in soil determines the full filling of the pore space restricting the diffusion of oxygen by 10^4-fold than in air (Drew and Armstrong 2002) and causing a stress, named waterlogging, with dramatic impact on plant growth and productivity. Climate change provisions provide that, as a consequence of anomalous weather patterns, waterlogging could be a more exceeding problem for many plant communities.

Under oxygen absence (anoxic soil) or under severe hypoxic conditions, the cytochrome oxidase activity of the plants became oxygen limited with a consequent reduction of ATP and pH of the cytoplasm, carbohydrate starvation, and accumulation of the toxic products due to a switch to a fermentative pathway (Drew 1997; Geigenberger 2003; Bailey-Serres and Chang 2005). Within short-time (few minutes or hours), these toxic effects caused severe damages to the plant growth and ultimately leads to death of many plant species. However, the supply of small amount of oxygen (hypoxic soil) stimulated several acclimative mechanisms which allowed the plants to survive to the transient waterlogging.

In waterlogging soils, root system represented the first and more sensitive target of plants which could be seriously damaged in its form and function. Significant inhibition of the root growth, exposed to waterlogging stress were observed in *Arabidopsis* (van Dongen et al. 2009), *Trifolium glomeratum* (Gibberd et al. 1999), wheat (Malik et al. 2002), maize (Wei and Li 2000; Qiu et al. 2007), woody species (Poot and Lambers 2003; Nicoll and Ray 1996; Nicoll and Coutts 1998; Coutts and Philipson 1978). At the same time, the root system was also able to engender several adaptative responses to waterlogging that could enhance the plant survival in flooded soils. The root adaptation mechanisms were addressed to improve the cellular energy status and reduce the accumulation of toxic end products that acidify the cytosol or damage membrane integrity.

To provide sufficient oxygen for maintaining the root respiration and, consequently, the ATP production it was essential to improve the root energy status and, ultimately, the root growth in anaerobic and chemically reduced soils. Aerenchyma, a plant tissue containing enlarged gas spaces, is an important trait for the root growth and function which provides a low-resistance pathway to obtain the oxygen from the atmosphere to the flooded below-ground organ. The development of aerenchyma has been associated with the tolerance to waterlogging in many plant species (Colmer et al. 1998). Evans (2003) distinguished two types of aerenchyma: (1) the schizogeneous derived from a differential cell expansion and specific pattern of cell separation with subsequent creation of cell spaces and (2) the lysigenous produced by the death and dissolution of the root cortical cells. While the former was common in various wetland species as *Rumex* (Laan et al. 1989), the lysigenous was typical of many crop species as soybean (Thomas et al. 2005), rice (Kaway et al. 1998), maize (Drew et al. 1979; Gunawardena et al. 2001), wheat (Huang et al. 1994), and pasture species (Gibberd et al. 2001; Ashi-Smiti et al. 2003). However, there was a third type of aerenchyma defined secondary aerenchyma, a white spongy tissue filled with gas spaces, which was found in stem, hypocotyls, tap roots, and root nodules of *Glycine max* (Shimamura et al. 2003), *Lotus uliginosus* (James and Sprent 1999), and *Sesbania rostrata* (Shiba and Daimon 2003). Generally, the aerenchyma was constitutively expressed in rice and wetland species. Recently, Seago et al. (2005) described the pattern of aerenchyma formation in 85 species representing 41 families of wetland plants. On the other hand, the aerenchyma development was also induced by flooding and other stresses, e.g., nutritional and drought in many field crops. For example, soybean, very sensitive species to flooding stress during the vegetative stage developed a secondary aerenchyma in stems, roots, and root nodules within few weeks of stress (Shimamura et al. 2003). Complex physiological and molecular mechanisms were involved in the development of aerenchyma in plants subjected to the flooding stress (Colmer 2003a; Evans 2003). In maize roots, the hypoxia conditions (3–12% oxygen) promoted the ethylene biosynthesis which triggered a signal transduction cascade involving Ca^{2+} and protein kinases, inducing a programmed cell death in target cells of the root cortex (Drew et al. 2000).

The genetic variability of the plant species in the differing tolerance to waterlogging was associated with the aerenchyma formation which determined higher root porosity. Indeed, the superior tolerance to waterlogging of *Trifolium tomentosum*, *T. fragiferum* and *T. repense* than *T. subterraneum* var. *subterraneum* and *T. glomeratum*, more sensitive species were due to the development of aerenchyma in adventitious roots

(Gibberd et al. 1999). The different ability to form aerenchyma and, hence, to exhibit a greater tolerance to the waterlogging stress were observed among the genotypes of soybean (Bacanamwo and Purcell 1999), maize (Zaidi et al. 2004), wheat (Boru et al. 2003), and woody species (Aguilar et al. 1999). Further, the wetland *Rumex* species tolerant produced a higher root porosity than sensitive ones (Laan et al. 1989).

Beside the aerenchyma, other root mechanisms which improved the presence of oxygen in plant tissues subjected to the flooding stress, such as the aerotropic roots and extensive lateral roots (Gibberd et al. 2001), the herringbone-type root architecture (Bouma et al. 2001), the emergence of adventitious roots (Mergemann and Sauter 2000) and the anatomical barriers such as exoderism (Colmer 2003b) were suggested to be part of the basis of tolerance to waterlogging. The recovery of the root cellular energy status in oxygen deficiency conditions was obtained through different metabolic adaptative mechanisms: the switch to a fermentative pathway (Sachs et al. 1996; Chang et al. 2000), the global depression of ATP-consuming processes (van Dongen et al. 2009), the death of metabolically intensive tissues as root tip (Subbaiah and Sachs 2000; Subbaiah and Sachs 2001) and the induction of anaerobic protein synthesis (Lal et al. 1998; Mujer et al. 1993; Chang et al. 2000). During waterlogging, the roots were more prone to oxidative stress which caused the generation of "reactive oxygen species" (ROS), including superoxide anion radicals (O_2^-), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), alkoxy radicals (RO), and singlet oxygen ($O_{1/2}$) (Munné-Bosch and Peñuelas 2003). The production of ROS led to enhanced peroxidation of membrane lipids and degradation of nucleic acids, and both structural and functional proteins. In this respect, the induction of free radical scavenging enzymes observed in wheat roots subjected to hypoxia determining tolerance to the waterlogging stress (Biemelt et al. 1998).

Proteomic and genomic analysis supported the morphological and physiological mechanisms that made up the root acclimative responses to the waterlogging stress. Indeed, the roots stressed by water shortage exhibited a large-scale reprogramming of gene expression and metabolism: (1) up- and downregulation of genes in *Arabidopsis* that mainly encoded proteins involved in fermentative process and energy-consuming processes (transport, lipid and secondary metabolism), respectively (van Dongen et al. 2009), (2) a quantitative trait locus containing the ethylene response factor-like genes was regulated by submergence in rice (Fukao et al. 2006), and (3) several quantitative trait loci associated with waterlogging tolerance were detected in maize plants (Qiu et al. 2007).

5 Root and Salinity

Salinity reduces plant growth and yield by two mechanisms, osmotic stress and ion cytotoxicity (Munns and Tester 2008). Munns 2002 proposed a two-phase model of salt injury where growth is initially reduced by osmotic stress and then by Na^+ toxicity. According to this biphasic model, growth is first reduced by the decrease in soil osmotic potential (ψ_o), caused by salt outside the plant rather than within it. The induced osmotic stress is controlled by inhibitory signals from the roots and, genotypes differing in salt resistance, respond identically in this first phase. Ionic stress develops over time and is due to a combination of ion accumulation in the shoot and to an inability to tolerate ions that have accumulated. The adaptation responses to ionic stress by plants are of two distinct types: Na^+ exclusion and tissue tolerance, and is the result of different abilities by plants to exclude or to sequester toxic ions into vacuoles. In this second phase of growth reduction, genotypes varying in salt resistance may respond differently. Secondary stresses induced by salinity, such as nutritional imbalances and oxidative stress, are also responsible of reduced plant growth.

It is difficult to separate the osmotic effect from specific ion effects that overlap during the development of salinity stress, thereby some uncertainty exists regarding the relative importance of both mechanisms. Rengasamy (2010) conducted a pot experiment on wheat growth where the plants were exposed to NaCl or to a

Hoagland nutrient solution at different salinity levels. The results evidenced that the osmotic effect is continuous but, at low level of salinity, the ionic effect may be significant in reducing growth and, the application of nutrients (Hoagland solution), alleviates the salinity stress on plants. However, above a threshold value of soil solution salinity, the osmotic effect becomes the dominant mechanism limiting the growth. Although the term salinity implies high concentration of salts in soil NaCl contributes the most part in soil salinity and this explains why all plants have evolved some mechanisms to regulate NaCl accumulation or exclusion. Moreover, the specific-ion toxicity of NaCl is not only the result of an excessive Na uptake, but a combined contribution of both Na^+ and Cl^- as well. In fact Cl^- concentrations may be higher than those of Na^+ ions, cations that can be adsorbed by soil particles. Generally, anions like Cl^- are repelled from soil surface and retained in soil solution where they can accumulate also at large amount, controlling the overall salt concentration of the soil solution. For most species Na^+ appears to reach a toxic concentration before Cl^- does; however, for some crops, such as soybean, citrus, and grapevine, Cl^- is considered to be the more toxic ion (Storey and Walker 1999).

5.1 Hydroponic vs. Soil Systems

The majority of works regarding salt effects and developing selection criteria for improved salt tolerance in plants has been done using solution culture, assuming that responses in hydroponics mimic those in soil. In a recent study on barley, Tavakkoli et al. (2010) show that the effects of salinity on plants differed between the hydroponic and soil systems. The salt concentration in the rhizosphere may increase, as a result of decreasing water content in the vicinity of the roots, due to the high transpiration demand and low hydraulic conductivity of soil. This does not occur in solution culture where no ion gradients will build up and neither depletion nor salt accumulation in the rhizosphere will occur. In soil sudden changes of salt concentration are unlikely because of the soil-buffering capacity, associated with the cation exchange soil complex (Vetterlein et al. 2004). Thus, plants in soil have more time to adapt to the increase of salinity than plants in hydroponic system. This is of a particular importance for cellular homeostasis adjustments that require ion uptakes and compatible solute accumulation. A result consistent with the two systems is the more significant negative effect of osmotic stress on plant growth, in comparison with the specific ion effects (Tavakkoli et al. 2010). This agrees with assumptions by Munns (2005) and Rengasamy (2010) which indicated that the biggest reduction in growth is caused by the osmotic stress and a relatively smaller effect is due to the genetic differences in ion exclusion.

5.2 Soil Constraints on Root Growth in Saline Environment

The evaluation of the average soil salinity and water content of a specific soil layer cannot be considered comprehensive to calculate the effective soil solution salinity roots are exposed to. In fact, it does not consider aspects concerning interactions between roots and soil at the root/soil interface. Driven by transpiration of the shoot, saline soil solution moves from the bulk soil to the root surface where water uptake occurs but most ions are excluded. Consequently, rhizospheric soil can be up to 15 times more saline than the bulk soil and this gradient is also more expressed under conditions of higher ET demand. The osmotic water potentials of the soil solution contacting the root surface are significantly lower than the bulk soil and this gradient initiates a flow of soil solution directed to the root surface (mass flow). Then, the increase in soil salinity, as result of evaporation, occurs at the soil interface, while the site of separation of salts from the soil water, due to root water uptake, takes place at the soil–root interface.

In many saline soils a deterioration of the structure leads some physical constraints that in the root zone appear principally in the form of compaction and crusting. Low porosity restricts rates of water and nutrient uptake by roots as well

as gas exchange, whereas high soil strength directly inhibits root elongation and expansion. Soil oxygen movement to roots is critical to maintain adequate respiration for plant growth. Under anoxic conditions some bacteria shift metabolic pathways so as to utilize alternative terminal electron acceptors and produce some substances, such as hydrogen sulfide, that are toxic for plants. Roots need nutrition, water aeration, and low mechanical strength to grow and function in the soil environment. The study of interactions between root properties (morphology and activity) and soil conditions are relevant to assess the water supply of plants and the salt tolerance of plants. If root growth and physiological processes in the root are affected, adverse leaf water status and top growth can occur through both hydraulic and biochemical signals.

5.3 Na^+ Uptake and Accumulation in Roots

In most plants, roots should exclude 98% of the salt in soil solution allowing only 2% to be transported to the shoot. Then roots filter out most of the salt in the soil while taking up water and play a fundamental role in protecting the plants from excessive uptake of salts. Furthermore, roots have a remarkable ability to control their Na^+ and Cl^- concentration, which is rarely much higher than in external solution. (Munns 2005)

Unidirectional influx and efflux provide the two main components of the currently accepted model of Na^+ uptake in plants. Na^+ ions passively enter the cell, down the ion's electrochemical potential gradient, and exit the cell via a secondarily active proton-driven sodium with a probable Na^+:H^+ stoichiometry of 1:1 (SOS1; Shi et al. 2002). This process consumes significant cellular energy. Recently, Malagoli et al. (2008) showed that the energy predicted to drive active Na^+ efflux in rice roots was much greater than the measured one. This discrepancy may indicate the involvement of more Na^+-specific transport systems and interestingly, a sodium–potassium–chloride transporter has recently been discovered in *A. thaliana* (Colmenero-Flores et al. 2007). Then, it was suggested a possible mechanism in which active Na^+ efflux is energized differently from current models, possibly via its coupling to passive fluxes of ions other than protons.

The compartmentation in root vacuoles of remaining Na^+ is achieved by tonoplast Na^+/H^+ antiporters. A passive leakage of Na^+ back to the cytosol (possibly via tonoplast nonselective cation channels) requires a constant resequestration of Na^+ into vacuoles (Apse et al. 1999). This mechanism allows plants to minimize or delay the toxic effects of high concentrations of salts, so genotypes with a poor ability to sequester salts have a greater rate of leaf death. Therefore, an efficient sequestration system may improve tissue tolerance by plants, perhaps by reducing cytosolic Na^+ concentrations. As the water moves from the soil across the root cortex ions are transported by this stream toward the stele. Some X-ray microanalysis on roots of wheat plants showed that the root cortex is the main barrier to Na^+ transport into the stele, rather than the endodermis (Läuchli et al. 2005) and the highest concentration of Na^+ was in the cell layer of pericycle. Similar results show substantial sequestering of large amount of Na^+ and Cl^- in vacuoles of pericycle cells in grapevine roots, grown at relatively low salinity (25 mM NaCl), suggesting an important role of pericycle in the radial transport of Na^+ and resulting xylem loading (Storey et al. 2003).

5.4 How Salinity Is Sensed in Roots

The perception of salinity is achieved by both ionic and osmotic stress signals in plants. The responses of root cells are finalized to maintain their own correct functionality, despite the condition of elevated Na^+ concentration. Long distance signals to shoots are activated in the form of hormones or their precursors; in fact the reduction of leaf growth under salinity is independent of carbohydrate supply and water status (Turka and Demiral 2009). ABA plays a central role in root-to-shoot and cellular signaling, but gibberellins

are also involved. ABA can inhibit leaf elongation by lowering the content of active GA, as observed in barley leaves (Munns et al. 2006). Root growth is usually less affected by salinity than leaf growth. Root elongation rates recover remarkably well after exposure to NaCl or other osmotica and, unlike leaves, the recovery takes place despite turgor. Changes in wall properties must occur, but the mechanism is unknown. With time, reduced initiation of new lateral or seminal roots is evident.

Signals within root cells are likely independent from ABA. Plants respond directly and specifically to the addition of Na^+ within seconds. Then, a plasma membrane protein must be the sensor, but this is still obscure. The first recorded response in roots is an increase in $[Ca^{+2}]_{cyt}$ from an influx across the plasma membrane and also from the tonoplast. This perturbation in Ca^{+2} level activates salt stress signaling, sensed by a protein (SOS_3) that interacts with a protein kinase, identified as SOS_2. The complex SOS_3/SOS_2, enabling the phosphorylation, activates the membrane bound Na^+/H^+ antiporter, SOS_1, that is responsible of Na^+ efflux. The discovery of the SOS (Salt-Overlay-Sensitive) pathway in *Arabidopsis* clarified how Na^+ (ionic stress) is sensed and the relationship between ion homeostasis and salinity tolerance. However, *Arabidopsis* is a glycophite species, sensitive to moderate levels of NaCl, and the adaptive responses to Na^+ in this plant should be extrapolated with caution. In fact, if *Arabidopsis* remains a useful model to study and discover plant Na^+ transport processes, the identification of signaling pathways in salt tolerant species is more relevant to define adaptive rather than dysfunctional responses to salinity. The relationship between Na^+ tolerance and Na^+ accumulation is different in *Arabidopsis* and cereals (Tester and Davenport 2003). More work is necessary for the identification of the different mechanisms that are fundamental to specific aspects of salinity tolerance, and also the evaluation of the time of exposure and the severity of salt treatment are important, because they determine the physiological and molecular changes that are detected.

5.5 Root Form and Function in Saline Environment

Root system is the main interface between plants and their environment, and shows a high degree of plasticity in its development in response to local heterogeneity of the soil. On the level of the individual root and the entire root system, various morphological parameters such as length, section, surface area, root hairs are used as potential indicator of root plasticity. Moreover, responses of biomass allocation patterns and structural traits such as SRL, RTD, and root diameter distribution, are associated with acquisition capacities for below-ground resources and respond to stresses and environmental changes. Therefore, some morphological modifications, at the individual root level can affect the structural and physiological characteristics of the entire root system and this can change water uptake and nutrient supply by plants.

Rice is considered as a moderately salt-sensitive crop, although a large variability exists among cultivars, as well as between developmental stages (Bahaji et al. 2002). A delay in the emergence of primary, adventitious, and lateral roots and a subsequent inhibition of root development, in terms of number and length were common responses to osmotic and saline treatments. However, some specific NaCl responses were detected and concerned lateral root development. In particular, lateral roots were thicker, as well as more densely arranged, and more irregular spaced than those of control plants. Furthermore, some bifurcations were occasionally noticed in primary and adventitious roots of NaCl stressed rice seedlings. In rice under salinity silicon can accumulate to high levels and it reduces Na^+ loading to xylem in plants. X-ray microanalysis of root transverse sections showed that the greatest silicon deposition was in the endodermis. Silicon deposition restricted the movement of water and ions through the apoplast so the Na^+ uptake was reduced by blocking the influx through the apoplastic pathway. It has been reported a positive role for silicon in reduction of salt stress in many crop grasses including wheat, maize, and barley (Munns 2002; Munns et al. 2006; Flowers 2004).

Wang et al. (2009) showed that high salt exposure suppressed lateral root initiation and organogenesis in *Arabidopsis thaliana*, resulting in the abortion of lateral root development but, on the other hand, salt stress markedly promoted lateral root elongation. The lateral root shaping is considered a prime example of developmental plasticity because both number and placement of lateral roots are highly responsive to external cues. This indicates that there must be a signal transduction pathway that interprets complex environmental conditions and makes the "decision" to form a lateral root at a particular time and place. Auxin plays a key role in shaping plant architecture and it mediates responses to a broad range of external signals. Histochemical staining, physiological experiments using transport inhibitors and genetic analysis revealed that the quantity of auxin and its patterning in roots were both greatly altered by exposure to high concentrations of salt stress and auxin transport pathway is important for adaptive root system development under salt stress (Malamy 2005). Root hairs can make up 70–80% of the root surface area. They play an important role in nutrient uptake, and root hair number and density generally increase as a consequence of a nutrient stress (Glory and Jones 2000). Wang et al. (2008) showed that in *Arabidopsis thaliana* root hair number and density decreased significantly under salinity, in a dose-dependent manner, and they reported a physiological mechanism for root hair development in response to salt stress. They hypothesize that salt stress may affect cell-fate specification and the reduction in root hair number is likely caused by a decrease in the epidermal cells differentiating into trichoblasts. The inhibition was sensitive to ions but not to osmotic stress, and was considered an adaptive mechanism to avoid excessive ion uptake, by reducing the absorptive area when ion disequilibrium occurs in roots. Furthermore, because of high sensitivity of root hairs toward salt, they suggest a possible role of root hair alteration as an early indicator of salt stress and plant response.

Salinity stress is also responsible for thickening of roots. Some studies were carried out on growth and changes in structure of root cells in *Kikuyu* seedlings grown in Hoagland nutrient solution with different salt concentrations (Panuccio et al. 2002, 2003; Muscolo et al. 2003). The cross sections of the primary structure of *Kikuyu* grass roots exposed to 50–100 mM NaCl did not show significant changes in the cortex growth and stele development; in contrast, 200 mM NaCl caused a significant reduction in the relative volume of the endodermis around the central cylinder, a thickness of the Casparian band and an increase in the number and diameter of root metaxylem vessels. These anatomical modifications may increase the mechanical resistance and decrease the root permeability to avoid the toxic effects of ions in excess. *Lens culinaris* has always been considered as a salt-sensitive species, but the *microsperma* landrace "Ustica" is a genotype that behaves like a salt-tolerant one because of its adaptation to the particular environment of the homonymous little island (North of Sicily). Some studies have been conducted to evaluate salt effects and plant responses in Ustica seedlings, grown for 20 days in microcosms, using agrilite as solid substrate and in the presence of different salt concentrations (0, 50, 100, 200 mM NaCl) (unpublished data). Various morphological root parameters such as length (cm), diameter (mm) and surface area (cm^2) were tested, by using an image analysis system. The results were also compared with those of a commercial cultivar (Eston), as they are valuable parameters when describing and comparing root systems. In both cultivars, the length of lateral roots was more affected than that of primary roots, but to different extent (Fig. 8.1). The parameter "root length" is considered more important than the "root weight" to indicate the root functionality, because it expresses the potential for solute and water uptake (Ryser 2006). In Eston seedlings, exposed to 100 mM NaCl, no lateral roots were expressed while seedling of Ustica showed an inhibition of the lateral root length even though the number was not significantly influenced. Generally, a water supply reduction in plants brings to a lower lateral root production (Fig. 8.1). The SRL values were higher in Ustica than in Eston seedlings (Table 8.1). SRL is the length-to-mass ratio; it is believed to characterize economic aspect of the

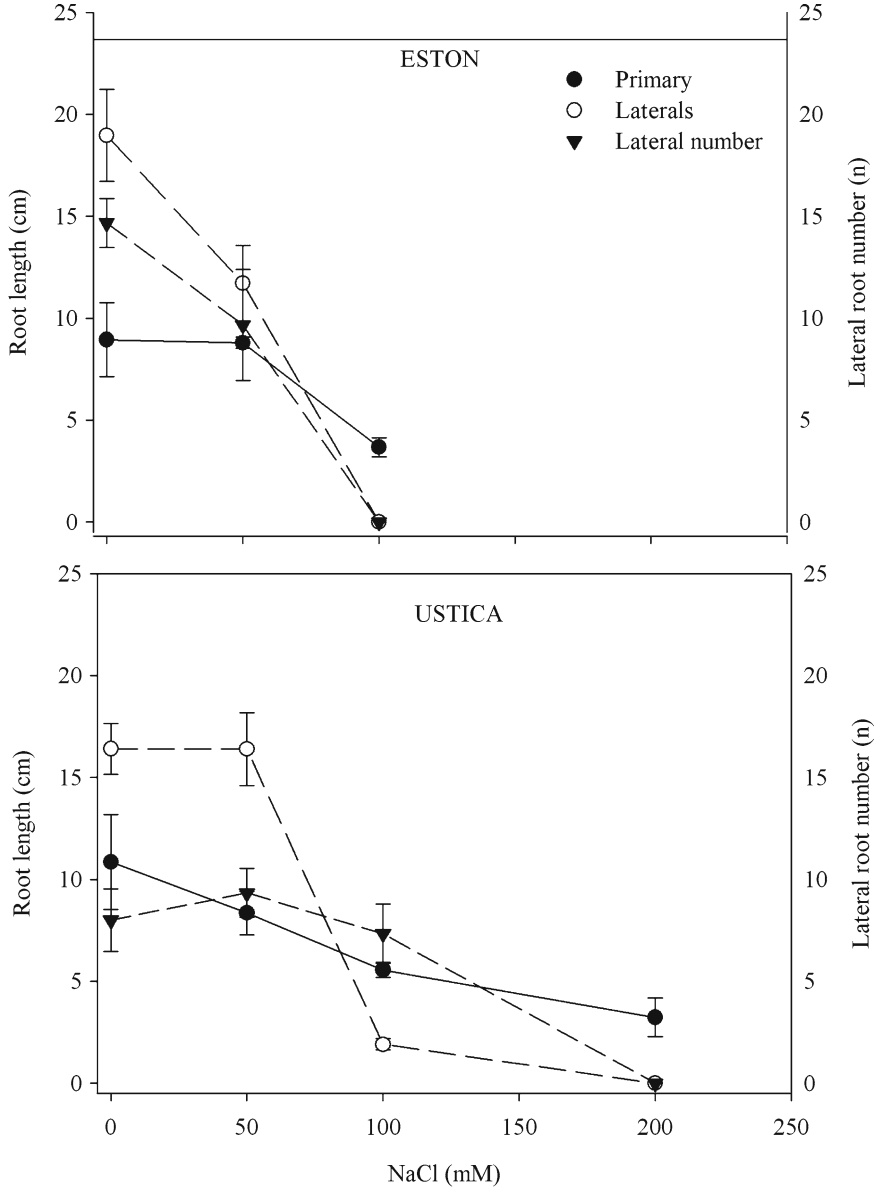

Fig. 8.1 Effects of salinity (0–200 mM NaCl) on primary and lateral root length and on lateral number of a landrace (Ustica) and a commercial cultivar (Eston) of *Lens culinaris* seedlings

Table 8.1 Effects of salinity (0–200 mM NaCl) on specific root length (cm g^{-1}) and root tissue density (g cm^{-3}) of a landrace (Ustica) and a commercial cultivar (Eston) of *Lens culinaris* seedlings

	NaCl (mM)	Specific root length (cm g^{-1})	Root tissue density (g cm^{-3})
Ustica	0	8.0 ± 1.9	48.1 ± 6.1
	50	10.6 ± 2.9	42.0 ± 9.3
	100	2.8 ± 0.3	73.1 ± 1.9
	200	4.5 ± 2.0	82.2 ± 10.7
Eston	0	4.9 ± 0.3	47.9 ± 1.7
	50	3.5 ± 0.5	63.3 ± 4.7
	100	1.1 ± 0.1	106.1 ± 11.2
	200	–	–

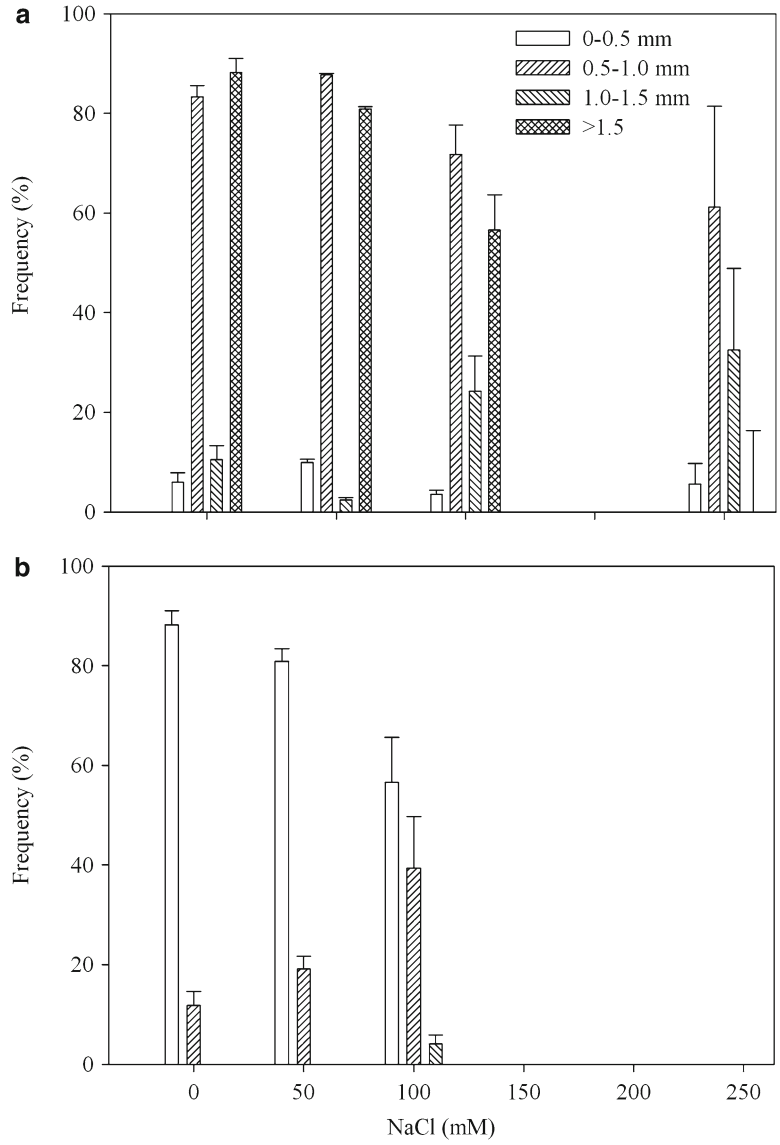

Fig. 8.2 Effects of salinity (0–200 mM NaCl) on the frequency (%) of different root diameter classes of primary (**a**) and lateral roots (**b**) of *Lens culinaris* landrace (Ustica)

root system and is frequently used as indicator of root fineness. Then, higher SRL results from longer and thinner roots per unit construction cost (root mass) and this root apparatus is more effective in water and nutrient uptake (Fitter 2002). SRL is a complex parameter that includes variations in root diameter and RTD, which respond to environmental conditions differently (Ryser 2006). In the Ustica variety, a salinity increase leads to an increase in SRL (Table 8.1), due to thicker lateral roots and the root diameter distribution was shifted toward larger diameter classes (Fig. 8.2a, b). Root diameter distribution is usually expressed as the mean diameter but sometimes it does not necessarily characterize a response of root system structure adequately. In fact, fine and coarse roots show different responses, indicating that root diameter classes should be considered as functionally distinct and regarded separately to fully understand stress responses of root systems. It is known that roots with a smaller root diameter can contact a larger soil volume per

unit root surface area; however, the maintenance carbon cost of producing finer roots may be higher as these will have to be replaced more frequently (Fitter 2002). In Ustica plants, coarse roots, for both principal and lateral prevailed under high salinity conditions. This result can be explained by considering that under salinity, the construction costs per root length should be minimized because of the onset of growth-limiting conditions, and the root development resulted further inhibited to counter water stress and ion toxicity due to the salt around the root. Apart from the effects on root biomass production, contrasting root morphological responses of ecotypes to salt treatments might be partially responsible for dissimilar abilities to tolerate salinity. Structural and morphological differences in roots certainly play an essential role for nutrient and water uptake by plants from saline soil and the study of these parameters can help to determine different mechanisms underlying salt toxicity and the way plants can cope with saline conditions. Some modifications of root morphology should not be considered a simple growth stopping, but rather an induced reorientation of growth which is related to stress avoidance. This information could be considered an important tool in studies that involve salt tolerance improvements in plants.

6 Conclusion and Future Perspectives

As detailed above, the root system may have a fundamental role in relieving the disturbances caused by the variables of the climate change on the plant growth, development, and production. However, an exciting challenge will be to understand the following key aspects regarding the impact of the root system on plant adaptation to the climate change:

1. An increase of the knowledge on the root responses to the interactive effects of the climate variables change (high temperature vs. drought and/or salinity and/or drought) that usually occur together
2. An greater understanding, in an integrated view, of physiological processes (development, growth, metabolic) involved in root responses to the warmer, drought, and salinity environments
3. A study of changes in proteins, metabolites, and other compounds inside the root cells by advanced genomic techniques for better understanding the molecular mechanisms implemented by the plant in response to temperature, water availability, and salinity change
4. An increase of the knowledge on the genetic diversity of the root system in plant response to climate variables change

An improved understanding of these aspects together with genomics, proteomics, and transcriptomic approaches are likely to pave the way for engineering roots that can withstand and give satisfactory economic yield under climate change.

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Reactive Oxygen Species and Nitric Oxide in Plants Under Cadmium Stress: From Toxicity to Signaling

9

Luisa M. Sandalio, Maria Rodríguez-Serrano, Dharmendra K. Gupta, Angustias Archilla, Maria C. Romero-Puertas, and Luis A. del Río

Abstract

The toxicity of heavy metals as a result of increasing environmental pollution in living organisms has become a major focus of research in recent decades. Among the heavy metals cadmium is one of the most dangerous heavy metals because of its high mobility in plants. It causes severe disturbances in plant metabolism that affect photosynthesis and water/nutrient balance, and it also causes oxidative damage. Although there is an enormous literature on the tolerance and accumulation of cadmium in plants, very little research has been performed on the molecular mechanisms and signaling events underlying plant responses to Cd toxicity. The dual role as both oxidative damage inducers and signaling molecules of ROS and NO in heavy metal toxicity has been demonstrated by many workers. In this chapter, we review the contribution of different ROS and NO sources in cells and their role in regulating cellular responses to Cd.

Keywords

Cadmium stress • ROS • NO • Photosynthesis • Metal transporters • GSH metabolism • Signaling

1 Introduction

Heavy metals such as Cd, Hg, Pb, and Al are major environmental pollutants, particularly in industrial areas. The heavy metals are generated as a result of anthropogenic activities such as metal working industries, cement factories, smelting plants, refineries, traffic, and heating systems (Sanitá di Toppi and Gabbrielli 1999). Much of the arable soil around the world has been moderately contaminated by Cd through the

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use of phosphate fertilizers, sludge, and irrigation water (Sanitá di Toppi and Gabbrielli 1999). In polluted soils, Cd is generally present as a free ion or in other soluble forms, and its mobility depends on pH as well as the presence of chelating substances and other cations. Cadmium has a toxic impact on all living organisms by entering the food chain and accumulating by humans and animals (Nordberg 2004). Prolonged exposure to Cd by humans can cause renal dysfunction, lung damage, acute gastrointestinal problems, depression of immune system, increased cancer risk, and anemia (Nordberg 2004). The accumulation of cadmium in plants causes chlorosis, growth reduction, and even cell death. The cellular toxicity of this metal results from its various direct and indirect effects on cell metabolism and can be explained by its chemical characteristics. Cd can bind to SH groups of proteins and enzymes, leading to misfolding, enzyme inhibition, and interferences in redox regulation. Cadmium can also displace other cations from proteins and enzymes, which affects their functioning (Van Assche and Clijsters 1990). Like most heavy metals, cadmium induces oxidative stress by generating reactive oxygen species which causes oxidative damage to biomolecules such as membrane lipids, proteins, nucleic acids, etc. (Sandalio et al. 2009).

Cadmium is one of the most dangerous heavy metals in nature, and at low concentrations it adversely affects the plant growth and development. Strong evidence have shown that Cd-induced generation of reactive oxygen species plays an important role in cellular toxicity, and the effects produced are dose- and species-dependent (Benavides et al. 2005; Sandalio et al. 2009). However, ROS are double-faced molecules acting as signal molecules that regulate a large gene network involved in cell response to biotic and abiotic stress. Nitric oxide (NO) is a gaseous reactive molecule with a pivotal signaling role in many developmental and cell response processes (Besson-Bard et al. 2008). This molecule can also interfere with the ROS metabolism. There are many reports that showed the role of NO in the alleviation of the toxicity caused by heavy metals including Cd and As (Xiong et al. 2010). Several defense strategies to avoid metal toxicity have been developed by plants, which include preventing the entry of metal through exudation of metal-complexing agents (citrates and phytosiderophores) by roots and metal immobilizing pectic sites and histidinyl groups in the cell wall (Sanitá di Toppi and Gabbrielli 1999; Clemens 2006). A second line of defense involves the induction of specific peptides called phytochelatins (PCs) which chelate the metal. PC–Cd complexes are transported into the vacuole to protect cells from toxicity (Cobett 2000). The isolation of an Arabidopsis cad1 mutant, which is defective in PC activity and hypersensitive to Cd, has demonstrated the importance of this mechanism in defending plants against Cd (Howden et al. 1995). Cd and other metals can also be complexed by metallothioneins and nicotianamine (Sharma and Dietz 2006).

2 Cadmium Toxicity in Plants

The toxic effects of cadmium on several plant species have been reported by different authors (Sanitá di Toppi and Gabbrielli 1999; Sandalio et al. 2001; Schützendübel et al. 2001; Benavides et al. 2005), although the mechanisms involved in cadmium toxicity are still not fully understood. Cadmium inhibits seed germination, decreases plant growth, induces premature senescence, and can even trigger cell death in cell suspension cultures (Fotjová and Kovařik 2000; McCarthy et al. 2001; Rodríguez-Serrano et al. 2009; De Michele et al. 2009). At cellular levels, Cd produces alterations in membrane functionality by inducing changes in lipid composition and by promoting lipid peroxidation (Ouariti et al. 1997; Hernández and Cooke 1997; Sandalio et al. 2001); it also produces disturbances in photosynthesis by affecting CO_2 fixation and by inhibiting PSII photoactivation because of competition with essential Ca^{2+} sites (Faller et al. 2005; Baryla et al. 2001). Cadmium toxicity is associated with modifications in both the uptake and distribution of macro- and micronutrients (Hernández et al. 1998; Rogers et al. 2000; Sandalio et al. 2001; Tsyganov et al. 2007) and can therefore compete with other cations for

protein- and transporter-binding sites (Clemens 2006). Cd uptake occurs through plasma membrane transporters similar to those used for other cations such as K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+} or Cu^{2+} (Clemens 2006). Cadmium reduces Ca^{2+} content, which can then affect the activity of calmodulin-dependent proteins (Rivetta et al. 1997; Rodríguez-Serrano et al. 2009). Cd tolerance and Ca^{2+} homeostasis in a Cd-resistant pea mutant (SGECdt) have been observed to be interrelated (Tsyganov et al. 2007), while in radish and Arabidopsis seedlings, calcium has been reported to alleviate Cd toxicity by reducing Cd uptake (Rivetta et al. 1997; Suzuki 2005). The role of oxidative stress in Cd toxicity has been established in different plant species by analyzing oxidative damage to proteins and lipids as well as by studying disturbances in antioxidative defenses caused by this metal (Benavides et al. 2005; Sandalio et al. 2009; Remans et al. 2010). Although Cd is a bivalent cation unable to participate in redox reactions in the cell, most transcriptome studies show upregulation of genes encoding proteins involved in defense against oxidative stress and ROS production (Suzuki et al. 2001; Zhao et al. 2009). These results suggest that oxidative stress is one of the primary effects of Cd exposure. Reactions involving oxygen free radicals are an intrinsic feature of plant senescence and stimulate the process of oxidative deterioration that leads to cell death (del Río et al. 2009). Cd induces senescence and cell death in both cell culture and plant tissues characterized by the induction of the glyoxylate cycle, protein oxidation, and proteolytic activities (McCarthy et al. 2001; Romero-Puertas et al. 2002). Senescence is considered to be a type of plant programmed cell death (PCD), and various studies have indicated that Cd induces PCD in cell cultures (Fotjová and Kovařik 2000; De Michele et al. 2009). There are evidence to support the possibility of dose dependence and intensity of the onset of the senescence process and the final cell death event (De Michele et al. 2009). Condensation of chromatin, fragmentation of DNA, as visualized by TUNEL assay, and induction of SAG12 expression are some of the symptoms of PCD and have been observed in Arabidopsis and tobacco cell cultures exposed to Cd (Fotjová and Kovařik 2000; De Michele et al. 2009). Cadmium-dependent senescence and PCD are regulated by ROS and NO, although the mechanisms involved are not fully understood (Yakimova et al. 2006; De Michele et al. 2009; Rodríguez-Serrano et al. 2009). Lipid signaling and Ca^{2+} also play an important role in Cd-induced cell death (Yakimova et al. 2006).

3 Sources of ROS in Plants Exposed to Cadmium

The reactive oxygen species are mainly singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) which are by-products of normal aerobic metabolism such as respiration and photosynthesis. Their steady-state levels are determined by the interplay of different ROS-producing and ROS-scavenging mechanisms. This balance is maintained by enzymes such as superoxide dismutase (SOD), which remove O_2^- radicals, and catalase (CAT), peroxidase (POX), and peroxiredoxin, which decompose H_2O_2 and use metabolites such as glutathione (GSH) and ascorbate (ASC), to control ROS accumulation in different subcellular compartments. An excess of ROS is dangerous mainly because of reactions with lipids, proteins, and nucleic acids, giving rise to lipid peroxidation, membrane leakage, enzyme inactivation and DNA breaks or mutations, which can cause severe damage to cell viability. Subtle control of ROS production enables these species to act as signaling molecules which are involved in the regulation of processes such as mitosis, tropism, cell death and cell response to biotic and abiotic stresses. Compared with other ROS, H_2O_2 is a relatively long-lived molecule that is able to diffuse across cell membranes and acts as a signaling molecule during growth and development (Van Breusegem and Dat 2006). However, although we have a clear understanding of the toxic effects of ROS induced by metals as well as detoxification mechanisms, information on their role in regulation and signal transduction under metal stress remains quite limited.

The electron transfer chains associated with chloroplasts and mitochondria are the main sources of ROS generation. However, this view has changed and the oxidative metabolism of peroxisomes is now seen as a very important source of ROS under different stress conditions (del Río et al. 2006, 2009). In peroxisomes purified from pea leaves, a Cd-dependent increase in the H_2O_2 concentration was observed, mainly as a result of the activation of glycolate oxidase, a key enzyme in the photorespiration cycle (Romero-Puertas et al. 1999). In pea leaves, it has been demonstrated through the use of a cytochemical approach that Cd-dependent H_2O_2 production occurs in peroxisomes, in the outer mitochondrial membrane, and mainly in the plasma membrane, where the NADPH oxidase (NOX) is the main source of ROS (Romero-Puertas et al. 2004). In peroxisomes, H_2O_2 was located in close contact with other organelles, which suggests possible cross-talk with other cell compartments (Romero-Puertas et al. 2004). In mitochondria, the Cd-dependent H_2O_2 produced could be because of increased O_2^- production at the complex III site of the electron transport chain, as reported in animals treated with Cd (Wang et al. 2004) and also suggested for soybean roots (Heyno et al. 2008). H_2O_2 was also observed in the tonoplast from bundle sheet cells and plasma membrane from epidermal and transfer cells (Romero-Puertas et al. 2004). Cd-dependent superoxide radical accumulation was demonstrated in the tonoplast from bundle sheet cells and plasma membrane from mesophyll cells, although the source has not been identified (Romero-Puertas et al. 2004). Accumulation of both H_2O_2 and O_2^- was also observed in vascular tissues from Cd-treated pea plants using confocal laser microscopy, electron microscopy, and cytochemistry (Romero-Puertas et al. 2004; Rodríguez-Serrano et al. 2006, 2009). This ROS accumulation is associated with lignifications processes which are highly active in vascular tissue under physiological conditions and are also induced in response to metal toxicity (Schützendübel et al. 2001; Rodríguez-Serrano et al. 2009). Results using different inhibitors and modulators of signal transduction demonstrated that the earliest control point in ROS production induced by Cd is at the level of protein phosphorylation/dephosphorylation. A comparative transcriptomic study using different metals and sodium chloride in *Arabidopsis thaliana* showed that Cd specifically induced genes coding kinases (Zhao et al. 2009), which demonstrates the importance of these processes in regulating cell response to Cd. Calcium ions are also important in the regulation of ROS production induced by Cd, and cGMP is also involved in this process, probably as result of a transient increase in Ca^{2+} concentration (Romero-Puertas et al. 2004).

NOXs are located in the plasma membrane and catalyze the production of O_2^-, which can be converted into H_2O_2, spontaneously or in the reaction catalyzed by SOD. Ten genes encoding NOXs in Arabidopsis have been described and are termed respiratory burst oxidase homologs A–J (rbohA–J) given their homology with the catalytic subunit gp91 phox (Nox2) of the NOX complex of mammalian phagocytes (Torres and Dangl 2005). The role of NOX as the main source of ROS under Cd stress has also been demonstrated in tobacco cell cultures (Olmos et al. 2003; Garnier et al. 2006; Horemans et al. 2007) and alfalfa roots (Ortega-Villasante et al. 2005). In Arabidopsis plants, the analysis of transcript levels of different NOXs shows a transient increase in the expression of rbohF in response to Cd, while the expression of rbohC and rbohD remained unchanged (Horemans et al. 2007). However, the contribution of NOXs to cadmium-induced ROS production is a subject of debate (Heyno et al. 2008). In tobacco cell cultures, Cd induced cell death, which was preceded by three successive waves of ROS production. The first wave was because of an NOX followed by an accumulation of O_2^- and fatty acid hydroperoxides (Garnier et al. 2006). Before the first oxidative burst induced by Cd, a rapid and transient induction of cytosolic Ca^{2+} concentration takes place, which requires protein phosphorylation and IP3-mediated release of calcium from internal stores (Garnier et al. 2006). Downstream, protein phosphorylation, calmodulin, and Ca^{2+} may directly regulate NtrbohD activity (Garnier et al. 2006). The disturbances caused by Cd in the mitochondrial

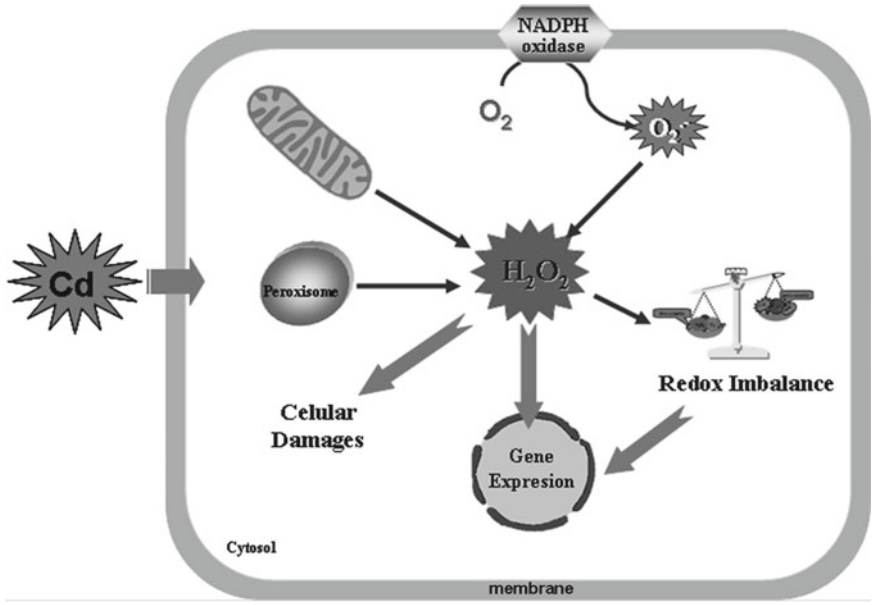

Fig. 9.1 Sources of reactive oxygen species activated in response to cadmium. Cd-dependent ROS production takes place in different compartments: plasma membrane-associated NADPH oxidase, electron transport chain from mitochondria and peroxisomes (mainly glycolate oxidase). Overaccumulation of H_2O_2 produces redox imbalance and oxidative damage, but also can regulate gene expression in order to improve plant survival

electron transport chain stimulate a second wave of ROS production possibly because of an increase in the semi-ubiquinone radical concentration (Garnier et al. 2006). A third wave of ROS coincides with cell death and involved membrane peroxidation as a result of increase in ROS production caused by mitochondria (Garnier et al. 2006). Remans et al. (2010) have recently demonstrated a Cd-dependent induction of NOX and differential regulation of gene expression by Cd and Cu in Arabidopsis plants and have suggested a link between NOX and lipoxygenase gene expression. A diagram showing the different subcellular locations of ROS production is provided in Fig. 9.1.

The inhibition of antioxidative enzymes may also lead to a cadmium-mediated increase in the level of cellular ROS (Sandalio et al. 2001, 2009; Romero-Puertas et al. 2002; Schützendübel and Polle 2002; Benavides et al. 2005). One of the consequences of plant cell exposure to cadmium is the rapid consumption of GSH for sequestration of the metal and synthesis of PCs. This limits the GSH level required to maintain the redox balance of the cell which then increases ROS accumulation (Romero-Puertas et al. 2007a, b).

4 NO Production in Plants Exposed to Cadmium

Nitric oxide is a simple gaseous signaling molecule which, in many plant tissues, regulates a wide range of physiological and biochemical processes as well as plant responses to biotic and abiotic stresses (del Río 2011; Delledonne 2005; Siddiqui et al. 2010). An increasing number of studies have reported the role played by NO in plant response to heavy metals including cadmium, although the source of NO and its role in metal toxicity and plant responses are not yet clearly established (Xiong et al. 2010). NO can be generated enzymatically by nitrate reductase and nitric oxide synthase (NOS)-like activities and can also be produced nonenzymatically by reduction of apoplastic nitrite under acid conditions and by reduction of nitrite to NO in the mitochondria (Neill et al. 2008; del Río 2011).

There is still some uncertainty concerning NOS in plants. Although there is strong evidence to show the presence of L-arginine-dependent NOS activity in plants (Barroso et al. 1999; del Río 2011), the only NOS from the plant kingdom to be fully characterized so far is the enzyme from the *Ostreococcus tauri* green alga (Foresi et al. 2010). NOS activity has been shown to be present in peroxisomes from pea leaves (Barroso et al. 1999). This enzyme uses L-arginine as substrate and requires NADPH, Ca^{2+}/calmodulin, BH_4, FAD, and FMN, although its gene has not yet been characterized (Corpas et al. 2004; del Río 2011). The generation of NO in peroxisomes by this NOS activity has also been reported by Corpas et al. (2004). In addition, chloroplasts have recently been identified as a source of NO via arginine and nitrite, although the enzyme involved has not been characterized yet (Jasid et al. 2006; del Río 2011). A nitrite-NO oxidoreductase enzyme (Ni-NOR) associated with root plasma membrane may also contribute to NO production (Stöhr and Stremlau 2006). However, there are other potential enzymatic sources of NO in plants (del Río et al. 2004; del Río 2011) such as xanthine oxidase which can produce NO under hypoxic conditions (Millar et al. 1998; Harrison 2002). Regardless of the source of NO involved, the mechanisms determining the effects of NO are far from being fully understood, while a number of downstream signaling pathways involving Ca^{2+}, cyclic GMP, and cyclic ADP-Rib have been described (Besson-Bard et al. 2008). NO is able to react with oxygen radicals such as O_2^-, generating peroxynitrite ($ONOO^-$), and also to control ROS levels in cells and vice versa (Delledonne et al. 2001). NO can also react with GSH to produce *S*-nitrosoglutathione (GSNO), which is regarded as a long-distance-signaling molecule and a natural reservoir of NO (del Río 2011). NO directly or indirectly can regulate gene expression and protein functions. It therefore reacts very rapidly with heme groups and thiols, thus regulating enzymatic activities (Moreau et al. 2010). The protein *S*-nitrosylation of cystein residues has been demonstrated to be very important in regulating the enzymatic activity of certain proteins (Lindermayr et al. 2006; Lindermayr and Durner 2009; Romero-Puertas et al. 2007b, 2008). Some studies of NO production during the exposure of plants to heavy metals have reached controversial conclusions. The cell cultures of soybean (Kopyra et al. 2006) and Arabidopsis (De Michele et al. 2009) exposed to Cd showed an increase in NO and was dependent on NOS-like activity (De Michele et al. 2009), whereas, in pea leaves and roots, prolonged exposure to 50 μM Cd reduced NO accumulation (Rodríguez-Serrano et al. 2006, 2009). Bartha et al. (2005) reported increased NO in the roots of *Brassica juncea* and *Pisum sativum* exposed to 100 μM Cd, Cu, and Zn. Besson-Bard et al. (2009) have shown that NO production in Cd-treated roots is related to Cd-induced Fe deficiency. The discrepancies observed in these results could be because of differences in Cd exposure duration, with NO increasing after a short period of Cd treatment and decreasing after a prolonged treatment (Fig 9.2). The metal concentrations, plant ages, and plant tissues used could also explain these discrepancies (Rodríguez-Serrano et al. 2009). Exogenously supplied NO has been demonstrated to alleviate heavy metal toxicity (Kopyra and Gwóźdź 2003; Hsu and Kao 2004; Yu et al. 2005; Wang and Yang 2005; Laspina et al. 2005; Xiong et al. 2010) possibly because of its ability to act as an ROS-scavenging antioxidant such as SOD and CAT (Wang and Yang 2005; Rodríguez-Serrano et al. 2006; Singh et al. 2008; Siddiqui et al. 2010). Exogenous NO application also affects root cell walls and helps in metal accumulation. NO cause increases in cytosolic Ca^{2+} concentrations by regulating Ca^{2+} channels and transporters, which may be involved in the signaling cascade that regulates gene expression under stress conditions (Besson-Bard et al. 2008). Recently, cross-talk between Cd, Ca^{2+}, ROS, and NO has been detected in pea leaves (Rodríguez-Serrano et al. 2009). The supply of exogenous Ca^{2+} to pea plants exposed to Cd reduced Cd-dependent O_2^- accumulation and restored NO accumulation to the level observed in control plants (Rodríguez-Serrano et al. 2009).

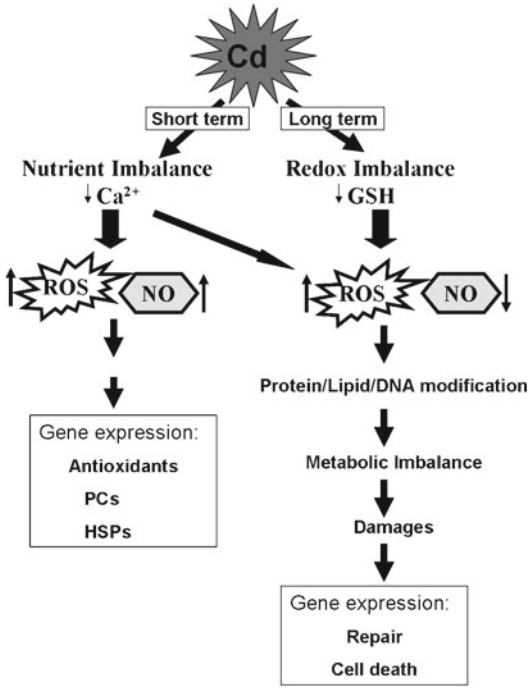

Fig. 9.2 Cadmium induces differential response in plants depending on the period of treatment. A short period of treatment produces oxidative and NO burst, which induces gene expression to prevent oxidative damages caused by the metal. Long-term treatment produces overaccumulation of ROS and a reduction of NO giving rise to severe damages. Gene regulation in long-term treatment is focused on repairing oxidative damages and cell death. *PCs* phytochelatins, *HSPs* heat shock proteins

5 Plant Responses to Cadmium

Information on molecular mechanisms and signaling events underlying plant transcriptional responses to Cd is rather limited as compared to research in the field of cadmium toxicity. The mechanism by which Cd modulates the levels of expression of most genes is not clearly understood, while our knowledge of global changes in the expression of Cd-responsive genes is also limited. A number of studies have been carried out involving both small-scale experiments and whole-genome approaches. Their findings suggest that gene expression is time- rather than dose-regulated in response to Cd and is differentially regulated in roots and leaves (Herbette et al. 2006; Ogawa et al. 2009). Genes regulated by cadmium can be categorized into different protein groups in terms of photosynthetic processes, signal transduction, and transcriptional regulation, cellular defenses, ROS detoxification and repair, hydric balance, metal transport, cell wall metabolism, sulfate and GSH metabolism and protein degradation.

5.1 Photosynthesis Regulation

The decrease in chlorophyll content has been considered to be one of the early symptoms of cadmium toxicity. The inhibition of chlorophyll biosynthesis has been suggested to be a primary event in Cd toxicity (Baryla et al. 2001). A substantial number of genes involved in photosynthesis were downregulated in the leaves of Arabidopsis plants grown with 5–50 μM Cd. For example, the genes involved in the photochemical process of photosynthesis, such as the chlorophyll synthesis pathway, glutamyl tRNA reductase, hydroxymethylbilane synthase, and Mg chelatase, some proteins of PSI and PSII, electron transporters, enzymes involved in Calvin cycle and Rubisco are downregulated (Herbette et al. 2006). Genes encoding enzymes in the pentose phosphate pathway were also downregulated by Cd (Herbette et al. 2006). These results have been corroborated by proteomic approaches (Álvarez et al. 2009) and correlate with the reduction observed in the photosynthesis net rates for different plant species (Sandalio et al. 2001; Faller et al. 2005). Downregulation of photosynthesis-related genes is a primary response under different stress conditions probably to avoid oxidative damage (Mittler 2002).

5.2 Signal Transduction and Transcriptional Regulation

Numerous genes involved in signal transduction were regulated in response to Cd in different plant species showing that signal transduction pathways are rapidly activated by the presence of Cd (Suzuki et al. 2001; Herbette et al. 2006; Ogawa et al. 2009). These include genes encoding

mitogen-activated protein kinases (MAPKs), calmodulins and calcium-dependent protein kinases (CDPKs) (Suzuki et al. 2001; Herbette et al. 2006; Ogawa et al. 2009), which suggests that Cd interferes with the Ca^{2+} signaling pathway, as demonstrated by Rodríguez-Serrano et al. (2009). MAPKs and CDPKs are involved in biotic and abiotic stress responses and participate in cross-talk with ROS production activities (Kobayashi et al. 2007). Transcription factors belonging to different families, such as WRKY, bZip, MYB, DREB, NAC, and AP2, are induced by Cd in different plant species (Herbette et al. 2006; Weber et al. 2006; Ogawa et al. 2009). The inductions by Cd of transcripts for bZIP, MYB, and zinc finger transcriptional factors have also been demonstrated in the root of the metal accumulator *B. juncea* (Fusco et al. 2005). Genes involved in hormone signaling, mainly ABA and ethylene and jasmonic acid, have also been shown to be regulated in response to Cd (Herbette et al. 2006; Minglin et al. 2005).

5.3 Cellular Detoxification and Repair

Several genes associated with cellular detoxification and repair have been shown to be induced by treatment with cadmium. Chitinases and heat shock proteins (HSPs) are induced in response to heavy-metal stress and are regarded as a second line of defense under these stress conditions (Metwally et al. 2003; Békésiová et al. 2008; Rodríguez-Serrano et al. 2009; Zhao et al. 2009). Transgenic plants expressing fungal chitinases actually showed enhanced tolerance to metals (Dana et al. 2006), while chitinase isoforms are differentially modified by certain metals (Békésiová et al. 2008). Chitinases are regulated by ROS and are possibly part of the general defense response program of cells under heavy-metal stress (Békésiová et al. 2008; Rodríguez-Serrano et al. 2009). Other pathogenesis-related proteins (PRPs) are upregulated by Cd (Fusco et al. 2005; Rodríguez-Serrano et al. 2009). ROS-dependent up-regulation of PRP4A has been demonstrated in pea plants exposed to Cd, whose transcripts were specifically accumulated in palisade mesophyll cells, as evidenced by in situ hybridization (Rodríguez-Serrano et al. 2009). These results point to an overlap in the regulatory mechanisms underlying these processes, with ROS production being a common event in these situations.

HSPs are upregulated by heat stress and can act as molecular chaperones favoring the transport of proteins to organelles and preventing protein aggregation (Ma et al. 2006). Induction of HSPs by Cd has been observed in different plant species (Sanitá di Toppi and Gabbrielli 1999; Rodríguez-Serrano et al. 2009) and is regulated by H_2O_2 overproduction (Rodríguez-Serrano et al. 2009). The transcription factors involved in HSP expression can act as H_2O_2 sensors (Miller and Mittler 2006). In *B. juncea*, Cd upregulates a DNAJ HSP (BjCdR57), a chaperone involved in protein protection against stress, which confirms that protein denaturation is one of the effects of Cd toxicity (Suzuki et al. 2001; Fusco et al. 2005). GSH S-transferases catalyze the conjugation of xenobiotics with GSH and participate in the removal of ROS and are upregulated in response to Cd (Suzuki et al. 2002; Fusco et al. 2005; Ogawa et al. 2009). Antioxidative defenses such as glutaredoxin, thioredoxin, GSH reductase, monodehydroascorbate reductase, SOD, CAT, and POXs are upregulated by Cd in order to deal with oxidative damage caused by this metal (Lemaire et al. 1999; Herbette et al. 2006; Romero-Puertas et al. 2007a, b; Smeets et al. 2005; Ogawa et al. 2009). Enzymes involved in vitamin E biosynthesis are upregulated in response to Cu and Cd in Arabidopsis plants, while vitamin E-deficient mutants (vte1) showed enhanced oxidative stress and sensitivity to both metals, suggesting that Vitamin E also contributes to defense against heavy metals (Collin et al. 2008). The regulation of these antioxidative enzymes is mainly dependent on H_2O_2 (Romero-Puertas et al. 2007a, b; Rodríguez-Serrano et al. 2009), although GSH metabolism also plays an important role in controlling the gene regulation of antioxidants in response to Cd stress (Cuypers et al. 2011). The activity of glucose-6-P dehydrogenase (G6PDH), malic enzyme (ME), and

NADP isocitrate dehydrogenase (NADP-ICDH) is stimulated by Ni, Zn, and Cd (Van Assche and Clijsters 1990; León et al. 2002), while, in pepper cultivars with different levels of sensitivity to Cd, tolerance to this heavy metal was more dependent on the availability of NADPH than on its antioxidant capacity (León et al. 2002).

5.4 Metal Transporters

Some of the genes regulated by Cd, such as AtPcr1 (Song et al. 2004) and those belonging to the ABC, MATE, cation diffusion facilitator (CDF), heavy metal P-type ATPase (HMA) and ZIP families, are involved in Cd transport (Ogawa et al. 2009). Fe and Zn transporters are also often involved in Cd transport because of their low substrate specificity. The iron transporters ZIP, AtIRT1, OsIRT1111, and OsIRT2 as well as the Zn transporter OsZIP1 have been shown to transport Cd. The HMA family is also involved in Cd detoxification in addition to CDF transporters and natural resistance-associated macrophage protein (NRAMP) family transporters (Ogawa et al. 2009). Pleiotropic drug resistance (PDR) family proteins are involved in Cd tolerance via export out of the cytoplasm (Kim et al. 2007). AtPDR8 is a cadmium extrusion pump, while AtOSA1 could be involved in the signal transduction pathway in response to oxidative stress (Kim et al. 2007; Jasinski et al. 2008). Cd-binding proteins such as Cdl19 could be involved in maintaining heavy-metal homeostasis and/or detoxification (Suzuki et al. 2002).

5.5 Cell Wall Metabolism

The cell wall is one of the first structures to be directly exposed to Cd and has the ability to bind metals, which is regarded as a mechanism of metal tolerance. Most of the heavy metals associated with the cell wall are linked to polygalacturonic acids, whose metal ion affinities vary depending on the metal in question. The plant cell wall is mainly composed of cellulose and matrix polysaccharides, which are divided into pectins and hemicelluloses, both of which are rich in polygalacturonic acids (Xiong et al. 2010). Cellulose is a key component in plant cell walls, and it has been reported that NO affects the cellulose content of tomato roots in a dose-dependent manner. Low concentrations of sodium nitroprusside (SNP) increase cellulose content in roots, while higher concentrations have the opposite effect (Correa-Aragunde et al. 2008). Exogenous NO increases Cd tolerance in rice plants by increasing pectin and hemicelluloses content in the root cell wall and by decreasing Cd accumulation in the soluble fraction of cells in rice leaves (Xiong et al. 2009). H_2O_2 may also trigger secondary defenses, causing cell wall rigidification and lignifications in Cd-exposed cells (Schützendübel and Polle 2002). The transcript levels of genes involved in cell wall metabolism are modulated in response to Cd. The proteins involved in lignification and extension were therefore upregulated (Fusco et al. 2005; Herbette et al. 2006), whereas expansins and pectin esterases were downregulated (Herbette et al. 2006).

5.6 Sulfate and GSH Metabolism

One of the best described mechanisms induced under heavy-metal toxicity is the chelation of the metal by PCs and GSH. PCs have the general formula $(\gamma\text{Glu-Cys})_n$-Gly (with $n = 2–11$) and are synthesized enzymatically through the transpeptidation of γGlu-Cys moieties of GSH onto another GSH molecule by the phytochelatin synthase (PCS) enzyme, which is known to be activated posttranslationally by a range of heavy metal metalloids (Grill et al. 2006). Chelation of metals by PCs and the compartmentalization of PC–metal complexes in vacuoles (Clemens 2006; Grill et al. 2006) are generally considered as first-line defense mechanisms.

The rate-limiting step for PCs and GSH biosynthesis is the availability of reduced sulfur to the roots. Various genes involved in the sulfate metabolism are induced in response to Cd, which include sulfate transporters from roots (Sultr1; 1; Sultr1; 2), enzymes involved in sulfate reduction

to sulfide (ATP sulfurylase), and those involved in PC biosynthesis (PC synthases) (Herbette et al. 2006; Ramos et al. 2007). One of the steps in PC biosynthesis is the synthesis of cysteine catalyzed by *O*-acetylserine(thiol)lyase (OASTL) which is upregulated by Cd (Fusco et al. 2005). Arabidopsis plants over expressing OASTL were highly Cd resistant, which suggests that cysteine pool required for GSH biosynthesis is one of the principal factors affecting Cd tolerance (Domínguez-Solis et al. 2001). A deficiency in the major OASTL isoform in the cytosol from Arabidopsis plants, OAS-A1, causes αH_2O_2 homeostasis imbalance (López-Martín et al. 2008).

5.7 Hydric Balance

The plant–water balance is also disturbed by Cd, and the stomatal opening is inhibited (Poschenrieder et al. 1989; Sandalio et al. 2001; Perfus-Barbeoch et al. 2002). Sequence analysis of Cd-responsive genes in the metal accumulator *B. juncea* revealed the induction of genes encoding aquaporins, which facilitates the movement of water through cellular membranes. In addition, other drought and ABA-responsive genes, such as BjCdR39 (the aldehyde dehydrogenase) and BjCdR55, (RNA-binding protein), are also upregulated by Cd, which confirms the existence of cross-talk between Cd-induced and water stress-induced signaling using ABA as a signal transducer. Stomatal closure, a symptom of water stress mediated by ABA, is one of the principal responses of higher plants to Cd (Sanitá di Toppi and Gabbrielli 1999).

5.8 Protein Degradation

Oxidative damage to proteins has been observed in different plant species exposed to Cd and is regarded as an oxidative stress marker (Sandalio et al. 2001; Pena et al. 2007; Djebali et al. 2008; Paradiso et al. 2008). Some of the proteins undergoing oxidative modification have been identified in pea leaves and include CAT, GR, Rubisco, and Mn-SOD (Romero-Puertas et al. 2004). Increased proteolytic activity in leaves following Cd treatment and more efficient degradation of the oxidized proteins have been observed (McCarthy et al. 2001; Romero-Puertas et al. 2004). Similar results have been reported by Pena et al. (2006) in *Helianthus annus* and by Djebali et al. (2008) in *Solanum lycopersicum*. A proteomic study of *A. thaliana* cells has also reported an increase in several proteases after Cd treatment (Sarry et al. 2006). Cd treatment has also been shown to increase polyubiquitinated protein accumulation (Pena et al. 2007). The proteasome–ubiquitin system is the major proteolytic pathway in eukaryotes and is also involved in the depredation of oxidized proteins (Pena et al. 2007). The plant proteasome was upregulated at transcriptional and translational levels under oxidative conditions caused by cadmium stress (Pena et al. 2006, 2007; Djebali et al. 2008; Polge et al. 2009). Using in vivo experiments with *A. thaliana* mutants, it has been demonstrated that 20S proteasomes are preferentially involved in the degradation of oxidized proteins (Kurepa et al. 2008). The remobilization of oxidized proteins may be a protective mechanism under stress conditions to prevent further damage to other macromolecules and to facilitate the recycling of amino acids for protein biosynthesis.

6 Signal Transduction Under Cadmium Stress

The response to heavy metals depends on a complex signal transduction pathway within the cell which begins with the sensing of heavy metal and converges in transcription regulation of metal-responsive genes (Sing et al. 2002), although much remains to be learned about the molecular components of metal-induced signal transduction. Various transcription factors (TFs) involved in the regulation of cell response to metal stress have recently been identified (Sect. 21.5.2). The modulation of different groups of TFs highlights the complex response of plants to Cd (DalCorso et al. 2008). ROS and NO are important players in the regulation of plant response from signal perception to the intracellular

transduction cascade, triggering the activation of genes involved in the induction of different metabolic pathways to deal with Cd toxicity. Hydrogen peroxide governs the transduction of cellular response in different abiotic stresses including those caused by heavy metals. The transduction of H_2O_2 signals into biologically relevant information is coordinated by a complex network of sensors and receptors, such as MAPKs, and transcription factors and is thought to be evolutionarily conserved (Vandenbroucke et al. 2008), although there are around 400 H_2O_2-responsive protein families in *A. thaliana* and may vary depending on the plant species in question (Vandenbroucke et al. 2008). There are several elements in the signal transduction pathway of ROS-sensitive plants which include the MAPK, MAPKK, MAPKKK, AtMPK3/6, AtANP1, NtNPK1, Ntp46MAPK, and calmodulin (Mittler 2002; Vanderauwera et al. 2009). The increase in H_2O_2 levels induced by Cd can be perceived by oxidative protein modifications. The protein thiol groups tyrosine, tryptophan, and histidine can be oxidised by H_2O_2 and O_2^-. The redox changes in the Cys residues of transcription factors directly regulate nuclear gene expression. However, transcriptional modifications may also require additional upstream sensing and transduction of ROS and ROS-derived signals, being involved MAPKs and several protein phosphatases (Vanderauwera et al. 2009). Salicylic and jasmonic acid as well as ethylene can also participate in signal transduction under Cd stress (Rodríguez-Serrano et al. 2009; Ogawa et al. 2009). Salicylic acid (SA) acts as an important signaling element in plants and has been observed to alleviate Cd-induced growth inhibition and oxidative damage (Metwally et al. 2003). Although this mechanism is not fully understood, it has been suggested that SA may induce H_2O_2 signals involved in Cd tolerance, such as repair processes, Cd binding and compartmentation (Metwally et al. 2003). The Cd-induced ethylene biosynthesis has been reported to occur in various plant species (Sanitá di Toppi and Gabbrielli 1999; Rodríguez-Serrano et al. 2006), although the molecular relationships between ethylene biosynthesis and Cd stress have yet to be clearly determined. Transcriptomic studies of *Arabidopsis* plants have detected Cd-dependent up-regulation of ACC oxidase and ACC synthase as well as the ethylene responsive factors ERF2 and ERF5 (Herbette et al. 2006). JA content increases in response to heavy metals in various plant species (Wang and Wu 2005; Rodríguez-Serrano et al. 2006, 2009). JA regulates genes involved in GSH and PCS in Arabidopsis plants under Cd treatment (Xiang and Oliver 1998). In different plant species, Ca^{2+}, calmodulin, CDPK and an MAPK act as signaling molecules which regulates cell response to cadmium stress (Romero-Puertas et al. 2004, 2007a, b; Herbette et al. 2006; Yeh et al. 2007; Rodríguez-Serrano et al. 2009). Several studies have provided genetic evidence for the importance of NO in gene regulation. Two studies involving large-scale transcriptional analysis of *A. thaliana* have revealed NO-dependent regulation of genes involved in signal transduction, disease resistance, stress response, photosynthesis, and basic metabolism (Grün et al. 2006); however, the intracellular signaling pathway involved has not yet been defined. Most of the information available relates to plant defense and wounding and suggests that NO and salicylic and jasmonic acids are interrelated (Grün et al. 2006). The activity of different nuclear regulatory proteins is dramatically affected by NO. Modification by *S*-nitrosylation can regulate the activity and function of some regulatory proteins and transcription factors. Although no plant transcription factor has been observed to be regulated by this process, some regulatory proteins could be S-nitrosylated (Grün et al. 2006). NO can regulate cell signaling by controlling Ca^{2+} homeostasis. Most Ca^{2+} channels are regulated by NO either directly through *S*-nitrosylation or indirectly through cyclic ADP-ribose (cADPR) involving GMP (Courtois et al. 2008). NO-dependent activation of protein kinases, MAPK, and CDPK has been reported in various plant species. The activation of these kinases by NO is thought to be involved in defense responses and/or cell death (Courtois et al. 2008).

7 Conclusion and Future Perspectives

A hypothetical model depicting some of the players involved in ROS, NO perception, and signal transduction pathways in response to cadmium is shown in Fig. 9.3. Cadmium promotes an increase of ROS production in different cell compartments and their accumulation could give rise to oxidative damages affecting to lipids and proteins. However, ROS can also trigger defense cellular responses indirectly acting as signaling molecules, by promoting changes in Ca^{2+} concentration, through GMP or by altering the redox status of several proteins, and further activating the MAPK cascade. But ROS can also directly regulate nuclear gene expression by affecting the redox state of transcription factor Cys residues. In this scenario, NO plays an important role in the regulation of cell responses to this metal, but further works are needed to better understand the coordinated role of ROS and NO in both toxicity and regulation of cell response to cadmium. The role of some hormones, such as JA and ET, in the cell response to Cd is also an interesting point which deserves more research in order to understand the cross-talk between hormone balance/ROS and NO in the regulation of plant defense against heavy metals. In response to Cd the up-regulation of some defense genes takes place, although some of them are also induced during pathogen attack, which suggests an overlap in the regulatory mechanisms governing these processes. However, the role of genes such as HSPs or chitinases in the mechanisms of tolerance to Cd has not been explored in depth so far, and they could be important in the development of

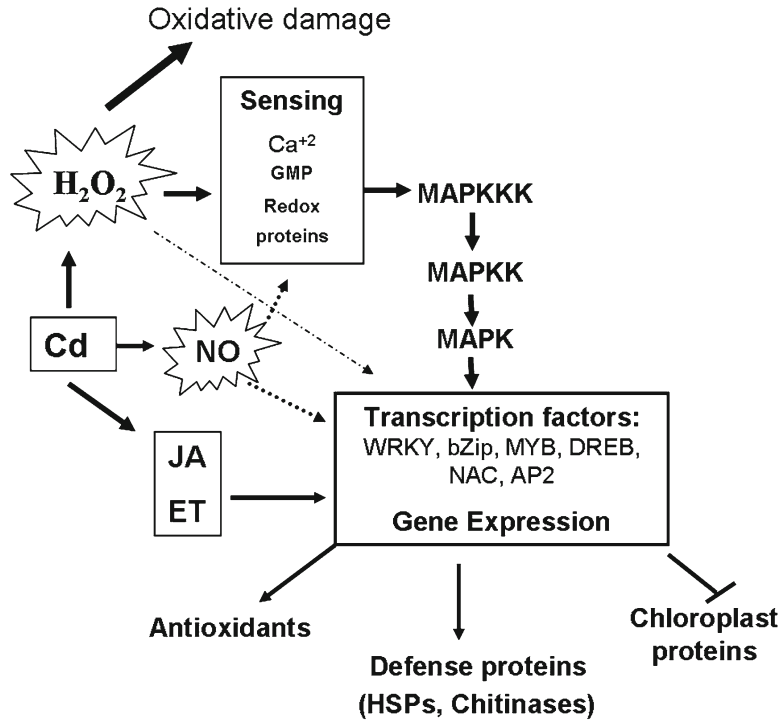

Fig. 9.3 Hypothetical model showing signal transduction of cell response to cadmium toxicity. Cd-dependent changes in H_2O_2 and NO levels can be perceived by changes in Ca^{2+} concentration, and oxidation or *S*-nitrosylation of proteins or transcription factors. The transcriptional response can also require more upstream transduction involving mitogen-activated protein kinases (MAPKs) and hormones such as jasmonic acid (JA) and ethylene (ET)

new strategies for phytoremediation. Further studies will be necessary to understand the role of the post-translational modification of proteins in the perception of metal toxicity and also in the transduction and regulation of cell response to Cd. An integrated study of all the players mentioned in this chapter at biochemical, molecular, and cellular levels is needed in order to understand the complex network involved in perception, transduction, and development of cell responses to cope with adverse conditions caused by heavy metals. This could allow the development of new and more efficient strategies for phytoremediation.

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Reactive Nitrogen Inflows and Nitrogen Use Efficiency in Agriculture: An Environment Perspective

10

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Abstract

Increased use of nitrogenous (N) fertilizer has significantly altered the global N-cycle and produced nitrogenous gases of environmental consequence. While nitrous oxide (N_2O) emissions contribute to global greenhouse gas accumulation and the stratospheric ozone depletion, degradation of groundwater quality by N use in agriculture is fundamentally a nitrate leaching problem. Despite these evident negative environmental impacts, consumption of N fertilizer cannot be reduced in view of the food security for teeming population in the developing countries. Various strategies, from agronomic to genetic engineering, have been tried to tackle this problem. Split application of N, use of slow-release fertilizers, nitrification inhibitors, and the use of organic manures are some agronomic techniques adopted. One of the important goals to reduce N-fertilizer application can be effectively achieved by choosing N-efficient (i.e., which can grow under low N conditions), ensuring their optimum uptake of applied N by application of adequate amounts of fertilizer nutrients in a balanced manner and knowing the molecular mechanisms for their uptake as well as assimilatory pathways. Newer approaches like quantitative trait locus and proteomics could also help us in understanding these processes fully, hence could contribute greatly in enhancing nitrogen use efficiency and reduction of N pollution in the environment.

Keywords

Reactive nitrogen • NUE • QTL • Proteomics • N pollution

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1 Introduction

Nitrogen (N) represents one of the most important nutrients found in terrestrial ecosystems. It is an important constituent of a number of complex organic molecules viz., proteins, nucleic acids, etc. Atmosphere is the main reservoir of nitrogen (N_2), which stores around one million times more N than contained in all the organisms. Oceans and organic matter in soil are the other major store houses of nitrogen. N is often considered as an important limiting nutrient for plant growth and development, despite its remarkable abundance in the atmosphere. This is the reason for the past half a century, supply of nitrogen through fertilizers has been an influential application for increasing the growth and yield of cultivated plants such as cereals. To meet the increasing demand for food, farmers apply more fertilizers in their bid to increase the agricultural productivity. Fertilizer nitrogen has provided food security particularly to developing nations including India, as the cereal production has kept pace with its ever-increasing population. Today, India occupies the third rank in the world in fertilizer N consumption and second in fertilizer N production (FAI 2008). The consumption of fertilizer nitrogen in India increased from a mere 55,000 metric tons in 1950–1951 to over 14.2 million tons in 2007–2008 and is still increasing (FAI 2008). With the current rate of N fertilization, the requirement of nitrogen will be 22–25 million tons/year in 2020 (FAI 2008). However, it is remarkable that utilization of applied fertilizer nitrogen in field by most cereal crops does not exceed 50% and around 70% of the total nitrogenous fertilizer is applied for rice and wheat cultivation (Abrol et al. 1999). Therefore, with the increase in agricultural food production worldwide in last 50 years, the N fertilization of crop plants has increased more than 20-fold (Shrawat and Good 2008). However, the use of this fertilizer is generally inefficient, as lesser amount of applied N (around 30–40%) is actually utilized by cereal crops, and the major part (60–70%) is lost from the plant–soil system which has caused severe impacts on the ecosystems of the non-agricultural neighboring bacteria, animals, and plants. As a result of leaching, the unused N fertilizer causes impacts like eutrophication of freshwater (London 2005) and marine ecosystems (Beman et al. 2005). In addition, gaseous augmentation of N oxides reacting and affecting with stratospheric ozone and the volatilization of toxic ammonia into the atmosphere (Stulen et al. 1998) has also been linked to unused N fertilizers. The toxic effects of nitrate are due to its endogenous conversion to nitrite and this ion has been implicated in the occurrence of methaemoglobinemia, gastric cancer, and many other diseases (Anjana et al. 2007).

Presently the human population is more than 6.5 million, which is expected to increase around 10 billion by 2025 (Hirel et al. 2007), therefore, the major challenge will be to reach a highly productive agriculture without degrading the quality of our environment. Efficient farming techniques and choosing plant varieties/genotypes that have better nitrogen use efficiency (NUE) could be the tools to tackle this problem. The development of such varieties/genotypes, through conventional plant breeding techniques or by using recombinant DNA technology, will be more proficient with a better understanding the physiological, genetic, and molecular bases of NUE among cereal crops. Therefore, there is an urgent need of a "second green revolution" that does not rely on exhaustive use of inorganic fertilizers rather would aim at improving crop yields in soils by developing varieties with better adaptation to low-fertility soils (Yan et al. 2006). In the present chapter, we have discussed the inflow and effects of reactive N in the environment and then summarized the strategies adopted to develop the crop varieties/genotypes with high NUE.

2 Reactive Nitrogen Inflow

Reactive nitrogen (Nr) is usually referred to all the nitrogen species that are biologically active, photochemically reactive, and radiatively important N compounds in the atmosphere and biosphere of the earth (Galloway 1995). Thus, Nr includes

reduced inorganic forms of N (NH_3, NH_4^+), oxidized inorganic forms (NO_x, HNO_2, N_2O, NO_3^-), and organic compounds (urea, amines, proteins, nucleic acids). There are numerous sources in environment that contribute to Nr and total nitrate content of natural waters, e.g., atmosphere, geological features, anthropogenic sources, atmospheric nitrogen fixation, and soil nitrogen. However, detailed hydro geological investigations conducted have indicated a heterogeneous pattern of nitrate distribution. Soils with low water-holding capacity (sandy soil) and high permeability, movement of pollutants like chloride and nitrate is much quicker than in clayey soil. This is probably the main cause for high nitrates in areas with sandy soil. Vegetables account for more than 70% of the nitrates ingested in the human diet. The remainder of nitrate in a typical diet comes from drinking water (21%), meat and meat products (6%) (Prasad 1999).

The form of added N plays a role in regulating N losses and influencing NUE. Among these forms, NO_3 is the most susceptible to leaching, NH_4 the least, and urea moderately susceptible. Ammonia and urea are more susceptible to volatilization loss of N than fertilizers containing NO_3. Urea is the most widely used N fertilizer in India. The studies showed the importance of selecting ammonium-based N fertilizer early in the season to reduce N leaching due the mobility of urea and nitrate source in irrigated rice and wheat systems (Prasad and Prasad 1996). Nitrate containing fertilizers when applied to rice proved less efficient because nitrate is prone to be lost via denitrification and leaching under submerged soil conditions in normal and alkali soils (Prasad 1998). In saline soils, however, it is beneficial to use NO_3 containing N fertilizers as it compensates the adverse effects of Cl^- and $SO4^{2-}$ on absorption of NO_3 by plants (Choudhary et al. 2003).

Nitrogen losses from soil–plant system. Once inorganic N has appeared in the soil, it can be absorbed by the roots of higher plants or still metabolized by other microorganism during nitrification. This process is carried out by a specialized series of actions in which a few species of microorganisms oxidize NH_4^+ to NO_2 or NO_2^- to NO_3^-. Ammonium ion reacts with excess hydroxyls in soil solutions, which leads to N losses to the atmosphere by NH_3 volatilization (Wood et al. 2000). This represents an important source of N loss in agricultural soils under favorable conditions. Due to extensive use of N fertilizers and nitrogenous wastes, the amount of N available to plants significantly exceeds the N returned to the atmosphere by gaseous losses of N through volatilization and denitrification (Martre et al. 2003). Minimizing drying of surface soil and providing additional source of urease enzyme can minimize NH_3 volatilization. A portion of this excess N is leached out in the soil profile as NO_3^- or carried in runoff waters. These are conductive conditions for N losses in agricultural soils, thus reducing the NUE (Delgado et al. 2001). With transport of N in water ways and neighboring ground-water systems, the N concentration could exceed the levels acceptable for human consumption. Nitrate in soil profile may be leached into groundwater when percolating water moves below the rooting depths of crop and provides leaching potential. Paramasivam et al. (2002) have reported a potential leaching of NO_3^- in arid regions and sandy soils. Losses of N by leaching are affected by local differences in rainfall, water-holding capacity of soil, soil-drainage properties, and rates of mineralization of soil organic N (Delgado et al. 1999). Processes such as adsorption, fixation, immobilization, and microbial assimilation of added NH_4-N in soils are of great importance as they affect NUE and have the corresponding environmental repercussions (Kissel et al. 2004).

In many field situations, more than 60% of applied N is lost due in part to the lack of synchrony of plant N demand with N supply. The remainder of the N is left in the soil, or is lost to other parts of the environment through leaching, runoff, erosion, NH_3 volatilization, and denitrification. The cereal NUEs are 42% in developed and 29% in developing countries (Raun and Johnson 1999). Many ^{15}N studies have reported N fertilizer losses in cereal production from 20 to 50% with higher values in rice than in wheat (Ladha 2005). Prasad (1998) reported that apparent recovery of N applied to wheat varies from 40 to 91%. It has been estimated that rice and wheat N recovery efficiency ranging from 30 to 40% are occurring

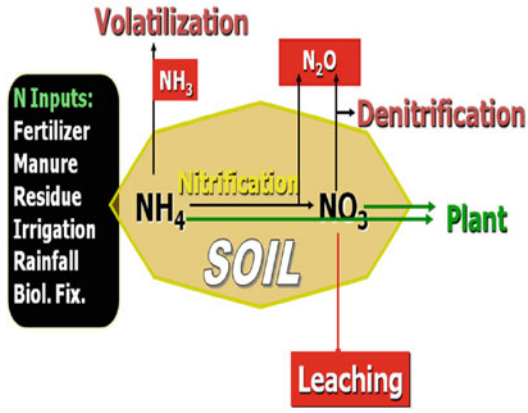

Fig. 10.1 Diagrammatic representation of N inflow and N loss in ecosystem

in irrigated conditions. An N recovery efficiency exceeding 40% is expected to occur in response to improved N management practices. In a rice–wheat cropping system of Punjab, recovery of ^{15}N by the first wheat crop was 30–41%, the soil at wheat harvest retained 19–26%, and the succeeding rice recovered 5.2% of the 120 kg N ha^{-1} applied (Singh and Singh 2001). Total losses of applied N (not recovered from soil–plant system) were about 42% in rice and 33% in wheat grown on a typical sandy loam soil in north-west India.

The main causes of for low N recovery are usually attributed to (1) ammonia volatilization, (2) denitrification, (3) leaching, and (4) runoff and erosion (Fig.10.1). Loss of N via NH_3 volatilization can be substantial from surface-applied urea in both rice and wheat, which can exceed 40%, and generally greater with increasing soil pH, temperature, electrical conductivity, and surface residue (Singh et al. 2003; Choudhary et al. 2003). Water management in rice and wheat fields influences the extent of N losses due to nitrification–denitrification and NH_3 volatilization. Available research results from ideal rice soils suggest that NH_3 volatilization rather than denitrification is an more important gaseous loss mechanism for fertilizer N applied to continuously flooded, puddled rice soils of the tropics. The picture is quite opposite in highly permeable porous soils under rice. There exist two mechanisms in such soils due to which losses due to denitrification assume more importance than NH_3 volatilization losses. Firstly, in porous soils under rice it is difficult to maintain continuous flooding. Rather there occur very frequent alternate aerobic–anaerobic cycles, which lead to very fast formation of nitrate under aerobic conditions and their subsequent denitrification under anaerobic conditions that develop due to application of irrigation (Singh and Singh 2001). Secondly, due to high permeability of coarse textured porous soils, urea as such is rapidly transported to subsoil where even after it is hydrolyzed to NH_4, it is not prone to losses via NH_3 volatilization (Sangwan et al. 2004a). Sangwan et al. (2004a, b) have shown that NH_3 volatilization losses from urea increases with the increase in soil salinity, sodicity, and the rate of N applied. The losses of fertilizer N as NH_3 in rice decreased with increasing floodwater depth and depth of placement (Singh et al. 1995a), and with the application of organic manures (Sihag and Singh 1997). Alkalinity, pH, and NH_3 concentration in flood water control the extent of NH_3 loss from flooded soils (Singh et al. 2003). Sarkar et al. (1991) reported a loss of 15–20% of applied N when urea was broadcast in a wheat field. Prasad (1999) reported a marked reduction in the loss of applied N when the urea was deep placed as compared with surface broadcast on a moist soil. They have reported 13.5% N losses as ammonia after 1 week of urea application under submerged conditions. The high pH or alkalinity resulted in high losses of ammonia by volatilization, which can be nearly 60% of applied N at field capacity. Submergence decreases pH as well as losses to 35% of applied N. The reclamation of sodic soils using gypsum has been found to decrease N losses through ammonia volatilization (Choudhary et al. 2003). The timing of fertilization and irrigation could further influence the losses of urea applied to porous soils. If applied on the wet soil surface following irrigation, as much as 42% of the applied ^{15}N was lost, most likely due to volatilization (Sangwan et al. 2004a). Singh et al. (1995b) showed that application of urea before irrigation increased the NUE by 20% as compared to its surface application after irrigation or broadcast application and surface mixing of urea at field capacity in a clay loam soil.

In nonideal porous soils under rice, there exist every possibility that applied urea N is preferentially lost via denitrification rather than NH_3 volatilization. Direct measurement of denitrification losses made by Aulakh et al. (2001) showed that denitrification is a significant N loss process under wetland rice amounting to 33% of the applied N. In excessive N fertilizer application (i.e., at rates in excess of that needed for maximum yield in cereal crops), NO_3 leaching can be significant, particularly from the coarse-textured soils. Residual N is then available in soil profile for potential leaching. High levels of NO_3-N in the region's groundwater have been reported by Singh et al. (1995b). There is not much information available on leaching losses of N. In a pot culture study, the leaching loss was 11.5% of the applied urea N and was reduced to 8.7% when urea was coated with neem cake (Prasad and Prasad 1996). In a field study at Pantnagar on a silty clay loam soil, 12% of the applied N was lost by leaching and these losses were reduced to 8% when urea was blended with neem cake (Singh et al. 1995b).

3 Nitrogen Removal by Crops

From the human nutrition point of view, rice and wheat are the most important cereals and their production in north-west India in rice–wheat cropping system, which covers about 10 million hectares, is the backbone of the India's food security (Prasad 2005). Rice–wheat cropping system produces 5–14 ton/ha/year grain and this depends heavily on nitrogen fertilization which ranges from 100 to 150 kg N/ha/crop or even more, especially in rice. From the animal nutrition point of view, maize, sorghum, and pearl millet stovers which contain 27–51% of nitrogen harvested by the crop in stover are more important both for milch as well as draught cattle. On the contrary, rice and wheat straw is low in nitrogen content and is a poor protein source. Nevertheless, they meet majority calorie requirements of the cattle. Also most sorghum and pearl millet is grown in rainfed areas where nitrogen application rates are low and even response to N application is low. Nitrogen removal per metric ton as well as its percentage in grain in pulses depends very much upon the plant stature and its vegetative growth. For example, Prasad (2004) reported a removal of 50.6 kg/ton in chickpea and 92.1 kg/ton in pigeon pea; these are the two major pulse crops in India. Most of this nitrogen is obtained by N fixation by *Rhizobia* as very little fertilizer N is applied to pulses. Again depending upon the plant stature and vegetative growth, 63.3% of total N removed by chickpea was contained in its grain, while the values for pigeon pea, a tall and heavily fertilized plant, was 31.6%. The protein-rich pulse foliage is widely used for enriching rice or wheat straw fed to cattle. Before the mechanization of Indian agriculture which is even now limited mostly to north-western India, draught animals were the major source of farm power and the Indian agriculture provided a characteristic "humans–animals–crops" ecosystem where man survived on the grains and the animals on the straw/stover. Taking an average N contribution by grain legumes at 30 kg N/ha, about 0.66 million metric tons of N is annually added to soil on 22 million hectares occupied by them. Another 0.34 million metric tons N may be added by leguminous trees and plants in forests and grasslands, and by leguminous oilseed crops such as groundnut. Thus the N contribution of legumes in Indian soils can be roughly estimated at least at 1 million metric tons, it is likely to be much more. In addition, some N is added by rains and use of N-fixing biofertilizer such as *Azotobacter*, *Azospirillum*, *Acetobacter*, Blue-green algae, and *Azolla*.

4 Concept of NUE

NUE at the plant level is its ability to utilize the available nitrogen (N) resources to optimize its productivity (Raghuram et al. 2006). As a concept, NUE includes N uptake, utilization, or acquisition efficiency, expressed as a ratio of the total plant N, grain N, biomass yield, grain yield (output) and total N, soil N, or N-fertilizer applied (input) (Pathak et al. 2008). NUE is quantified based on apparent nitrogen recovery using physiological and agronomic parameters. Agronomic

efficiency is an integrative index of total economic outputs relative to the available soil N (native and applied). Apparent nitrogen recovery is related to the efficiency of N uptake; physiological NUE deals with N utilization to produce grain or total plant dry matter. NUE in the context of photosynthesis is called as photosynthetic nitrogen use efficiency (PNUE), which is determined by the rate of carbon assimilation per unit leaf nitrogen (Kumar et al. 2002). The most suitable way to estimate NUE depends on the crop, its harvest product, and the processes involved in it.

5 Strategies for Minimizing N Pollution in Agriculture

Various strategies were adopted to minimize the N loss from the agricultural fields. Split application of N, use of slow-release fertilizers, nitrification inhibitors (NIs), and the use of organic manures are some agronomic techniques used. Bulk of the fertilizer nitrogen in India is broadcast on surface and both surface runoff (on sloppy lands) and ammonia volatilization lead to N losses. This can be easily overcome by deep placement of N a few centimeters below soil surface. For example, Sarkar (2005) showed that in wheat surface broadcast application of urea as band or top dressing caused 15–20% loss of N due to agriculture volatilization. Surface broadcast application followed by its mixing with top soil reduced the volatilization loss to 10%, while side band placement of urea reduced it further to only 5%. Thus the farmers need to be told about the advantage of incorporation in surface soil or if possible its placement using a *ferti-drill* or a pore in upland crops. Split application is a well-established technique for increasing NUE. In wheat and maize, studies with ^{15}N showed that application of 40 kg N/ha as basal followed by 60 kg N/ha at crown root initiation (CRI) gave significantly higher yield than all basal application and other split application combinations (Sachdev et al. 2000; Narang et al. 2000). Havangi and Hegde (1983) showed in pearl millet also two or three split applications were found to be better than a single application. In rice, two split applications are recommended for short and medium duration varieties, while three split applications are recommended for long duration varieties (Prasad 1999). Another way is NIs, these are a group of chemicals that are toxic to *Nitrosomonas* sp. and *Nitrosomonas* sp. involved in the conversion of NH_4 to NO_2^- as well as to *Nitrobacter* sp. involved in the conversion of NO_2 to NO_3 and therefore, inhibits nitrification, which reduces losses due to leaching and denitrification. The most widely tested NIs are 2-chloro-6-trichloromethyl pyridine (N-serve), 2 amino-4-chloro-6 methyl pyrimidine (AM), dicyandiamide (DCD), and sulfathiazole (ST) (Prasad and Power 1995). Research on the use of NIs for reducing N losses and increasing NUE from the soil was initiated in India by Prasad (1999) at the Indian Agricultural Research Institute (IARI), New Delhi, with a field experiment on rice. Treatment of ammonium sulfate with N-serve significantly increased rice yield and nitrogen uptake by the rice crop. Prasad (2005) showed from a laboratory experiment that N losses due to denitrification could be considerably reduced by treating ammonium sulfate with NIs N-serve and AM. Prasad and Prasad (1996) showed through field experiments that treatment of urea with NIs, N-serve, and AM significantly increased rice yield and N uptake. Das et al. (2004) showed the effect of N-serve and AM on nitrification under field capacity moisture (upland) and water-logged (low-land paddy) conditions at New Delhi. Both the NIs were effective in retarding nitrification. The nitrification rate (nitrates expressed as percentage of total mineral N) after 40 days of incubation was 78% with N-serve at 2 ppm and 76% with AM at 10 ppm (mg/kg) as against 100% with untreated urea. Slow-release N fertilizers (SRFs) were developed with an aim to slowdown the dissolution of applied N so that most of it is taken up by crop plants rather than be subjected to N-loss mechanisms. There are two kinds of SRFs, namely, coated fertilizers and inherently slow dissolution rate materials. The examples of coated SRFs are sulfur-coated urea (SCU) (developed by TVA, USA), lac-coated urea (developed by Indian Lac Research Institute), polymer-coated urea, and to some extent neem cake-coated urea. The other

kind of slow-release fertilizers are generally urea–aldehyde condensates, e.g., urea-form (urea and formaldehyde products developed in USA), isobutylidene diurea (IBDU, urea and isobutyraldehyde product developed in Japan and USA), and CD-urea (urea and crotonaldehyde product developed in Germany) (Prasad 2005). After 20 days of incubation under field capacity conditions, the mineral N (NH_4^+ NO_3^-) in soil was 67, 43, 31, and 27 ppm (mg/kg soil) with urea, oxamide, IBDU, and SCU, respectively. As would be expected under submerged conditions, NO_3-N was not detected and the NH_4^+-N content in soil after 20 days of incubation was 67, 61, 46, and 15 ppm with urea, oxamide, IBDU, and SCU, respectively. Thus, of the three SRFs oxamide released, the N the fastest and SCU the slowest.

6 Physiological and Molecular Aspects for Improving NUE

NUE at the plant level is its ability to utilize the available nitrogen (N) resources to optimize its productivity. In terms of agriculture, it is the optimal utilization of nitrogenous manures or fertilizers for plant growth, yield, and protein content, as atmospheric nitrogen gas is not utilized by higher plants, except symbiotic legumes. The inherent efficiency of the plant to utilize available N for higher productivity needs to be tackled biologically (Abrol et al. 1999; Abdin et al. 2005). This includes uptake, assimilation, and redistribution of nitrogen within the cell and balance storage and current use at the cellular and whole plant level. Moreover, since N demand and its actual availability tend to vary in time, space, and environmental conditions, the regulation of plant nitrogen metabolism must be responsive to nutritional, metabolic, and environmental cues.

6.1 Regulation of Nitrate Uptake

Plants have evolved an active, regulated, and multiphasic transport system making their NO_3^- uptake scheme efficient enough to transport sufficient NO_3^- to satisfy total nitrogen demand of the plant in face of varying external NO_3^- concentrations. Plants can also take up other forms of nitrogen, such as amino acids and ammonium ions. Root NH_4^+ uptake is carried out by both high-affinity and low-affinity NH_4^+ transporters that are encoded by a multigene family (Glass et al. 2002). However, nitrate is the most abundant form of nitrogen available to the plant roots in aerated soils. Nitrate influx is an active process driven by the H^+ gradient and can work against an electrochemical potential gradient (Vidmar et al. 2000). The uptake involves high- and low-affinity transport systems, also known as HATS and LATS, respectively (Forde 2000). One of the high-affinity systems is strongly induced in presence of NO_3^- and is known as inducible high-affinity transport system (or iHATS), while the second high-affinity system (the cHATS) and LATS are constitutively expressed (Aslam et al. 1993; Glass and Siddiqi 1995; Forde 2002). The Km values of iHATS, cHATS, and LATS for nitrate are in the ranges of 13–79 μM, 6–20 μM, and >1 mM, respectively.

The iHATS is a multicomponent system encoded partly by genes of the NRT2 family or nitrate–nitrite porter family of transporters. Recently, two dual affinity transporters have been identified in *Arabidopsis*, AtKUP1, and AtNRT1.1, of which the latter is induced as HATS by phosphorylation at threonine residue 101. This family of transporters is recognized as being exceptional in both the variety of different substrates which its members can mobilize (oligopeptides, amino acids, NO_3^-, chlorate) and in the ability of individual transporters to handle substrates of very different sizes and charges. Nitrate acts as a regulator for its own uptake, a specific property which is not seen in other ion transport systems such as phosphate, sulfate, etc. On exposure of the cells to external NO_3^-, the uptake capacity increases after a lag period of 0.5–1.5 h and reaches a new steady state after 4–6 h. Use of RNA and protein synthesis inhibitors provided early evidence that induction of the iHATS involves gene expression and the synthesis of new transporter protein (Aslam et al. 1993). The evidence that the inducer of iHATS is indeed

nitrate ion and not its downstream metabolite came from NR-deficient mutants of *Arabidopsis* and *N. plumbaginifolia* (Krapp et al. 1998; Lejay et al. 1999). Studies in the last decade have shown that enhancing the uptake of N by overexpressing transporters may not necessarily improve NUE. For example, transgenic overexpression of a CHL1 cDNA (representing the constitutive HATS) driven by the cauliflower mosaic virus 35S promoter in a chl1 mutant, recovered the phenotype for the constitutive phase but not for the induced phase (Liu et al. 2003). Similarly, the NO_3^- contents in transgenic tobacco plants overexpressing the NpNRT2.1 gene (encoding HATS), were remarkably similar to their wild-type levels, despite an increase in the NO_3^- influx. These findings indicate that genetic manipulation of nitrate uptake may not necessarily lead to associated improvement in nitrate retention, utilization, or NUE, though it remains to be seen whether different plants respond differently to the overexpression of different transporters (Pathak et al. 2008). Light as an important abiotic factor is known to enhance NO_3^- uptake in a number of plant species and diurnal changes in nitrate uptake have been observed (Anjana et al. 2007). These changes seem to be linked to the imbalance between nitrate uptake and reduction due to the light regime and as well as to the rate of photosynthesis in shoots. Reduced nitrate uptake during darkness could be reversed by exogenous supply of sugars (Raghuram and Sopory 1995). Recent evidence on the upregulation of AtNRT1.1 gene expression by auxin (Li et al. 2007) suggests that nitrate transporters may also be regulated by hormones.

6.2 Physiology of Nitrate Reduction in Crops

A portion of the nitrate taken up is utilized/stored in the root cells, while the rest is transported to other parts of the plant. Due to the abundant availability of photosynthetic reductants, leaf mesophyll cells are the main sites of nitrate reduction. This is initiated by the NAD/NADP-dependent NR enzyme, which converts nitrate to nitrite by catalytic reaction in the cytosol. Nitrite is transported into the chloroplast, where it is further reduced into ammonium ion by a ferredoxin-dependent NiR. Being the first, irreversible, and often rate-determining step of the N-assimilatory pathway, nitrate reduction has been a favorite step for physiological and biochemical approaches to optimize fertilizer N use.

6.3 Developing Plants with Transport Gene Systems Using Genetic Engineering Tools

Plants receive N from the soil in the form of nitrate or ammonia, however, some may utilize amino acid as an important sources of N. Specific transporters located in the root cell membrane are responsible for uptake of N from the soil. Subsequent to its uptake, NO_3^- is assimilated via a series of enzymatic steps. Nitrate reductase being the first enzyme in nitrate assimilatory pathway and thus an important gene for manipulation. NR activity in leaf blades, express either as seasonal average or converted into seasonal input of reduced N, has been related to total reduced N, grain N, and grain yield of cereals. The pattern of nitrate assimilation from different plant parts, viz. the main shoot of wheat, developing ear of wheat plants grown at different soil N levels, and in the leaf blades at different stages of growth has revealed a direct positive correlation between increasing NR activity and increasing rates of nitrogenous fertilization. Most plant tissues have the capacity to assimilate nitrate, though their NR activity varies widely. Several endogenous as well as exogenous factors have been found to influence the expression of NR genes at both translational as well as transcriptional levels.

Andrews et al. (2004) reported that overexpression of either the NR or the NiR gene often affects N uptake by increasing mRNA levels in the plants. However, this does not seem to increase the growth or yield of plants, irrespective of N source. It is believed to be due, in part, to the complex regulation of both NR and the

pathway as a whole. Transcriptional regulation of NR has only minor influence on the levels of free amino acids, ammonium, and nitrate, whereas posttranslational regulation of NR strongly affects these compounds (Lea et al. 2006). The light/dark conditions affect NR activity; heterotrophic nitrate assimilation in darkness is closely linked to the oxidative pentose phosphate pathway and the supply of glucose-6-phosphate. Under photoautotrophic conditions, glucose-6-phosphate dehydrogenase is inhibited by reduction with thioredoxin in light, thus replacing the heterotrophic dark nitrate assimilatory pathway with regulatory reactions functioning in light. These studies as well as bioenergetic calculations have indicated that both yield and N harvest or protein can be increased to some extent with adequate nitrogen supply by altered management practices, thus improving the fertilizer NUE. Genotypic differences in the NR levels also provide insight in the relation of varietal differences in N assimilation. The genotypic differences in NR expression have been reported in corn, wheat, sorghum, and barley. In sorghum, a positive relationship between decline in the height of the plant and enhancement of NR activity was observed, though no such relationship was evident in tall and dwarf cultivars of wheat, *T. aestivum*. Wheat genotypes revealed over twofold variability in NR activity, which supports genetic findings that the enzyme level is highly heritable, its differences are reflected in N harvest and that hybrids could be bred with predictable NR levels by selecting parents appropriately. In the high NR genotypes, higher levels of NR activity were found under low N levels, often with significantly higher N concentration in the grains. They also have sustained activity at later stages of growth, such as flag leaf emergence and anthesis. The reasons for these genetic differences are not fully understood, except that the regulation operated at the level of gene expression and that low levels of NADH might limit NR activity in low NR genotypes. Similarly, overexpressing NiR genes in *Arabidopsis* and tobacco resulted in increased NiR transcript levels but decreased enzyme activity levels, which were attributed to posttranslational modifications.

6.4 Glutamine Synthetase and Glutamate Synthase (GOGAT) Gene Systems

Glutamine synthetase (GS) catalyzes the critical incorporation of inorganic ammonium into glutamine. In higher plants, it is represented by two groups of protein – the cystolic and plastidic forms (Miffin and Habash 2002). Cystosolic GS (GS1) is known to be encoded by a complex multigene family, whereas plastidic GS (GS2) is encoded by a single gene. Glutamate synthase (Glutamine (amide): 2-oxoglutarate aminotransferase, GOGAT) catalyses the reductive transfer of the amide group of glutamine (produced by GS) to 2-oxoglutarate (α-keto glutarate) to form two glutamate molecules (Lea and Ireland 1999). GS/GOGAT pathway is of crucial importance since the glutamine and glutamate produced are donors of amino groups for the biosynthesis of major N-containing compounds, including amino acids, nucleotides, chlorophylls, polyamines, and alkaloids (Lea and Ireland 1999; Hirel and Lea 2001). A direct correlation was reported between an enhanced GS activity in transgenic plants in some cases, which is depicted by an increase in biomass or yield by transforming novel GS1 construct. Similarly, Kozaki and Takeba (1996) constructed transgenic tobacco plants enriched or reduced in plastidic glutamine synthetase (GS2, a key enzyme in photorespiration). Ectopic expression of GS1 has been shown to alter plant growth (Fuentes et al. 2001; Oliveira et al. 2002) and the overexpression of GS1 in transgenic plants could cause the enhancement of photosynthetic rates, higher rates of photorespiration and enhanced resistance to water stress (Fuentes et al. 2001). The overexpression of soybean cytosolic GS1 in the shoots of *Lotus corniculatus* was reported to accelerate plant development, leading to early senescence and premature flowering, particularly when plants were grown under conditions of high ammonium (Vincentz et al. 1993). Man et al. (2005) provided additional empirical evidence for enhanced nitrogen-assimilation efficiency in GS1 transgenic lines. However, differences in the degree of ectopic GS1 expression have been reported (Fuentes et al. 2001) and attributed to

positional effects, effectiveness of chimeric constructs, or differences in growth conditions. This may be due to lack of correlation between the enhanced expression of GS1 and concomitant growth (Vincentz et al. 1993; Ortega et al. 2001). A significant increase in leaf area, plant area, plant height, and dry weight has been recorded in poplar trees transformed with conifer gs1a gene. Striking differences were observed at low nitrate concentration. Furthermore, higher rates at ^{15}N incorporation into the transgenic plants demonstrate that the transformed plants have increased NUE (Man et al. 2005). Transgenic overexpression and antisense technology have been employed recently to modulate the expression of NADH-GOGAT in alfalfa and rice plants (Yamaya et al. 2002). The studies on transgenic rice plants expressing antisense RNA for either GS1 or NADH-GOGAT point towards the possible involvement of GS1 in the export of N via phloem in senescing leaves. On the other hand, in case of developing leaf blades and spikelets, NADH-GOGAT was implicated in the utilization of glutamine transported from senescing organs (Yamaya 2003). While these genes appear to be good candidates for improving NUE in the short run, the degree of improvement may vary with the crop and cropping conditions. Therefore, the utility of transgenic overexpression of N-assimilatory genes for major improvements of NUE remains uncertain, though the possibility that different crops respond differently cannot be ruled out yet.

6.5 Other Gene Systems Regulating N Metabolism and Their Manipulation

Enzymes like asparagine synthetase (AS), that catalyzes the formation of asparagine (Asn) and glutamate from glutamine (Gln) and aspartate. In higher plants, AS is encoded by a small gene family (Lam et al. 1998). Together with GS, AS is believed to play a crucial role in primary N metabolism. The observation made by Carvalho et al. (2003) that the levels of AS transcripts and polypeptides in the transgenic nodules of *Medicago truncatula* increase when GS is reduced suggests that AS can compensate for the reduced GS ammonium assimilatory activity. However, it was also demonstrated that GS activity is essential for maintaining the higher level of AS. Thus, GS is required to synthesize enough Gln to support Asp biosynthesis via NADH-GOGAT and AspAT (Carvalho et al. 2003). A reduction in GS activity in transgenic *Lotus japonicas* is also correlated with an increase in Asn content (Harrison et al. 2007), supporting the hypothesis that when GS becomes limiting, AS may be important in controlling the flux of reduced N into plants. With the aim of increasing Asn production in plants and to study the role of AS, several researches have attempted to clone AS genes and to examine the corresponding gene expression in plants. Lam et al. (2009) showed overexpression of the ASN1 gene in *Arabidopsis* and demonstrated that the transgenic plants have enhanced soluble seed protein content, enhanced total protein content, and better growth on N-limiting medium. *Arabidopsis* plants overexpressing the ASN2 gene accumulate less endogenous ammonium than wild-type plants when grown on medium containing 50-mM ammonium. This study indicates that signaling processes may provide an attractive route for metabolic engineering. In comparison to GS/GOGAT enzymes, the physiological role of glutamate dehydrogenase (GDH) has been less clear (Dubois et al. 2003). In an attempt to investigate the role of GDH by expressing a bacterial gdhA gene from *E. coli* in tobacco, Ameziane et al. (2000) found that biomass production is consistently increased in gdhA transgenics, regardless of whether they are grown under controlled conditions or in the field.

7 Signaling and Regulation of Nitrogen Metabolism

It is a well-known concept in signal transduction that whenever multiple genes are subject to transcriptional regulation by a common signal, it is mediated through a regulatory sequence that exists in all the genes that respond to the signal.

These signature sequences, commonly known as response elements, are identified by mutations that abolish their function, and their conserved nature as revealed by homology comparisons. Early experiments in transgenic Nicotiana plants using GUS gene fused to NR and NiR promoter sequences clearly demonstrated for the first time that nitrate induction of gene expression requires some sequence(s) associated with the NR and NiR promoters (Raghuram et al. 2006). Subsequent studies in transgenic tobacco incorporating the 5′ flanking regions of the nitrate reductase genes NR1 and NR2 (designated NP1 and NP2), in case of *Arabidopsis thaliana*, demonstrated that 238 and 330 bp of NP1and NP2, respectively, are sufficient for nitrate-dependent transcription (Lin and Demain 2006). These nitrate-responsive elements (NREs) are composed of several copies of a core A[G/C]TCA sequence motif preceded by an ~7-bp AT-rich sequence present in the 5′ flanking regions of nitrate reductase (NR1 and NR2) genes. This particular sequence motif was also found to be very well conserved in the 5′ flanking regions of NR and NiR genes from eight other plants. Sarkar (2003) compared the flanking sequences of all available plant nitrate-responsive genes and found that the NRE core sequence (A[C/G]TCA) was present in multiple copies on both strands in all the known nitrate-responsive genes in many dicots, monocots, and cyanobacteria. Though most of the NREs examined contained both the core sequence and a proceeding AT-rich sequence, there were some cases which had GC-rich regions or did not reveal any AT/GC bias. A more detailed bioinformatic analysis of the entire *Arabidopsis* genome in our lab revealed that the proposed NREs are randomly distributed, with no difference between nitrate-responsive genes and the presumably nonresponsive genes and intergenic regions in the rest of the genome (Raghuram et al. 2006). These findings raise doubts on the validity of the proposed NRE as comprising of (A[C/G]TCA) elements preceded by AT-rich sequence. Further work in this area will need a combination of bioinformatic and experimental approaches to redefine the NREs that mediate the expression of all nitrate-responsive genes in all plants. The discovery of NREs is important, as it provides an end point for nitrate signal transduction.

8 QTL Approach to NUE

NUE in plants is a complex quantitative trait that depends on a number of internal and external factors in addition to soil nitrogen availability, such as photosynthetic carbon fixation to provide precursors required for amino acid biosynthesis or respiration to provide energy. Although this trait is controlled by a large number of loci acting individually or together, depending on nutritional, environmental, and plant developmental conditions, it is possible to find enough phenotypic and genotypic variability to partially understand the genetic basis of NUE and thus identify some of the key components of yield for marker-assisted breeding. Thus the development of molecular markers has facilitated the evaluation of the inheritance of NUE using specific quantitative trait loci (QTLs) that could be identified. In maize, Hirel et al. (2001) and Masclaux et al. (2001) analyzed recombinant inbred lines for physiological traits such as nitrate content, NR and GS activities. When the variation in these traits and yield components were compared, it was found that there was a positive correlation between nitrate content, GS activity, and yield. When the loci that govern quantitative traits were determined on the map of the maize genome, the positions of QTLs for yield components and the locations of the genes for cytosolic GS (GS1) coincided. In maize, studies on different genotypes or populations of recombinant inbred lines based on NUE components, chromosomal regions, and putative candidate genes have hinted at some factors that might control yield and its components directly or indirectly, when the amount of N fertilizers provided to the plant is varied (Hirel et al. 2007).

Similar results were obtained in rice by Obara et al. (2001), confirming the earlier indications that the GS1 enzymatic activity in the leaf cytosol is one of the major steps controlling organic matter reallocation from source to sink organs

during senescence and for grain-filling in cereals. Previous studies have already demonstrated that when GS1 is over expressed in *Lotus*, nitrogen remobilization was prematurely induced leading to early senescence of the plant (Vincentz et al. 1993). In rice (Yamaya et al. 2002) and wheat (Habash et al. 2001), preliminary investigations with enhanced or decreased GS1 activity indicated that grain yield and grain nitrogen content were modified. In other species such as tobacco (Migge et al. 2000) or poplar (Gallardo et al. 1999), overexpression of GS2 or GS1 significantly increased plant biomass production at early stages of plant development. With these experiments, two out of seven QTLs for GS1 protein content were detected in different regions from other physiological and biological traits. In maize, QTLs for the activities of acid-soluble invertase and sucrose-phosphate-synthase were detected in the regions where each structural gene was mapped (Ishimaru et al. 2001).

Thus, quantitative studies of genetic variability for NUE using molecular markers and combining agronomic and physiological studies will be increasingly used in the future to identify new genes or loci involved in the regulation of these metabolic pathways and their interconnection with carbon assimilation and recycling and to select genotypes that assimilate or remobilize nitrogen more efficiently.

9 Proteomics Approach to NUE

The ability of crop plants to cope up with the variety of environmental stresses depends upon a number of changes in their proteins, which may be up- and downregulated as a result of altered gene expression. Under a stressful condition, the modifications in the expression levels of these proteins could provide us valuable information about the nature of stress factor as well as the physiological and molecular state of a biological system. Hence, provides us some clues to understand the nature of defensive mechanism and adaptability, besides stress monitoring in these biological systems.

Proteomic-based technologies have been recently applied for the systematic analysis of the induced gene products in a number of plant species subjugated to a wide range of abiotic and biotic challenges. Proteome analysis is becoming a powerful tool in the functional characterization of plants. Due to the availability of vast nucleotide sequence information and based on the progress achieved in sensitive and rapid protein identification by mass spectrometry, proteome approaches open up new perspectives to analyze the complex functions of model plants and crop species at different levels. Improvements in proteomic technology regarding protein separation and detection, as well as mass spectrometry-based protein identification, have an increasing impact on the study of plant responses to salinity stress (Parker et al. 2006; Qureshi et al. 2007; Caruso et al. 2008). Proteomics has provided valuable information in various fields of plant biology. Construction of several plant protein databases is in progress for *Arabidopsis*, rice, maize, and some trees, where different genetic, cellular, and physiological information is available, such as expression in various organs or tissues, response to treatments, cellular localization, and genetic bases (Thiellement et al. 1999). Recent advances in MS techniques will facilitate protein identification so that in the future this will not be a limiting factor in the interpretation of variations detected on 2D gels. By providing information on affected and unaffected proteins, large-scale protein identification will simplify determining the consequences of mutations, plant transformation, or natural polymorphism for plant metabolism, as well as interpreting the effects of protein changes on development, or in response to biotic and abiotic stress. Studies in *Saccharomyces cerevisiae*, for which hundreds of proteins have been identified, show the power of the proteomic approach in the study of the regulation of metabolic pathways. Schiltz et al. (2005) studied that during seed filling, the accumulation of proteins in the seeds relies on the nitrogen supply from the mother plant, and a proteomic approach was used to study the mobilization of proteins from the leaves to the filling seeds in pea. Two contrasting N-responsive wheat varieties have differential expressions of root as well as leaf

proteins when grown under controlled conditions at different N levels (Bahrman et al. 2004, 2005). These proteins were grouped into two categories, one involved in carbon metabolism and the other associated with other pathways and functions like thiol-specific antioxidant proteins, etc. This study revealed that levels of gene expressions are modified with the varying levels of nitrate supply, even if only a few polypeptides appear, disappear, or change. Sarry et al. (2006) have demonstrated the protein level changes associated with nitrogen and sulfur metabolism, and their interaction. With the help of high throughput proteomic tools, they were able to detect various enzymes including ATP sulfurylase, sulfite reductase, cysteine synthase, S adenosylmethionine synthase, glutamine synthase, aspartate aminotransferase, GDH, etc., involved directly or indirectly in S and N metabolism. Recently a study for the detection of low nitrogen-responsive proteins in cultivated rice species was done by Kim et al. (2009). Studies at constructing 2-D gel reference map for use in comparative proteomics among cultivars for N-responsive proteins might provide an insight for precise identification of potential molecular protein markers to assist the breeders for screening N-efficient genotypes and help in understanding how crop adapts to low N availability. Correlations between the level of expression and NUE might bring information on the possible role of the genes involved in nitrogen metabolism.

10 Conclusion and Future Perspectives

Present review provides an overview of plant nutriomics, which is still at a conceptual stage. Although considerable efforts are in progress with the aim at enhancing plant nutrient efficiency through molecular and genetic approaches. We have focused here largely on nitrogen with which we have been working on along molecular biology lines. Crop response to N and NUE is very low in developing countries including India. Use of NIs and slow-release nitrogen fertilizers and efficient crop and fertilizer management can significantly increase NUE. It is clearly evident that optimizing the plants, NUE goes beyond the primary process of uptake and reduction of nitrate, involving quality of events, including metabolite partitioning, secondary remobilization, C–N interactions, as well as signaling pathways and regulatory controls outside the metabolic cascades. Despite the various attempts to manipulate each of the above steps in some plant or the other, we are far from finding a universal switch that controls NUE in all plants. However, transgenic studies, QTL, and proteomics approaches seem to increasingly suggest that the enzymes of secondary ammonia remobilization are better targets for manipulation, followed by regulatory processes that control N–C flux, rather than the individual genes/enzymes of primary nitrate assimilation. There is an urgent need of large-scale, co-ordinated research on plant nutriomics, involving sincere efforts from both national and international researchers to develop the nutrient-efficient, high-yielding, and stress-tolerant genotypes/varieties that will contribute to both environmental safety as well as food security worldwide.

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Arbuscular Mycorrhizal Symbiosis and Other Plant–Soil Interactions in Relation to Environmental Stress

11

Patrick Audet

Abstract

In this chapter, focused on the arbuscular mycorrhizal (AM) fungi and their mostly mutualistic association with the vast majority of herbaceous plant species, we examine the cellular, molecular, and physiological mechanisms by which the mycorrhizal symbiosis can enhance plant stress tolerance in relation to a number of abiotic environmental stressors, such as macro- and micronutrient deficiency, drought, and metal toxicity. Overall, the primary mechanisms of interaction discussed here include: (1) the enhanced uptake of macro- and micronutrients and water; and (2) the stabilization of the soil architecture via mycorrhizal-enhanced soil aggregation and metal biosorption processes. A key facet of this analysis involves the identification of direct vs. indirect benefits of interactions, and their distinctive impacts toward plant development as well as the proximal growth environment. Accordingly, due to the significant and widespread effects of these direct and indirect processes toward plant physiological and soil ecological function, it is suggested that the mycorrhizal symbiosis should constitute an extrinsic stress tolerance strategy that could complement the inherent resistance mechanisms of plants when subjected to an array of potential stressors, and also buffer the growth environment. For this reason, it is recommended that future studies take into account such multitrophic interactions (e.g., above- and belowground relationships) to better depict physiological and ecological phenomena in relation to environmental stress.

Keywords

Mutualism • Macro- and micronutrients • Drought • Soil stabilization

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1 Introduction

1.1 Intrinsic vs. Extrinsic Stress Tolerance

In the field of plant physiology, *environmental stress* (or *strain*) refers to a state or event causing a deviation in plant growth relative to its optimal development (Larcher 1987, 2003). As shown in other chapters of this volume, potential environmental stress factors are many (e.g., radiation, temperature, water, nutrient availability) and often grouped according to their abiotic or biotic origins (Fig. 11.1). From this definition, such stressors can be either localized (root vs. shoot predation) or systemic (heat shock, frost), and typically incur a complex cascade of physiological effects ranging

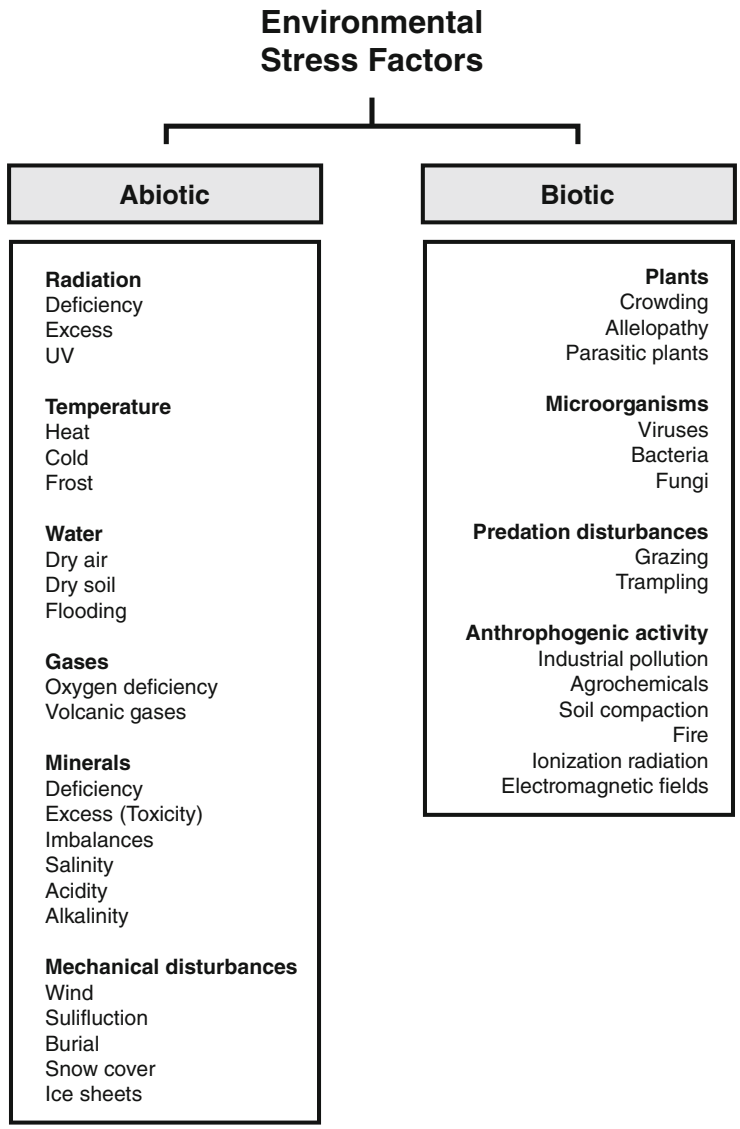

Fig. 11.1 Abiotic and biotic environmental stress factors (adapted from Marschner 1995)

from temporary (reversible) to permanent (irreversible) adaptive responses depending on the relative intensity of the given stress factor. In order to increase their survivorship and reproductive success, plants have developed a remarkable array of stress *tolerance* (endurance), *resistance* (acclimation), and (or) *avoidance* (prevention) mechanisms to circumvent a number of environmental challenges and avoid any permanent associated-stress injuries. Further to these more conventional descriptors of adaptive stress tolerance (e.g., tolerance, resistance, and avoidance), two alternative descriptors have been proposed relating to plant investment (or resource and energy allocation) in either *intrinsic* (e.g., metabolic) or *extrinsic* (e.g., symbiotic) tolerance strategies[1] (Audet and Charest 2007b, 2008). Here, intrinsic stress tolerance refers to plant investment in inherent (or built-in) metabolic systems that are inducible when subjected to stress. For example, the production of metallothioneins which bind ion-free radicals to prevent metal-induced cellular oxidative stress (Chaps. 9, 20, and 21), the production of secondary metabolites to thwart herbivores (Howe and Jander 2008; Mole 1994), the dispatch of heat shock proteins to prevent enzyme denaturing under temperature extremes (Chaps. 5–7), or the production of anti-microbial proteins to inhibit viral, fungal, and (or) bacterial pathogens (Dangl and Jones 2001; Fritig et al. 1998; Ganz and Lehrer 1999), to name just a few. More broadly, intrinsic stress tolerance can also include constitutive systems such as the processes of cell lignification, the development of trichomes and glandular hairs, or the exudation of resins and waxes which, together, offers mechanical defenses to some of these environmental stressors (Bhuiyan et al. 2009; Duke 1994; Langenheim 2003; Wagner 1991). By contrast, extrinsic stress tolerance refers to plant investment in external systems, particularly *symbiotic mutualism*, to circumvent environmental stress. In this regard, the symbiotic mutualism encompasses an intimately co-operative relationship between two different species (referred to as *symbionts*) contributing to the mutual benefit of both individuals (Leung and Poulin 2008). Notably, the balance between the benefits of association among the symbionts is critical for defining the symbiotic mutualism since symbiotic relationships are believed to function along a continuum ranging from parasitism to mutualisms (Boucher et al. 1982; Bronstein 2001; Johnson et al. 1997). From this definition, it can be argued that plants have developed the widest assortment of mutualism in the natural world, whereby host plants typically exchange essential resources (e.g., plant carbohydrates, soil nutrients) and (or) ecological services (e.g., pollination services, shelter) with individuals from another species to reciprocally enhance their tolerance to environmental stressors and thereby increase their survivorship (Boucher 1988; Boucher et al. 1982). Examples of plant mutualism include plant–pollinator interactions with insects, birds, and mammals to ensure plant fertilization and sexual reproduction (Rønsted et al. 2005, 2008; Wiebes 1979), plant–fungus (*mycorrhizal*) interactions to increase the root system's resource acquisition capacity (Douds and Johnson 2007; Marschner and Dell 1994), and plant–rhizobial interactions for the fixation of inorganic soil nitrogen (Long 1996, 2001; Young and Johnston 1989). Among these interactions, the mycorrhizal symbiosis is considered to be one of the most widespread and well-studied ecological associations having key implications at the scale of plant physiological and whole-ecosystem function. In this chapter, focused on the arbuscular mycorrhizal (AM) fungi and their symbiotic association with herbaceous plant species, we examine how some plants invest in mycorrhizal symbiosis as an extrinsic stress tolerance strategy in relation to a number of abiotic environmental stressors, such as macro- and micronutrient deficiency, drought, and metal toxicity. It is also suggested that such an investment can contribute

[1] In their review of "Heavy metal tolerance in plants," Antonovics et al. (1971), and later Baker and Walker (1990), were first to allude to and distinguish between internal and external tolerance mechanisms. Similarly to the investment of plant resources toward intrinsic versus extrinsic strategies proposed here, the internal and external tolerance mechanisms suggested by Antonovics et al. refer to central (e.g., metabolic) and peripheral (e.g., rhizospheric) processes, respectively, which can impact plant development when faced with critical metal toxicity conditions.

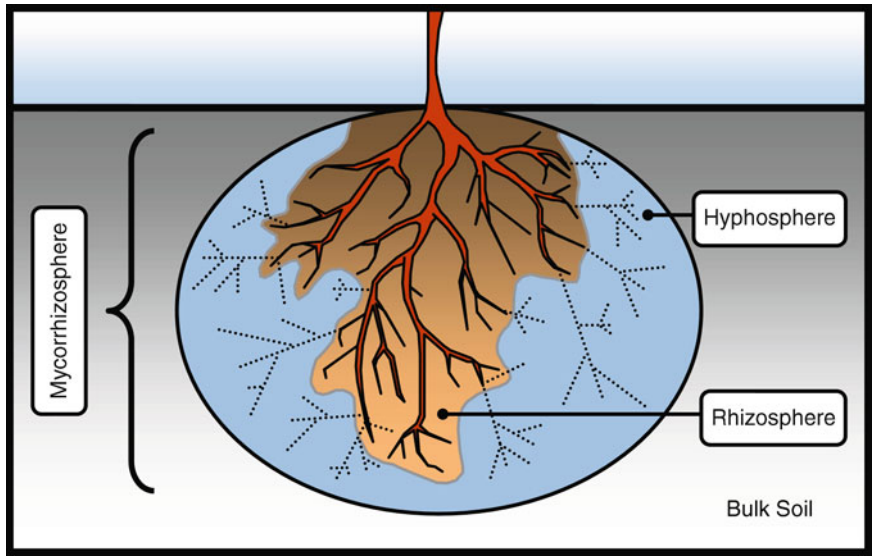

Fig. 11.2 Defining the mycorrhizosphere and its zone of influence (adapted from Beare et al. 1995)

in shaping plant development by influencing edaphic conditions within the proximal growth environment.

1.2 Mycorrhizae and the AM Symbiosis

Aptly referred to as "fungus roots" (Frank 1885), the mycorrhizae are mostly nonpathogenic soil fungi living intimately with terrestrial plant roots that, together, form a symbiotic mutualism characterized by the direct exchange of plant carbohydrates for soil resources, such as mineral nutrients and water (Allen 1991). The mycorrhizae are ubiquitous organisms having adapted to and successfully colonized nearly all known terrestrial ecosystems by forming symbiotic associations with the broad majority of all plant families (Peterson et al. 2004). For this reason, the mycorrhizal fungi are classified into three primary assemblages depending on their respective morphologies and specific plant hosts: the ectomycorrhizae (primarily associated with *Pinaceae*, *Fagaceae*, *Betulaceae*, and *Salicaceae* species), the endomycorrhizae (associated with the majority of angiosperms and some gymnosperms), and the ectendomycorrhizae (primarily associated with *Orchidaceae* and *Ericaceae* species). A common feature among all mycorrhizae is the development of the *mycorrhizosphere*[2] (Fig. 11.2) consisting in the combined zones of influence of the roots (*rhizosphere*) and extraradical hyphae (*hyphosphere*), and encompassing a highly active and multilateral interface between the host plants, mycorrhizal fungi, and proximal soil environment (Duponnois et al. 2008; Garbaye 1991). In line with the notion of plant investment in extrinsic systems for the purpose of stress tolerance, plant investment toward the development of the mycorrhizospheric network involves a considerable plant carbon allocation (occasionally representing up to and possibly well over 20% of the plant's total carbon budget) which is required for

[2] Fitzpatrick's (1984) characterization of the micromorphology of soils indicates that the pedosphere (e.g., soil realm) is constituted of four essential "spheres": atmosphere (e.g., soil air), biosphere (e.g., litter and microorganisms), lithosphere (e.g., rocks and minerals), and hydrosphere (e.g., soil water). Accordingly, Beare et al. (1995) have subclassified arenas of interaction to identify further interfaces within the pedospheric framework, such as the drilosphere (e.g., worm castings), detritusphere (e.g., saprotrophs), rhizosphere (e.g., plant roots), mycorrhizosphere (e.g., combined roots and extraradical hyphae), etc.

actively sustaining the symbiotic infrastructure and maintaining the functional viability of the mycorrhizal symbiont (Douds et al. 2000; Tinker et al. 1994). In exchange, this extrinsic investment provides the host plant with a number of ecological services typically pertaining to the enhancement of the plant's resource acquisition capability and the stabilization of the proximal soil environment.

Falling within the grouping of the endomycorrhizae, the AM fungi and their symbiosis with herbaceous plants are particularly well studied in the field of plant physiology and mycology, and widely recognized for benefiting host plants when subjected to various environmental stressors (Table 11.1). Having originated an estimated 450 million years ago, the AM fungi comprise species of the *Glomeromycota* phylum which are believed to form associations with up to 90% of all herbaceous plants (Redecker et al. 2000; Remy et al. 1994; Schüßler et al. 2001). As characterized by a unique morphology consisting of intra- and extraradical hyphae, arbuscules, vesicles, and spores (Fig. 11.3), the AM fungi behave in a peculiar manner compared to other mycorrhizal phyla since they penetrate between the cortical cells of vascular plant roots in order to develop an intracellular exchange network (Garcia-Garrido et al. 2009): a behavior believed to be rooted in ancient parasitic origins (Purin and Rillig 2008). Following reciprocal signaling processes between the symbionts resulting in the successful colonization of host roots (Harrison 1999, 2005; Vierheilig and Piché 2002), the intraradical hyphae proliferate within the root architecture to interact with roots cells across a slender periarbuscular zone formed between the fungal arbuscular structures and plant cell membranes. It is across the periarbuscular zone where soil resources (e.g., phosphorus, nitrogen, or mineral nutrients) and plant carbohydrates (e.g., glucose, hexose) are actively exchanged between the symbionts (Hahn and Mendgen 2001). Meanwhile, the extraradical hyphae typically scavenge beyond the root depletion zone to form an expansive mycorrhizospheric network, thereby increasing the host roots' resource acquisition capabilities and zone of influence compared to the rhizosphere alone (Koide 1991, 2000; Koide and Elliott 1989). With the development of this active and bidirectional symbiotic exchange network, the AM fungi then shift their developmental allocation to the production of extraradical spores and vesicles which are involved, respectively, in fungal reproduction and lipid storage (Bago et al. 2000; Dalpé et al. 2005). Under these circumstances, the AM fungi are generally considered to be "true" mutualists due to their *host obligate* status which requires that they maintain an active symbiosis in order to ensure an influx of plant carbon allocations for the completion their life cycle (Johnson et al. 1997; Jones and Smith 2004).

As stated previously, numerous advances have been made over the past decades demonstrating the beneficial role of the AM fungi in plant physiology and soil ecology; this being attributed especially to the dynamic function of the mycorrhizosphere in relation to various edaphic processes (Fig. 11.4). In order to accurately depict such dynamic interactions, the classification of the mycorrhizospheric processes presented here distinguishes specifically between two types of interaction depending on the nature of the benefits of association being either direct or indirect. In the present context, the direct benefits of interaction refer to processes that directly enhance the plant health status as mediated by the dynamics of bidirectional exchange between the symbionts described above (Cushman and Beattie 1991; Schwartz and Hoeksema 1998). For example, the process of mycorrhizal *enhanced uptake* in which the extraradical hyphae increase the uptake of limiting soil resources in exchange for plant carbohydrates, then enabling the host plant to supplement its nutrient status when subjected to deficiency conditions. Alternatively and occasionally overlooked from a plant physiological perspective, the indirect benefits[3] of interaction refer to processes that indirectly enhance the plant growth or survival status by altering the proximal growth environment thereby providing

[3] The notion of "indirect benefits" derived from mutualism has previously been used within the context of species community structure.

Table 11.1 Summary of the impact of AM symbiosis on plant physiology and soil ecology

Mechanism		Target	Description	Reference
Direct benefit	Enhanced resource acquisition capability	Essential soil resources (nonmetals, metals, and water)	Preferential uptake of nitrogen (NO_3/NH_4) and phosphorus (P_i)	Bolan (1991)[a], Chapman et al. (2006)[a], Schachtman et al. (1998)[a], Smith et al. (2003)[a], and George et al. (1995)[a]
			Mobilization and uptake of trace essential elements having low bioavailability (e.g., Zn, Ni, Co, Cu, Mn, Fe) particularly under nutrient-deficiency conditions	Jeffries et al. (2003)[a], Koide (1991)[a], and Marschner and Dell (1994)[a]
			Enhanced water use efficiency and drought recovery	Augé et al. (2001), Augé (2001)[a], and Montaño et al. (2007)[a]
Indirect benefit	Soil structure stabilization	Metal bioavailability	Metal binding due to negatively charged surface constituents of extraradical hyphae (e.g., carboxyls, hydroxides, oxy-hydroxides, sulfhydryls); Reduction of plant metal uptake to delay phytotoxicity, particularly at high soil exposure levels (e.g., Zn Pb, Cd, Ni)	Leyval et al. (1997)[a], Galli et al. (1994)[a], Gadd (1993)[a], Meharg (2003)[a], and González-Guerrero et al. (2009)[a]
		Soil retention capacity	Enhanced soil aggregation properties; increased water and nutrient retention capacity, decrease of nutrient leaching	Augé et al. (2001), Augé (2001)[a], Bearden (2001), Bearden and Petersen (2000), Miller and Jastrow (1990)
	Mycorrhizal exudation	Soil microbia	Promotion of beneficial bacteria (i.e., nitrogen fixation), competitive exclusion of soil pathogens	Chapman et al. (2005)[a], Barea et al. (1998)[a], and Johanson et al. (2003)[a]
		Metal bioavailability	Precipitation of metal–ligands, modulation of soil pH	Leyval et al. (1997)[a], Galli et al. (1994)[a], and Gadd (1993)[a]

[a] Denotes review publications

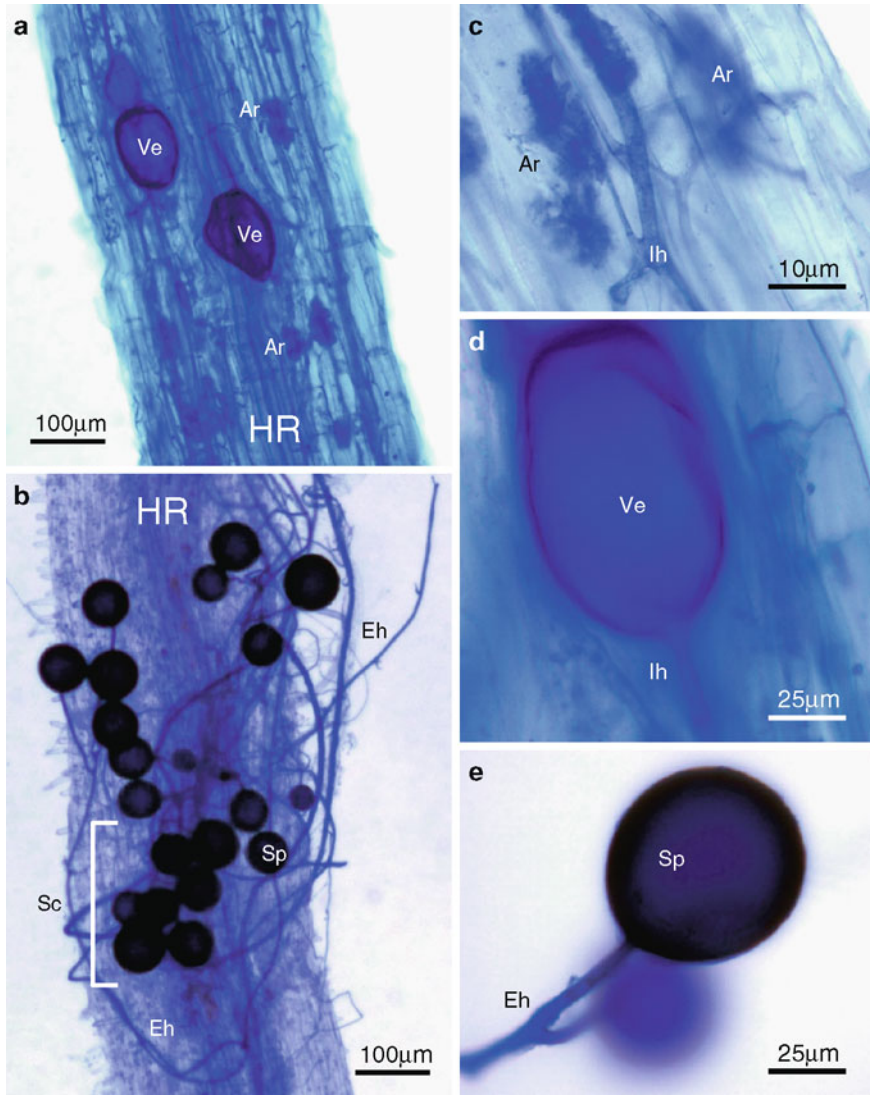

Fig. 11.3 Defining structures of an arbuscular mycorrhizal fungus (*Glomus intraradices* Schenck & Smith) in association with Ri T-DNA carrot roots (*Daucus carota* L.) grown under aseptic conditions. Shown from (**a**) to (**e**) are: vesicles (Ve), arbuscules (Ar), host roots (HR), spores (Sp), spore clusters (Sc), extraradical hyphae (Eh), and intraradical hyphae (Ih). Roots are stained with an aniline blue 0.02% dye solution and observed under a compound microscrope

more favorable developmental conditions (Bertness and Callaway 1994; Stachowicz 2001; Müller and Krauss 2005). For instance, the process of mycorrhizospheric-enhanced soil aggregation which contributes in stabilizing the proximal growth environment to increase its resource retention capacity. A key aspect of the indirect benefits of interaction is the notion that such processes can benefit host plants as well as nonassociated species within the mycorrhizosphere's zone of influence, unlike the direct benefits which suggest an intimate exchange occurring exclusively between the symbionts. By distinguishing between the direct and indirect benefits of interaction, it is intriguing that a combination of such AM-induced mycorrhizospheric processes can complement many intrinsic tolerance mechanisms when subjected to a broad

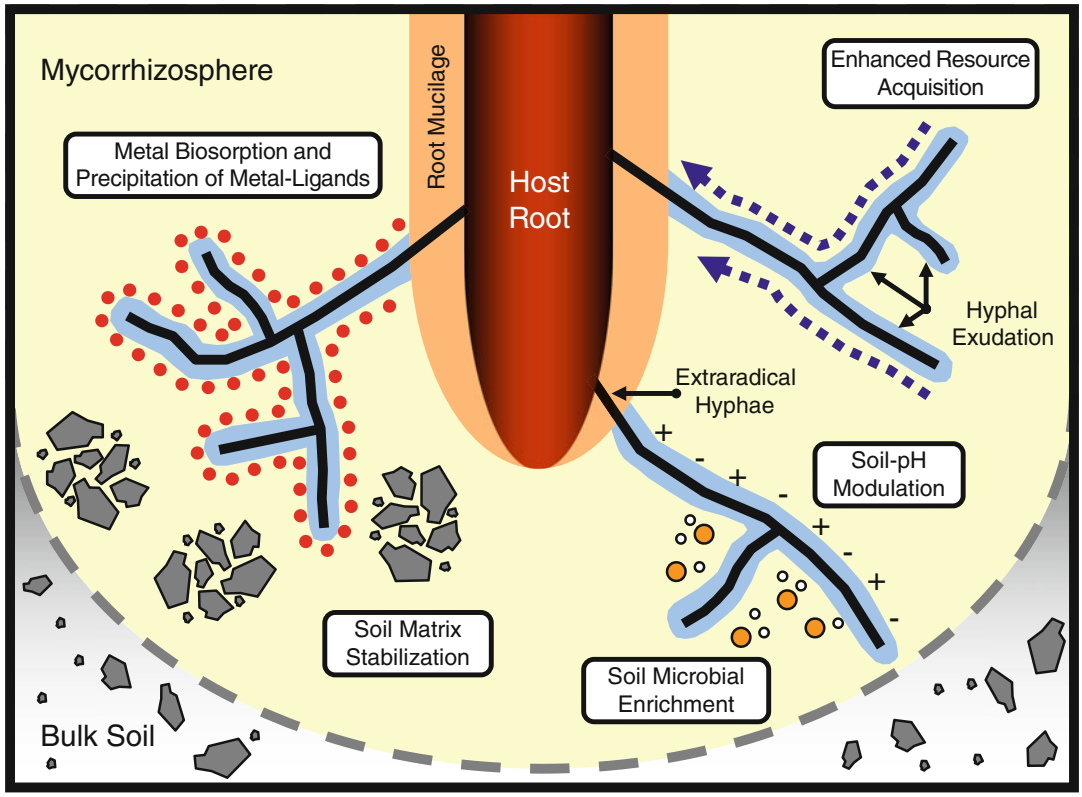

Fig. 11.4 Summary of potential mycorrhizospheric interactions

range of environmental conditions and abiotic stress. For this reason, it is considered that these processes likely play a key role in enhancing plant stress tolerance, as well as shaping the proximal growth environment to increase the soil's resilience, in relation to a number of potential ecological stressors.

2 Direct Benefits of Association

2.1 Macro- and Micronutrient Uptake

2.1.1 N Acquisition

Plant productivity in temperate agro-ecosystems is most commonly limited by soil nitrogen (N) bioavailability (Vitousek and Howarth 1991; Chapin et al. 2002). Due to its principal role in protein biosynthesis and nucleic acid metabolism, N deficiency typically results in stunted plant growth and increased leaf senescence thereby detrimentally affecting the plant's photosynthetic potential and overall growth yield. A particular environmental challenge regarding plant N assimilation exists with regard to the source of N in soils, whether it is in the form of nitrate (NO_3^-) which is readily assimilated in roots and (or) shoots but easily leached from the rhizosphere or, instead, in the form of ammonium (NH_4^+) which can be more abundant than the former but requires detoxification prior to its assimilation (Gutschick 1981; Bloom 1997). The primary benefit of mycorrhizal associations pertains to the increase in belowground surface area (e.g., roots and extraradical hyphae) which enhances the host plant's soil resource acquisition capability compared to the rhizosphere alone. In this regard, two complementary mechanisms describing the role of AM fungi in plant N assimilation have been presented suggesting that the

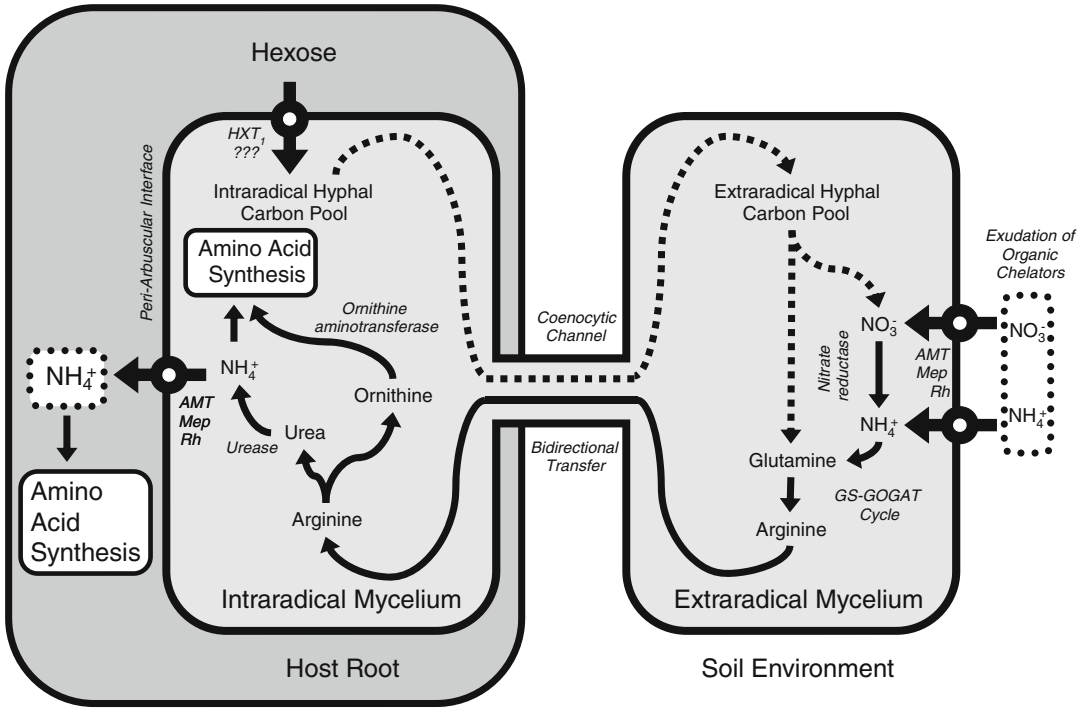

Fig. 11.5 AM fungal nitrogen uptake and transfer pathway (adapted from Govindarajulu et al. 2005; modified according to Jin et al. 2005, Chalot et al. 2006, and Cruz et al. 2007). Bidirectional transfer is indicated by solid and dashed lines. Refer to text for abbreviations

mycorrhizosphere contributes first by generally increasing the plant N acquisition capability (both NO_3^- and NH_4^+ sources), and second by specifically increasing the uptake of NH_4^+ as a result of fungal pre-assimilation and detoxification processes (Chapman et al. 2005; Marschner and Dell 1994). Together, these mechanisms are believed to increase the bioavailability of N within and often beyond the rhizosphere's resource depletion zone to improve plant stress tolerance when subjected to N-deficiency conditions. Among others, Haystead et al. (1988), Faure et al. (1998), and Subramanian and Charest (1997, 1998, 1999) investigated these hypotheses using greenhouse experimental systems in the objective of assessing the nutritional status of plants in relation to the amendment of NO_3^- and NH_4^+ fertilizers, and then comparing the N assimilation pathways of AM vs. non-AM plants by measuring the activity of key assimilation enzymes: namely, nitrate reductase (NR), glutamine synthase (GS), glutamate dehydrogenase (GDH), and glutamate synthase (GOGAT). Collectively, these studies have shown that AM-colonized rye grasses (*Lolium perenne*), "field" clover (*Trifolium repens*), and maize (*Zea mays*) all gained considerable increases in total nitrogen uptake leading to an overall greater amino acid composition compared to non-AM plants. Notably, these physiological effects coincided with increases in the activity of NR, GDH, and GS-GOGAT assimilation enzymes measured in the AM roots and shoots. As predicted, the AM–plant nutritional status was supplemented by increasing the overall uptake of N, especially by increasing the uptake of its less labile form, [NH_4^+]. More recently, the studies of Toussaint et al. (2004), Govindarajulu et al. (2005), Jin et al. 2005; Chalot et al. (2006), and Cruz et al. (2007) have corroborated these general findings using *in vitro* culture tools to further characterize the AM fungal uptake, assimilation, and translocation pathways as soil-N travels from the extraradical hyphae to the host root (Fig. 11.5).

Accordingly, it has been elucidated that NO_3^- and NH_4^+ are actively taken up by extraradical hyphae via transporters of the AMT/Mep/Rh protein superfamily (Khademi et al. 2004). Having reached the cytosol of the extraradical mycelium, NO_3^- and NH_4^+ are converted to glutamine and then arginine via the fungal NR, GDH, and GS-GOGAT enzyme cycles. In this form, arginine travels to the intraradical mycelium through cytoplasmic streaming via coenocytic channels to be further broken down through the action of ornithine aminotransferase and urease, thereby releasing ornithine, urea, and ultimately NH_4^+. Finally, the NH_4^+ is either catabolized via AM fungal amino acid synthesis or transferred to roots across the periarbuscular interface apparently via ammonium transport proteins. To complete the bidirectional exchange between the symbionts, plant carbohydrates in the form of hexose are transferred to the AM fungus via putative transporters (HXT1, potentially among others – Hahn and Mendgen 2001) and likely mediated via plasma membrane H^+-ATPases ($GmHA_{1\text{-}5}$ – Ferrol et al. 2000). Once in the fungal cytosol, the hexose is converted to trehalose, glycogen, and glucose for usage in various fungal metabolisms. As such, the characterization of the AM nitrogen uptake pathway provides key evidence as to the active role of AM fungi in supplementing the plant N nutritional status in the enhancement of plant nutrient stress tolerance.

2.1.2 P Acquisition

After soil-N, plant productivity in temperate agroecosystems is limited by phosphorus (P) bioavailability[4]: a key bioenergetic constituent (e.g., ATP) and cellular structural component (e.g., phospholipids, DNA, RNA). In this regard, P deficiency is prevalent in areas of high rainfall due to extensive nutrient leaching and (or) acidic soil conditions conducive to reciprocal antagonisms (also known as phosphorus-induced micronutrient deficiencies) which can cause leaf chlorosis and stunted growth (Cleveland et al. 2002; Mengel and Kirkby 2001). Similarly to the case of N assimilation described above, it has been hypothesized that the AM fungi hold a significant role in plant P acquisition by increasing the plant's soil resource uptake capacity, particularly by enhancing the uptake of phosphates and inorganic P which typically have slow soil diffusion rates (Marschner 1995; Picone et al. 2003; Saito 2000). In this regard, these physiological mechanisms are among the most thoroughly investigated in the study of the AM symbiosis and have been well reviewed by Bolan (1991), Koide (1991), George et al. (1995), Schachtman et al. (1998), and Smith et al. (2003). The consensus from these studies is that host plants benefit from an investment in AM symbiosis for the supplementation of their P nutritional status, especially when subjected to soil-P deficiency, due to the expansive mycorrhizosphere's ability to increase soil–surface contact and reduce soil–P diffusion distances. Consistent with the notion of plant investment in extrinsic systems to circumvent environmental stress, the mycorrhizal investment is often inversely correlated with the bioavailability of soil–P such that AM root colonization and symbiotic activity are believed to be highest under low P conditions (Smith et al. 2003, 2004). Consequently, this relationship would suggest that P bioavailability is a key factor dictating the plant's relative symbiotic investment (or *mycorrhizal responsiveness*) in order to maximize the reciprocal benefits of association (Graham et al. 1991; Janos 2007; Tawaraya 2003). Under such environmental conditions, soil–P can be actively taken up by the extraradical hyphae and efficiently transferred to host roots (Fig. 11.6 – Schachtman et al. 1998; Smith et al. 2003; Javot et al. 2007). This process is characterized by the activity of extraradical hyphae which increase the solubility of soil-P due to the exudation of organic chelators to then facilitate the uptake of both organic (P_{org}) and inorganic (P_i) forms of P. Coinciding with these events, a number of AM fungal phosphate

[4] Unlike temperate environments, plant productivity in tropical agro-ecosystems is primarily limited by phosphorus bioavailability followed by less labile soil micronutrients due to their slow diffusion rates and subsequently low bioavailability to plants. In addition, the high rates of plant photosynthesis and evapo-transpiration in this ecosystem typically cause the bioavailable nutrient pool to be rapidly assimilated (Brams 1973; Baligar and Bennett 1986; Ewel 1986).

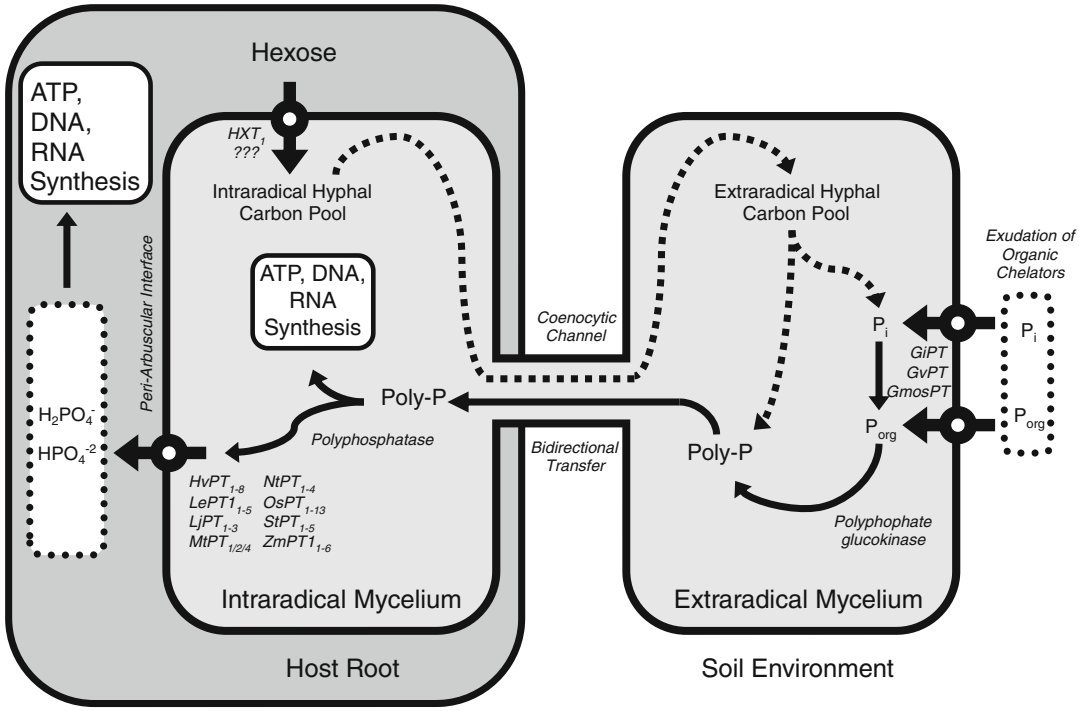

Fig. 11.6 AM fungal phosphorus uptake and transfer pathway (adapted from Schachtman et al. 1998; modified according to Smith et al. 2003 and Javot et al. 2007). Bidirectional transfer is indicated by solid and dashed lines. Refer to text for abbreviations

transporters have been isolated from the mycelium of a number of AM fungi and found to be upregulated under P-deficiency conditions, such as: *Glomus intraradices* (GiPT – Maldonado-Mendoza et al. 2001), *G. mosseae* (GmosPT – Benedetto et al. 2005), and *G. versiforme* (GvPT – Harrison and van Buuren 1995). Once taken up into the cytosol of the extraradical mycelium, the P_{org} and P_i are converted to polyphosphate complexes (poly-P – e.g., glucose-6-phosphate) through the enzymatic activity of polyphosphate glucokinase (Capaccio and Callow 1982; Cox et al. 1980). In this more stable cytosolic form, the poly-P complexes are either stored in fungal vacuoles or transferred to the intraradical mycelium through cytoplasmic streaming via coenocytic channels: a process which is putatively linked with H^+-ATPase co-transport (Gauthier and Turpin 1994). Here, the polyphosphate complexes can be broken down by polyphosphatases for use in AM fungal ATP, DNA, RNA, and (or) phospholipid syntheses, or transferred to the host plant across species-specific phosphate transporters in exchange for plant carbohydrates in the form of hexose. To date, advanced molecular analyses have identified an array of phosphate transporters (Table 11.2) isolated especially in the periarbuscular interface region of various model study organisms, such as barley (*Hordeum vulgare*), deervetches (*Lotus japonica*), tomato (*Lycopersicon esculentum*), alfalfa (*Medicago truncatula*), rice (*Oryza sativa*), potato (*Solanum tuberosum*), and maize (*Zea mays*). Accordingly, it has been reported that these species also experience an increase in phosphorus assimilation activity (e.g., acid phosphatase, alkaline phosphatase, and H^+-ATPase) in their roots which can contribute in increasing P assimilation and P nutritional status following the enhanced uptake and transfer of soil-P from the extraradical hyphae to the host plant (Capaccio and Callow 1982; Dexheimer et al. 1982; Schwab et al. 1991), which was later

Table 11.2 Summary of known AM fungal and plant phosphate transporters (from Javot et al. 2007)

Taxon	Nomenclature				Reference
Arbuscular mycorrhizal fungi					
Glomus intraradices Schenck & Smith	GiPT				Maldonado-Mendoza et al. (2001)
Glomus mosseae (T.H. Nicolson & Gerd.) Gerd. & Trappe	GmosPT				Benedetto et al. (2005)
Glomus versiforme (P. Karst.) S. M. Berch	GvPT				Harrison and Van Buuren (1995)
Plant host					
Hordeum vulgare L.	$HvPT_1$ $HvPT_2$ $HvPT_3$				Smith et al. (1999), Rae et al. (2003), and Glassop et al. (2005)
Lotus japonicus L.	$LjPT_1$ $LjPT_2$ $LjPT_3$				Maeda et al. (2006)
Lycopersicon esculentum L.	$LePT_1$ $LePT_2$	$LePT_3$ $LePT_4$	$LePT_5$		Daram et al. (1998), Liu et al. (1998a), Rosewarne et al. (1999), and Nagy et al. (2005)
Medicago truncatula L.	$MtPT_1$ $MtPT_2$ $MtPT_4$				Karandashov et al. (2004), Liu et al. (1998b), and Harrison et al. (2002)
Oryza sativa L.	$OsPT_1$ $OsPT_2$ $OsPT_3$ $OsPT_4$	$OsPT_5$ $OsPT_6$ $OsPT_7$	$OsPT_8$ $OsPT_9$ $OsPT_{10}$	$OsPT_{11}$ $OsPT_{12}$ $OsPT_{13}$	Paszkowski et al. (2002) and Guimil et al. (2005)
Solanum tuberosum L.	$StPT_1$ $StPT_2$	$StPT_3$ $StPT_4$	$StPT_5$		Nagy et al. (2005), Leggewie et al. (1997), Rausch et al. (2001), and Karandashov et al. (2004)
Zea mays L.	$ZmPT_1$ $ZmPT_2$ $ZmPT_3$				Glassop et al. (2005), Wright et al. (2005), and Nagy et al. (2006)

corroborated by molecular analyses of phosphate transporter activity (Karandashov and Bucher 2005). As in the case of AM–plant N uptake, the ongoing characterization of the mycorrhizal P uptake and translocation pathways provide key evidence as to the active and intricate role of AM fungi in the enhancement of plant nutrient stress tolerance.

2.1.3 Micronutrient (Metal) Uptake

Analogous to the processes of AM–plant N and P acquisition, there is a considerable body of literature describing the beneficial role of the AM symbiosis in micronutrient uptake, particularly soil-metal[5] deficiency conditions (Jeffries et al. 2003; Koide 1991; Marschner and Dell 1994).

[5] Macro- and micronutrients are alternatively classified according to their physicochemical properties to define them, respectively, as either non-metals (nitrogen, sulfur, phosphorus, boron, chlorine) which have a negative valence, or metals (potassium, calcium, magnesium, iron, manganese, zinc, copper, molybdenum, nickel) which have a positive valence (Foy et al. 1978; Larcher 2003).

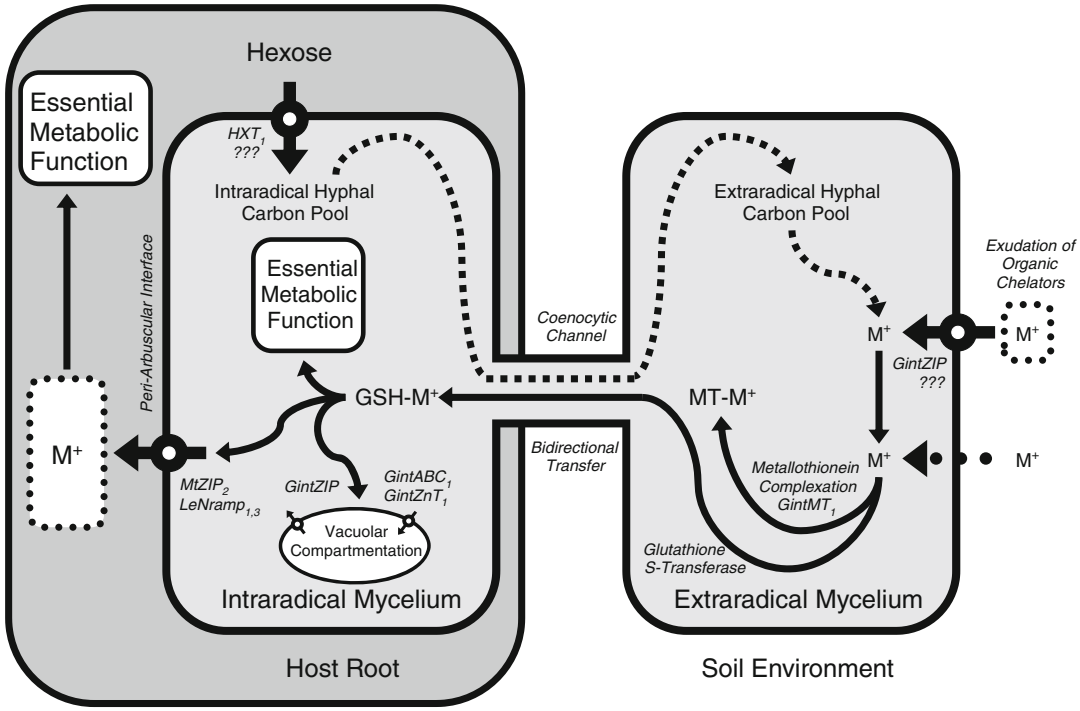

Fig. 11.7 AM fungal micronutrient (metal) uptake and transfer pathway (adapted from Meharg 2003; modified according to Göhre and Pazkowski 2006 and González-Guerrero et al. 2009). Bidirectional transfer is indicated by solid and dashed lines. Refer to text for abbreviations

In this regard, soils of temperate environments (typically classified as alfisols and vertisols) and tropical environments (ultisols and oxisols) can suffer from suboptimal elemental compositions and (or) nutrient imbalances due to long-term weathering and soil erosion resulting in plant zinc (Zn), nickel (Ni), cobalt (Co), copper (Cu), manganese (Mn), and (or) iron (Fe) nutritional deficiencies and (or) mutual antagonisms (Blinkley and Vitousek 1989; White and Zasoski 1999). As described previously, the AM symbiosis contributes in circumventing nutrient deficiency stress by increasing nutrient bioavailability in the mycorrhizosphere due to an increased resource acquisition capability which helps in supplementing the plant nutritional status, especially poorly labile metal nutrients (Eckhard et al. 1994; Liu et al. 2000; Marschner 1998; Rengel et al. 1999; Sharma et al. 1994). A commonality in the mode-of-action seems to exist regarding the general AM–plant metal uptake pathway (Fig. 11.7) as metal nutrients are taken up, translocated, and transferred from the extraradical hyphae to the host roots, as depicted in reviews by Meharg (2003), Göhre and Pazkowski (2006), and González-Guerrero et al. (2009). These recent studies have reported that the exudation of organic chelators by the extraradical hyphae contributes in solubilizing metal ions in the mycorrhizosphere followed by the mobilization of metal–chelator complexes across fungal transporters, such as GintZIP in *Glomus intraradices* (González-Guerrero et al. 2009). Free metal ions may also be taken up passively across trans-membrane ion channels depending on the soil metal concentration gradient. In the cytosol, metal ions are typically bound and (or) sequestered by metallothionein proteins or glutathione complexes. Notably, it has been shown that this course-of-action can correspond with an upregulation of $GintMT_1$ (encoding for fungal metallothioneins in *G. intraradices* – González-Guerrero et al. 2005, 2007; López-Pedrosa et al. 2006) as well as an increase in glutathione

S-transferase activity (González-Guerrero et al. 2009), which could represent critical steps in limiting internal stress due to the production of reactive oxygen species in the fungal cytosol (González-Guerrero et al. 2010a). Subsequently, the production of such less reactive metal complexes enables the AM fungi to store excess metal ions into their vacuolar compartments (via GintZIP, $GintABC_1$, or $GintZnT_1$ membrane transporters), integrate them into their essential metabolic function, or transfer them to the host root across the periarbuscular interface (via $MtZIP_2$ or $LeNramp_{1,3}$) (González-Guerrero et al. 2005, 2007, 2009, 2010b). The identification of ion transporters common to both plants and fungi which are putatively involved in the regulation of metal uptake (Burleigh et al. 2003; López-Millán et al. 2004) could support the perspective that this process is actively co-modulated by both symbionts and provides further evidence as to the fundamentally mutualistic nature of the association, as suggested regarding the symbiotic transfer of P. Altogether, these combined processes characterize the complex role of the mycorrhizosphere in plant mineral nutrition contributing by modulating plant nutrient uptake and thereby enhancing the host plant's physiological status compared to non-AM plants, particularly when subjected to a number of environmental stress factors.

2.2 Plant Water Relations

Besides the significant role of the AM symbiosis in plant mineral nutrition, there is a considerable body of literature describing the beneficial effects of the AM mycorrhizosphere in plant water relations across a broad range of water stress, for instance, from amply watered to droughted conditions (Augé 2001, 2004). Yet, unlike the dynamics of AM–plant nutrient acquisition described above, the specific mechanisms underlying the direct impact of the mycorrhizosphere on plant stress tolerance under such environmental conditions remain slightly ambiguous. A central question in this regard considers whether the AM symbiosis benefits plants more by enhancing their intrinsic drought resistance (i.e., survival at low internal water content) or, rather, by increasing their drought avoidance (i.e., maintenance of high internal water content) when subjected to a low external water potential, such as drought and drought recovery conditions. As outlined in recent reviews by Augé (2001, 2004), a wide array of AM–plants in association with a number of *Glomus* and allied AM species (refer to Augé 2001 for a more comprehensive list of plant and AM fungal species interactions) have been shown to develop a variety of beneficial physiological responses compared to non-AM plants, with such responses ranging from relatively higher stomatal conductance, leaf transpiration, and (or) osmotic potential (Allen et al. 1981; Augé 2000; Augé et al. 2003, 2007, 2008). Among AM–plants, such enhanced metabolic and physiological functions under strained water conditions have subsequently been linked to an increased photosynthetic potential due to a generally larger leaf area, relatively greater water potential (i.e., water content) in roots and shoots, and an increased overall growth status observed under both greenhouse and field conditions (Cho et al. 2006; Khalvati et al. 2005). Altogether, these physiological responses contribute to an increased AM–plant stress tolerance occurring especially, but not exclusively, during both drought and drought recovery conditions. In addition to imposing a direct physiological stress toward host plants as symptomatically expressed by a general loss of turgidity and down regulation of various essential metabolisms (Hsiao 1973; Nautiyal et al. 1994), strained water relations often also cause alterations in nutrient bioavailability in the soil solution which leads to significant nutrient imbalances and potential antagonisms between metal ions. This potential correlationality between environmental stressors (e.g., both water and nutrient deficiency) represents an important challenge faced by experimental investigators in determining the role(s) of AM fungi in plant water relations, particularly when attempting to distinguish the specific mechanisms of AM-enhanced plant stress tolerance (Koide 1993). Similar to the notion of enhanced mycorrhizospheric uptake regarding AM–plant nutrient acquisition, it has

been suggested that the mycorrhizosphere should play a fundamental role in enhancing both the nutrient and water acquisition capabilities of host plants by increasing their resource acquisition pool compared to the rhizosphere alone; this, again through the general mechanism of actively scavenging the proximal soil environment for essential resources (Koide 1993). More specifically, extraradical hyphae could contribute directly in circumventing water deficiency conditions by penetrating soil micropores to improve hydraulic conductivity due to the expansive mycorrhizosphere, and thereby enhance plant stress avoidance during drought stress and drought recovery (Miller and Jastrow 1990). Accordingly, analyses comparing AM and non-AM root conductivity among plants under drought and amply watered conditions have also shown improvements in AM–plant water uptake that often coincide with increases in the host plant's mineral nutrition. Here, an improved N, P, and (or) micronutrient status could further benefit AM–plants by circumventing internal mineral deficiencies that may otherwise detrimentally affect their intrinsic drought resistance mechanisms, such as the accumulation and maintenance of high foliar concentrations of soluble sugars and free polyamines (Augé 2001, 2004). This improved mineral status has also been linked with lower amino acid accumulation in AM than non-AM plants which suggests that the former are generally less metabolically strained under these conditions (Augé 2001). The consensus from these overall findings suggests that AM colonization primarily contributes by bolstering intrinsic plant resistance mechanisms by circumventing internal deficiencies. In addition, water conductance and nutrient uptake are improved due to an increased bioavailable pool of soil resources within the mycorrhizosphere (Cho et al. 2006; Khalvati et al. 2005). Together, these mycorrhizospheric processes can increase AM–plant resilience in relation to stressful water relations by enabling a relatively more *stable* (or less strained) metabolic function, which is typically manifested through an increased overall photosynthetic potential and relative growth potential during drought stress and drought recovery. While these physiological effects provide a significant environmental advantage to AM vs. non-AM plants when subjected to adverse water conditions, plants having overall increased photosynthetic activities and stomatal conductances also tend to have higher rates of evapo-transpiration. Ironically, this effect can impose further stress on the plants themselves by increasing the rate of soil-drying in the proximal soil environment. In adding further complexity to the role of the AM symbiosis in plant water relations, these plants could be more vulnerable compared to less photosynthetically active plants under such environmental conditions due to complications stemming from accelerated soil-drying. Nevertheless, investigations into the role of the mycorrhizosphere in stabilizing the proximal soil environment could shed light into these matters, especially regarding the impact of extraradical hyphae in soil aggregation and biosorption processes which can buffer a number of edaphic factors such as the water and nutrient retention capacities. In fact, such "indirect" mycorrhizospheric processes could represent equally important components of plant stress tolerance and ecosystem function compared to the "direct" processes presented here.

3 Indirect Benefits of Association

3.1 Soil Structure Stabilization

3.1.1 Soil Aggregation

Notwithstanding the direct role of the AM symbiosis in plant resource acquisition, the mycorrhizosphere also provides significant indirect benefits of interaction which can buffer and (or) stabilize the soil matrix. In this regard, processes such as mycorrhizal-induced soil aggregation and metal biosorption (Figs. 11.8 and 11.9) are considered, here, to fall within the category of *indirect benefits* since plant investment in the mycorrhizosphere can provide key ecological services which are not directly associated with the intimately co-modulated mechanism of resource exchange. Accordingly, it can also be argued that non-associated species could benefit

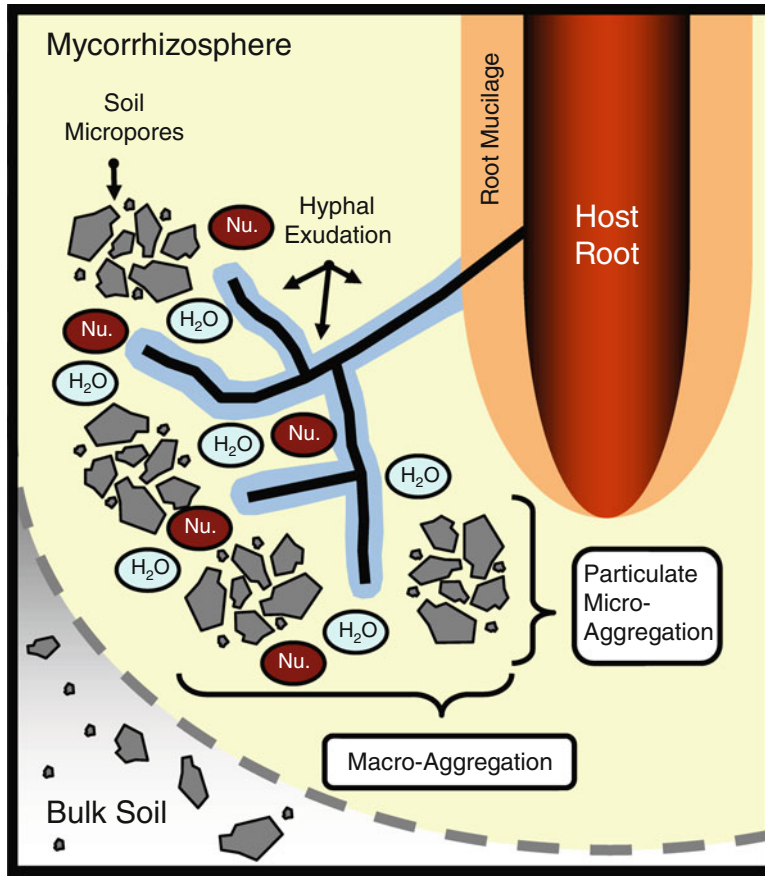

Fig. 11.8 Mycorrhizal-induced micro- and macroaggregate formation indicating affinities for binding soil nutrients and water. (adapted from Miller and Jastrow 1990 and Rillig and Mummey 2006)

from these processes due to their proximity to the mycorrhizosphere's zone of influence and its broad effects on the soil growth environment. Coincidentally, it has been suggested that such edaphic interactions should influence ecosystem function at various hierarchical scales due their biogeochemical implications ranging from the micro- (e.g., soil water and nutrient retention, enhanced resource acquisition) to the macroscopic levels (e.g., whole plant functionality, species abundance and distribution) (Beare et al. 1995; Rillig and Mummey 2006; Rillig et al. 2010). In this regard, and further to the role of the extraradical hyphae in directly improving nutrient uptake and hydraulic conductivity (as discussed previously), the proliferation of extraradical hyphae and their penetration into soil micropores can significantly enhance the aggregation properties of soils to then improve their overall water and nutrient holding capacities (Piotrowski et al. 2004): a process akin to the enmeshment of roots throughout soils during rhizospheric expansion (Angers and Caron 1998). More specifically, the greater degree of mycorrhizal branching and ramification produces localized compression forces within the proximal soil environment which serve to increase the formation of micro- and macroaggregates (Fig. 11.8) (Miller and Jastrow 1990; Rillig and Mummey 2006). Consequently, mycorrhizospheric expansion can directly increase the rate of soil cluster formation and enhance the overall resilience of the soil structure in relation to stress (e.g., soil drying, flooding, compaction, nutrient leaching).

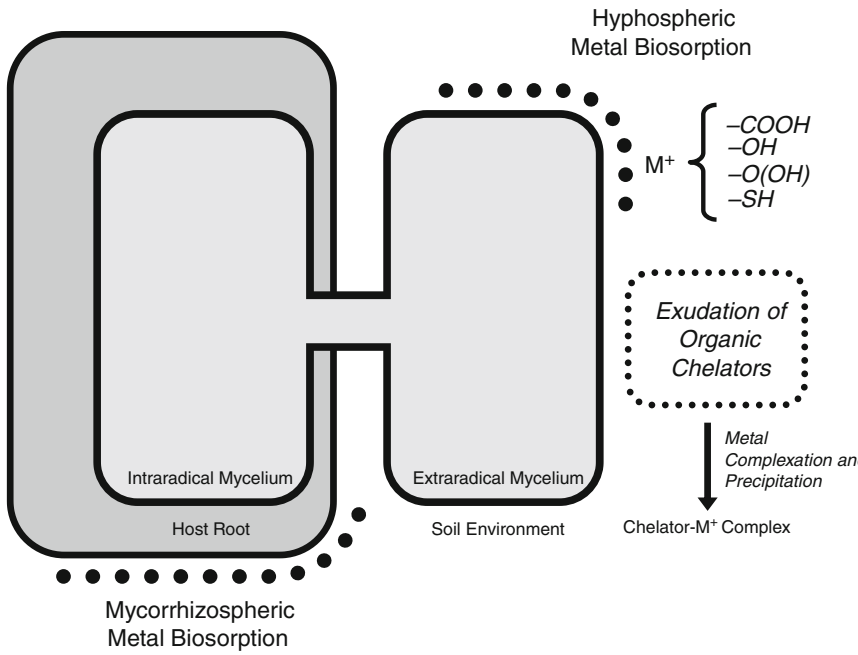

Fig. 11.9 Mycorrhizal-induced metal biosorption and metal complexation (adapted from Apak 2002, Gadd 1993, Galli et al. 1994, González-Chavez et al. 2002, and González-Guerrero et al. 2008)

Alternatively, under such conditions, bulk soils tend to have a comparatively lower particulate binding capacity and subsequently lower aggregation potential resulting in the relatively more rapid collapse of their matrix structure. In addition to the physical entanglement of soil aggregates by the mycorrhizosphere, mycorrhizal-induced soil aggregation can also be attributed to the exudation of organic acids by extraradical hyphae (Bais et al. 2006; Bertin et al. 2003; Rovira 1969). Fundamentally, these mucilage exudates – typically consisting in polysaccharides and other extra-cellular polymeric compounds – contribute to nutrient chelation for mineral solubilization within the mycorrhizosphere as well as protection of the extraradical mycelium from desiccation. Further to these essential roles, such organic exudates also adhere to soil particles and permit the physical entanglement of micro- and macroaggregates leading to the development of soil clusters within the mycorrhizosphere. Glomalin and glomalin-related soil proteins, considered to be effective biochemical markers of AM fungal growth and mycorrhizosphere development, are also believed to bind soil particles in the same manner leading to increased structure stability (Driver et al. 2005; Purin and Rillig 2007). Altogether, this improved potential for soil cluster formation can increase the incidence of micropores within the soil architecture leading to an overall increased colloidal surface area. As a result, the mycorrhizosphere can have a higher affinity for retaining water molecules as well as metal and nonmetal ions compared to bulk soil. Taking into account the processes of hyphal proliferation and chelator exudation, Augé (2004) and Rillig and Mummey (2006) have likened the mycorrhizospheric network to an essential skeletal structure and the production of mycorrhizal-derived organic compounds as the "glue" which, together, contribute in holding together the soil matrix. Consequently, from a biogeochemical perspective, the mycorrhizospheric network should play central role in enhancing soil water and nutrient retention. When subjected to environmentally stressful conditions, these enhanced soil stabilization properties can significantly increase the soil's resilience to then buffer the

growth environment for plants and associated soil microorganisms. Notably, under drought stress, the development of soil aggregates increases water and nutrient retention to delay the effects of soil drying (Auge 2004; Rillig and Mummey 2006); meanwhile, these aggregates also increase water infiltration during drought recovery due to the more *hydratable* (or water stable) soil matrix (Rillig et al. 2010). This mycorrhizal-induced structural advantage benefits plant stress tolerance by increasing the soil's water storage capacity and increasing its resilience. Likewise, the increased water retention capacity within the mycorrhizosphere can also impact plant stress tolerance in relation to nutrient stress (e.g., reciprocal ion antagonisms leading to deficiency) since soil nutrient bioavailability is closely correlated with soil water potential. As a result, nutrient bioavailability may be increased within the mycorrhizosphere due to a greater retention capacity; meanwhile nutrient losses are decreased due to a reduction in leaching.

3.1.2 Metal Biosorption

The metal-binding capacity of soil is primarily dictated by its essential composition, whereby soils having a higher proportion of organic matter (e.g., humic and fluvic acids) typically tend to have a greater retention capacity and redox potential than other soil types (Bohn 1971; McBride 1994). Further to the role of the mycorrhizosphere in stabilizing the soil's structural matrix, the extraradical hyphae have also been shown to increase the biosorption potential of soils. This is attributed primarily to the preferential binding of metal ions to negatively charged mycelial and root surface constituents (Fig. 11.9), such as carboxyl, hydroxide, oxy-hydroxide, and sulfhydryl groups (Apak 2002; Gadd 1993; Galli et al. 1994; González-Chavez et al. 2002, 2008). Similar analyses of non-mycorrhizal fungi suggest that phenolic polymers and melanins should also be effective metal binding sites even among AM fungi (Baldrian 2003; Fogarty and Tobin 1996). Likewise, the exudation of organic chelators within the mycorrhizosphere (described above) has been shown to result in an enhanced binding capacity due to the formation of metal–ligand complexes and precipitates in the soil solution. For these reasons, the general processes of metal biosorption, including ion-exchange (i.e., CEC), metal complexation, and metal–ligand precipitation and crystallization occurring on and within the fungal cell wall (Gadd 1993; Galli et al. 1994), represent significant mechanisms regarding the modulation of metal bioavailability within the mycorrhizosphere. As in the case of mycorrhizal-induced soil aggregation, these enhanced metal biosorption properties can improve the soil's resilience by increasing its nutrient retention capacity, while reducing nutrient losses due to leaching (Giller et al. 1998; Leyval et al. 1997). Notably, there is considerable evidence suggesting that, when essential and nonessential metals occur at exceedingly high exposure levels representing potentially toxic metal conditions, such metal biosorption properties can significantly reduce the bioavailability of metals in the soil solution to reduce plant metal uptake and then delay the onset of metal phytotoxicity (Audet and Charest 2007b). In this regard, a wide array of plant species (refer to Audet and Charest 2007b for broad list plant species) subjected to increasingly high metal concentrations, both essential (e.g., Cu, Fe, Mn, Ni, and Zn) and nonessential elements (e.g., Cd, Co, Cr, and Pb), have repeatedly been shown to incur considerably lower (up to 50%) metal uptake among AM (*Gl. caledonium*, *Gl. intraradices*, *Gl. mosseae*, and a consortium of unidentified *Glomus* species) than non-AM plants; an effect often coinciding with an increased plant growth and (or) health status. As proposed by Leyval et al. (1997), and later Audet and Charest (2006, 2007a, b, 2008, 2009), Hildebrandt et al. (2007), and Giasson et al. 2008, these findings suggest that mycorrhizal-induced metal biosorption could represent a significant extrinsic plant stress avoidance strategy, whereby excess soil metals are bound and precipitated in the soil solution as well as sequestered in fungal tissues instead of being transferred to host roots. As such, plant investment in this extrinsic stress avoidance mechanism could complement known intrinsic plant detoxification mechanisms, for instance, metallothienin and phytochelatin metabolisms (Cobbett 2000; Cobbett and Goldsbrough 2002) by reducing

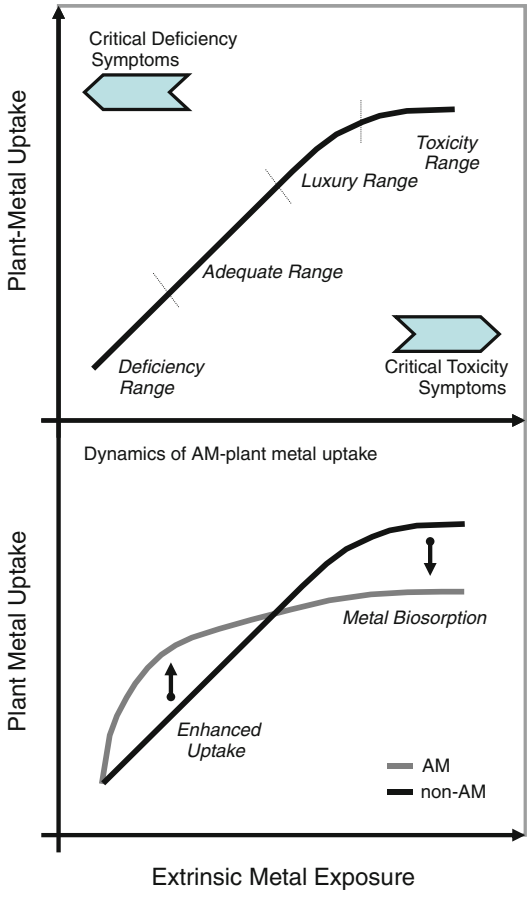

Fig. 11.10 Dynamics of AM–plant metal uptake as characterized by the mycorrhizospheric processes of enhanced uptake and metal biosorption. (adapted from Audet and Charest 2007b, 2008, 2009)

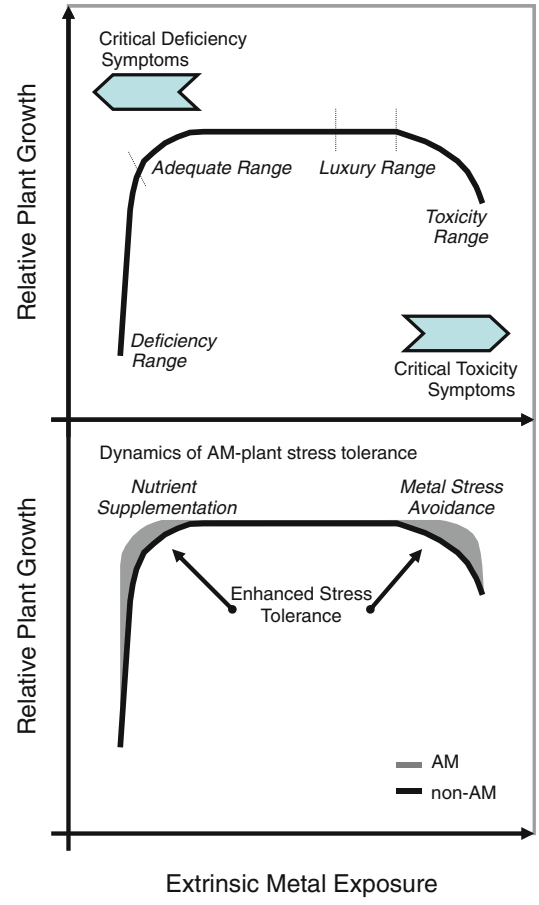

Fig. 11.11 AM–plant relative plant growth in relation to metal exposure corresponding to the "Dynamics of AM-plant metal uptake" (adapted from Audet and Charest 2007b, 2008, 2009)

AM–plant metal uptake and subsequently reducing cellular oxidative stress and other physiological challenges associated with metal toxicity conditions. This entire perspective has been summarized by Audet and Charest (2007b, 2008, 2009) who described plant metal uptake (Fig. 11.10) and relative plant growth (Fig. 11.11) by AM and non-AM plants using a conceptual modeling strategy based on meta-analytical, *in vitro*, and greenhouse culture systems. When taking into account the many different roles of AM fungi in plant physiology and ecosystem function, the mycorrhizosphere is first believed to increase AM–plant metal uptake to supplement the plant nutritional status under nutrient deficiency conditions. Subsequently, the role of the mycorrhizosphere seems to shift toward the modulation of soil nutrients due to metal biosorption processes which can delay the effects of metal phytotoxicity when subjected to potentially toxic conditions. Here, the combination of the *enhanced uptake* and *metal biosorption* mechanisms occurring simultaneously and (or) independently causes a distinct metal uptake profile among AM than non-AM plants in relation to a wide range of metal exposure, albeit depending on the soil's fundamental composition and inherent properties. As such, AM–plant growth and stress tolerance are often significantly improved as evidenced by an increased growth status whether under trace or toxicity conditions. As for conditions within the proximal growth environment, the mycorrhizal-

induced processes of metal biosorption and metal–ligand complexation are also believed to influence a number of edaphic factors, for instance, the soil pH and redox potential (Christie et al. 2004; Fourest and Roux 1992; Joner et al. 2000; Leyval et al. 1997). When considering the case of phosphorus and nitrogen acquisition by mycorrhizae, the extraradical hyphae are believed to cause the moderate alkalinization of the growth substrate due to their selective depletion of nutrients and specific exudation of organic chelators, unlike roots that tend to acidify it (Bago et al. 1996; Eckhard et al. 1995; Gahoonia and Nielsen 1992; Li et al. 1991; Rufyikiri et al. 2004). Similar outcomes have been reported by Li and Christie (2001) and Audet and Charest (2010 and unpublished results) who investigated the impact of the rhizosphere (roots), mycorrhizosphere (roots and extraradical hyphae), and hyphosphere environments (strictly extraradical hyphae) in relation to increasing soil–Zn exposure levels. Although these facets of plant–AM–soil interactions should require further and more in-depth investigation, these studies indicate preliminarily that the presence of roots and (or) extraradical hyphae should play an essential part in shaping edaphic conditions due (in part) to differential nutrient depletion zones caused by AM and non-AM plants, their exudation of organic chelators, and their retention (or biosorption) of metals within the proximal growth environment. These factors are relevant to the bioavailability of metal and non-metal nutrients in soils since the process of hyphal alkalization could favor metal biosorption and contribute in reducing metal bioavailability and toxicity, whereas root acidification may facilitate leaching by increasing metal solubility (Apak 2002; Bradl 2004; Tack et al. 1996).

3.2 Biotic Interactions

3.2.1 Biodiversity of Beneficial and Nonbeneficial Soil Microflora

Although this chapter has focused primarily on identifying the role(s) of the AM symbiosis in benefiting plant tolerance when subjected to a number of abiotic stressors (e.g., macro- and micronutrient deficiency, drought, metal toxicity), the role of the mycorrhizosphere toward biotic soil interactions is still worthy of mention – albeit discussed only briefly in the present context. In addition to shaping growth parameters such as soil nutrient bioavailability and other edaphic factors, mycorrhizal proliferation and exudation can also significantly impact belowground biodiversity by influencing soil microbial communities within the proximal soil environment (Brussaard et al. 1997; Newsham et al. 1995a, b; Wardle et al. 1998, 2004). In turn, such subsidiary mycorrhizospheric interactions can have important consequences toward aboveground species biodiversity (i.e., plant species abundance and distribution) due to the mutual feedback existing between above- and belowground symbionts (Bever 1999, 2003; Bever et al. 1997; Klironomos 2002; van der Heijden et al. 1998). As such, the AM fungi are believed to develop their own soil microflora apparently due to the exudation of organic compounds and chelators within the mycorrhizosphere (Andrade et al. 1997; Fitter and Garbaye 1994; Frey-Klett et al. 2007; Linderman 1988); this, in addition to their modulation of edaphic factors (as described previously) which can provide more favorable soil pH and nutrient bioavailability conditions for the development of such a microflora (Cavagnaro et al. 2006; Deubel and Merbach 2005; Villegas and Fortin 2001). This is not surprising considering that AM fungal spores are known to harbor their own internal bacterial flora within their sporocarps, meanwhile the extraradical hyphae maintain their own extensive array of bacterial biofilms (Andrade et al. 1997; Bianciotto and Bonfante 2002; Fitter and Garbaye 1994; Frey-Klett et al. 2007). Although the ecological role(s) of these bacteria in AM fungal development have yet to be fully understood, various *in vitro* analyses have demonstrated a number of interactive behaviors ranging from stimulated spore germination, induced hyphal branching, and enhanced sporulation due to the presence of volatile bacterial metabolites (Azcón-Aguilar and Barea 1992; Barea 1997; Bianciotto and Bonfante 2002; von Alten et al. 1993). In response, the AM fungi seem to reciprocate this ulterior "symbiotic" association by enriching the

mycorrhizosphere environment with carbon in the form of organic exudates (Antoun and Prevost 2001; Artursson et al. 2006). For this reason, it is possible that such bacteria (also known as *mycorrhiza helper bacteria* – Garbaye 1994; Frey-Klett et al. 2007) and their exudates are fundamentally involved in AM fungal development and mycorrhizospheric function, and then potentially impacting plant health status due to their role as growth promoters (Bianciotto and Bonfante 2002; Bianciotto et al. 1996a, b; Söderberg et al. 2002). In terms of their potential effects on plant stress tolerance and extrinsic growth conditions within the mycorrhizosphere, the mycorrhiza helper bacteria are generally considered to influence edaphic parameters similar to the extraradical hyphae themselves: for instance, by increasing the solubilization of soil nutrient via chelation and (or) contributing in the modulation of nutrient bioavailability via metal biosorption and soil aggregation processes (Cavagnaro et al. 2006; Deubel and Merbach 2005; Villegas and Fortin 2001). Accordingly, these mechanisms should benefit plant stress tolerance by indirectly improving nutrient bioavailability and uptake, particularly when subjected to suboptimal soil nutrient conditions. Moreover, the AM fungi could also benefit plant stress tolerance in relation to biotic stressors by inhibiting and (or) impeding soil-borne pathogens (Azcón-Aguilar and Barea 1996; Barea et al. 1998; Newsham et al. 1995a, b). In this regard, St. Arnaud and Elsen (2005) have meticulously summarized the interaction outcomes of a number of AM fungi cultured under *in vitro* conditions in the presence of soil bacteria (Table 11.3), other soil fungi (Table 11.4), and nematodes (Table 11.5). This meta-analysis as to the impact of the AM fungi on soil-borne pathogens, and *vice versa*, suggests that their interaction can be highly variable (i.e., having positive, neutral, or negative outcomes under experiment conditions); however, the AM fungi are still widely recognized for benefiting plant growth in relation to a number of highly persistent and destructive pathogens (Azcón-Aguilar and Barea 1996; Barea et al. 1998; Newsham et al. 1995a, b). Harrier and Watson (2003, 2004) as well as Mukerji and Ciancio (2007) have outlined a number of potential mechanisms (particularly in the context of integrated pest and disease management) in which AM fungi can interact with such soil microorganisms within the proximal growth environment to then enhance plant stress tolerance, which include: the competitive exclusion (or mechanical inhibition) of nonbeneficial soil microorganisms by competing for sites of infection and (or) colonization; the subsequent alteration of root architecture and anatomical structure due to AM colonization; the activation of plant defense responses such as antibiotics and phytoalexins due to AM–plant signaling; the modification of soil physicochemical parameters such as soil pH and nutrient bioavailability as well as alteration in carbon partitioning (or rhizo-deposition); and, finally, the increase in damage compensation due to an enhanced nutrient status. Although some of these perspectives have been described only briefly in this sections, it is evident that such mycorrhizospheric processes leading to the development of a beneficial belowground species biodiversity profile can then indirectly influence the composition of aboveground species biodiversity. Likewise, further experimental investigation to better elucidate specific mechanisms of interaction would certainly highlight the impact of AM fungi toward plant stress tolerance in regards to biotic stress and other biodiversity interactions.

4 Conclusions and Future Perspective

4.1 Assessing AM–Plant Interactions in Plant Stress Tolerance

In this chapter, it has been reported how the AM symbiosis is a widespread ecological association which is deeply rooted in the essential function of the vast majority of herbaceous plant species, as well as the function of soils and associated soil microorganisms. By describing some of the mechanisms underlying these plant physiological and soil ecological functions, it is clear that a number of such AM fungal processes are fundamentally involved in enhancing the stress tolerance of

Table 11.3 Interactions between AM fungi and bacteria (from St. Arnaud and Elsen 2005)

Bacteria	AM fungi	Interaction outcome[a]	Reference
Azospirillum brasilense	*Glomus intraradices*	N	Hildebrandt et al. (2002)
Bacillus chitinosporus; B. pabuli and other spore-associated bacteria	*G. clarum*	P, n,N	Xavier and Germida (2003)
Clavibacter michiganensis ssp. michiganensis	*G. intraradices*	N	Filion et al. (1999)
Corynebacterium sp.	*G. versiforme*	P	Mayo et al. (1986)
Escherichia coli	*G. intraradices*	N	Hildebrandt et al. (2002)
Paenibacillus validus	*G. intraradices*	P	Hildebrandt et al. (2002)
Pseudomonas sp.	*Endogone sp.*	P	Mosse (1962)
Pseudomonas sp.	*G. versiforme*	P	Mayo et al. (1986)
P. aeruginosa	*G. intraradices*	P	Villegas and Fortin (2001, 2002)
P. chlororaphis	*G. intraradices*	P	Filion et al. (1999)
P. fluorescens	*Gigaspora margarita*	P	Bianciotto et al. (1996a, b)
P. putida	*G. intraradices*	P, n	Villegas and Fortin (2001, 2002)
Rhizobium leguminosarum	*Gi. margarita*	N	Bianciotto et al. (1996a, b)
Serratia plymutica	*G. intraradices*	P	Villegas and Fortin (2001, 2002)
Streptomyces avermitilis	*G. mosseae*	N	Tylka et al. (1991)
S. griseus	*Scutellospora heterogama*	P	Tylka et al. (1991)
S. orientalis	*Gi. margarita*	P	Tylka et al. (1991)
S. orientalis	*G. mosseae*	P	Mugnier and Mosse (1987) and Tylka et al. (1991)
S. orientalis	*S. heterogama*	P, N	Tylka et al. (1991)
Spore-associated bacteria	*G. versiforme*	P	Tylka et al. (1991)
Unidentified soil bacteria	*G. mosseae*	P	Azcón (1987, 1989)

P Positive, *N* negative, *n* neutral
[a]Refers to the impact of the bacterial species toward the AM fungus (e.g., promotion of spore germination and hyphal growth)

plants and bolstering the resilience of soil in relation to a number of abiotic environmental stressors due especially to the dynamic function of the mycorrhizosphere. When taking into account the significant allocation of plant carbohydrates required for the development and maintenance of an expansive and prolific mycorrhizospheric infrastructure, it has been suggested that the AM symbiosis should represent an extrinsic plant stress tolerance strategy which could inherently complement other intrinsic plant stress tolerance strategies. To further depict the roles of the mycorrhizosphere in ecosystem function, the present description of the AM symbiosis has distinguished between the direct vs. indirect dynamics of interaction. Accordingly, this distinction refers to AM-induced activities which either directly benefit host plant due to intimately co-modulated pathways or, alternatively, activities which indirectly benefit the symbionts (potentially including non-associated species) due to their effects in buffering the proximal growth environment. This perspective is intriguing since it presumes that a number of essential mycorrhizospheric processes could occur simultaneously and (or) independently, and thereby have fundamental ecological functions across different trophic levels. This could account, in part, for the widespread abundance and distribution of mycorrhizal associations within the majority of terrestrial ecosystems, especially those subjected to highly stressful and (or) extreme environments. Still, there are so far very few modeling strategies depicting these combined, multilateral effects especially across a broad or continuous spectrum of stress (e.g., from trace to toxicity conditions or from droughted to amply water conditions). For instance, the case for AM–plant metal uptake is

Table 11.4 Interactions between AM fungi and other fungi (from St. Arnaud and Elsen 2005)

Fungus	AM fungi	Interaction outcome[a], P, n, N	Reference	Fungus	AM fungi	Interaction outcome	Reference
Alternaria alternate	*G. mosseae*	N	McAllister et al. (1996)	*Pyrenochaeta terrestris*	*Gi. margarita*	n	Chabot (1991)
Aspergillus fumigatus	*G. mosseae*	N	Calvet et al. (1992)	*Rhizoctonia solani*	*Gi. margarita*	n	Chabot (1991)
A. niger	*G. mosseae*	n	McAllister et al. (1996)	*Rhodotorula mucilaginosa*	*G. mosseae*	P	Francchia et al. (1998)
Bipolaris sorokiniana	*Gi. margarita*	P, n, N	Chabot (1991)	*Sclerotinia scletotiorum*	*Gi. margarita*	n	Chabot (1991)
Fusarium equiseti	*G. mosseae*	N	McAllister et al. (1996)	*Thievaliopsis basicola*	*Gi. margarita*	n	Chabot (1991)
F. oxysporum f. sp. Chrysanthemi	*G. intraradices*	N	Benhamou et al. (1994) and Filion et al. (1999)	*Trichoderma aureoviride*	*G. mosseae*	P	Calvet et al. (1992)
F.o. chrysanthemi	*G. intraradices*	P, N	St. Arnaud et al. (1996)	*T. harzianum*	*G. intraradices*	P	Filion et al. (1999)
F. solani	*Gi. margarita*	N	Chabot (1991)	*T. harzianum*	*G. mosseae*	P	Calvet et al. (1992)
F. solani	*G. mosseae*	P, n	McAllister et al. (1996)	*T. harzianum*	*G. mosseae*	n	Francchia et al. (1998)
Gaeumannomyces graminis	*Gi. margarita*	n	Chabot (1991)	*T. harzianum*	*G. intraradices*	N	Rousseay et al. (1996)
Gliocladium roseum	*G. mosseae*	n	Francchia et al. (1998)	*T. koningii*	*G. mosseae*	N, n	McAllister et al. (1996)
Ophiostoma ulmi	*Gi. margarita*	n	Chabot (1991)	*T. pseudokoningii*	*G. mosseae*	n	Francchia et al. (1998)
Paedilomyces farinosus	*G. mosseae*	P, n	Francchia et al. (1998)	*Verticillium albo-atrum*	*Gi. Margarita*	n	Chabot (1991)
Penicillium decumbens	*G. mosseae*	N	Calvet et al. (1992)	*V. dahliae*	*Gi. margarita*	n	Chabot (1991)
Phytophthora sp.	*Gi. margarita*	n	Chabot (1991)	*Wardomyces inflatus*	*G. mosseae*	N	Francchia et al. (1998)
P. nicotianae	*G. intraradices*	N	Lioussanna et al. (2003)	Unidentified soil fungi	*G. mosseae*	P	Azcón-Aguilar et al. (1986)
Pythium ultimum	*Gi. margarita*	n	Chabot (1991)				

P Positive, *N* negative, *n* neutral

[a]Refers to the impact of the bacterial species toward the AM fungus

Table 11.5 Interactions between AM fungi and nematodes (from St. Arnaud and Elsen 2005)

Nematode	AM fungi	Interaction outcome[a]	Reference
Globodera pallida	*Glomus sp.*	P	Ryan et al. (2000)
Radopholus similis	*G. intraradices*	N	Elsen et al. (2001)
Pratylenchus coffeae	*G. intraradices*	N	Elsen et al. (2003)

P Positive, *N* negative, *n* neutral
[a]Refers to the impact of the bacterial species toward the AM fungus

noteworthy since the AM fungi apparently hold two antithetical roles in plant metal uptake (i.e., enhanced uptake vs. metal biosorption) having different predicted outcomes depending on the soil metal conditions. From this viewpoint, a few lingering questions (among other) have yet to be fully addressed regarding the fundamental role(s) of the AM symbiosis in plant stress tolerance and ecosystem function, such as:

> How do these combined mycorrhizospheric processes benefit plant stress tolerance? Under what environmental conditions do they occur? How are they regulated by the host plants, or the AM fungi?

Considering the abundance of high-quality data available within the published literatures which were developed primarily using reductionist strategies, future investigations could benefit from more holistic approaches in order to bridge our current mechanistic understanding of mycorrhizospheric function with other known soil ecological processes to better depict the impact of the AM symbiosis in whole-ecosystem function; in other words, taking into account multitrophic interactions and making attempts to quantify such relationships at the physiological and ecological levels. Such an approach would highlight the interconnectedness of above- and belowground species, as well as proving beneficial in re-assessing the balance between the costs of maintaining the symbiosis vs. the benefits of association among the symbionts. As mentioned previously, this resource allocation balance has been critical for plant physiologists and mycologists alike in defining the symbiotic mutualism since it is widely believed to function along a continuum potentially ranging from parasitism to mutualisms (Bronstein 2001; Johnson et al. 1997; Jones and Smith 2004). And so, an assessment of plant–AM–soil interactions using quantifiable descriptors could provide an insightful analysis of this continuum of interaction.

Another potential topic of concern regards the quantification of the mycorrhizosphere in relation to plant *mycorrhizal dependence*. With exception to the characterization of AM–plant nutrient uptake and symbiotic transfer using molecular tools, we have yet to quantitatively define the mycorrhizosphere's and (or) hyphosphere's actual zones of influence (e.g., their capacity to bind metals or stabilize the soil structure) other than by indirect methodologies. Such a quantification of the mycorrhizosphere could help address issues such as:

> How prolific is the mycorrhizosphere compared to the rhizosphere? What are its biogeochemical properties and subsequent area of influence? How prolific is the mycorrhizosphere in relation to the plant's carbon allocation investment?

In turn, it may be feasible to quantitatively assess the mycorrhizal dependency (or resource allocation and extrinsic investment) of plants in relation to a wide-range stressors and broad-spectrum of environmental stress. Altogether, the potential focus-shift of future research objectives toward assessing the combined mycorrhizospheric impact in whole-ecosystem function as well as the quantification of the mycorrhizosphere in relation to plant symbiotic investment could highly benefit the effective integration of mycorrhizal technologies into agro-ecosystem management practices, especially sustainable agriculture and environmental remediation (Brussaard et al. 1997, 2007; Gosling et al. 2006; Jeffries et al. 2003; Mäder et al. 2002) – an intricate topic deserving of more lengthy discussion. Nevertheless, in the present context, it can be concluded that the AM fungi have a quintessential role in plant stress tolerance which could translate well toward enhancing the

stress tolerance and stress resistance of whole ecosystems, particularly in the current *era of climate change* and in relation to anthropogenically derived environmental stressors.

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MicroRNAs and Their Role in Plants During Abiotic Stresses

12

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Abstract

Abiotic stresses have been considered as the growth-limiting factors affecting plants. Nutrient deficiency, drought, salinity, cold, submergence, and hypoxia are some of the severe types of abiotic stresses. Interdisciplinary research has been carried out to find stress-regulating mechanisms. MicroRNAs (miRNAs) are the newly discovered, 18–24 nucleotides long molecule of the genome. They have been considered as the key players against plant stress. They have been identified in plants, animals, humans, and even microbes. miRNAs have been shown to regulate various stress-responsive genes, proteins and transcription factors, thus helping to counteract adverse conditions. Various stress-inducible miRNAs have been identified and well characterized. Most of these miRNAs have been conserved among plants. This conservative nature has become the basis of development of computational methods of miRNA identifications, in additional to the traditional cloning approach. Presence of computational strategy has further simplified the miRNA prediction. Using this approach various stress-responsive miRNAs have been predicted, annotated and functionally validated from cotton, grapes, rice, maize, and soyabean. This chapter reviews the expanding world of miRNAs, methods unveiling miRNAs from various organisms, and specifically stress-induced miRNAs.

Keywords

miRNA • ESTs • GSSs • Drought • Salt • Nutrient stress • Hypoxia • Submergence • Plant responses

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1 Introduction

Gene regulation is the basic and foremost phenomenon assisting the possible survival of multicellular organisms in the adverse environmental conditions and varying evolutionary changes. Gene regulatory mechanisms are highly complex, diverse, variously linked, and flexible (Szymanski et al. 2003). This regulatory mechanism has been found to work at various important levels of genome function like chromosome segregation, transcription, RNA processing, and translation (Carthew and Sontheimer 2009). It has been the area of intense research in the recent years and has lead to the discovery of new mechanism termed as RNA interference (RNAi). RNAi was initially studied in plants followed by fungi and was later studied in almost each and every eukaryotic organism. It has been considered as the conserved mechanism of gene regulation among all eukaryotes. Most important feature of RNAi is the presence of RNA as a signal molecule that triggers gene regulation. These signaling RNA molecules are actually 22–25 nucleotides (nts) long small RNAs (sRNAs) (Mao et al. 2009).

sRNAs came into scenario when the hidden facts regarding eukaryotic gene regulation were explored. These are actually a part of noncoding RNAs (ncRNAs). ncRNAs are characterized as the RNA molecules lacking significant open reading frames that code for RNAs, not proteins. Genes coding for ncRNAs have been explored and annotated from certain genomes. This suggests that ncRNAs have a role to play during the cellular development, physiology, and maintenance. But still a lot is to be discovered about ncRNAs and genes encoding them. It has been expected that with the generation of sRNA libraries, EST analysis and new algorithms, sooner or later more and more ncRNAs and respective genes will be revealed. Thus, the horizons of plant genomics will be widened (MacIntosh et al. 2001).

ncRNAs are variously classified depending upon their origin and functions, out of which sRNAs have been extensively studied (Erdmann et al. 2001; Kim 2005; Rymarquis et al. 2008). sRNAs differ from rest of the RNAs of genome because of their small length and capability to bind to the Argonaute (AGO) family proteins (Ghildiyal and Zamore 2009). It has been observed that sRNAs mostly repress the expression of the target genes and hence, the phenomenon is also defined as RNA silencing. They have a sequence-specific mode of action. They direct the final effector proteins, i.e., AGO proteins to the target molecules by the approach of base-pairing interactions (Carthew and Sontheimer 2009).

Several classes of sRNAs have emerged and a number of them have been identified and functionally characterized. On the basis of their origin, structure and functional identifications, three main categories of sRNAs have been identified: short interfering RNAs (siRNAs), microRNAs (miRNAs), and piwi-interacting RNAs (piRNAs). Out of these three, siRNAs and miRNAs have been broadly studied among plants whereas piRNAs, found in mammalian testes, are still under exploration. The piRNAs are reported to have single-stranded RNA precursors, while miRNAs and siRNAs have double-stranded RNA precursors (Carthew and Sontheimer 2009). Both of these sRNAs have been characterized as riboregulators. They have the ability to act on DNA as well as on RNA (Vaucheret 2006).

miRNAs are 20–22 nts and are more abundant in the plant system. They act posttranscriptionally through mRNA degradation or translational repression. Unlike miRNAs, siRNAs are 21–24 nts long and act at both transcriptional and post-transcriptional levels by carrying out DNA methylation, histone modification, and mRNA degradation (Sunkar and Zhu 2007). It has been mentioned that siRNAs and miRNAs have the tendency to adopt similar mechanism for gene regulation. But the kind of mechanism they adopt depends upon the level of complementarity between the target gene and sRNAs (Zeng et al. 2003). As far as their biogenesis is concerned, they differ on the basis of kind of precursors, mechanism of synthesis and maturation, formation of RNA-induced silencing complex (RISC) and finally mode of action on the target gene (Bartel 2004).

miRNAs are endogenous by origin. Genes encoding miRNAs are part of the host's genome and termed as miRNA genes. miRNA genes form a fold-back stem-loop structure which is around 10–100 nts long. This structure is known as pri-miRNAs. The double-stranded region created by the fold-back acts as a marker for Dicer-Like (DCL) enzyme, that cleaves pri-miRNAs and synthesizes mature miRNAs against the specific endogenous target mRNAs. Mature miRNAs bound to AGO proteins of RISC. This binding directs miRNAs toward the target mRNA, thus repressing the gene expression. siRNAs are endogenous as well as exogenous sRNAs. It has been found that siRNAs precursors are double-stranded RNAs but longer than miRNAs precursors. siRNAs precursors are again acted upon by certain unusual Dicer ribonuclease, giving rise to mature siRNAs (Zeng et al. 2003; Eckardt 2004).

It has been observed that among the known sRNAs, miRNAs have emerged as one of the important regulatory molecules of plant and animal system (Carrington and Ambros 2003; Bartel 2004). This very fact has initiated an era of evolutionary miRNA research in the scientific fraternity. It has lead to the identification and characterization of various miRNAs from plants as well as animals. This research, in turn, has enhanced the development of several new and novel experimental and computational approaches for miRNA prediction, isolation, and characterization. The miRNA study has revealed surprising facts related to genomics. It has also well played a part to understand metabolomics, proteomics, and stress biology. So, it can be concluded that miRNAs have initiated and well supported the era of interdisciplinary research.

2 miRNAs Isolation and Characterization

With the initiation of miRNA research, a lot of work has been carried out since then. Fifteen classes of miRNAs have been identified and well characterized from the model plant *Arabidopsis* (Bartel and Bartel 2003). Twenty miRNA families have been computationally predicted in case of *Oryza*, out of which 14 miRNAs have been experimentally isolated and characterized (Sunkar et al. 2005). Similarly, eight novel miRNAs have been isolated and characterized from *Medicago* (Szittya et al. 2008). miRNAs have been identified by cloning as well as computational approaches. The two strategies for miRNAs identification are:

2.1 In Silico Prediction and Characterization

The very first miRNAs were experimentally isolated and cloned by biochemical and genetic approaches (Lee et al. 1993). But in cases of low expression and tissue-specific miRNAs, cloning is not an appropriate approach. As a result, it has been replaced by the method of computational predictions and experimental validations (Zhang et al. 2006). Since then computational approaches have dominated the field of miRNAs research. The traditional computational strategy was based on the decoded genome sequences of certain model species. In that case only completely sequenced genomes could be analyzed for miRNAs prediction, leaving behind the species with unsequenced genomes (Zhang et al. 2005). Because of the inefficiency associated with the traditional method, presently a new approach has been developed. It has been reported that within same kingdom as well as sometimes in between different kingdoms, miRNAs show evolutionary conservations. It gave the idea that comparative genomics could be a powerful strategy to identify miRNAs. So, this conservative nature was employed to predict miRNAs (Zhang et al. 2007). Most recently, genomic survey sequences (GSSs) and expressed sequence tags (ESTs) have been adopted for mining novel, undiscovered miRNAs (Zhang et al. 2005).

Various steps involved in the in silico miRNAs prediction are: (1) miRNAs prediction using ESTs and GSSs analysis, (2) identification of potential miRNAs, and (2) miRNAs target identification.

2.1.1 miRNAs Prediction Using ESTs and GSSs Analysis

It has been known that ESTs are the cDNA sequences of the expressed genes. In case of organisms with incomplete genomic draft, ESTs have been considered as an alternative tool for gene discovery. Also, ESTs and expressed genes are actually obtained from true gene expression. Thus it justifies the use of ESTs to predict miRNAs for species with undiscovered genomes (Matukumalli et al. 2004; Zhang et al. 2005). National Center for Biotechnology Information (NCBI) contains 22,165,266 entries of ESTs from various organisms in its EST database (Boguski et al. 1993). ESTs could be obtained from EST database of NCBI, dbEST (http://www.ncbi.nlm.nih.gov/dbEST/). Initially, redundancy within the ESTs is removed by using software CAP3 (http://pbil.univlyon1.fr/cap3.php). This software presents the overlapping sequences as contigs and nonoverlapping sequences as singletons. The contigs are used for miRNAs prediction. These processed sequences, i.e., contigs are submitted to miRNA-finder (http://bioinfo3.noble.org/mirna/). This software predicts the possible miRNAs from the query sequence. In order to further remove the redundant and overlapping miRNAs from the predicted ones, a reference set of miRNAs is used. This reference miRNAs set belongs to any of the closely related organisms and can be obtained from miRNA registry database (http://miRNA.sanger.ac.uk) (Manila et al. 2009). A computational tool MicroHARVESTER (http://www.ab.informatik.uni tuebingen.de/bribane/tb/index/php) has been designed to search homology between the predicted miRNAs and previously detected miRNAs (Dezulian et al. 2006). While using GSSs, the complete sequence is used as miRNA precursor sequence. Overlapping as well as protein coding sequences is removed. The remaining nonprotein sequences are used for further proceedings (Sunkar and Jagadeeswaran 2008). The predicted miRNAs are then analyzed to determine the potential miRNAs among them.

2.1.2 Identification of Potential miRNAs

Potential miRNAs are predicted on the basis of data obtained from their secondary structures. For the predicted miRNAs or pre-miRNAs, secondary structure is determined by using software MFOLD 3.1 (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) (Mathews et al. 1999; Zuker 2003). In order to screen the miRNAs, following criteria are followed (Xie et al. 2007; Sunkar and Jagadeeswaran 2008):

1. Pre-miRNA sequences should fold into an appropriate stem-loop hairpin secondary structure that contains around 22 nts mature miRNA sequence within one arm of the hairpin.
2. Predicted mature miRNAs are allowed to have only 0–3 nts mismatch in comparison to the previously known plant mature miRNAs.
3. Predicted secondary structures should have higher negative minimal free energies (MFEs) and minimal free energy index (MFEIs) than the other different types of RNAs.
4. No loop or break is allowed in the miRNAs sequence.
5. miRNAs sequence should have 30–70% A + U content.
6. miRNAs should have no more than six mismatches with the opposite miRNA* sequence in the other arm.

miRNAs satisfying the following parameters are thus considered as the potential miRNAs.

2.1.3 miRNAs Target Prediction

It has been known that plant miRNAs have the tendency to bind to the protein coding regions of their mRNA targets. The binding of miRNA to target mRNA is based on the nature of complementarity between them, either perfect or nearly perfect (Xie et al. 2007). This reveals the usage of homology search to determine miRNA targets (Zhang et al. 2007). A software for miRNAs target prediction based upon above said approach is miRU2 (http://bioinfo3.noble.org/miRU2/). This tool has been applied for the prediction of several miRNA targets (Fig. 12.1) (Manila et al. 2009). Presently, computational approaches are being further explored to ease the process of novel

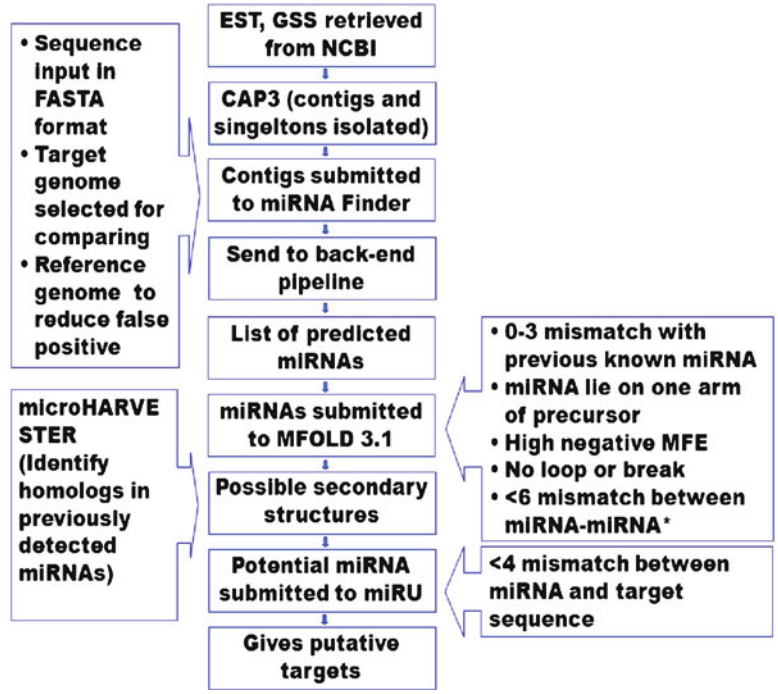

Fig. 12.1 Flowchart showing computational tools and related criteria to predict potent miRNAs

miRNAs identification. One of such modified computational approach is miRTRAP. miRTRAP stands for miRNA Tests for Read Analysis and Prediction. This tool simplifies the systemic and whole-genome prediction of miRNAs by making use of high-throughput sequencing data. This tool utilizes a system of binary decisions that are based on biochemical mechanisms behind miRNA biogenesis (Hendrix et al. 2010). This program has been able to predict 400 putative miRNAs loci from simplest chordate *Ciona intestinalis*. No doubt in silico prediction provides an efficient and easy method for miRNA prediction and target identification. For organisms with incomplete genomic draft, in silico methods offer a promising approach to study genetic regulations. But an important fact to note is that all the data obtained by in silico predictions require experimental validations.

2.2 sRNA Library Preparation and miRNA Characterization

The experimental approach of miRNAs identification has been termed as RNomics. It deals with the preparation of cDNA libraries of sRNAs (Huttenhofer et al. 2005). In order to develop sRNA libraries, a number of strategies have been adopted. Various commercial kits are available that specifically isolate sRNAs fraction from the sample. Other method is to isolate total RNA and then purification of sRNAs fraction from the large RNA fractions. sRNAs fraction is usually resolved on 15% polyacrylamide gel and then eluted. sRNAs range from 18 to 26 nts in length, as a result before cloning, adaptors are ligated to them. Before ligations, sRNAs are often polyadenylated to avoid circularization of linker RNAs (Devor et al. 2009). Ligation of adaptors on both

5′ and 3′ ends helps in the visualization of sRNAs in the further steps. Ligated product is reverse-transcribed using adaptor-specific primers. Resulting PCR product is used for cloning purpose and cloned into the desired vector (Sunkar et al. 2005). Prepared sRNA clones are then sequenced.

Sequencing of clones using BLAST analysis against Genbank genome assemblies helps to determine its nature, whether it is a transposon, any degraded product or already known noncoding RNAs except miRNAs/siRNAs. Candidates with perfect matches against genome sets are further used for fold-back secondary structure predictions using MFOLD 3.1 software (Zuker 2003). sRNA sequences are folded with flanking sequences in five contexts: (1) 300 bp upstream and 20 bp downstream, (2) 150 bp upstream and 20 bp downstream; (3) 150 bp upstream and 150 downstream, (4) 20 bp upstream and 150 downstream, and (5) 20 bp upstream and 300 bp downstream (Lu et al. 2005). Candidate clones satisfying all the criteria are considered as potential miRNAs. Further target for these miRNAs is determined using software miRU2. sRNAs isolation and cloning is a laborious job. Also it does not always ensure that each clone is a miRNA. As a result, in silico prediction of miRNAs and their experimental validation outshine the above technique.

3 miRNAs Annotation Criteria

With the increasing rate of sRNA discovery, there is an increased requirement for miRNA annotations. A specific criterion has been managed for the purpose of annotations that include experimental as well as computational data. It has been considered that any sRNA should fulfill two conditions to be recognized as miRNA. These two conditions are expression and biogenesis criteria. Where expression criteria stands for the identification of miRNA by cloning or hybridization; biogenesis criteria includes folding of predicted precursors into a defined stem-loop hairpin structure, phylogenetic miRNA conservation, and increased precursor accumulation on decreased Dicer activity (Ambros et al. 2003). With the development of more sophisticated techniques for miRNAs prediction, it has been observed that some miRNAs followed the criteria but some not. This has led to the generation of new annotation parameters. These parameters are divided as: primary criteria and ancillary criteria.

Primary criterion defines the fundamental features of miRNAs. It states that biogenesis of around 21 nts miRNA/miRNA* duplex should occur by an excision of a qualifying stem-loop precursor. These criteria have been considered as the most necessary and sufficient parameters for miRNAs annotation. In order to fulfill this parameter, both miRNA as well as miRNA* should satisfy the conditions. In case of miRNA*-deficient clones, the potential miRNA candidate should be isolated and sequenced from multiple, independent libraries. During sequencing, often very low abundance of one or two sequencing reads of putative miRNA is obtained. In such cases, even RNA gel blots fail to satisfy the qualifying conditions of primary criteria. It is so because detection of sRNAs via blotting is unable to determine whether it is a miRNA, siRNA, or a decay product of larger precursors. To help out in such cases low depth sequencing and extensive blot analysis using multiple probes is utilized.

In addition to primary criteria, certain other features have also been determined for miRNAs annotation. However, satisfaction of ancillary parameters is not essentially required but fulfilling these features would further enhance the significance of miRNAs annotation. Ancillary criteria include miRNAs conservation, their targets, Dicer-like 1 (DCL1) dependence, RNA Dependent RNA polymerases (RDRs) independence. Conservation of stem-loop secondary structure and mature miRNA sequence among lineages is a sufficient proof for miRNAs annotation. Target prediction is not a necessary miRNAs annotation. There could be cases where conserved miRNAs are target-less or nonconserved miRNAs have well characterized targets. So, in both cases, functional characterization of miRNAs is not required for its annotation. Plants require DCL1 enzyme for miRNAs generation but not in all cases. The *dcl1* mutant has also been reported

to possess miRNAs. So, DCL1 dependence cannot be a strict annotation requirement. Similar is with RDRs. RDRs generate dsRNA precursors. These precursors lead to siRNAs biogenesis and hence, RDR dependence is an essential feature of siRNAs. But miRNAs are not synthesized from RDR-generated dsRNA molecules. As a result, RDR independence should not be a necessary condition for miRNAs annotation (Meyers et al. 2008). This can be summed up as: the primary criterion, but not the ancillary, is a kind of eligibility for sRNAs to be annotated as miRNAs.

4 miRNAs Nomenclature Parameters

The process on submission of novel miRNAs to miRBase registry requires miRNAs to be well named and numbered. Certain rules have been formulated to ensure proper nomenclature of miRNAs (Griffiths-Jones et al. 2008; Meyers et al. 2008). The rules are discussed as follows:

1. A three-letter prefix "miR" is written in every miRNAs name. This is followed by a number denoting the order in which they were discovered, e.g., miR1, miR2. In an organism, miRNA genes are named as "mir," e.g., mir1, mir2 in animals and MIR1, MIR2 in plants. Identical miRNAs are given the same numerical designations, no matter from which organism they were isolated.
2. Similar or identical miRNAs, originating from a common locus should be assigned that number followed by a sequential alphabetical suffix, e.g., MIR172a, MIR172b.
3. miRNA is assigned a three-letter prefix that specifies the genus and species-generating miRNA, e.g., miRNA from *Brassica napus* designated as bna-miR167a, bna-miR167b.
4. In some cases, miRNA genes encode similar miRNAs, that for certain historical reasons have been assigned distinct identifiers. They are thus grouped under same families, e.g., miR156/157, miR170/171 families.
5. While naming miRNAs derived from the same arm of stem-loop, miRNA designations should also consider the number of mismatches among the miRNAs. Mismatches from zero to four are considered as typical and are acceptable up to four.
6. miRNAs derived from same sequence but having different targets can be classified under different families.
7. In case miRNAs originating in equal proportion from same miRNA/miRNA* duplex, suffixes 5p and 3p are used. 5p and 3p signify the end, either 5′ or 3′, giving rise to the miRNA sequences.

5 Role of miRNAs in Abiotic Stress

Abiotic stresses have already been known as the primary plant growth-limiting factors. Abiotic stress is a broad term including various physiochemical stresses like salinity, drought, temperature, and oxidative stress (Vinocur and Altman 2005). These stresses have been reported to reduce the average yield of most of the crops by 50% (Tuteja and Sopory 2008). It has been observed that plants possess the ability of monitoring fluctuations occurring around its niche and have the inbuilt capacity to respond against them. Plants survive in unfavorable stress conditions by carrying out variations at their physiological and genetic grounds. Various genes, proteins, transcription factors, DNA histone-modifying enzymes, and several metabolites have been reported responsible for plant stress tolerance (Cushman and Bohnert 2000; Vinocur and Altman 2005; Chinnusamy and Zhu 2009). Now-a-days, molecular studies are being done to decode the mechanism by which plants cope with these adverse conditions. These studies have unveiled that sRNAs, mostly miRNAs present within the plant system, have the ability of regulating stress-responsive factors (Sunkar and Zhu 2004). It has been reported that miRNAs either upregulate or downregulate in response to stress. Various miRNAs specifically responding to abiotic stresses have been reported from various plants as *Arabidopsis*, *Oryza*, *Nicotiana*, *Brassica*, *Gossypium*, etc. Role of several miRNAs in various kind of stresses are discussed.

5.1 miRNAs in Response to Nutrient Stress

Plants often grow in nutrient-deprived soils, where amount of macronutrients is less than the required concentration. In such cases, plants sense the internal and external mineral ion concentration and adapt in response to the nutrient deficiency. Various miRNAs have been reported to act during nutrient stress.

5.1.1 Phosphorus Starvation

Phosphorus is the structural unit behind nucleic acids, cellular membranes and energy currency ATP (Chiou 2007). Phosphorus is taken via plant roots in the form of inorganic phosphate [Pi(HPO_4^{2-})] (Marschner 1995; Schachtman et al. 1998). It has been found that external phosphorus concentration is 100–1,000 times lower than internal concentration (Marschner 1995). It has lead to the development of adaptive responses by plants to maintain phosphate homeostasis. With the discovery of miR395 involvement during sulfate stress, it has been considered that miRNAs do play a role in nutrient starvation. So, afterwards miR399 was reported to act specifically in response to Pi deprivation (Fujii et al. 2005; Bari et al. 2006). Affymetrix GeneChip analysis in *Arabidopsis* has revealed that At2g33770 is the target gene of miR399. miR399 was reported to target multiple sites on the 5′ untranslated regions (UTRs) of At2g33770 mRNA encoding a ubiquitin-conjugating E2 enzyme AtUBC24 (Sunkar and Zhu 2004; Kraft et al. 2005). It was reported that during low Pi concentration, miR399 was highly expressed with lower accumulation of UBC24 mRNA transcript and vice versa during higher Pi content. Thus, it is demonstrating a direct relation between E2 and miR399.

Role of miR399 was further confirmed by generating transgenic *Arabidopsis* overexpressing miR399. The transgenics showed an enhanced expression of miR399 both in decreased and increased Pi levels. Elevated levels of miR399 accumulation suggested a successful processing and expression of precursor RNAs. Further a detectable loss in the expression of E2 RNA was observed. The transgenics experimentally validated that miR399 down regulated the E2 mRNA levels (Chiou et al. 2006). The same study proved that overaccumulation of miR399 was responsible for the increased uptake of Pi. In wild plants, Pi normally constitutes 0.2% of plant dry matter (Schachtman et al. 1998). While in transgenics overexpressing miR399, Pi level was reported to increase up to 1.8–2.0%. The plants also showed symptoms of Pi toxicity like chlorosis, necrosis (Delhaize and Randall 1995; Shane et al. 2004). It was further reported that overaccumulation of Pi in transgenics was due to the increased uptake of Pi (Chiou et al. 2006).

5.1.2 Sulfur Deficiency

Cysteine and methionine are two important sulfur-containing amino acids. Animals and humans cannot synthesize them, so they are essential part of their diets. In plants, sulfur is a component of important anti-cancerous secondary metabolites (Talalay and Fahey 2001). Sulfur concentration keeps changing; as a result plants initiate their sulfate transport system for an enhanced uptake of sulfur. Sulfur is taken by the plants in the form of inorganic sulfate (Nikiforova et al. 2006). It has been reported that miR395 has a role in sulfur starvation (Kawashima et al. 2009). miR395 has been observed to accumulate in sulfur deficient conditions. It has been found to regulate the activities of low-affinity sulfate transporter (SULTR2;1) and ATP sulphurylases (APS1, APS3 and APS4) (Jones-Rhoades and Bartel 2004; Allen et al. 2005). ATP sulphurylases are the key enzymes of sulfate assimilation pathway and SULTR2;1 is a transporter helping sulfate translocation from roots to shoots (Takahashi et al. 2000; Allen et al. 2004; Jones-Rhoades and Bartel 2004). miR395-mediated cleavage of APS1, APS4, and SULTR2;1 has been reported both in roots and leaves whereas for APS3 in the leaves only (Kawashima et al. 2009). While studying the relationship between miR395 and ATP sulphurylases, a negative correlation has been determined (Jones-Rhoades and Bartel 2004); whereas for SULTR2;1 contradictory results were obtained. It was found that in sulfate-deficient conditions, in leaves with miR395 upregulation, SULTR2;1 mRNA was downregulated. But at the same time, roots

showed upregulation both in the levels of miR395 as well as SULTR2;1. Later, it was found that a transcription factor Sulfur Limitation 1 (SLIM1) was actually responsible for the induction of miR395. This was further experimentally validated by carrying out miR395 and SULTR2;1 mRNA expression analysis in *slim1* mutants (Kawashima et al. 2009).

5.1.3 Copper Maintenance

Copper is an essential micronutrient required for photosynthesis, ethylene perception, respiratory electron transport, and oxidative stress protection (Marschner 1995). Plant proteins requiring copper as an important cofactor are plastocyanin, laccases, and intracellular proteins formed by Cu, Zn-superoxide dismutase (SOD). Plastocyanin is an essential protein of electron transport chain, laccases are required for wound healing, lignin synthesis, stress responsiveness and Cu, Zn-SOD for regulating reactive oxygen species (ROS) (Abdel-Ghany and Pilon 2008).

It has been reported that CSD1, CSD2, and CSD3 are three Cu, Zn SOD encoding genes, out of which CSD1 and CSD2 have been considered as two important isoforms. Copper deficiency has been shown to downregulate the expression of CSD1 and CSD2. Later it has been proved that a miR398 is responsible for their downregulation (Yamasaki et al. 2007). It has been observed that copper deficiency causes upregulation of miR398 that resulted in the reduced expression of above two genes. This also gives a view of miR398 role toward oxidative stress. It has been found that oxidative stresses causes transcriptional downregulation of miR398. This downregulation is responsible for posttranscriptional CSD1 and CSD2 mRNA accumulation, thus providing tolerance to oxidative stress. By generating CSD2 overexpression *Arabidopsis* transgenics, it has been proved that plants neglecting miR398-mediated suppression of CSD2 are more tolerant to high light, heavy metals, and oxidative stress (Sunkar et al. 2006).

Further experimentation has shown that three more miRNAs, miR397, miR408, and miR857 are accumulated during copper starvation. Computational analysis has revealed that these miRNAs target laccase and plastocyanin mRNAs (Sunkar et al. 2005; Yamasaki et al. 2007). Cleavage site analysis has experimentally validated the computational predictions. Expression analysis data of miR397, miR408 and miR857 in presence of varying copper concentrations have evidenced an inverse relationship between these miRNAs and their target genes. It has been considered that miRNA-mediated downregulation of the respective genes offer a significant mechanism of copper homeostasis (Abdel-Ghany and Pilon 2008).

5.2 miRNAs in Response to Cold and Related Stresses

Cold stress is a house of other secondary stresses. Cold stress refers to variation in temperature ranges causing freezing ($T<0$°C) and chilling ($T<20$°C). This stress itself causes direct damage to the genetic potential of plant by causing inhibition of plant secondary metabolism. In addition, cold stress is responsible for causing further damage due to osmotic, oxidative, and other kind of abiotic stresses (Chinnusamy et al. 2007). Microanalysis has revealed that 17% of cold upregulated genes code for transcription factors. On the contrary, only 7% of cold downregulated genes code for transcription regulators. This has given a view that plants undergo cold acclimatization via post-transcriptional downregulation of genes. miRNAs have been observed as an efficient genetic regulators, so they might have a role to play in cold stress. Later, it was hypothesized that the miRNAs targeting growth and development responsive genes might be cold-responsive. Various miRNAs have been found responsive to cold and its related stresses. It has been reported that miR393, miR397b, and miR402 are upregulated in response to cold, ABA, dehydration, and salt stress; whereas miR389a.1 is downregulated under similar set of conditions. The cold-specific upregulation has also been observed in case of miR319c. Oxidative stress-responsive miR393 has been found to be upregulated during cold stress. miR393 targets E3 ubiquitin ligase mRNA causing their cleavage during cold acclimatization.

An F-Box protein similar to glucose repression resistance 1 (GRR1) has been known to undergo miR393-mediated cleavage (Sunkar and Zhu 2004). Sugar has been considered as a signaling molecule regulating the growth and developmental processes during various stresses. miRNA-mediated targeting of sugar-related gene suggests that miR393 has the ability of integrating sugar signaling with cold and other related stresses (Sunkar et al. 2007). Most recently, 18 novel cold-responsive miRNA families have been reported from rice. Most of these have been shown to undergo downregulation. Out of these, three families have been characterized as miR167, miR319, and miR171. miR167 and miR319 have been observed to show downregulation in their expression pattern, whereas miR319 has shown variable-expression profiles (Lv et al. 2010).

5.3 miRNAs in Response to Mechanical Stress

Plants have various stress-sensing mechanisms. It has been observed that plants undergo certain developmental changes in xylem and cambium to increase its mechanical support. Two types of woody tissues, tension wood and opposite woods, synthesized in response to stress are termed as reaction woods (Wu et al. 2000). This long-term deposition of supporting woody tissues is one of the most critical plant defense mechanism against mechanical stress. In order to have a better understanding of plant developmental changes, miRNA networks ruling xylem tissues were explored in one of the species of *Populus* (Lu et al. 2005). Experimental analysis has revealed the presence of 21 novel miRNAs in the developing xylem and phloem tissues. Presence of miRNAs suggests their role in cambium differentiation activities. The cloned miRNAs were designated as miR156, miR472, miR160, miR164, miR171, miR473, miR477, miR478, miR479, and miR480. A comparative expression analysis of miRNAs was carried out in tension-stressed and normal-developing xylem tissues. A detectable difference was observed in the expression patterns of most of the miRNAs. Depending upon the expression patterns, miRNAs were categorized as:

1. Type A miRNAs: suppressed by tension and compression stresses. This type includes miR156, miR162, miR164, miR475, miR480, and miR481.
2. Type B miRNAs: upregulated in tension and compression stresses. This type includes miR408.
3. Type C miRNAs: upregulated specifically in compression tissues. This type includes miR159, miR476, and miR479.
4. Type D miRNAs: suppressed only in compression tissues. This type includes miR160 and miR172.
5. Type E miRNAs: attenuated induction specifically by tension stress. This comprises of miR168.

It has been reported that type A and B miRNAs have the ability of counteracting against overall mechanical stress without differentiating between compression and tension stresses. Type C, D, and E miRNAs have been considered responsible for the specialized regulatory mechanisms behind development of reaction woods (Lu et al. 2005).

5.4 miRNAs in Response to Salt Stress

Salt stress has been considered as one of the most severe abiotic stress. Plants have the ability of altering their genetic profile in order to counteract against salinity. miRNAs microarray analysis has revealed the presence of 98 miRNAs belonging to 27 miRNA families during varying salt treatments (Ding et al. 2009). It has been observed that most of the miRNAs responding to salt stress are directly involved in the regulation of transcription factors (TFs). From *Zea mays*, miR159a/b, miR164a/b/c/d and miR1661/m have been cloned that targets TFs Myb, NAC1 and homeodomain-leucine zipper protein (HD-ZIP) (Jones-Rhoades and Bartel 2004). Other salt-responsive TFs targeted by miRNAs were MADS-box proteins and zinc-finger proteins (Fang et al. 2006). Further experimentation has lead to the cloning of miR474,

miR395, and miR396 family miRNAs from *Zea mays*. miR474 and miR395 were reported to target negative regulators of salt tolerance. They were upregulated during salt stress, causing suppression of the respective factors. On the contrary, miR396 was reported to downregulate in presence of salt stress.

It has been observed that during salt stress miR395, miR474 initiated a salt-induced nonspecific pathway for the maintenance of continuous energy supply. Both of these miRNAs were reported to upregulate in salt-stressed plants, thus targeting the key enzymes of plant energy transduction pathways, NADP-ME and NAD-ME (Cheng and Long 2007; Ding et al. 2009). It has already been known that salt stress causes the excess accumulation of ROS in plants. It has been experimentally validated that miR528 from *Oryza sativa* targets Cu/Zn SOD, causing its upregulation and scavenging ROS (Kim et al. 2007). Another miRNA family, miR169 has been known to be induced by high salinity. It causes cleavage of CCAAT box-binding transcription factor and thus plays role in transcriptional regulation of large number of genes (Zhao et al. 2009).

5.5 miRNAs in Response to Drought Stress

Drought stress is considered as a moderate loss of water content. This stress causes extensive water loss, stomatal closure, hampered gas exchange, disruption of cellular metabolism, and leads to death in severe cases (Jaleel et al. 2009). Genome-wide miRNA profiling has revealed 30 drought-responsive miRNAs from *Oryza sativa*. Out of these, 16 miRNAs namely miR156, miR159, miR168, miR170, miR171, miR172, miR319, miR396, miR397, miR408, miR529, miR896, miR1030, miR1035, miR1050, miR1088, and miR1126 were downregulated in response to drought stress. The remaining miRNAs miR159, miR169, miR171, miR319, miR395, miR474, miR845, miR851, miR854, miR896, miR901, miR903, miR1026, and miR1125 were significantly upregulated during drought stress. It has been considered that these cloned miRNAs are actually responsible for the physiological and developmental changes occurring during drought stress. miR854 and miR170 have been confirmed as novel drought-induced miRNAs from rice. Rest of the miRNAs requires experimental verifications (Zhou et al. 2010).

5.6 miRNAs in Response to Hypoxia

Hypoxia is a state of reduced availability of oxygen. But it should not be confused with flooding or water logging. Till date very less is known about hypoxia and its responsive signaling cascade. Recently, a study has been carried out to predict the role of TFs and miRNAs in response to hypoxia. More than 1,900 TFs and 180 miRNAs primary transcripts from *Arabidopsis* roots have been exposed to hypoxic conditions via quantitative PCR. It has been found that TFs do play a significant role to regulate the expression of hypoxia-induced genes, whereas only single miRNA, miR391 showed a potent activity against hypoxia. Thus, it has been concluded that TFs are the potent transcriptional regulators of hypoxia and miRNAs are a minor players (Licausi et al. 2010).

5.7 miRNAs in Response to Submergence

Submergence of plants or flooding of soil causes adverse anaerobic conditions. Plants carry out several morphological, physiological, and metabolic changes to survive the adversity but the underlying mechanism remains unknown. It has been thought that miRNAs might have submergence-responsive roles. Microarray analysis has resulted into the prediction of 39 submergence-responsive miRNAs. It has been found that these miRNA-targeted enzymes are involved in carbohydrate and energy metabolism, ROS elimination pathways. Most of the targeted mRNAs possessed *cis*-acting elements that are considered as an essential requirement for anaerobic responses (Zhang et al. 2008).

6 Conclusion and Future Perspective

In the recent times of scientific research, efficient work has been done in the direction of exploring miRNAs, their mechanism of action and applications. But many questions still remain unanswered. miRNAs not only influence plants but humans, animals and microbes as well. It was a microbe from where miRNAs were first isolated. miRNAs have a broad area of influence. Talking about plants, miRNAs play an effective role to maintain plant's growth, development, stress tolerance and cellular integrity. It has been well known that miRNAs either upregulate or downregulate to influence the expression of genes. miRNAs help plants to survive the stressful conditions. An important fact to notice is that from the beginning of miRNAs research till date, scientists are still keenly involved in discovering more and more sRNAs and finding their various applications to help plant maintenance. Development of overexpression transgenics and knockout mutants has further expanded the knowledge bank. The field of miRNA research is blooming and is going to grow in the years to come. Discoveries of many more miRNAs and invention of technologies using these wonder molecules will help to fight adversity. Thus miRNAs can be rightly pronounced as "*the miracle of genome*."

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Transcription Factors Involved in Environmental Stress Responses in Plants

13

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Abstract

Environmental stresses such as drought, high salinity, high temperature, and cold stresses are the major factors which cause significant losses to the yields of economically important crops. Sequence-specific binding transcription factors (TFs) are known to be involved in regulation of stress responses. In this chapter, we review our current knowledge about the functions of various TFs in stress responses, including the major DREB/CBF, AREB/ABF, and HSF TFs, and their signaling cascades. Understanding the mechanisms of how the TFs act as "master regulators" in the signal transduction networks involved in the conversion of stress signal perception to stress-responsive gene expression will enable us to develop stress-tolerant crops by genetic engineering of the major TFs.

Keywords

Drought • Salinity • Cold, DREB • Transcription factors • Heat shock proteins, plant responses

1 Introduction

Environmental stresses such as drought, salinity, and extreme temperatures adversely affect the growth and production of plants, and have been considered as major stressors because of severe desertification worldwide (Vinocur and Altman 2005). It has been reported that abiotic stresses may cause more than 40% yield loss for most of the major crop plants (Bray et al. 2000; Manavalan et al. 2009; Tran and Mochida 2010; Hadiarto and Tran 2011). Over the last several decades, botanists have been seeking various approaches including traditional breeding, promotion of cultivation environment, and adoption of rational cropping system to reduce the crop loss caused by these stresses.

However, conventional methods are known to face with difficulties to improve such complex

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stress-related traits which are considered to be controlled by quantitative loci. For example, breeding strategies based on genetic variants, natural mutations, and intergeneric crosses often meet with the difficulties in eliminating undesired linkage drag (Rommens et al. 2007). In contrast with traditional breeding, genetic engineering takes an advantage in connecting the tolerance trait directly to determinant gene loci by manipulating expression level of key stress-associated genes (Ashraf 2010). Recent advances in functional genomics have enabled biotechnologists to identify and characterize many stress-responsive functional and regulatory genes which are key components in abiotic stress signaling pathways in various plant species (Chinnusamy et al. 2007; Nakashima et al. 2009). Among the regulatory proteins, transcription factors (TFs) have been known to play crucial roles in signal transduction by receiving the upstream signal and activating the expression of downstream stress-inducible genes.

In this chapter, we focus on the regulation of stress-responsive TFs under various abiotic stresses and summarize the recent advances in enhancing stress tolerance of plants by genetic engineering using TFs.

2 DREB1/CBF TFs and Cold Stress Response

Cold stress adversely affects all aspects of cellular function. The DREB1/CBF regulon is one of the most important regulatory systems at transcriptional level in cold signal transduction in plants. The DREB1/CBF TFs, which specifically bind to the dehydration-responsive *cis*-element (DRE), belong to the AP2_EREBP family that is unique to plants (Nakashima et al. 2009). The DREB1/CBF TFs have been the most extensively studied TFs in the last 20 years aimed at improving plant tolerance to various abiotic stresses, including cold stress (Thomashow 2001; Shinozaki et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2006; Nakashima et al. 2009). It has been shown that *DREB1* genes are promising candidates for development of stress-tolerant plants by manipulation at transcriptional level (Shinozaki and Yamaguchi-Shinozak 2000; Thomashow 2001). Among the *DREB1* genes, the *DREB1B/CBF1* and *DREB1A/CBF3* genes were first isolated using yeast one-hybrid screening by two independent research groups (Stockinger et al. 1997; Liu et al. 1998). These two proteins specifically recognize and bind to the DRE motif containing the core sequence A/GCCGAC located in the promoters of cold and water stress-inducible genes (Yamaguchi-Shinozaki and Shinozaki 1994; Baker et al. 1994; Liu et al. 1998). In cold signal transduction pathway, the *Arabidopsis* DREB1A/CBF3, regulated positively by the Inducer of C-repeat binding factor Expression 1 (ICE1) and negatively by the MYB15, functions as a switch to control expressions of downstream *COR* genes (Chinnusamy et al. 2007). In addition to ICE1 and MYB15, members of the calmodulin binding protein transcriptional activator (CAMTA) family target directly the CM2 motif found in the promoters of the *CBFs*, and CAMTA3 is a positive regulator of *DREB1C/CBF2* expression, which provides evidence of a link between calcium signaling and CBF-dependent cold accumulation (Doherty et al. 2009).

The *Arabidopsis* genome contains six *DREB1/CBF* genes (Sakuma et al. 2002), including the well-studied *DREB1B/CBF1*, *DREB1A/CBF3*, and *DREB1C/CBF2*, which lie in tandem on chromosome 4 and are rapidly induced by cold and slightly by high-salinity stresses but not by dehydration (Liu et al. 1998; Gilmour et al. 2000; Nakashima et al. 2000). It should be noticed that although the transcripts of *DREB1s/CBFs* were specifically induced by cold stress, the majority of their downstream genes respond not only to cold stress but also to dehydration and high salinity. The enhanced tolerance to drought, high salinity, and freezing stresses was observed in transgenic *Arabidopsis* plants overexpressing *DREB1B/CBF1* or *DREB1A/CBF3* under control of the cauliflower mosaic virus (CaMV) 35S promoter (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000), indicating that DREB1s/CBFs target multiple genes which respond to various stresses. Genomewide analyses of the *DREB1A* transgenic *Arabidopsis* plants using both cDNA and GeneChip microarrays

identified more than 40 target genes of DREB1A/CBF3, such as *RD29A*, *Cor15B*, *Kin1*, *Kin2*, *RD17*, *LEA14*, *AtGolS3,* and *STZ/ZAT10* (Seki et al. 2002; Fowler and Thomashow 2002; Maruyama et al. 2004). These target genes contain DRE or DRE-related core sequences in their promoter regions, suggesting that the DREB1A/CBF3 TF could bind to these motifs and directly activate expressions of these genes in plants (Maruyama et al. 2004). The majority of the target genes of DREB1A/CBF3 encode TFs, phospholipase C, RNA-binding proteins, sugar transport proteins, desaturase, LEA proteins, osmoprotectant biosynthetic proteins, which are known to play important roles in plant acclimation to stresses. However, constitutive overexpression of *DREB1/CBF* genes adversely affects plant growth, leading to severe growth retardation even under optimal conditions (Liu et al. 1998; Kasuga et al. 1999). To overcome the negative impact of DREB1 TFs on growth and development, Kasuga et al. (1999) used the stress-inducible *RD29A* promoter instead of the constitutive 35S promoter. With this strategy, the *DREB1/CBF* genes have been successfully used to promote the tolerance in a number of transgenic plants to various abiotic stresses (Kasuga et al. 1999, 2004). The potential application of *DREB1/CBF* genes in genetic engineering of stress-tolerant plants has led to isolation and functional studies of DREB1/CBF orthologs in many plant species such as rice, maize, *Brassica napus*, tomato, wheat, rye, oat, sorghum, ryegrass, and dwarf apple (Jaglo et al. 2001; Gao et al. 2002; Dubouzet et al. 2003; Qin et al. 2004; Zhao and Bughrara 2008; Yang et al. 2010). The availability of *DREB1/CBF* genes in various plant species implies that the *DREB1/CBF* regulon is ubiquitous in higher plants.

DREB1/CBF genes have been widely studied in rice, which is not only an important crop worldwide but also a model for monocotic plants. Interestingly, the *OsDREB1A–D* genes, which were cloned as *DREB1* orthologous genes from rice, showed different stress-inducible expression patterns in comparison with their *Arabidopsis* counterparts (Dubouzet et al. 2003). Both *OsDREB1A* and *OsDREB1B* expressions were induced by cold stress. In addition, *OsDREB1A* was also induced by salt stress. Additionally, *OsDREB1C* displayed a constitutive expression pattern, whereas *OsDREB1D* transcripts were undetected in plants (Dubouzet et al. 2003). Overexpression of *OsDREB1A* in *Arabidopsis* conferred freezing and high salinity tolerance, implying that this gene may have similar function as that of *AtDREB1A*, and play an important role in stress tolerance in rice. Furthermore, Ito et al. (2006) generated transgenic rice plants independently overexpressing the *Arabidopsis DREB1A*, *1B,* and *1C* using the 35S promoter, and the *OsDREB1A* and *1B* using the maize Ubi promoter. Under the normal conditions, all the transgenic plants exhibited growth retardation and enhanced stress tolerance to drought, high salinity, and cold stresses in comparison with control plants. Microarray and RNA-blot analyses confirmed that 12 genes were up-regulated by either OsDREB1A or AtDREB1A in rice. Among the 12 up-regulated genes, 8 genes contained DRE sequences in their promoters, implying that they could be direct target genes of OsDREB1A and AtDREB1A proteins. Additionally, the majority of these downstream genes were induced not only by dehydration, but also by cold and high salinity.

It is worthy to mention that, in another independent study Oh et al. (2005) did not observe any negative effects on the growth of transgenic rice plants in which the *AtDREB1A/CBF3* was constitutively overexpressed using the maize Ubi promoter. These transgenic rice plants exhibited enhanced stress tolerance as well. This phenomenon was explained that stress-associated genes were not up-regulated under unstressed conditions in the transgenic plants (Oh et al. 2005).

In maize, Kizis and Pagès (2002) isolated two members of the AP2-EREBP TF family, and named *DBF1* and *DBF2*. Phylogenetic analysis indicated that these two genes were classified into the A-6 and A-4 subgroups of the DREB family proteins, respectively. Although both proteins are capable to bind to the DRE motif, only DBF1 functions as transactivator to activate DREB-dependent gene expression. Overexpression of *DBF1* gene in *Arabidopsis* led to enhanced

tolerance of transgenic plants to dehydration and salt stresses (Saleh et al. 2006). A maize DREB1 orthologous TF encoding gene, named *ZmDREB1A*, was identified by Qin et al. (2004). Its transcripts were increased greatly at 4°C and induced slightly by high salinity stress. An enhanced tolerance to dehydration and cold stresses was observed in transgenic *Arabidopsis* plants overexpressing *ZmDREB1A* which was accompanied with a dwarfed phenotype correlated to expression levels of the transgene. In two independent *ZmDREB1A* transgenic lines, downstream genes of DREB1 TFs, such as *COR*, *KIN1*, *KIN2*, *RD29A,* and *RD17*, were found to be upregulated. These data indicated that DREB1 regulon might play a conserved mechanism to protect different plant species under adverse conditions.

Skinner et al. (2005) reported that barley (*Hordeum vulgare*), an important crop and a diploid Triticeae plant, contains at least 20 *DREB/CBF* genes comprising three multigene phylogenetic subgroups designated HvCBF1, HvCBF3, and HvCBF4 subgroups. In transgenic *Arabidopsis*, the temperature-independent CBFs, the members of the HvCBF1- and HvCBF3-subgroups, activated *COR* genes at warm temperature, whereas the cold-dependent CBFs, HvCBF4-subgroup TFs, did not. HsDREB1A from wild barley (*Hordeum spontaneum*) shows a high degree of sequence conversation to that of barley (*H. vulgare*) (James et al. 2008). Eight *CBF* genes from another cereal, the Einkorn wheat (*Triticum monococcum*), have dramatically different levels of induction after exposure to cold stress (Vágújfalvi et al. 2005). Differences in *CBF* expression were generally associated with a variation in frost tolerance. Pellegrineschi et al. (2004) introduced *RD29Apro:AtDREB1A* construct into wheat, and screened 12 T2 transgenic lines exhibiting water-deficit stress tolerance. The *AtDREB1A* wheat plants displayed substantial tolerance to water stress in comparison with control plants under experimental greenhouse conditions. In the Triticeae, rye (*Secale cereale*) is one of the most low-temperature-tolerant species. Therefore, it has been considered as an excellent model to study and compare expression profiles of DREB/CBF-type TF encoding genes under cold stress. Eleven *ScCBF* genes spanning all four main subgroups were cloned. Several *ScCBF* genes were found to be expressed at warmer acclimation temperatures and be repressed at the end of an 8-h dark period at warmer temperatures, implying that the *ScCBF* genes might have not only temperature-dependent but also light-regulated diurnal response in rye (Campoli et al. 2009).

In horticultural plants, progress has been made in studies on isolation, functional and transformation analyses of *DREB1* genes. The growth and yield of tomato (*Lycopersicon esculentum*), which is originated from subtropical area and sensitive to cold, are adversely affected by chilling. As a means to provide a solution to this negative impact, transgenic tomato plants overexpressing *AtDREB1B/CBF* gene were constructed (Hsieh et al. 2002). As expected, transgenic tomato exhibited enhanced tolerance to chilling by showing higher survival rate under stressed conditions in comparison with control plants. However, the growth rate, the fruit and seed numbers of transgenic plants tolerant to freezing were not observed to be improved. Three *DREB1/CBF* homologous genes, *LeCBF1/2/3*, were cloned and characterized in tomato (Zhang et al. 2004). Among these three genes, only expression of *LeCBF1* is induced by chilling stress. Overexpression of *LeCBF1* in *Arabidopsis* improved tolerance of transgenic plants. However, the same authors demonstrated that neither overexpression of *AtDREB1A/CBF3* nor that of *LeCBF1* in tomato improved freezing stress tolerance. Recently, a dwarf apple *MbDREB1* was isolated and functionally characterized (Yang et al. 2010). Expression of *MbDREB1* was induced by cold, drought, salt stress, and exogenous ABA treatment. In comparison with wild-type plants, transgenic *Arabidopsis* overexpressing *MbDREB1* showed increased tolerance to low temperature, drought, and salt stresses. These data imply that *MbDREB1* might increase plant tolerance to low temperature, drought, and salt stresses in both ABA-dependent and ABA-independent manners in apple. It has also been reported that *RD29pro:DREB1A* construct was introduced into *Chrysanthemum* (Hong et al. 2006a, b). As expected, transgenic plants showed stronger resistance to drought, salt, and low temperature.

DREB1/CBF-type genes, *BnCBF5*, *7*, *16*, and *17* from *B. napus* were cloned by Gao et al. (2002). Deduced protein sequences of *BnCBF5*, *7*, and *16* are very similar to the *Arabidopsis CBF1* whereas that of *BnCBF17* is different because of the presence of two extra regions in its acidic domain. All the *BnCBF* genes are induced by low temperature. Additionally, expression of *BnCBF5*, *7*, and *16* is also slightly responsive to salt stress, but that of *BnCBF17* is not. Furthermore, overexpression of either *BnCBF5* or *BnCBF17* improved tolerance of transgenic *Brassica* to freezing (Savitch et al. 2005).

Overall, the DREB1/CBF TFs regulate cold stress signaling at transcriptional level without posttranscriptional regulation. Overexpression of *DREB1/CBFs* can improve tolerance to cold stress in various transgenic plants. Noticeably, the adverse effect caused by overproduced DREB1/CBFs should be minimized by using inducible or controllable promoter such as *RD29A* promoter instead of the constitutive 35 S. Investigation of several upstream regulators of the DREB1/CBFs, such as ICE1 and MYB15, may provide a deeper understanding of DREB1/CBF function in cold acclimation.

3 DREB2 and Drought/High Salinity Stress Response

Although *DREB2A* was isolated from the yeast-one-hybrid screening together with *DREB1A* (Liu et al. 1998), its function has been less reported than that of *DREB1A* gene. The reason might be that because overexpression of *DREB2A* led to neither enhanced tolerant phenotype nor alteration in expression of stress-inducible genes. Domain analysis of DREB2A in *Arabidopsis* protoplasts revealed that DREB2A contains a negative regulatory region and the deletion of this region activated DREB2A protein. The deleted protein was called DREB2A-CA (Sakuma et al. 2006a). Overproduction of DREB2A-CA protein in *Arabidopsis* enhanced water stress tolerance and caused a dwarf phenotype. However, no obvious tolerance to freezing was observed. As is the case for *DREB1s*, overexpression of *DREB2A-CA* using stress-inducible *RD29A* promoter eliminated the dwarfism and maintained a better tolerance (Sakuma et al. 2006a).

Further analyses of expression and sequences demonstrated differences in stress-responsive expression profiles and the AP2-EREBP domains between DREB1-type and DREB2-type TFs which might lead to their different response to different stresses and the discrepancy in their downstream target genes. In *Arabidopsis*, *DREB1* genes are induced greatly by cold or slightly by salt stress; however, *DREB2* genes are greatly increased by salt and dehydration stresses (Liu et al. 1998; Sakuma et al. 2002). Recently, it was reported that *DREB2* genes are induced by heat stress (Sakuma et al. 2006b; Qin et al. 2007; Lim et al. 2007). It was also found that downstream target genes of DREB2A were different from those of DREB1A. This might be explained by the fact that the DNA-binding domains of DREB1A and DREB2A are slightly different, which causes a high affinity of DREB1A to A/GCCGACNT sequences and the preferential binding of DREB2A to ACCGAC motifs (Sakuma et al. 2006a).

Interestingly, in addition to drought and salt inducible genes, overexpression of *DREB2A-CA* in *Arabidopsis* also up-regulates heat shock related genes (Sakuma et al. 2006b). Consequently, the *DREB2A-CA* transgenic plants displayed enhanced thermotolerance. Thus, DREB2A was recognized to play an important regulatory role in response not only to drought but also to high temperature stress. Microarray analysis of *DREB2A-CA*-overexpressing plants identified heat shock protein 70 (*HSP70*), *HSP18.2,* and heat shock A3 factor encoding gene (*HSFA3*) among the up-regulated downstream genes (Sakuma et al. 2006b). The DREB2A protein was shown to bind to the DRE sequence located in the promoter of the *AtHSFA3* and activate its transcription (Schramm et al. 2008; Yoshida et al. 2008). Additionally, Chen et al. (2009) presented data in regard to the regulation of *HSFA3* by DREB2C in the heat shock signal transduction cascade. Similar to DREB2A, DREB2C interacts with two DREs located in the *HSFA3* promoter. Deletion analysis of DREB2C indicated that its transactivation region is located in the C terminus.

A DREB2A orthologous TF encoding gene, the *ZmDREB2A*, was isolated from maize. Expression analysis revealed that *ZmDREB2A* is strongly induced by high salinity, slightly by drought, and unchanged by heat stress (Qin et al. 2007). Interestingly, in contrast with *Arabidopsis DREB2A*, *ZmDREB2A* gene contains 53 bp intron, causing a premature termination of translation. This fragment was shown to be involved in an alternative splicing under stress conditions. This phenomenon was also found in other monocotic plants, such as wheat and barley (Xue and Loveridge 2004; Terashima and Takumi 2009). Constitutive overexpression of *ZmDREB2A* in *Arabidopsis* enhanced water-deficit stress tolerance but caused dwarfism. Using the *RD29A* promoter instead of the 35 S for overexpression of the *ZmDREB2A* diminished plant growth retardation (Qin et al. 2007). In contrast with overexpression of *ZmDREB2A*, that of a *Populus euphratica DREB2* gene, designated *PeDREB*, under the control of 35 S promoter improved salt stress tolerance but did not cause dwarf phenotype (Chen et al. 2009).

Matsukura et al. (2010) performed a comprehensive analysis of all five *DREB2*-type genes (*OsDREB2A*, *OsDREB2B*, *OsDREB2C*, *OsDREB2E,* and *OsABI4*) in rice. Among them, only *OsDREB2A* and *OsDREB2B* exhibited abiotic stress-inducible expression. OsDREB2B showed nuclear specific localization and the highest transactivation activity. In transgenic *Arabidopsis*, overexpression of *OsDREB2B* upregulated expression of AtDREB2A target genes, including both the drought and heat shock-inducible genes. Noticeably, *OsDREB2B* has functional and nonfunctional transcripts similar to its orthologues in the grass family. In the study on DREB2-type TFs in diploid progenitors and hexaploid wheat lines, it was found that allopolyploidization during wheat polyploid evolution could inhibit efficiently alternative splicing of *WDREB2* transcripts (Terashima and Takumi 2009). These data suggest that *DREB2* genes in grass family play an important role in response to drought and heat shock stress which is controlled by alternative splicing.

In contrast with alternative splicing found for *DREB2* genes in grass, AtDREB2 protein was shown to undergo posttranscriptional regulation by degradation caused by DRIPs (DREB2A-interacting proteins), which are C3HC4 ring domain-containing proteins. The DRIPs function as E3 ubiquitin ligases and are able to mediate DREB2A ubiquitination and proteolysis under favorable growing condition, which probably restricts the negative effects of DREB2A protein on plant growth and development (Qin et al. 2008).

Collectively, research on DREB2 TFs indicates that the DREB2 regulon forms an ABA-independent pathway which functions in both osmotic and heat shock responses (HSR). Alternative splicing and posttranscription regulation are the two mechanisms which control the stress-related DREB2 functions in grass species and *Arabidopsis*, respectively.

4 AREB TFs and ABA-Dependent Drought and High Salinity Stress Response

Under water stress conditions, such as cold, high salinity, and drought, endogenous ABA levels in plants increase, which triggers stomatal closure to avoid environmental stress damage (Leung and Giraudat 1998). In *Arabidopsis* and rice, many drought and high salinity-inducible genes also respond to ABA (Seki et al. 2002; Rabbani et al. 2003). The key transcriptional regulators of ABA-dependent gene expression are ABA-responsive element binding TFs (AREBs/ABFs) which belong to the basic-leucine zipper (bZIP) TF family (Finkelstein et al. 2005; Choi et al. 2005). AREBs/ABFs control ABA-dependent gene expression by binding to *cis*-acting regulatory elements which share the C/TACGTGGC consensus, designated as ABREs, in the promoters of ABA-inducible genes (Busk and Pages 1998). ABRE element is usually coupled with other sequences to realize its function. ABRE and GC-rich coupling elements, including coupling element 1 (CE1) and coupling element 3 (CE3), function together as a complex in the regulation of gene expression in wheat (Shen et al. 1996). The core sequence of known coupling elements, which is A/GCGT, is similar to that of ABREs (Hobo et al. 1999a). Narusaka et al. (2003)

reported that the DRE/CRT may also function as a coupling element of ABRE in response to ABA in *Arabidopsis*.

In *Arabidopsis*, cDNAs encoding AREBs/ABFs were successfully isolated using yeast one-hybrid system (Choi et al. 2000; Uno et al. 2000). Further genomic sequence analyses indicated that the *Arabidopsis* genome contains at least 75 distinct bZIP TFs, among which 13 members are identified as AREB/ABFs (Bensmihen et al. 2002; Jakoby et al. 2002). Among these AREB/ABFs encoding genes, *AREB1/ABF2*, *AREB2/ABF4*, and *ABF3* are induced by ABA, drought, and high salinity in vegetative tissues but not in seeds (Fujita et al. 2005). However, *ABI5*, *AREB3*, *DPBF2,* and *EEL* (Enhanced Em Level) genes were expressed during seed maturation (Finkelstein and Lynch 2000; Lopez-Molina and Chua 2000; Bensmihen et al. 2002).

Different from DREB1/CBF TFs, AREB/ABF proteins contain many target sequences of protein kinases, and modification of AREB/ABF by phosphorylation is required to transform the native but inactive AREB/ABF TFs into their active form. A number of reports have revealed that phosphorylation of the AREB1 protein by SnRK2, a sucrose nonfermenting protein (SNF1)-related kinase, is important for its activation. Recent research showed that SnRK2.2/SnRK2D and SnRK2.3/SnRK2I are involved in the phosphorylation of AREB1 (Fujii et al. 2007). A strong lines of evidence indicated that OST1/SnRK2.6/SnRK2E (an ABA-activated protein kinase, homologue of SnRK2.2/SnRK2D and SnRK2.3/SnRK2I) was also able to directly target ABF/AREBs to phosphorylation, and three of SnRK2 kinases cooperatively phosphorylate AREB1 in response to water stress or ABA signals (Fujii et al. 2007, 2009; Yoshida et al. 2010). Therefore, it was the reason why transformation of an unphosphorylated or inactive *AREB1* gene into *Arabidopsis* has failed to cause remarkable improved expression of downstream genes and ABA-related phenotype (Fujita et al. 2005). In addition to the SNF-type kinases, Ca^{2+}-dependent protein kinases, such as the CPK4 and CPK11, also phosphorylate ABF1 and ABF4/AREB2 (Zhu et al. 2007).

An independent study indicated that deletion of a fragment from 61 to 318 aa region between the transactivation and the DNA binding domain transforms the inactive AREB1 into a constitutive active form, which can promote its target gene expression, even in the absence of ABA (Fujita et al. 2005). Transgenic *Arabidopsis* plants overexpressing the active form of AREB1 under the control of 35 S promoter were shown to be hypersensitive to ABA and displayed enhanced drought tolerance. Microarray and RNA-gel blot analyses of transgenic plants discovered eight genes as downstream target genes of the active AREB1. These up-regulated genes were classified into two groups: the first group consists of genes encoding functional proteins, such as LEA or LEA-like proteins, including RD29B, At3g17520, and the other contains regulatory proteins, including RD20 and HIS1-3 (Fujita et al. 2005). Concurrently, the same lab demonstrated that overproduction of a phosphorylated AREB1 protein in *Arabidopsis* improved *RD29B* and other ABA-inducible gene expression in plants (Furihata et al. 2006). Taken together, these results support that AREB1 protein requires posttranscriptional modification to modulate its downstream gene expression.

In addition to AREB1, other AREB/ABF TFs also contribute to ABA-dependent stress response. ABI3, a member of B3 transcriptional regulators, interacts with ABI5 to enhance its action. An AP2-type TF, ABI4, and some MYC/MYB-type TFs (AtMYC2 and AtMYB2) function as positive regulators of ABA response (Yamaguchi-Shinozaki and Shinozaki 2006).

In rice and barely, the homologues of *Arabidopsis* AREB1/ABF2, the TRAB1, and HvABI5 encoding genes, respectively, were found to respond to ABA treatment and drought stress in seedlings (Hobo et al. 1999b; Casaretto and Ho 2003). Zou et al. (2008) reported the cloning of *OsABI5* and that its expression was induced by ABA and high salinity, but was down-regulated by drought and cold stresses in seedlings. Overexpression of *OsABI5* caused high sensitivity of transgenic rice to salt stress. However, down-regulation of *OsABI5* enhanced stress tolerance, but negatively influenced rice

fertility. Microarray analysis indicated that, among 89 *OsbZIP* genes surveyed, 26 genes were up-regulated and 11 genes were down-regulated under dehydration, high salinity, and cold conditions (Nijhawan et al. 2008).

In conclusion, AREB/ABF regulon is a common ABA-dependent signaling pathway in plants, functioning in response to dehydration and high salinity stresses. Phosphorylation of AREB/ABF TFs, such as AREB1, may be required for their full activation.

5 HSFs and HSP-Related Heat Stress Response

Heat stress is one of the key environmental stresses in fields around the world, especially in tropical areas. As sessile organisms, plants can acquire better thermotolerance against unavoidable high temperature challenges by a prior high temperature treatment (Sun et al. 2002). In this process, heat acclimation involves the accumulation of HSPs that are molecular chaperons (Sun et al. 2002), which are important for cellular homeostasis in cells under both optimal and adverse growth conditions (Kim and Schöffl 2002; Hartl and Hyer-Hartl 2002; Port et al. 2004; Wang et al. 2004). Expression of HSP encoding genes is regulated by a large family of HSFs which bind to the heat shock elements (HSE) "GAANNTTC" in the promoters of HSP encoding genes (Schöffl et al. 1998; Nover et al. 2001; von Koskull-Döring et al. 2007).

While there are only a few HSF encoding genes in yeast, *Drosophila* and vertebrates (yeast and *Drosopholia* have only 1; vertebrates possess 4), plants have multiple HSF encoding genes (for instance tomato, *Arabidopsis*, rice and soybean have 18, 21, 25, and 34 genes, respectively) divided into three classes of A, B and C (Nover et al. 1996, 2001; Nakai 1999; Baniwal et al. 2004; Kotak et al. 2004; Xing et al. 2005). Identification and detailed functional analyses of HSFs of *Arabidopsis* and tomato have revealed that the majority of class-A HSFs contain the AHA motifs vital for transcriptional activation of their downstream genes, while the class-B and C HSFs have no transactivational activity, and they function as repressors or co-activators (Kotak et al. 2004).

In the plant kingdom, cloning and genetic analysis of HSF encoding genes have been pioneered from tomato (Nover et al. 1996, 2001; Lyck et al. 1997). The excellent work of Mishra et al. (2002) revealed that HSFA1a functions as a master regulator of HSR in tomato. In their study, plants with tenfold overexpression of *HSFA1* (OE) and cosuppression (CS) plants were produced. Under normal conditions, major developmental parameters of OE and CS plants are similar to that of wild type. However, CS plants and fruits were extremely sensitive to elevated temperatures because of reducing and lacking of heat-inducible HSFs and synthesis of HSP chaperons. These results indicated that HSFA1 has a unique function as master regulator for induced thermotolerance in tomato. In contrast with HSFA1a, tomato HSFA2 or HSFB subgroup members could not function independently. HSFA2 needs interaction with HSFA1 for efficient nuclear import (Scharf et al. 1998), and HSFB1 has been identified as a co-activator of class-A HSFs and other TFs for transactivation (Bharti et al. 2004).

Multiple plant HSFs display diversity in their biological functions. Completion of *Arabidopsis* genomic sequence has enabled us to find and analyze HSFs and their target genes such as HSPs encoding genes at whole genome scale. In *Arabidopsis*, 44 HSPs and 21 HSFs were identified (Swindell et al. 2007). Additionally, Guo et al. (2008) reported that the *Arabidopsis* genome contains 22 HSFs, and in contrast with TAIR annotation of HSFs, an additional HSF has been found to be probable member of HSFA2 class. Unlike tomato HSF system, no master HSF regulator has been found, suggesting that there may be more complicated HSF regulation in *Arabidopsis*. A number of studies on the functions of *Arabidopsis* HSFs have been conducted. HSFA1a and HSFA1b play important roles in the induction of some HSP encoding genes in the early phase of HSR and in acquired thermotolerance (Lohmann et al. 2004). *Arabidopsis* HSFA2, HSFA3, and HSFA7a also play critical role in this process (Charng et al. 2007; Larkindale

and Vierling 2008; Schramm et al. 2008; Yoshida et al. 2008). There are five different *HSFB* genes in *Arabidopsis* genome. The HSFB3 and HSFB4 function at the early stage after heat shock, and HSFB1, HSFB2a, and HSFB2b are considered to function as late HSFs (Lohmann et al. 2004; Busch et al. 2005).

Although functionally similar to *HSFA1a*, several plant HSF encoding genes are HS-inducible genes themselves, such as *HSFA2* proteins from tomato and *Arabidopsis*, which encode the major HSFs in thermotolerant cells (Scharf et al. 1998; Morimoto 2002; Busch et al. 2005). According to the data gained from transcriptome analyses, six HSF encoding genes, *HSFA2*, *HSFA4a*, *HSFA7a*, *HSFB1*, *HSFB2a*, and *HSFB2b*, are strongly up-regulated in *Arabidopsis* leaves by heat shock treatment at 37°C for 1 h (Busch et al. 2005). Among these up-regulated genes, function of *AtHSFA2* was analyzed in detail. Loss-of-function studies of *AtHSFA2* supported the direct evidence that *AtHSFA2* is essential for acquired thermotolerance (AT) after long recovery but not short recovery (Charng et al. 2007). On the other hand, ectopic overexpression of *OsHSFA2e* led to increased thermotolerance not only in the cotyledons but also in rosette leaves, inflorescence stems, and seeds in transgenic *Arabidopsis* (Yokotani et al. 2008).

In addition to heat stress, numerous abiotic and biotic stressors can induce expression of HSF encoding genes (Swindell et al. 2007), suggesting that HSFs may play crucial role in crosstalk between multiple stress response pathways. Recently, it was reported that overexpression of *AtHSFA2* conferred not only increased tolerance of transgenic *Arabidopsis* to heat and osmotic stresses but also enhanced callus growth (Nishizawa et al. 2006; Yokotani et al. 2008). High-level overexpression of the *Arabidopsis HSFA2* gene revealed that HSFA2 plays, in addition to its role in heat and salt stress tolerance, an important role in cell proliferation (Ogawa et al. 2007). Interestingly, AtHSFA2 was shown to be one of the regulatory components of cytoplasmic protein response (CPR) (Sugio et al. 2009) and target of sumoylation which may regulate AtHSFA2 transcriptional activity (Cohen-Peer et al. 2010).

There is an ongoing effort to elucidate functions of the HSFs. However, detailed analyses of the HSFs are still mainly restricted to tomato and *Arabidopsis* (Panchuk et al. 2002; Baniwal et al. 2004; Zhang et al. 2009). During recent several years, functional analyses of HSFs from other species have been conducted. In rice, a mutant of *HSFA4* exhibited a potted leaf phenotype under elevated temperature (Yamanouchi et al. 2002). It has been reported that *GmHSFA1*, a heat shock TF, was cloned and characterized from soybean (*Glycine max*), and its overexpression conferred enhanced thermotolerance of transgenic soybean plants (Zhu et al. 2006). Zhu et al. (2009) cloned a *HSF* gene named *BhHSF1* from *Boea hygrometrica*. Their study revealed that this gene may play dual functions in mediating heat stress tolerance and growth retardation via regulation of target genes related to stress protection and mitotic cell cycle. A *HSFA2* from lily, an important ornamental flower, has recently been isolated by Xin et al. (2010). This gene was found to be induced exclusively under heat shock. Ectopic overexpression of the lily *HSFA2* obviously improved thermotolerance of transgenic *Arabidopsis*.

Recently, a CaM-binding protein kinase (CBK) was identified as an interacting protein of HSFA1a in *Arabidopsis* (Liu et al. 2008). The knockout lines of *AtCBK* failed to acquire thermotolerance and accumulate downstream HSP encoding genes, implying that the activity of HSFs can be modulated at posttranscriptional level (Liu et al. 2008). This case is similar to that of CAMTA regulation for cold induction.

Taken together, HSFs form a group of key regulators, controlling gene expression, thereby playing critical roles in heat signal transduction pathway. Manipulation of single *HSF* can change expression of a series of heat-inducible genes which may lead to thermotolerance.

6 Other Stress-Related TFs

In addition to the major TFs mentioned earlier, many other TFs are involved in plant response to abiotic stress.

AtMYC2 and AtMYB2 proteins are thought to function as transcriptional activators in the ABA-responsive gene expression of the *RD22* (Abe et al. 1997). Overexpression of *AtMYC2* increased the ABA-responsive expression of *RD22*. In plants overexpressing both *AtMYC2* and *AtMYB2*, the expression of *RD22* was induced by ABA significantly earlier than in *35S:AtMYB2* or *35S:AtMYC2* plants. At the same time, the *AtADH1* transcript in plants overexpressing both two genes accumulated to high level under normal condition, implying that MYC2 and MYB2 act together as transcriptional activators in the ABA-responsive expression of *AtADH1*. Furthermore, microarray analysis of *35S:AtMYC2/MYB2* transgenic plants demonstrated that the up-regulated genes possess the MYC (CANNTG) and MYB (C/TAACNA/G) recognition sequences in their promoter regions (Abe et al. 2003).

Cys-2/His-2-type zinc-finger and zinc-finger homeodomain (ZFHD) proteins have been identified to be important as transcriptional regulators in plant abiotic stress responses. Unlike the above-mentioned TFs, Cys-2/His-2-type zinc-finger TFs were observed to regulate target genes by repressing their expressions in plants (Sakamoto et al. 2004). Overproduction of Zat10/STZ, a Cys-2/His-2-type zinc-finger TF, in *Arabidopsis* enhanced drought stress tolerance (Sakamoto et al. 2004). Zat12 was also shown to be involved in response to both cold and oxidative stresses (Iida et al. 2000; Rizhsky et al. 2004; Vogel et al. 2005). As for ZFHD TFs, an *Arabidopsis* ZFHD protein, the ZFHD1, was characterized by a putative DNA-binding homeodomain in the C terminus which binds to the 62-bp region in the promoter region of *ERD1* (early responsive to dehydration 1) gene. Expression of the *ZFHD1* gene is induced by dehydration, high salt, and ABA treatment. Overexpression of *ZFHD1* in transgenic plants conferred drought stress tolerance (Tran et al. 2007).

The NAC gene family, which is specific to plant kingdom, was originated from the names of three TFs (1) NAM (no apical meristem, *Petunia*), (2) ATAF1-2, and (3) CUC2 (cup-shaped cotyledon, *Arabidopsis*) (Nuruzzaman et al. 2010). *Arabidopsis* and rice contains 117 and 151 nonredundant NAC TFs, respectively, which are characterized by the conserved N-terminal region known as the NAC domain (Ooka et al. 2003; Nuruzzaman et al. 2010). NAC proteins play critical role in plant developmental processes such as formation of apical shoots and in disease defense. Several NAC TFs were found to be regulators of abiotic stress response (Tran et al. 2010). Three NAC genes have been isolated from *Arabidopsis* by yeast one-hybrid screening (Tran et al. 2004). These NAC TFs were found to bind to a CATGTG motif in the promoter of *ERD1*. Expression of these three *NAC* genes was induced by drought, high salinity, ABA, and methyl jasmonate. Following this study, Hu et al. (2006) reported the isolation of a rice *NAC* gene, named *SNAC1*, whose expression was found to be induced by drought, salt, cold, and ABA treatment. The *35S:SNAC1* transgenic rice showed significantly increased drought resistance under field conditions and strong tolerance to salt stress. Another NAC TF encoding gene, *OsNAC6*, was also reported to be induced by cold, drought, and high salinity (Nakashima et al. 2007). When *OsNAC6* was overexpressed in rice under the control of the maize Ubi promoter, the transgenic plants were more tolerant to both drought and high salt stresses accompanied by retarding of growth. *Lip9* (low temperature inducible protein 9) promoter was subsequently used to minimize the adverse effect on plant growth. Recent research has revealed that *Arabidopsis* ATAF1, a NAC family member, plays important roles in both abiotic and biotic stress responses. *ATAF1* overexpression lines exhibited enhanced drought tolerance, implying a positive regulation in plant drought response (Wu et al. 2009b). Recent studies indicated that some NAC members are membrane-associated proteins and involved in stress responses (Kim et al. 2006). It is worthy to mention that a membrane-bound bZIP TF, the bZIP28, was found to be involved in regulation of heat-stress-associated gene expression (Gao et al. 2008).

The WRKY TFs, which are regulators of downstream genes including *NAC* genes, were reported to be involved in the ABA signaling pathway (Zou et al. 2004). Transient expression

study using aleurone cells revealed that OsWRKY24 and OsWRKY45 acted as repressors of an ABA-inducible promoter; however, OsWRKY72 and OsWRKY77 were shown to be activators of the same promoter (Xie et al. 2005). Overexpression of *OsWRKY11* using heat shock-inducible *HSP101* promoter led to enhanced heat and drought tolerance in transgenic rice (Wu et al. 2009a). In *Arabidopsis*, overexpression of either *AtWRKY25* or *AtWRKY33* increased salt tolerance (Jiang and Deyholos 2009). In *B. hygrometrica*, the *BhGolS1* gene is inducible by both dehydration and ABA (Taji et al. 2002). The promoter of *BhGolS1* encoding galactinol synthase, which plays an important role in drought and cold tolerance, contains four W boxes. Chromatin immunoprecipitation demonstrated that they are bound in vivo by the early dehydration- and ABA-inducible BhWRKY1 (Wang et al. 2009). These data provide direct evidence linking a dehydration-inducible WRKY TF with a downstream target gene that plays a vital role in drought response (Rushton et al. 2010).

Additionally, some nuclear TFs were identified to be important regulators of abiotic stress response. Nelson et al. (2007) identified a TF from the nuclear factor Y family, the AtNF-YB1, whose overexpression improved drought tolerance in *Arabidopsis*. ZmNF-YB2, an ortholog of AtNF-YB1, also has similar function in maize. The *Arabidopsis* NF-X1 has a heat-inducible expression pattern and promotes both acquired thermotolerance and salt tolerance (Larkindale and Vierling 2008; Hua 2009).

7 Conclusions and Perspectives

Owing to the power of genetics and genomics, more and more abiotic stress signaling pathways and key TFs acting as molecular regulatory switches are identified. The regulatory functions of the TFs involved in the stress signaling pathways have been elucidated. Commonly, each group of TFs responds mainly to a certain abiotic stress. However, a number of TFs, such as DREB2A, MBF1c, NAC, and NF-X1, play dual or multiple roles in different signaling pathways. Up-to-date, a plenty of valuable TFs have been identified and isolated for our original intention of studying their function in stress responses and using them to improve tolerance of plants to environmental stresses by genetic engineering. Importantly, the biological functions of some TF encoding genes have been characterized in transgenic plants. These findings have provided us a better understanding of transcriptional regulatory mechanisms in plants exposed to environmental stresses and a bright perspective in improvement of stress tolerance of plants by manipulating gene expression.

On the other hand, there is a long way to go for our ultimate goal of enhancing plant tolerance to environmental stresses by manipulating key stress-associated TFs in a field condition because of the complexity of regulatory systems in plant itself and uncontrollable testing conditions. The challenge ahead is to elucidate more detailed regulatory mechanism at transcriptional level, find internodes of multiple signals, and improve gene transfer strategies and evaluation systems. All these efforts will lead to an essential advance in development of stress-tolerant plant cultivars.

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Plant Signaling Under Abiotic Stress Environment

14

Parvaiz Ahmad, Renu Bhardwaj, and Narendra Tuteja

Abstract

Abiotic stress is one of the major factors that negatively affect crops yield; therefore, development of stress-tolerant crops is essential for future food security. The stress stimuli are perceived by the plasma membrane and different signals get activated. In response to the stress, expression of many genes gets altered which plays an important role in the transmission of the signals. Various chemicals are also responsible for these signals like calcium (Ca^{2+}), nitric oxide (NO), sugars, abscisic acid (ABA), brassinosteroids (BRs), ethylene, jasmonates (JA), salicylic acid (SA), and auxins. Ca^{2+} acts as a secondary messenger to perceive the environmental stimuli and transduce them into downstream effectors in order to bring about changes leading to adaptations to stressful conditions or developmental effects. The phytohormones have a role in tolerance and adaptations to plants under abiotic stress. During abiotic stress, cross talk between different signaling pathways is very common. In the present review, we elucidated the role of these chemicals in plant signaling under abiotic stress. The signal transduction pathway involving mitogen-activated protein kinases (MAPK) under abiotic stress is also discussed.

Keywords

Abiotic stress • ABA • Ca^{2+} signaling • CaM • CDPK • MAPK • Phytohormones

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1 Introduction

Plants are forced to exposure to various abiotic stresses including salt, drought, extreme temperatures (cold and heat), UV radiations, etc., since they are unable to move to more favorable places. These abiotic stresses adversely affect the plant

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metabolic activities and are also responsible for crop loss to a greater extent (Mahajan and Tuteja 2005; Tuteja 2007a). During stress, the upregulation of many genes has been reported which helps the plant to withstand the stress conditions and lead to plant adaptation (Tuteja 2009a).

Plants perceive the external and internal signals and are used to regulate various responses for its development. Exposure to stimuli causes membrane depolarization of the plant cells with 10–30 s, which is coordinated with early Ca^{2+} influx (reviewed by Tuteja and Sopory 2008). Signals are perceived by the membranes and therefore membrane events are the likely routes for signal generation and transduction (Tuteja and Sopory 2008). Different signaling pathways can operate independently to each other and can modulate other pathways (Kaur and Gupta 2005). Sometimes, components of pathways are dependent on each other and can cross talk among them.

Different molecules have been reported to have a role in signal transduction. The present review throws light on various signaling molecules and pathways like calcium and MAPK signaling pathways during abiotic stress. We also tried to cover the role of phytohormones such as ABA, SA, JA, BR, etc., in abiotic stress signaling.

2 Calcium Signaling

Ca^{2+} regulates a range of activities within the cell such as cell division and elongation, cytoplasmic streaming, photomorphogenesis, and plant defense against environmental stresses (Song et al. 2008; Tuteja 2009b; Kader and Lindberg 2010). It functions as the central node in overall signaling web and has a promising role in stress tolerance (Tuteja and Sopory 2008). During abiotic stress, Ca^{2+} acts as a second messenger under various stress conditions (Knight 2000). Ca^{2+} signatures have a leading role in numerous physiological processes such as regulation of stomatal apertures in plants (Allen et al. 2001). Ca^{2+} signatures change according to the nature of stress (Kiegle et al. 2000), duration of stress (Plieth et al. 1999), earlier exposure to any stress (Knight et al. 1997), and the type of tissue exposed to stress (Kiegle et al. 2000). For studying the role of calcium and its dynamics, various pharmacological and transgenic approaches have been utilized. The calcium reporter protein aequorin has proved to be very useful for such studies in revealing calcium fluxes within one cell and in different tissues.

Calcium-binding proteins act as stress sensors, get conformationally transformed upon binding with Ca^{2+}, which facilitates their interaction with downstream effector molecules (Clapham 2007; Gifford et al. 2007; Tuteja 2009b). Elongation factor (EF)-hand motif, which generally occurs in pairs, is the most common motif present in these Ca^{2+}-binding proteins and helps in high-affinity binding of Ca^{2+}. It has a helix-loop-helix structure and is reported in about 250 proteins of *Arabidopsis* (Day et al. 2002). In plants these EF-hand proteins comprise three different categories, namely, CDPKs (Ca^{2+}-dependent protein kinases), CaMs (calmodulins) and CMLs (CaM-like proteins), and the CBLs (calcineurin B-like proteins) (Fig. 14.1). Of these, only CDPKs act as "responders," as they are capable of directly transducing signals through their catalytic activity. CaMs/CMLs and CBLs are only sensors for regulating the downstream targets. CMLs, CDPKs, and CBLs are restricted to plants and some protists, whereas CaM is universal to all eukaryotes.

2.1 Calcium-Dependent Protein Kinases

CDPKs have been reported from several plants (Kawasaki et al. 2001; Seki et al. 2002; Ozturk et al. 2002; Asano et al. 2011) and are specific for a particular osmotic stress response. Studies on salt-tolerant and salt-sensitive rice varieties revealed specific CDPKs to be induced earlier with a sustained expression in the tolerant variety in comparison to the sensitive variety (Kawasaki et al. 2001). *Arabidopsis* CDPKs are also highly specific (Sheen 1996). Out of several CDPKs tested, only AtCDPK1 and AtCDPK1a were able to transcriptionally activate selected reporter

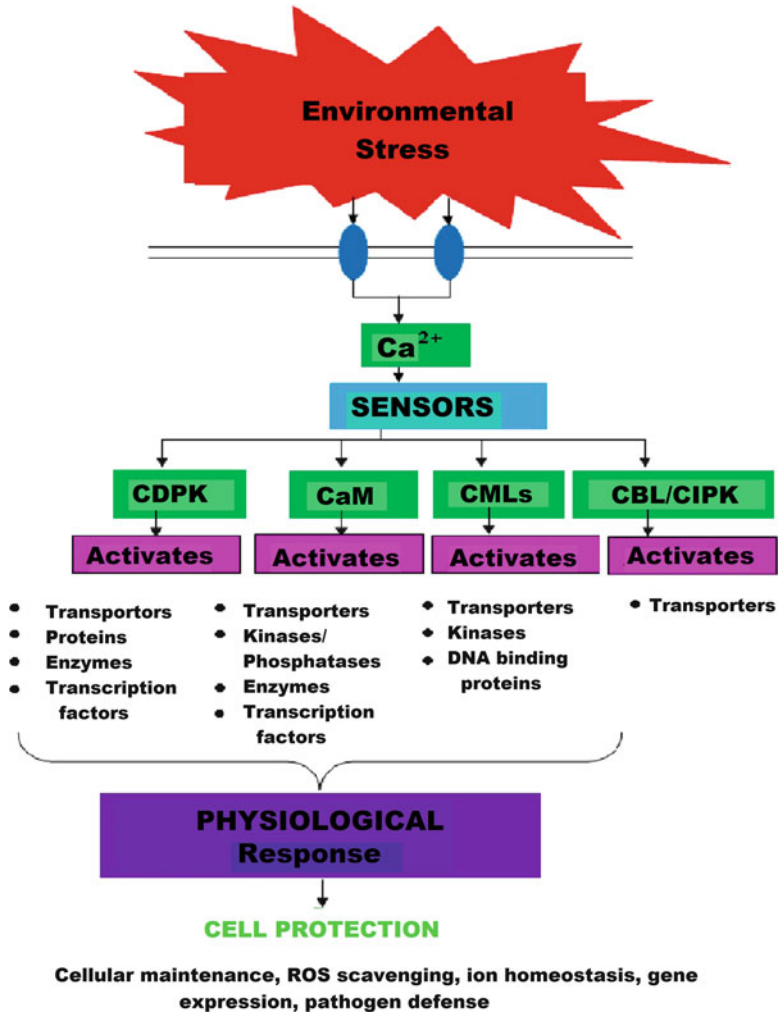

Fig. 14.1 Calcium-dependent signaling. Environmental stress responses are perceived by cell surface which in turn increases cytosolic Ca^{2+} concentration. Ca^{2+} got activated by cytosolic Ca^{2+} and regulates downstream targets leading to physiological responses

genes. CDPKs are five domain-containing proteins, and range from ~40 to 90 kDa in size. Their catalytic domain consists of a highly conserved serine/threonine kinase region, whereas, their N-terminal variable domain ranges from 21 to 185 amino acids in length (Klimecka and Muszynska 2007). Adjacent to the kinase domain is a pseudosubstrate-containing autoinhibitory junction domain that interacts with the active site and inhibits its kinase activity. Next to the autoinhibitory domain is the CaM-like domain (CLD) which is responsible for its Ca^{2+}-binding activity. Their C-terminal domain is relatively short and variable.

Plants have a large family of Ca^{2+}-dependent protein kinase (CDPKs) and have an important role in signaling during abiotic stresses like drought, wounding, and cold (Fig. 14.2). Induction of CDPK by osmotic stress has been shown by many workers in various plants (Kawasaki et al. 2001; Seki et al. 2002; Ozturk et al. 2002; Witte et al. 2010; Franza et al. 2011; Wurzinger et al. 2011). Induction of CDPK exists in longer duration in tolerant varieties of rice than in the salt-sensitive variety (Kawasaki et al. 2001). Martin and Busconi (2001) have reported that cold stress activates the membrane-associated CDPK in rice plants. Saijo et al. (2001) have also

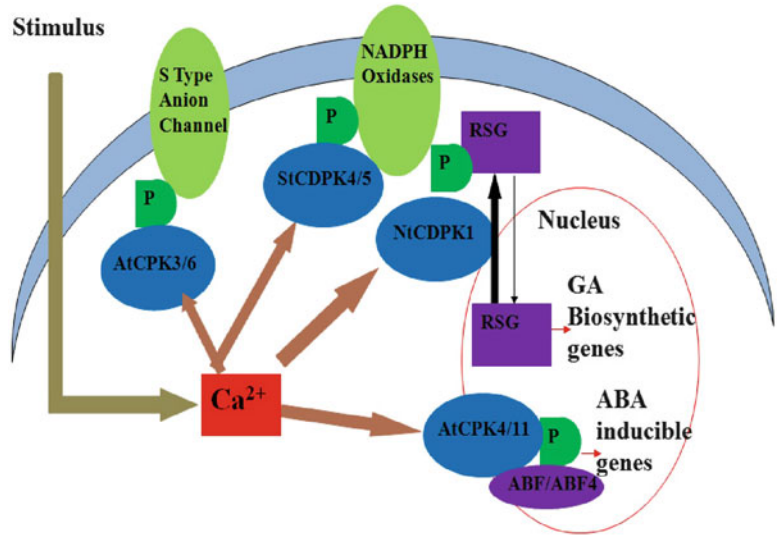

Fig. 14.2 Signaling through CDPK and protein phosphorylation by Ca^{2+} signatures

reported that overexpression of OsCDPK7 in rice provides cold and osmotic stress tolerance in these plants. Sheen (1996) has demonstrated that CDPK is involved in signal transduction. In his experiment, he demonstrated that in protoplast of maize leaf the expression of stress-responsive HVA1 was induced by active AtCDPK1. Sheen (1996) has also reported that AtCDPK1 activation is blocked by a protein phosphatase type 2C (AtPP2CA). Enhanced induction of CBF1, RAB18, RC12A, and LT178 (i.e., RD29A) gene expression has been observed in AtPP2CA-silenced *Arabidopsis* plants during cold and ABA treatment (Tahtiharju and Palva 2001). Romeis et al. (2001) also reported that pathogen infection also activated CDPK in plants.

2.2 Calmodulin and Other Calcium-Binding Proteins

Elevated levels of calcium within a cell activate various calcium-binding proteins which in turn induce specific kinases. On the basis of function, calcium-binding proteins are classified into two groups: (1) trigger proteins and (2) buffer proteins. The trigger proteins are activated when they bind with Ca^{2+} and after that they interact with other proteins and alter their activity. The trigger-type CaBPs are calmodulin (CaM), CaM-binding proteins, Ca^{2+}-dependent protein kinase, and phosphatase (Reddy 2001).

Stress causes increase in inositol 1,4,5-triphosphate (IP3). Activation of phospholipase C (PLC) results in hydrolysis of PIP2 to IP3. IP3 is regarded as the activator of vacuolar calcium channels in plants. During stress, the increase in cytosolic Ca^{2+} is due to the activation of IP3-dependent calcium channels (Braam 2005; Boudsocq and Laurière 2005). Furthermore, calcium-binding proteins (CaBP) or calcium sensors recognize and translate the information provided in calcium signatures (Tuteja and Mahajan 2007) and pass the information downstream for regulation of gene expression.

These CaBPs are partially responsible in modulating the intracellular calcium levels. The calcium-binding proteins can be regulated either in a cell- or in a tissue-specific manner. NaCl-inducible Ca^{2+}/calmodulin-dependent protein kinase in pea (PsCCaMK) was reportedly specific to roots (Pandey et al. 2002). A calmodulin-binding transcription activator family was shown to be specific only to the multicellular organisms (Bouche et al. 2002). Other examples of osmotic stress-activated calcium-binding proteins include

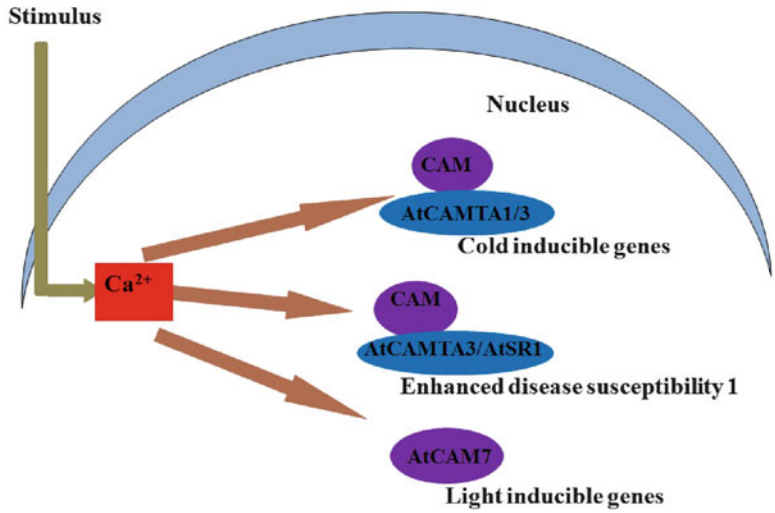

Fig. 14.3 Ca^{2+} signatures induce transcriptional responses

Arabidopsis protein AtCP1, the membrane-associated rice protein OsEFA27, and the *Arabidopsis* counterpart RD20 (Frandsen et al. 1996; Jang et al. 1998). Some CaBPs can be negative regulators of osmotic stress also; one example is the calmodulin-binding protein in *Arabidopsis*, AtCaMBP25. Although it is upregulated by osmotic stress, its overexpression renders plants sensitive to osmotic or salt stresses and its antisense transgenics show improved tolerance (Perruc et al. 2004).

Calmodulin, an important CaBP, is a small acidic protein and is responsible for the regulation of intracellular Ca^{2+} levels. Increased Ca^{2+} concentration activates calmodulin which then induces specific kinases. Calmodulin is a very important calcium-binding protein in Ca^{2+} signaling and has been found to be involved in biotic and abiotic stresses (Fig. 14.3) (Reddy 2001; Tuteja and Mahajan 2007; Tuteja and Sopory 2008). Plants have been found to possess unique Ca^{2+} sensors like calmodulin-like proteins (CMLs), and *Arabidopsis* contains 50 such proteins (Tuteja and Sopory 2008). The calmodulin-like proteins differ from calmodulin in having more than 148 amino acids and also have one to six EF-hand motifs. These CMLs have been found to play a role as Ca^{2+} sensor during stress in plants (Vanderbeld and Snedden 2007). Certain Ca^{2+}-binding proteins do not contain EF-hand motifs, such as calreticulin, annexins, calnexin, phospholipase D (PLD), and pistil-expressed Ca^{2+}-binding proteins.

PLD activity has been reported to be involved in ethylene and ABA responses, synthesis of α-amylase in aleurone cells, closing of stomata, responses to pathogens, leaf senescence, and drought tolerance (reviewed by Tuteja and Sopory 2008).

Annexins have been reported to be involved in biological membrane organization and functions (Tuteja and Mahajan 2007). The exact function of annexins is not clear yet but are thought to play a role in secretary processes and they have ATPase and peroxidase activities (Tuteja and Mahajan 2007). Annexins have been regarded to have a role in stress responses (Gorecka et al. 2007a). Annexin At1 of *Arabidopsis thaliana* (AnnAt1) plays a vital role in pH-mediated cellular responses to environmental stress (Gorecka et al. 2007b).

Calnexin (CNX) is an important calcium-binding protein and is an endoplasmic reticulum (ER) type 1 integral membrane protein. CNX behaves as a molecular chaperone and has a leading role in the recognition of misfolded proteins, lectin-like activity, and Ca^{2+} binding (Sarwat and Tuteja 2007). Till date, the role of calnexin in stress has not been reported but it is considered

that it has a role in ER stress response in plants (reviewed by Tuteja and Sopory 2008).

In plants, another group of Ca^{2+} sensors is the SOS (salt overly sensitive) family and is responsible for calcium-mediated pathway for salinity stress tolerance (Mahajan et al. 2008). Zhu (2003) has isolated *sos* mutants (*sos1*, *sos2*, and *sos3*) from *Arabidopsis* which are hypersensitive to salt. The *sos* mutants have been shown to accumulate more proline under salt stress which gives protection to the salt-stressed plants (Liu and Zhu 1998). Cloning and characterization of *sos* genes (*SOS1–SOS3*) has opened new doors for ion homeostasis and plant tolerance to salt. It has been reported that *sos1*, *sos2*, and *sos3* function in a common pathway leading to salt tolerance (Halfter et al. 2000; Zhu 2000). *Sos1* is activated by *sos3–sos2* complex and has been reported by many workers. Shi and Zhu (2002) demonstrated that if *sos1* alone was allowed to express in yeast cells, slight enhancement in salt tolerance was observed. However, if *sos1* was expressed with *sos3* and *sos2* the yeast cells showed more tolerance to salt. Qiu et al. (2002) have reported very low Na^+/H^+ exchange activity in *sos* mutant plants in comparison with wild type in plasma membrane vesicles. Addition of activated *sos2* protein to isolated membrane vesicles of mutant plants showed that the exchange activity increased in *sos2* and *sos3* but remains unaffected in *sos1* mutants. The results lead to the conclusion that Na^+/H^+ exchange activity of *sos1* was stimulated by *sos3* and *sos2* (reviewed by Xiong et al. 2002). *sos3–sos2* also regulate Na^+ transporter AtHKT1, which is a salt tolerance effector (Uozumi et al. 2000). Homologs of HKT1 in other plant species revealed that it can be either a K^+ transporter or a Na^+/K^+ cotransporter (Horie et al. 2001; Liu et al. 2001). Rus et al. (2001) demonstrated that mutation in AtHKT1 of *Arabidopsis* suppressed the salt hepersensitivity phenotype of *sos3*, leading to the concept that activity of AtHKT1, the Na^+ influx transporter, may be inhibited by *SOS3* (reviewed by Xiong et al. 2002).

As compared to caltractin and calmodulin, *sos3* binds with Ca^{2+} with low affinity and has three EF-hand motifs (Ishitani et al. 2000). In *sos3* mutation, one of the EF-hand motifs gets mutated, thus preventing it to bind Ca^{2+} (Ishitani et al. 2000). *sos4* and *sos5* have recently been characterized (Mahajan et al. 2008). *sos4* encodes a pyridoxal (PL) kinase which has a role in the biosynthetic pathway of pyridoxal-5-phosphate, which is an active form of vitamin B6. Sos5 is a putative cell surface adhesion protein and has a role in normal cell expansion. Under salt stress, *sos5* has been found to have a role of maintenance of cell wall integrity (reviewed by Tuteja and Sopory 2008).

2.3 Calcineurin B-Like Proteins

These proteins possess four Ca^{2+}-binding EF-hand domains and have significant identity to calcineurin B subunit and neural calcium sensor from yeast and animals (Kudla et al. 1999). The CBL protein family and their corresponding kinases (CIPKs) together form a complex and dynamic Ca^{2+} decoding signaling network (Fig. 14.4). Together they show Ca^{2+}-binding functionality and kinase activity (Mahajan et al. 2006a).

CIPKs have a conserved N-terminal kinase domain and C-terminal regulatory domain, separated from the kinase domain by a variable junction domain. A conserved NAF domain present in the divergent regulatory domain is required for interaction with CBLs. CBL binds to the NAF domain of CIPKs, releases the C-terminal (autoinhibitory) domain from the kinase domain, in turn transforming the kinase into its active state (Guo et al. 2001; Mahajan et al. 2006a).

Bioinformatic analysis shows a number of components of both proteins in the families of CBLs and CIPKs; 10 CBLs and 26 CIPKs in *Arabidopsis*, 10 CBLs and 30 CIPKs in rice (Albrecht et al. 2001; Kolukisaoglu et al. 2004; Weinl and Kudla 2009). However, several species of green algae possess single CBL and CIPK genes, other plant species can have multiples, like *Physcomitrella* contains four CBLs and seven CIPKs and the fern *Selaginella moellendorfii* has five CBLs and five CIPKs (Batistic and Kudla 2009; Weinl and Kudla 2009); thus showing evolutionary complexity of these CBL and CIPK protein families from lower to higher organisms.

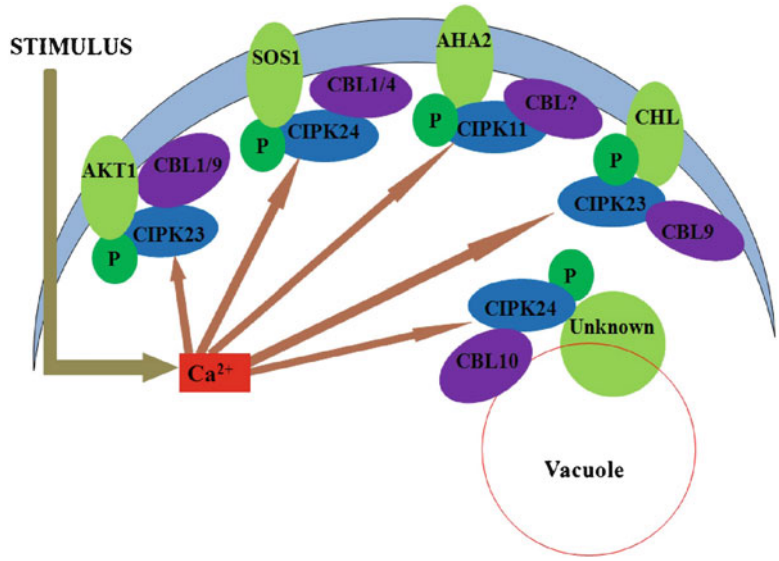

Fig. 14.4 Signaling through CBL/CIPK and protein phosphorylation by Ca^{2+} signatures

CBLs are found to be localized all through the cell. In *Arabidopsis* itself, four CBLs are present at the plasma membrane, four at the vacuolar membrane, and two in the cytoplasm and nucleus (Batistic et al. 2008; Cheong et al. 2007; D' Angelo et al. 2006; Kim et al. 2007; Weinl and Kudla 2009). The pea CBL was reported to be exclusively localized in the cytosol whereas pea CIPK is localized in the cytosol and the outer membrane (Mahajan et al. 2006b). The pea CBL and CIPK were reported to be coordinately upregulated in response to high NaCl, cold, wounding and also in response to calcium and salicylic acid, whereas drought and abscisic acid had no effect on the expression of these genes (Mahajan et al. 2006b).

Our knowledge has been greatly facilitated by the reverse genetic approaches. A CIPK3 loss-of-function (LOF) mutant showed its involvement in regulating ABA-induced gene expression and ABA responses during seed germination (Kim et al. 2003). Through these studies, CBL1 has came out to function in an ABA-independent manner in controlling responses to drought, cold, and salinity (Albrecht et al. 2003; Cheong et al. 2003), whereas its closely related Ca^{2+} sensor CBL9 renders plants hypersensitive to ABA (Pandey et al. 2004). Interestingly, when CIPK1 complexes with CBL1, it mediates the ABA-dependent pathway (D' Angelo et al. 2006). CBL1 and CBL9 activate CIPK23, and the complex regulates the activity of the shaker-like K^+ channel ARABIDOPSIS K^+ TRANSPORTER1 (AKT1) and thus contributes in K^+ homeostasis within the cell (Kudla et al. 1999). This complex also has a role in stomatal regulation under drought conditions.

CBL–CIPK network is a central and critical system functioning in response to a broad variety of stimuli in order to decode Ca^{2+} signals. Each CBL and each CIPK can make alternative protein interactions and are part of multifunctional signaling component, thus determining the flow of information (Mahajan et al. 2006a).

3 Signaling Through MAPK Kinases

3.1 Historical Background

Sturgill and Ray (1986) for the first time discovered MAPK in animal cells and named it as microtubule-associated protein-2 kinase (MAP-2 kinase). It was renamed as mitogen-activated protein kinase (MAP kinase) 1 by Rossomando et al. (1987)

as mitogen was found to activate the group of proteins and this kinase was found to be related to these proteins (reviewed by Sanan-Mishra et al. 2006; Sinha et al. 2011). In 1990, it was reported as serine/tyrosine kinase that belonged to a multigene family (Gotoh et al. 1990). MAP kinase genes (MsERK1) in plant system was for the first time reported from alfalfa in 1993 (Duerr et al. 1993) and D5 kinase in pea (Stafstrom et al. 1993). After that they were reported from various plants like tobacco, *Arabidopsis*, etc. (Jonak et al. 1994). Three major groups of MAPKs are found in yeast and animals: (1) extracellular signal-regulated kinases (ERK) (Cobb et al. 1994), (2) c-Jun amino (NH_2)-terminal kinases or stress-activated protein kinases (JNS/SAPK) (Davis 1994), and (3) high osmolarity glycerol response or p38 kinases (Hog/p38) (Landry and Huot 1995). MAP kinase genes reported in plants belong to the ERK subfamily (Hirt 2000) and transmit a broader range of stimuli (Ligterink and Hirt 2001). Stafstrom et al. (1993) reported that the first MAP kinase gene isolated from pea has 41% identity with plant cdc2 kinases and other kinases involved in osmosensing. MAP kinases generally function as a cascade in which MAPKKK phosphoralates and activates MAPKK which in turn activates MAPK. All the three kinases are interlinked together and are also called extracellular receptor kinases (Hirt 2000; Sanan-Mishra et al. 2006). Different plant MAPKs recognize different substrates (Jonak et al. 2002) because of the high similarity in catalytic domain and little similarity in N-termini. The activation domain of most of the plant MAPKs contains TEY (Thu-Glu-Tyr) sequence and is similar to ERK/MAP kinases group of mammals and yeast (Hirt 2000). The activation domain of some MAPKs possesses TDY (Thr-Asn-Tyr) sequence and is closer to the p38/Hog group of mammals and yeast (Tena et al. 2001). No plant MAPK has been found which has TPY (Thr-Pro-Tyr) sequence at its activation domain. There are three functionally linked protein kinase viz.: MAP3K, MAP2K, and MAPKs. 60 MAP3Ks, 10 MAP2Ks, and 20 MAPK have been reported in *Arabidopsis thaliana* by Group et al. (2002).

3.2 MAP3Ks

It is also known as MAPKKKs or MEKKs. MAP3Ks constitute a diverse family of kinases and are divided into two subfamilies viz. MEKK1 and RAF-like kinases. The MEKK1-like subfamily members are similar to mammalian MEKK1 and to yeast STE11 and BCK1 and RAF-like kinases are similar to mammalian RAF1 MAPK (Group et al. 2002). About 8–10 algal MAP3Ks are found in *Chlamydomonas* and *Volvox* and about 40–60 in *Sorghum* and *Populous*. One of the important things in MEKK-like kinases is having a conserved catalytic domain and *Arabidopsis* has ten members in this group. *Arabidopsis* has been reported to have 80 putative MAPKKKs whereas rice has 75 members (Rao et al. 2010). Plant species having homologs of MAPKKKs have been identified, including the MEKK-like protein kinases, oxidative stress-activated MAP triplekinase 1 (OMTK1) from alfalfa (Nakagami et al. 2004), ANP1, ANP2, ANP3 (Kovtun et al. 2000), YDA (Lukowitz et al. 2004) from *Arabidopsis*, NPK1 (Nicotiana protein kinase 1) from tobacco (Nishihama et al. 2001), Raf-like protein kinase, EDR1 (enhanced disease resistance 1), and CTR1 (constitutive triple response 1) from *Arabidopsis* (Frye et al. 2001; Kieber et al. 1993). MEKK-like proteins have been found to participate in canonical MAP kinase cascades that activate downstream MAP2Ks (Rodriguez et al. 2010). The two RAF-like MAP3Ks are CTR1 and EDR1, and these two RAF-like MAP3Ks are found to participate in ethylene-mediated signaling and defense responses (Frye et al. 2001; Huang et al. 2003). It has also been reported that CTR1 and EDR1 do not participate in a canonical MAPK cascade (Rodriguez et al. 2010).

3.3 MAP2Ks

It is also known as MEKs and MKKs and is divided into four groups viz. Groups A, B, C, and D (Hamel et al. 2006). MKK1 and MKK2 belong to Group A and act upstream of the MAPK MAK4 (Ichimura et al. 1998). There are several reports about the

involvement of MKK2 in response to cold and salinity stress and apart from this both MKK1 and MKK2 mediate innate immunity responses (Meszaros et al. 2006; Qiu et al. 2008). Group B includes MKK3. One of the distinguishing features of this group is nuclear transfer factor (NTF) domain (Hamel et al. 2006). Steggerda and Paschal (2002) reported that NTF enhances the nuclear import of cargo proteins, which suggests that plant MAP2Ks with NTF domains are involved in cytoplasmic nuclear trafficking. MKK3 has been found to participate in cascades that are elicited by pathogens and are dependent on jasmonic acid (JA) signaling (Doczi et al. 2007; Takahashi et al. 2007). Group C has MKK4 and MKK5 and Group D has rest of the kinases from MKKs 7–10. In general, all plant phyla appear to use a more limited number of MKKs compared to other MAPK components. Rodriguez et al. (2010) have reported a single MKK each for the algae *Chlamydomonas* and *Volvox*. This indicates that the same MAP2K may function in several different MAPK modules. Genetic analysis has shown that closely related pairs of plant MAP2Ks have similar functions, e.g., MKK1 and MKK2 are proposed to activate MAPK MPK4 (Qiu et al. 2008), whereas MKK4 and MKK5 act upstream of MPK3 and MPK6, apparently in a redundant manner (Asai et al. 2002). In rice system, MKK genes exhibit differential regulation under different abiotic stresses (Kumar et al. 2008).

3.4 MAPKs

These are also known as MPKs and have been divided into four groups (A–D) (Group et al. 2002). The activation domain of Groups A, B, and C contains TEY (Thu-Glu-Tyr) sequence similar to ERK kinases of animals. The activation domain of Group D contains TDY (Thr-Asn-Tyr) sequence (Rodriguez et al. 2010). MPK3 and MPK6 belong to Group A and have been reported to have a role in developmental processes and also shows responses against biotic and abiotic stresses (Zhang and Klessig 2001; Seo et al. 2007; Sinha et al. 2011). MPK4 belongs to Group B and has a role in pathogen defense and abiotic stress responses (Andreasson et al. 2005; Brodersen et al. 2006; Qiu et al. 2008). The characteristic feature of Group D MAPKs is a C-terminal docking domain that may act as a docking site for MAP2Ks (Yoo et al. 2008).

3.5 MAPKs and Abiotic Stress Signaling

The mitogen-activated protein kinase (MAPK/MPKs) cascades transducer environmental cues into intracellular responses. The stimulated plasma membrane receptors activate MAP kinase kinase kinase and then the sequential phosphorylation ensues as MAP3Ks activate downstream MAP kinase kinase that in turn activates MAPKs (Sinha et al. 2011) (Fig. 14.5). MAPKs then target various effector proteins in the cytoplasm or nucleus, which include other kinases, enzymes, or transcription factors (Khokhlatchev et al. 1998; Sinha et al. 2011; Wurzinger et al. 2011).

Abiotic stress is responsible for the activation of MAPK genes and increased MAPK activity. MEKK1, MKK2, MPK4, and/or MPK6 have been activated during salt, drought, and cold stress (Teige et al. 2004; Sinha et al. 2011). Temperature stress (26–38°C) induces 50 kDa MAP kinase in tomatoes. Induction of MPK3 during cold and salinity in *Arabidopsis* has also been reported by different workers. Ichimura et al. (2000) reported that MPK4 and MPK6 are activated during low-temperature and osmotic stress. The role of several MAP kinases in response to salinity has also been reported by Sanan-Mishra et al. (2006). Matsuoka et al. (2002) demonstrated the role of MAPK kinase (MKK1) in abiotic stress signaling. Multiple abiotic stresses such as wounding, cold, drought, and salinity activated MKK1 which activates downstream MPK4 (Matsuoka et al. 2002). In *Arabidopsis* a specific MAPKK kinase, ANP1, was activated by H_2O_2. This ANP1 activates MPK3 and MPK6 and its positive regulator nucleoside diphosphate kinase (NDP) 2 (Moon et al. 2003). Overexpression of ANP1 shows tolerance to heat shock, freezing, and salt stress tin transgenic plants (Kovtun et al. 2000). Plants

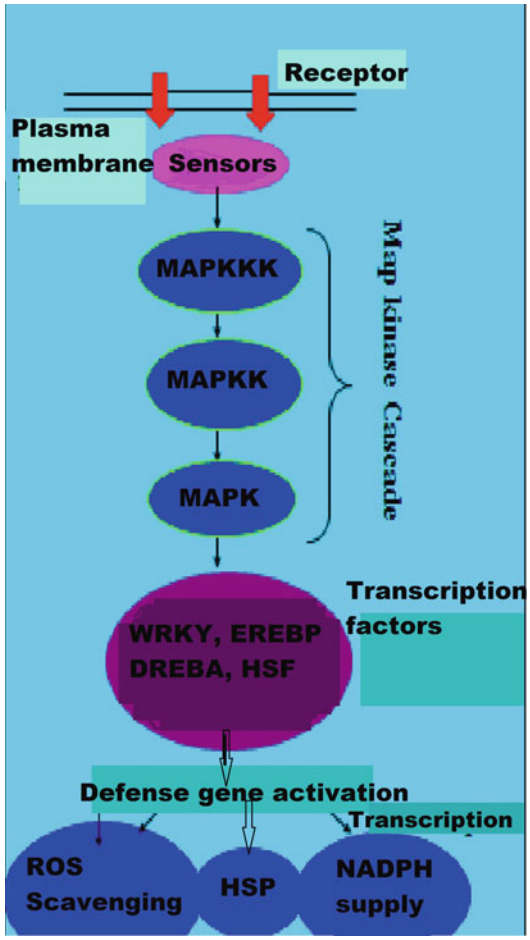

Fig. 14.5 The MAP kinase signal transduction pathway (Adopted from Ahmad et al. 2008)

expressing AtNDPK2 show reduction in H_2O_2 accumulation and tolerance to multiple stresses like cold, salt, and oxidative stress. Munnik et al. (1999) demonstrated that a 46 kDa MAP kinase named SIMK activated during moderate hyperosmotic stress in alfalfa. At severe hyperosmotic stress, a smaller kinase gets activated and the activation of SIMK was not observed at severe hyperosmotic stress suggesting that the two kinases function at different stress levels. Mikolajczyk et al. (2000) showed that a salicylic acid-induced protein kinase (SIPK) was activated in tobacco cells under hyperosmotic stress. In tobacco, MAP kinase is activated by multiple stresses like hyperosmotic, hypoosmotic, salicylic acid, and fungal elicitors. A 40 kDa kinase was activated in *Arabidopsis* by salt stress in a calcium and ABA-independent manner (Hoyos and Zhang 2000). MPK4 acts as a negative regulator in plant defense mechanisms. Tang et al. (2005) demonstrated that *Arabidopsis* EDR1 acts as negative regulator of disease resistance and drought. The *edr1* mutant containing a kinase-deficient form of the EDR1 gene exhibits enhanced resistance to pathogens. The *edr1* mutants could also enhance stress responses and spontaneous necrotic lesions under drought conditions (Tang et al. 2005). Accumulation of H_2O_2, superoxide anions, and hydroxyl radicals during abiotic stress causes oxidative burst in cells (Samuel and Ellis 2000). Plants can withstand this oxidative stress by production of antioxidant enzymes like catalase, which decompose H_2O_2 in the cells. Xing et al. (2008) demonstrated that AtMKK1 mediates ABA-induced CAT1 expression in *Arabidopsis thaliana*. The *Arabidopsis* mutants *mkk1* and *mpk6* were altered in their responses to ABA and desiccation stress and the results showed that MKK1–MPK6 regulate H_2O_2 metabolism by CAT1 (Xing et al. 2008). Nakagami et al. (2004) reported that MEKK1–MPK4 cascades are an important part of ROS metabolism. During abiotic stress, two signaling events must occur to induce defense responses in plant cells: one is the inhibition of negative regulators such as EDR1 and the other is activation of positive regulators (Tena et al. 2001). LeMPK3 isolated from *Lycopersicon peruvianum* is homologous to AtMPK3 and is activated by UV-B radiations (Holley et al. 2003). Mayrose et al. (2004) showed that LeMPK3 is involved in mechanical stress and wounding in tomatoes. CbMAPK3 isolated from *Chorispora bungeana* shows identity of MPK3 and is activated by cold, salt, and ABA. H_2O_2 activated a novel MAPKKK OMTK1 in alfalfa which activates the MMK3 pathway. In tobacco, the overexpression of NtMEK2 stimulates the gene expression for defense and the generation of reactive oxygen species, which are led by the stimulation of two endogenous MAPKs, SIPK and WIPK (Yang et al. 2001; Ren et al. 2002). In rice system, Rao et al. (2011) showed activation of OsMPK3 and OsMPK4 by arsenic stress. While OsMPK3 was activated only in leaves, both OsMPK3 and MPK4 showed activation in roots. Recently, MPK3 from rice and pea were

reported to function as effector molecules of the stress-regulated beta subunit of pea heterotrimeric G-proteins (Bhardwaj et al. 2011).

H_2O_2 induces 2MAPK-like activities in *Arabidopsis* and are independent of ethylene and jasmonic acid (Grant et al. 2000). It is not clear that these MAPKs belong to AtMPK3 and AtMPK6. Jonak et al. (1999) have reported that AtMPK3 and AtMPK6 have similarity to wound and SA-induced protein kinases (WIPK and SIPK) of tobacco, respectively. It was also found that only SIPK is activated in tobacco by ozone and H_2O_2.

4 Nitric Oxide

Nitric oxide (NO) is an important endogenous plant signaling molecule that is responsible for several developmental and physiological processes (Neill et al. 2003, 2008; Tuteja et al. 2004; Delledonne 2005; Lamotte et al. 2005; Erdei and Kolbert 2008; Molassiotis et al. 2010). Extensive work on mammalian system has revealed that NO is a crucial signaling molecule in animals. The physiological functions in plants that are influenced by NO include reduction in seed dormancy (Libourel et al. 2006; Bethke et al. 2007), plant growth regulation and senescence (Mishina et al. 2007), floral transition suppression (He et al. 2004), stomatal movements (Bright et al. 2006; Garcia-Mata and Lamattina 2007), and tolerance to abiotic and biotic stress responses (Uchida et al. 2002; Modolo et al. 2005; Zhao et al. 2007; Floryszak-Wieczorek et al. 2007; Molassiotis et al. 2010). It has also been reported that externally applied NO or NO donors enhance plant tolerance to environmental stresses (Uchida et al. 2002; Garcia-Mata and Lamattina 2007; Zhao et al. 2007a, b).

4.1 Biosynthesis of NO in Plants

In animals, NO is synthesized via a pathway where l-citrulline is formed from l-arginine with the help of nitric oxide synthase (NOS) (Fig. 14.6). The electron donor in this reaction is NADPH and cofactors FMN, FADH, and tetrahydropterin are also used in this reaction. For the biosynthesis of NO in plants, two enzymes NOS and nitrate reductase (NR) are involved (Crawford 2006). There may be other sources for NO biosynthesis apart from these two enzymes (Arnaud et al. 2006). In higher plants, the genes responsible for the upregulation of NOS proteins are yet to be identified. A unique plant NOS (AtNOS1) was isolated from *Arabidopsis* which encodes a protein associated with NO synthesis (Guo et al. 2003). Overexpression of AtNOS1 increases NO synthesis in *E. coli*. Reduced root growth and guard cell NO synthesis has been found in the *Atnos1* mutant of *Arabidopsis* plant in response to ABA (Guo et al. 2003). Low amount of NO accumulation in *Atnos1* mutants was also reported by many workers (Zhao et al. 2007b; Bright et al. 2006; Zottini et al. 2007). Recently, AtNOS1 was found not to have NOS activity and was not required for the normal synthesis of NO, and it now appears that it is not in fact an NOS at all (Crawford et al. 2006; Zemojtel et al. 2006).

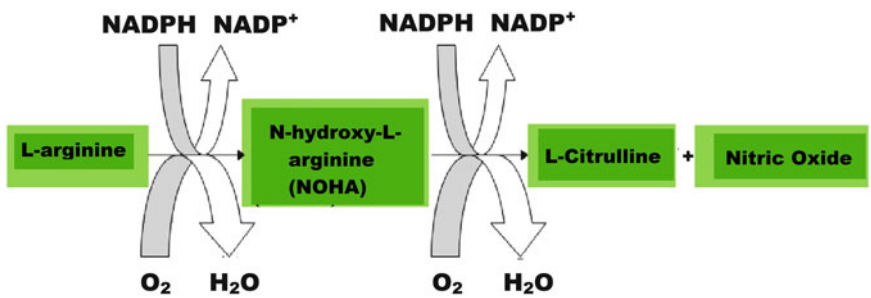

Fig. 14.6 Synthesis of NO from l-arginine catalyzed by nitric oxide synthase

AtNOS1 in the biosynthesis of NO is either indirect or regulatory and thus AtNOS1 was renamed as *Arabidopsis thaliana* nitric oxide-associated 1 (ATNOA1).

Another enzymatic source of NO generation is NAD(P)H-nitrate reductase (NR) that converts nitrite to nitric oxide (NO) (Yamasaki 2000; Rockel et al. 2002). The preliminary function of NR in plants is nitrogen assimilation, but in an NAD(P)H-dependent reaction NR can also convert nitrite to NO (Neill et al. 2003; Bright et al. 2006; Crawford 2006). Generation of NO by NR activity has been reported in different plants by different workers, e.g., cucumber (Haba et al. 2001), sunflower, maize (Rockel et al. 2002), and wheat (Xu and Zhao 2003). NR is encoded by two genes NIA1 and NIA2 in *Arabidopsis*. Double mutants (nia1nia2) deficient in NIA genes showed low NR activity and low NO production in guard cells while their stomata do not close in response to ABA or nitrite (Desikan et al. 2002). Modolo et al. (2006) have reported that due to the lack of NR activity, the nia1nia2 double mutants showed reduced levels of l-arginine and the exogenous l-arginine can restore NO generation in this mutant. Reduction in NOS-mediated NO production may be due to reduced levels of arginine. It is still to be identified how cooperation of two pathways of NO generation controls production of NO in plants.

Another source of NO is a plasma membrane-bound root-specific enzyme, nitrite:NO oxidoreductase (Ni-NOR). The electron donor in this enzyme is not NAD(P)H but cytochrome c and its optimum pH is more acidic than that of NR. The physiological role and genetic identity of this enzyme are not clear yet (Stohr and Stremlau 2006).

4.2 NO Signal Transduction

NO signaling involves cyclic GMP (cGMP)-dependent and -independent pathways such as protein nitrosylation. Pfeiffer et al. (1994) demonstrated that NO stimulated cGMP formation in spruce needles. It is also reported that in tobacco cGMP synthesis is required for NO signaling (Durner et al. 1998). NO activates the cGMP-dependent pathway leading to adventitious root formation in cucumber (Pagnussat et al. 2003). NO increases the cGMP content and inhibitors of cGMP synthesis through guanylate cyclase. It has also been observed that NO induces PCD in *Arabidopsis* and cGMP synthesis is also involved there. cGMP was suggested to be the likely target of NO signaling in guard cells. The inhibitor of cGMP synthesis ODQ attenuated ABA and NO-induced stomatal closure. Clarke et al. (2000) reported that the addition of cell-permeable cGMP analogue, 8-bromo cGMP relieved this inhibition. However, 8-bromo cGMP alone did not promote closing of stomata, suggesting that synthesis of cGMP is involved but is insufficient for stomotal closure (Neill et al. 2002). Desikan et al. (2004) reported that stomatal closure by H_2O_2 was not suppressed by ODQ which indicates that H_2O_2 and NO are different signaling pathways in terms of cGMP signaling. Neither guanylate cyclase nor a cGMP-dependent protein kinase has yet been isolated and cloned from plants (Neill et al. 2003). NO activates intracellular Ca^{2+} channels through cGMP/cADPR-dependent signaling pathway. Besson-Bard et al. (2008) demonstrated that calcium and MAP kinase can mediate NO signaling. K/Na ratios in *Populus eupharatica* have been regulated by NO and H_2O_2 (Zhang et al. 2007). Activation of MAP kinases by NO has been reported in *Arabidopsis* (Clarke et al. 2000) and tobacco (Kumar and Klessig 2000). Other signaling molecules like H_2O_2 (Samuel et al. 2000) and SA (Kumar and Klessig 2000) have been reported to activate MAPK in tobacco which indicates that MAPK cascades should be a focal point of convergence of both H_2O_2 and NO signaling pathway activated in response to various stresses.

5 Sugar as Signaling Molecule

In plants, the biomolecules are very much essential for plant growth and development. Among biomolecules, sugars serve as energy source and as structural component in plants. Koch (2004) reported that 80% of the CO_2 assimilated during

photosynthesis is used for synthesis of sucrose. Sucrose is the major transport form of organic carbon exported from the photosynthetic source to sink organs. Extensive losses in agricultural production have been observed due to environmental stresses (Bohnert et al. 2006; Mittler 2006). Sucrose is performing dual functions, one as transported carbohydrate in vascular part and the other as signaling molecule (Baier et al. 2004). Sugars are responsible for gene expression involved in plant metabolism viz. photosynthesis, glycolysis, N_2 metabolism, cell cycle regulation, etc. Soluble sugars like hormones can act as primary messengers and regulate signals that control the expression of different genes involved in plant growth and metabolism (Rolland et al. 2006; Chen 2007). Gupta and Kaur (2005) have reported that HXK is a sugar sensor in plants. Evolutionary conserved glucose sensor hexokinase 1 (HXK1) mixes different signals (nutrient and hormone signals) and use them for the expression of genes and plant growth in response to environmental stress (Cho et al. 2006). It is not fully understood how the HXK1 controls the gene expression for encoding proteins involved in photosynthesis. Rolland et al. (2006) reported two glucose signal transduction pathways in plants: hexokinase (HXK)-dependent and -independent. HXK-dependent system requires the phosphorylation of glucose while HXK-independent system do not need (Smeekens 2000). Recently, expression of specific photosynthetic genes by HXK1 nuclear complex has been observed and the process is glucose metabolism independent and requires two partners VHAB1 and RPT5B (Chen 2007). This leads to the conclusion that enzymes involved in metabolic activities can play a part in signal transduction by expression of genes in the nucleus. *Arabidopsis thaliana* mutant hsr8 (high sugar response 8) exhibited increased sugar-responsive growth and expression of genes (Li et al. 2007). Li et al. (2007) observed that hsr8 plants grown under light showed lower chlorophyll content and higher levels of starch and anthocyanin in response to glucose treatment. The hsr8 plant grown under dark showed glucose hypersensitivity. HXK transgenic *Arabidopsis* plants (AtHXK) have three distinct glucose signal transduction pathways. First is AtHXK1-dependent pathway, where the expression of gene was correlated with the AtHXK1-mediated signaling functions. The second pathway is glycolysis dependent and is regulated by catalytic activity of both AtHXK1 and the heterologous yeast HXK2. The third is AtHXK1-independent pathway, where the expression of gene was independent of AtHXK1 (Xiao et al. 2000). Hence, the role of HXK in sensing the sugar status is still under discussion (reviewed by Rosa et al. 2009).

Kempa et al. (2007) reported that MsK4 (*Medicago sativa* glycogen synthase kinase 3-like kinases) have been involved in stress signaling with carbon metabolism. MsK4 is located in plastids and is associated with starch granules. Kempa et al. (2007) have also reported that kinase activity of MsK4 is induced rapidly under high salt concentration. Overexpression of MsK4 in transgenic plants showed tolerance to salinity stress accompanied with more starch accumulation and modified carbohydrate content (Kempa et al. 2007). The protein kinases KIN10 and KIN11 connect abiotic stress signals, sugar signals, and developmental signals to regulate plant metabolism (Baena-Gonzalez et al. 2007).

Sucrose nonfermenting-1 (SNF-1)-related proteins, analogues of the protein kinase (SNF-1) yeast signaling pathway, have been reported in plants (Loreti et al. 2001). SNF-1-related proteins have a role in sugar sensing (Purcell et al. 1998).

6 Abscisic Acid in Signaling

Ohkuma et al. in 1963 for the first time purified and crystallized abscisic acid (ABA) from cotton fruits and named it as Abscisin II. It was also isolated from sycamore leaves and was named as dormin. Chemical characterization of both revealed identical nature of the two compounds and it was later named as abscisic acid. Apart from being having an inhibitory role in the plant system, the hormone is also known to possess a stress-protective function. ABA plays an important role in plant responses to drought and salt stresses (Tuteja 2007b; Bansal et al. 2011).

ABA has been shown to regulate many agronomical aspects of plant development like synthesis of proteins and lipids, seed desiccation tolerance and dormancy, and germinative, vegetative, and reproductive growth (Leung and Giraudat 1998; Rock 2000; Rohde et al. 2000). In addition, it mediates the responses of plants to many abiotic stresses like drought, salt, cold stress, and biotic stress like pathogen (Leung and Giraudat 1998; Rock 2000; Rohde et al. 2000; Shinozaki and Yamaguchi-Shinozaki 2000). This implies that ABA is involved in both long-term developmental processes as well as short-term physiological effects. Long-term processes involve changes in pattern of gene expression whereas short-term responses involve changes in the activity of various signaling molecules and fluxes of ion channels across the membranes. Both set of responses require the action of signaling elements which amplify the primary signal generated when the hormone binds to its receptors.

Signaling through ABA causes the production and accumulation of second messengers like Ca^{2+}, phosphatidic acid (PA), or reactive oxygen species (ROS) in the cell which play an important role in ABA signal transduction (Bansal et al. 2011). Reversible protein phosphorylation is an early and central mechanism that occurs in ABA signal transduction (Leung et al. 1997; Himmelbach et al. 2003; Sokolovski et al. 2005). This mechanism involves several protein kinases and phosphatases (Leung and Giraudat 1998; Finkelstein et al. 2002). For example, ABA-activated serine–threonine protein kinase (AAPK) which is a guard cell-specific protein kinase in *Vicia faba* or orthologous OPEN STOMATA 1/SNF1-RELATED PROTEIN KINASE 2.6 (OST1/SnRK2.6) which regulates ABA-stimulated closure of stomata (Li et al. 2000; Mustilli et al. 2002). Fujii et al. (2007) reported other SnRK2, SnRK2.2, and SnRK2.3 that regulate ABA response in germination, growth, and gene expression. PKABA1 is another protein kinase involved in suppressing the gibberellin (GA)-inducible gene expression in barlet aleurone layers (Gomez-Cadenas et al. 1999). Apart from the above-mentioned calcium-independent protein kinases, the role of several calcium-independent protein kinases has also been revealed in ABA signaling. These either belong to the CDPK or to the SnRK3 family. CDPK1 and CDPK1a have been reported to activate ABA-dependent promoters (Sheen 1996). Also, CDPK3 and CDPK6 possess a role in controlling the aperture of guard cells under abiotic stress (Mori et al. 2006). Zhu et al. (2007) reported the involvement of CPK4 and CPK11 which positively regulate ABA signal transduction in seed germination, seedling growth, and stomatal movements. The mechanism involved is probably the phosphorylation of abscisic acid responsive element-binding factor 1 (ABF1) and abscisic acid responsive element-binding factor 4 (ABF4). Finally, several reports confirm the involvement of mitogen-activated protein kinases (MAPKs) in ABA-dependent response to different stresses (Boudsocq and Laurière 2005; Zhang et al. 2006) and germination (Lu et al. 2002). ABA, in case of stress, regulates gene expression in both positive and negative manner (Chandler and Robertson 1994). Under stress conditions, the gene expression results in the production of transcripts that are responsible for hardening or stress tolerance.

7 Phytohormones as Signaling Molecules

7.1 Brassinosteroids

Brassinosteroids (BRs) are plant steroidal hormones having growth-promoting activities (Hacham et al. 2011). Grove et al. (1979) discovered the brassinolide (BL) (the most active form of BR) from the pollens of *Brassica napus*. BRs play significant role in seed germination, photomorphogenesis, root and stem elongation, vascular differentiation, senescence, flowering, and resistance to biotic and abiotic stresses (Clouse and Sasse 1998; Divi and Krishna 2010; Divi et al. 2010). The biosynthetic pathway of BRs was elucidated through chemical analysis and isolation of additional BR-biosynthetic mutants, defective in genes encoding proteins which catalyze

the plant steroid conversion to BR precursors (Asami et al. 2005). First BR biosynthesis inhibitor, brassinazole, is another powerful tool for elucidation of BR signaling pathway (Asami et al. 2000). Genetic, genomic, and proteomic approaches lead to the establishment of BR signaling pathway by providing an important role in the mechanism of receptor activation and regulating components by process of phosphorylation (Tang et al. 2010).

In *Arabidopsis*, extensive genetic screens for LOF BR signaling mutants are detected in one locus, BRI1 encoding LRR RLK (Clouse et al. 1996; Kauschmann et al. 1996; Li and Chory 1997; Noguchi et al. 1999). Phenotypes of BRI1 mutants are similar as that of BR-deficient mutants, but these are not rescued by the addition of BRs. Components of BR signaling pathway have been characterized in additional suppressor and gain-of-function screens, which involve second LRR RLK, the BRI1-associated receptor kinase-1 (BAK1) (Li et al. 2002); the glycogen synthase kinase-3 (GSK3)-like kinase, BR insensitive-2 (BIN2) (Li et al. 2001b; Li and Nam 2002), the serine/carboxypeptidase BRI1 suppressor-1 (BRS1) (Li et al. 2001a), the phosphatase BRI1 suppressor-1 (BSU1) (Mora-Garcia et al. 2004), and the transcription factors brassinazole-resistant 1 (BZR1) (Wang et al. 2002) and BZR2 (BRI1-EMS suppressor 1 (BES1)) (Yin et al. 2002).

Recently, BR signaling model has been refined by proteomic studies by identifying the components like BR signaling kinases (BSKs), which are not found in previous screens, generating a complete signaling pathway from an RLK to transcription factors in plants (Tang et al. 2008).

BRs regulate the signaling pathway identical to that of classic receptor tyrosine kinases (RTKs) and transform growth factor-β (TGF-β)-mediated signaling in plants (Feng and Derynck 1997; Schlessinger 2000, 2002). In *Arabidopsis*, genome sequence has more than 600 RLK members (Shiu et al. 2004) leading to identical signaling mechanisms. Plant RLKs and signaling pathways provide activation to signaling networks, which are controlled by plant hormones (Smet et al. 2009).

7.2 Ethylene

Ethylene is a gaseous plant hormone, plays significant role in developmental processes like seed germination, senescence, fruit ripening, root nodulation, leaf abscission, programmed cell death, stress, and pathogen attack (Johnson and Ecker 1998; Bleecker and Kende 2000; Binder et al. 2010). Ethylene has "triple response" effect on plant growth of etiolated dicotyledonous seedlings. This response leads to radial swelling of hypocotyl, inhibition of hypocotyls, and root cell elongation and exaggerated curvature of the apical hook. Genetic screens of *Arabidopsis* are based on the triple-response phenotype. More than dozen of mutants are divided into three distinct categories. Constitutive triple-response mutants, i.e., ethylene-insensitive overproduction (eto1), eto2, eto3, constitutive triple-response1 (ctr1) and responsive to antagonist1 (ran1)/ctr2; ethylene-insensitive2 (ein2), ein3, ein4, ein6 and tissue-specific ethylene-insensitive mutants, i.e., hookless1 (hls1), ethylene insensitive root1 (eir1) and various auxin-resistant mutants (Johnson and Ecker 1998; Bleecker and Kende 2000; Stepanova and Ecker 2000). Ethylene belongs to the family of membrane-associated receptors, which include ETR1/ETR2, ethylene response Sensor1 (ERS1)/ERS2 and EIN4 in *Arabidopsis* (Chang et al. 1993; Hua et al. 1995, 1998; Sakai et al. 1998). Ethylene attaches to its receptor by copper transporter RAN1-delivered copper cofactor. Functions of receptor are inactivated by the hormone binding (Hua and Meyerowitz 1998). EIN2, EIN3, EIN5, and EIN6 act downstream of CTR1 and positively regulate the ethylene response. EIN2 acts as an integral membrane protein, EIN3 acts as transcription factor and expression of intermediate target gene like ethylene response factor1 is regulated.

Ethylene belongs to the family of five receptors (ETR1, ETR2, ETS1, ERS2, and EIN4) and is divided into two subfamilies on the basis of structural similarities. Type-I subfamily contains ETR1 and ERS1 having amino-terminal ethylene-binding domain (which is also known as sensor domain) and carboxy-terminal histidine (His) kinase domain, whereas type-II subfamily

receptors contain ETR2, ERS2, and EIN4, which involve an amino-terminal ethylene binding domain and a degenerate His kinase domain. Receptors of ethylene negatively regulates the ethylene responses (Bleecker and Kende 2000; Chang and Stadler 2001). Dominant ethylene in sensitivity mutations in receptor ETR1 leads to signaling (Schaller and Bleecker 1995). LOF mutants have no ethylene response phenotypes. Recently, LOF mutations were isolated in ERS1 gene (Zhao et al. 2002; Wang et al. 2003) with etr1, etr2, ers2, and ein4 mutants. Double LOF etr1ers1 mutants possess strong constitutive-ethylene response phenotypes (Wang et al. 2003). These phenotypes are present in plants containing strong allele of ran1, which cause loss-of-function of all receptors of ethylene (Woeste and Kieber 2000). ETR possesses His kinase activity in vitro, which is important for receptor function (Gamble et al. 1998). For other aspects of receptor functionality like localization, protein stability or interaction with other factors, His kinase activity is essential.

In the mechanism of ethylene signaling, ethylene perception and signaling occurs at endoplasmic reticulum (Chen et al. 2002; Gao et al. 2003). For ER association, the amino-terminal membrane-spanning sensor domain of ETR1 is essential. ER localization of ETR1 is not affected by the introduction of etr1-1 mutations or BR application. CTR is found at ER (Gao et al. 2003). CTR1 contains an amino-terminal domain and carboxy-terminal kinase domain that is linked with Raf-like mitogen-activated protein kinase (MAPK). CTR1 interacts with His kinase domains of ETR1 and ERS1 (Clark et al. 1998). ER-associated CTR1 level inhibits due to removal of ethylene receptors and distribution of CTR1 and receptor interactions. CTR1–ETR1 interaction depends on two lines of evidences in vivo. Co-purification of ETR1 leads to affinity purification of CTR1 from the *Arabidopsis* ER-membrane fraction, which describes the ETR1 and CTR1 presence in the protein complex (Gao et al. 2003). Overexpression of amino-terminal domain of CTR1 causes LOFctr1 mutant phenotype. Type-I receptors, i.e., ETR1 and ERS1 play significant role in ethylene signaling. This role is not due to His kinase activity of type-I receptors.

7.3 Jasmonates

Jasmonates (JA) regulate plant growth and development. In the reproductive development of plants, jasmonate signaling plays an important role (Stintzi and Browse 2000; Avanci et al. 2010) by giving protection to plants from abiotic stresses (Traw and Bergelson 2003; Huang et al. 2004; Avanci et al. 2010; Lackman et al. 2011) and from pathogens and insects (Farmer and Ryan 1990; Engelberth et al. 2004; Smith et al. 2009; Ma et al. 2010). In *Arabidopsis*, three mutants namely *jar1*, *coi1*, and *jin1*, which are defective in JA response and one triple mutant defective in JA biosynthesis (*fad3-2fad-72fad8*) help in understanding the functioning of JA in plants (Staswick et al. 1992; Feys et al. 1994; Berger et al. 1996; McConn and Browse 1996). Disruption of biosynthetic pathway of JA causes susceptibility of plants to various insects and pathogens (Engelberth et al. 2004; Lewsey et al. 2010); for example, susceptibility of coil to *Alternaria brassicicola* and *Pythium mastophorum* (Feys et al. 1994). Oxo-phytodienoic acid, JA-amino acids, and JA-glucosyl are the intermediates of JA biosynthesis which act as the signaling molecule in JA pathway (Staswick et al. 2002).

7.4 Salicylic Acid

Salicylic acid (SA) is a naturally occurring phenolic compound having carboxylic acid group attached to the benzene ring. SA has important role in various aspects of plant development (Hayat et al. 2007, 2010). In mung bean, SA helps in the increase in yield and pod number (Singh and Kaur 1980). It also possesses tuber-inducing capacity in potato (Koda et al. 1992). It has positive influence on productivity and nitrogen content in maize (Singh and Srivastava 1978; Asthana and Srivastava 1978), flowering, and helps in reducing transpiration by regulation of

stomata (Khurana and Maheswari 1978; Larque 1979) and alleviation of abiotic stress (Ahmad et al. 2011).

SA signaling has been evaluated in case of plants exposed to abiotic stress. Plant tissues when exposed to abiotic stress release more superoxide anions which further increase the level of hydrogen peroxide (Doke et al. 1994; Ahmad et al. 2010). The increased level of hydrogen peroxide has the ability to stimulate the accumulation of SA (Ahmad et al. 2011). Hence, there is a connection between increase in H_2O_2 level and SA accumulation (Rao et al. 1997). Role of SA has also been described by many workers during cold tolerance in plants like maize, rice, wheat, cucumber, tomato, banana, pea, and mung bean (Janda et al. 1999; yDing et al. 2002; Kang and Saltveit 2002; Kang et al. 2003; Tasgin et al. 2003, Krantev et al. 2009; Popova et al. 2009; Khan et al. 2010). Joseph et al. (2010) have reviewed the exogenous application of SA ant its protective role in different plants under salt stress. Scott et al. (2004) showed the inhibitory effect of SA on the growth of *Arabidopsis* exposed to chilling conditions. Under low temperatures, the salicylate is reported to accumulate as free and glucosyl SA. Based on studies on various wild species and mutants in *Arabidopsis*, it was proposed that SA induces low-temperature growth inhibition. Wang and Li (2006) showed increase in cytoplasmic Ca^{2+} levels after the pretreatment of grape plants with SA. This increased Ca^{2+} helps in maintaining the integrity of plasma membrane during the stress conditions. Also, it was shown that SA-treated plants had higher levels of antioxidants like glutathione and ascorbic acid.

7.5 Auxins

Auxin, the dynamic plant hormone, controls the growth and developmental processes by modulating the levels of auxin/indole acetic acid proteins (Mockaitis and Estelle 2008; Iglesias et al. 2011). Exogenous application of auxin to plants causes alteration in the transcription of gene families, changes in the rate of cell division and cell elongation, range of electrophysiological responses, and changes of tissue pattern and differentiation (Berleth and Sachs 2001; Perrot-Rechenmann 2010). Auxin signaling initiates with the interaction of auxin receptors. Auxin is considered as a multifunctional hormone and the signal is transduced through several signaling pathways. Iglesias et al. (2010) reported that auxin signaling participates in the adaptive response against oxidative stress and salinity in *Arabidopsis*. For wild-type auxin response, a large screen for mutants with changed auxin sensitivity was used to define genes for normal functioning. AXR1, AXR2, AXR3, AXR4, and AXR6 are five different loci and TIR1 is the sixth one (Leyser 2002).

8 Conclusion and Future Perspectives

Plants often experience a variety of changing environmental conditions like light conditions, temperature variations, water and nutrient availability, CO_2 levels, etc. These changes often lead to stress which hampers the plant growth and development. Plants recognize and respond differently to different stresses for their survival. Plants can sense the external stimulus and lead to induction of defense mechanism. How plants cope with these demands is the subject of intensive research and in this review we tried to throw light on different signaling molecules during abiotic stress.

Calcium signaling is involved in the regulation of plant cell cycle progression in response to abiotic stress. Our knowledge regarding Ca^{2+} signaling processes in plants have increased tremendously because of the reverse and forward genetic approaches. Most of this research is focused on Ca^{2+} signal decoders. The versatility of Ca^{2+} ion signaling is amazing. Major contributor to this phenomenon is its unequal distribution which leads to rapid Ca^{2+} oscillations resulting in major concentration changes. Other contributors being highly evolved group of calcium sensors which sense these Ca^{2+} signatures and transduce them to downstream targets for phosphorylation and transcriptional responses. These sensors and

transducers together form intricate signaling networks. Ca^{2+}-regulated transcription factors also have an important role in this information processing. CAMTAs are the ones which are most emerging in this field. So, the important parameters which give specificity to this whole mechanism of sensing and decoding of Ca^{2+} signatures are brought out as the specific Ca^{2+}-binding affinity, the specific cellular concentration and subcellular localization, and the specific interaction affinities of Ca^{2+} decoders. Now the important question is how calcium sensor proteins read the codes in calcium signatures and decode them downstream in such a precise manner. In order to answer this there is a need to analyze the structures of these sensors, their structural dynamics, the parameters which lead to association and dissociation of these Ca^{2+} sensors based on fluorescence resonance energy transfer, and the mathematical modeling approaches. In plants, studies can also be utilized to detect the dynamics of protein interactions and protein complex formation. Additionally, an important but not fully exploited area is the reverse genetics approach for exploring the functioning of Ca^{2+} sensor proteins. Gene knockouts prepared either via crosses or RNAi approaches can be proved beneficial to untangle the functional redundancy issues within and between Ca^{2+} sensor families. The mutant complementation analyses are useful to study specific pathways which may get influenced by disrupting the gene encoding Ca^{2+} sensor. MAPK cascades transduce the environmental and developmental signals into adaptivet and programmed responses. Physiological and developmental processes including stress and hormonal responses, innate immunity, etc. are regulated by MAPK cascades. More research is needed to identify substrates of MAPKs and crosstalk with other signaling molecules.

Chemicals such as NO, ABA, SA, JA, BRs, ethylene, and auxins are reported to respond to various environmental stresses and are reviewed in this chapter. Phytohormones SA, JA, and ET play a central role in plant metabolism. SA is known to play a central role in defense against biotrophic pathogens and systemic acquired resistance. JA plays a central role in defense responses to necrotrophic pathogens and herbivores, whereas ET plays a great role in fruit ripening and senescence and modulates defense responses. Intensive research is needed to know the role of different phytohormones in signaling. The overall progress of research on chemical-regulated stress-responsive genes and their products reflects their central role in plant growth and development under stress conditions.

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Calcium Signalling in Plant Cells Under Environmental Stress

15

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Abstract

A change of intracellular calcium concentration is an early event in a large array of biological processes in plants, such as cell division, polarity, growth and development at normal conditions and under adaptation to abiotic and biotic stresses. This chapter focuses on calcium signalling induced by different types of abiotic stresses, such as salt, cold, anoxia, aluminium and heavy metal stress, while a minor part deals with biotic stress signalling. Most investigations, so far, concerned Ca2+ signalling in the cytosol; however, signalling in the nucleus and other cell compartments such as mitochondria, ER and cell wall have also been reported. The specific "signature" of calcium, including duration, amplitude and frequency of the signalling, induced by different stresses is essential for a change of the physiological function. Different stores for calcium take part in the signalling under various types of stress. Of special interest is a comparison of signalling in tolerant and sensitive species and cultivars.

Keywords

Abiotic stress • Aluminium • Anoxia • Calcium • Membrane transport • Signal transduction • Stress

1 Introduction

Calcium, (Ca^{2+}), is involved in the signal transduction of almost all abiotic and biotic stresses in plants. It also mediates growth and links hormonal signals in plants under stress (Marschner 1995; Reddy 2001; White and Broadley 2003; Desikan et al. 2005; Hepler 2005; Covington and Harmer 2007; Kudla et al. 2010). The signalling network is very complex since it processes multiple

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signals simultaneously. Signals not only operate in the cytosol but also in mitochondria, chloroplasts, ER and nucleus (Logan and Knight 2003; Xiong et al. 2004; Mazars et al. 2009). The cytosolic calcium is maintained at submicromolar level to prevent precipitation of calcium with phosphate and other anions, or binding to negatively charged macromolecules. Different types of stress usually cause a temporary increase in free cytosolic calcium, (Ca^{2+}cyt), concentration depending on the specific stimulus that induces it. Calcium is transported into the cytosol by channels in the plasma membrane, tonoplast, ER or mitochondria (Hetherington and Brownlee 2004; Dodd et al. 2010). The signal is transient or sustained and occurs as repetitive oscillations or spiking, or can last for a longer time. There is a great electrochemical potential difference for Ca^{2+} across the membrane systems. Regulation of calcium in the cytosol is important for the plant to maintain homeostasis through different channels, pumps and carriers (Dodd et al. 2010). An increase in Ca^{2+} concentration in the cytosol and nucleus is detected by many Ca^{2+}-sensing proteins, present in these compartments as free proteins or attached to membranes. These proteins are able to decode the information present within the different calcium spikes or oscillations and process this information into alteration of the cell function. Different signalling pathways are regulated by the transcription machinery and expression of downstream target genes. The downstream reactions can cause an adaptation, helping the plant to survive the specific stress. In addition, if the stress is severe, or continues for a long time, the response could be growth inhibition or cell death.

2 Sensors for Calcium

The role of Ca^{2+} as a second messenger molecule depends on its unique physical and chemical properties (Jaiswal 2001). The free Ca^{2+} ion rapidly binds to anions and easily forms insoluble salts with inorganic and organic macromolecular anions (Williams 2006). At high concentration in the cell cytosol, calcium becomes toxic and the Ca^{2+} concentration thus is well regulated. The Ca^{2+} concentration in the apoplast and vacuoles usually range between 0.1 and 1 mM while the endoplasmic reticulum (ER) lumen contains approximately 1 mM, but these concentrations can increase up to 50 mM (Tuteja 2007). On the other hand, the stress-induced Ca^{2+} elevations in the cytosol never exceed micromolar concentration and even a sustained signal may be toxic to the cell and cause apoptosis (Levine et al. 1996).

Plants have a large family of Ca^{2+}-sensor proteins. DeFalco et al. (2010) recently presented a detailed review of Ca^{2+} sensors. The different Ca^{2+}-sensor proteins detect the Ca^{2+} signals and transform them to downstream pathways, either by binding or activating the different targets. Together with channels, pumps and carrier proteins these sensor proteins regulate the free calcium concentration in the cell cytosol. The calcium binding proteins (CaBPs) can function as "trigger" proteins as well as "buffer" proteins (Tuteja and Sopory 2008). The former are activated by forming complex with Ca^{2+} and then affect other proteins of the signalling pathway, while the buffer proteins bind to an elevated concentration of Ca^{2+}, leading to a decrease of the free Ca^{2+} level. The trigger proteins identified so far are calmodulin (CaM), CaM-binding proteins, Ca^{2+}-dependent protein kinases (CDPKs) and phosphatases (Reddy 2001).

An increase in Ca^{2+}cyt concentration can be sensed by CaM, CDPKs and other calcium-binding proteins. These proteins can trigger downstream responses, which, in turn, can elicit specific physiological responses. The signals from CaM can induce mRNA and gene expression, either directly, or by binding to specific transcription factors (Tuteja and Mahajan 2007), that can be activated by phosphorylation or dephosphorylation. The CDPKs are Ser/Thr protein kinases found in plants and in some protozoans. Except for the N-terminal domain the CDPKs have a catalytic domain, an auto inhibitory region, and a calmodulin-like domain activated by calcium binding (Harper et al. 1991; Hrabak et al. 2003). The peroxisome-located CDPK may be involved in oxidative stress and lipid metabolism. In *Arabidopsis*, 9 CDPK isoforms have been

characterized (Dammann et al. 2003). The subcellular locations were detected by expression of green fluorescent protein (GFP) fusion to root tips of transgene plants and fluorescence microscopy. The isoforms, AtCPK3 and AtCPK4, being primarily soluble, showed a nuclear and cytosolic distributions, respectively, while all the others were membrane bound. Except for AtCPK1, which was targeted to the peroxisomes, all the others were targeted to plasma membrane, and these membranes were associated with the kinase by potential N-terminal acylation sites.

Plants possess calmodulin-like proteins (CMLs), which differ from CaM in possessing approximately 148 amino acids and also have 1–6 EF hand motifs (Reddy 2001). Some of the CMLs are suggested to take part as sensors for calcium during stress and developmental responses (Vanderbeld and Snedden 2007).

Protein phosphatases are involved in various stress signals in plants, for instance, at low temperature-induced signal transduction (Sharma and Deswal 2004). Cold stress induces protein phosphatase C (PP2B) and transcripts in the ice plant (Miyazaki et al. 1999). Calcineurin is a Ca^{2+}/CaM-dependent protein phosphatase (PP2B), belonging to the Ser/Thr-specific phosphatases. It has a structure similar to CaM with a catalytic subunit (CnA) and a regulatory subunit (Hashimoto et al. 1990). When the Ca^{2+} concentration is low in a cell, calcineurin becomes inactive, since it has an autoinhibitory domain in the active site, whereas at elevated Ca^{2+} concentration, Ca^{2+}/CaM binds to the CnA subunit of calcineurin, gets activated, causing a conformational change in the enzyme. Calcineurin activity is present in animal cells, but has not yet been detected in plant cells. However, calcineurin-like proteins may be involved in stomatal guard-cell opening, as calcineurin inhibitors block this process (Luan et al. 1993). Calcineurin-like activity was also shown in *Brassica juncea* subjected to low temperature (Sharma and Deswal 2004).

There are calcium-binding proteins with no EF-hand motifs, such as PLD (phopholipase D), annexins, calreticulin, calnexin and PCP (Pistil-expressed Ca^{2+}-binding protein). The PLDs can split the membrane phospholipids into a head group and phosphatidic acid (Wang 2001). Many different isoforms exist with various affinities for Ca^{2+} and difference in their modulation of phosphoinositides, free fatty acids and lysolipids. The regulators of PLD activity are substrate or products of PLC (phospholipase C). The latter enzyme cleaves phospholipids into IP_3 and diacylglycerol (DAG). These enzymes together with phospholipase A2 and DAG kinase are supposed to be involved in plant signalling under stress. The annexins have been found in both animals and plants. They contain 4–8 repeats of about 70 amino acids and bind to phospholipids in a Ca^{2+}-depending way (Tuteja and Mahajan 2007). They are supposed to be involved in the organization and function of membranes. The *Arabidopsis* annexin (AnnAt1) may have an important impact on pH-mediated cellular response to environmental changes and in ion homeostasis in plant cells (Gorecka et al. 2007). CaM can bind to and activate transcription factors. A family of transcription factors called calmodulin-binding transcription activators (CAMTA) is probably involved in stress-induced gene expression in *Arabidopsis*, mediated by calcium (Yang and Poovaiah 2002). All CAMTAs have a specific DNA-binding domain, as well as an IQ motif which binds CaM. In salt stress signals in plants, the salt overly sensitive (SOS) pathway is involved and is discussed in this chapter.

3 Transporters of Calcium

In higher plant cells, the free Ca^{2+} level necessary for maintaining a proper metabolism in the cytosol usually varies between 30 and 400 nM. By hormone action, light and when plants are subjected to biotic or abiotic stresses, calcium ions are transported passively into the cytosol by channels in the plasma membrane, tonoplast, ER, chloroplasts and mitochondria. Calcium signals not only generate in the cytosol, but also in organelles with a double membrane, such as mitochondria, chloroplasts and nuclei (Xiong et al. 2006). To restore the low resting concentration, Ca^{2+} has to be transported out against an electrochemical gradient into the apoplast, or into intracellular

organelles by Ca^{2+} ATPases or Ca^{2+}/H+ antiporters. CaBPs play a vital role in regulating the free Ca^{2+} concentration, whereas the calcium-transporter proteins have different affinities for Ca^{2+}. It was suggested by Hirschi (2001) that the Ca^{2+}-ATPases, with high affinity (K_m = 1–10 μM), although low capacity for transport, are responsible for maintaining the [Ca^{2+}]cyt homeostasis in resting cells, but that the Ca^{2+}/H^+ antiporters, with lower affinity (K_m = 10–15 μM) and high transport capacity, are active after Ca^{2+}cyt signals.

3.1 Ca^{2+} ATPases

In plants the major families of Ca^{2+} ATPases energized by ATP are P-type ATPases IIA and IIB (Sanders et al. 2002). In *Arabidopsis*, the PIIA clade ATPases, present in the ER named ECAs, and the PIIB clade of autoinhibited Ca^{2+} ATPases, named ACAs, have been identified (Boursiac and Harper 2007). The nucleotide specificity of the PIIA (ECA) type is broad, as they can react with GTP and ITP and are inhibited by erythrosine B (Axelsen and Palmgren 2001). The ECA type lacks the N-terminal autoregulatory domain, present in the ACA type. The ECA group seems to be less important for calcium homeostasis (Dodd et al. 2010). The ACA type along with Ca–CaM binding and a serine-residue phosphorylation site can be modulated by Ca^{2+}cyt, either through activation upon binding CaM or by inhibition upon phosphorylation by CDPK (Hwang et al. 2000). The isoforms of PIIB (ACAs) ATPases have different affinities for CaM and are active at different membranes in *Arabidopsis*, such as the plasma membrane, tonoplast and plastid membranes. It is suggested that they have different functions and react differently to different Ca^{2+} signals (Tuteja and Mahajan 2007). The expression of many Ca^{2+} ATPases increases under high salinity or high Ca^{2+}cyt, and some of them are only expressed under stress (Garciadeblas et al. 2001). ACA12 and ACA13 transcripts were upregulated by pathogen stress (Boursiac and Harper 2007) and ACA8 and ACA10 were differently regulated by cold (Schiott and Palmgren 2005).

3.2 Calcium/Proton Exchangers

The Ca^{2+}/H^+ antiporters energized by a proton-motive force have less affinity for Ca^{2+} than Ca^{2+} ATPases. The proton gradient is generated by the tonoplast while the H^+ ATPase and the proton-pumping pyrophosphatases pump protons into the vacuole. The Ca^{2+}/H^+ antiporters are called cation exchangers (CAXs) 1–6 (Shigaki and Hirschi 2006). When the membrane location has been identified, for instance for CAX1, CAX2 and CAX4, the location was in the tonoplast (Hirschi 2001). The CAX1 antiporter has high affinity and high specificity for Ca^{2+}, while CAX2 has high affinity for heavy metals and transports them into the vacuole. Especially CAX1 and CAX3 seem to be important for Ca^{2+} homeostasis. Mutants lacking CAX1, cax1, have reduced lateral roots and number, as well as reduced primary inflorescence length (Cheng et al. 2003). These mutants also have an increased capacity for cold acclimation and enhanced expression of CBF/DREB1 (Catala et al. 2003). CAX1 and CAX3 are expressed differently in the root and shoot, and the expression increases at a high Ca^{2+} supply (Hirschi 2001). In shoot, CAX1 expression is strong, while in roots, CAX3 expression dominates. It was shown that CAX3 mutants were more susceptible to salt stress (Zhao et al. 2008). The stoichiometry of the tonoplast antiporters was reported to be $3H^+/1Ca^{2+}$ (Sanders et al. 2002).

3.3 Calcium Channels

Calcium transport into the cytosol is mediated by different Ca^{2+}-permeable channels, such as depolarization-activated Ca^{2+} (DACC), hyperpolarization-activated Ca^{2+} (HACC) and voltage-independent (VICC) channels (Sanders et al. 2002). These channels can be activated by abscisic acid (ABA) and ROS, for instance in guard cells, and by ROS at the plasma membrane of root hairs during cell expansion (Foreman et al. 2003). In other membranes, calcium-permeable channels also take part in the signalling pathway. There are also voltage-dependent channels in the

tonoplast and ER (Sanders et al. 2002; Demidchik and Maathuis 2007). Moreover, ligand-gated channels in the tonoplast and ER transport Ca^{2+} into the cytosol. Results have shown that inositol triphosphate ($InsP_3$) and cADPR can mobilize Ca^{2+} from internal stores. However, receptors for these substances are not encoded by higher plants as in animal cells (Dodd et al. 2010).

Other types of Ca^{2+}-permeable channels are cyclic nucleotide-gated channels (CNGCs), glutamate receptor-like (GLR) channels, two-pore channels (TPCs) and annexins (Dodd et al. 2010 and references therein). Some of the CNGCs, besides permeable for monovalent cations, have also been found in the plasma membrane with both a nucleotide-binding domain and a CaM-binding one. The GLR channels are homologues of animal glutamate receptors and function as non-selective cation channels. *Arabidopsis* has 20 members of the GLR gene family and the channel is supposed to be a tetramer or pentamer (Davenport 2002). Addition of glutamate and five other amino acids, as well as glutathione, to *Arabidopsis* seedlings induces a Ca^{2+} spike in the cytosol, which is reduced in glr3.3 mutant (Qi et al. 2006).

The *Arabidopsis* genome contains of a single member of the TPC. This protein is supposed to be a homodimer, with two calcium-binding EF hands and a 14-3-3 binding domain and is probably voltage gated (Peiter et al. 2005). It has been suggested that during stress, annexins could form plasma membrane Ca^{2+}-permeable channels (Laohavisit et al. 2009). When annexin proteins were purified from maize and incorporated into planar lipid bilayers, they could function as Ca^{2+} channels. Also, it was proposed that calcium transport into the nucleus proceeds in different ways (Alonso et al. 2006).

4 Organ, Cell and Organelle-Specific Signalling

Different types of signalling occur in different plant organs, cell types and cell organelles. Moreover, root-to-shoot signalling often occurs under different types of stresses. The xylem is most likely involved in the transfer of signals, e.g. pH changes (Felle et al. 2005). To study calcium signalling in vivo, in intact plants and in different cell types, luminescence measurements of seedlings constitutively expressing the calcium-binding protein aequorin can be used (Plieth 2001). To investigate Ca^{2+}cyt changes in single living cells or protoplasts, fluorescent probes specific for calcium, such as Fura 2 and Indo 1, and two-wavelength photometry together with epifluorescence microscopy can be used. For confocal laser-scanning microscopy, the fluorescent probe Fluo 3 is more often used. The sub-cellular distribution of soluble calcium can also be detected by transmission electron microscopy in combination with potassium pyroantimonate precipitation method (Ma et al. 2009).

4.1 Calcium Signalling in Different Cell Types

To detect changes in Ca^{2+}cyt concentration in different root cells, Kiegle et al. (2000) used transgene *Arabidopsis*, so-called GAL4 expression in specific cell types. By fusing yellow fluorescent protein (YFP) to aequorin, the luminescence of targeted aequorin, reflecting the free calcium concentration, could be measured in the different cells when roots were subjected to salt, cold and osmotic stresses. Cold stress induced rapid peaks in all cell types tested. However, there were significant differences in their response to cold, osmotic (440 mM mannitol) and salt stresses (220 mM NaCl) in different cell types. The endodermis and pericycle cells displayed prolonged oscillations in Ca^{2+}cyt concentration when subjected to osmotic and salt stresses, different from other cell types (Kiegle et al. 2000).

4.2 Calcium Signalling in Different Organelles

Calcium-dependent events take place in the cytosol, as well as in the organelles containing millimolar concentration of calcium, mainly

sequestrated as bound calcium (Malho et al. 1998). Calcium signalling takes place in organelles originating from prokaryotes, such as mitochondria and chloroplasts (Johnson et al. 1995; Logan and Knight 2003; Xiong et al. 2006; McAinsh and Pittman 2009). The sub-cellular location of Ca^{2+} sensors plays an important role in defining their functions. Different organelles in the cell can communicate with each other by Ca^{2+}. For instance, Ca^{2+} can be exchanged between chloroplasts and cytosol (Sai and Johnson 2002), and between mitochondria and ER (Rizutto et al. 2009). The nucleus has the potential to generate and regulate Ca^{2+} signals of its own (Pauly et al. 2000; Mazars et al. 2009). Since the nucleus has a Ca^{2+} signalling system, this could originate from prokaryotes too, as speculated by Galon et al. (2010b). In maize leaflets under drought stress, the level of Ca^{2+} increased in cytoplasm, chloroplasts and nucleus and decreased in vacuoles and intercellular spaces (Ma et al. 2009). It is evident that calcium signalling in plant cells is very complex.

4.2.1 Apoplastic Signalling

Gao et al. (2004) reported both intracellular and extracellular pH and calcium changes in *Arabidopsis* mutants under salinity, drought (mannitol) and cold stresses. They used ratiometric pH-sensitive derivatives of GFP as pH indicator and fused it with luminescent aequorin for simultaneous calcium measurements. An *Arabidopsis* chitinase signal sequence was used to deliver the indicator complex to the apoplast. Luminescence measurements from the root showed that chilling stress induced different calcium and pH changes in the cytosol compared with apoplast. Calcium changes were much more distinct in the cytosol than in the apoplast. Osmotic stress (200 mM mannitol) hardly affected the apoplastic calcium, but gave pronounced transients in cytosolic calcium. On the other hand, repeated additions/removals of iso-osmotic NaCl (100 mM) induced calcium changes both in the apoplast and cytosol. The first NaCl addition showed a permanent increase of the cytoplasmic calcium, after a short transient peak, and this increase remained after several additions/removals. The amplitude of Ca^{2+}cyt response to hyperosmotic stimulus was much smaller as compared to hypo-osmotic stimulus. This finding corroborates result from experiments with tobacco culture cells (Pauly et al. 2001) showing that shrinking is less serious for the cells compared to swelling. Swelling could rupture the cell wall and/or membrane (Gao et al. 2004). Although calcium can activate cell-wall phosphatases (DeMarty et al. 1984) and apoplastic CaM plays an important role in signal transduction (Sun et al. 1994; Ma et al. 1999), it is still an open question whether the apoplast plays an important role in the signalling system under stress. Essah (2000) showed that external calcium had no effect on Na^+ toxicity in *Arabidopsis*.

4.2.2 Calcium Signalling and Transport in Chloroplasts

It is now more clear that organelles with double membranes, such as mitochondria, chloroplasts and nuclei can generate calcium signals on their own (Xiong et al. 2006). It was already shown during 1980 that light induces a calcium influx into chloroplasts of both wheat and spinach (Muto et al. 1982; Kreimer et al. 1985). Kreimer et al. (1985) suggested that the influx across the envelope of intact chloroplasts was linked to the photosynthetic electron transport. Later on Shabala and Newman (1999) reported, by use of ion-selective vibrating microelectrodes close to the leaf surface, that light also induced changes in H^+, K^+, Cl^- and Ca^{2+} concentrations in the mesophyll cells of beans. These ion fluxes were related to changes in plasma membrane potential and a calcium influx was considered as a main depolarizing agent in the electrical response to light. Calcium influx started within 5 s, while net fluxes of H^+, K^+, Cl^- did not begin until after 2 min. The initial alkalinization found in the medium was suggested to depend on CO_2 uptake by the photosynthesizing tissue, while the activation of the H^+ pump occurred 1.5–2 min later. Two different mechanisms for uptake of Ca^{2+} into chloroplasts were suggested; one by a potential-stimulated uniporter at the inner-envelope membrane and another by a H^+/Ca^{2+} antiporter in the thylakoids fuelled by ATP (Xiong et al. 2006).

Elevated temperatures induce many changes in gene expression leading to thermotolerance and improve the cell survival to high temperature. A report of Gong et al. (1998) suggests that cytosolic Ca^{2+} is also involved in heat-shock (HS) signal transduction. Use of transgene tobacco, where the aequorin protein was targeted both to cytosol and chloroplasts, showed that HS induced a prolonged, transient increase of Ca^{2+} in the cytosol and not in the chloroplasts. Inhibitor analysis suggested that Ca^{2+} was mobilized from both intra as well as extracellular sources. There are some reports showing that chloroplast can control the cytosolic Ca^{2+} transients involved in stomatal closure (Nomura et al. 2008; Weini et al. 2008).

4.2.3 Calcium Signalling and Transport in Mitochondria

Logan and Knight (2003) described the first successful targeting of aequorin to plant mitochondria, where they found, that the resting values of free Ca^{2+} concentration differed in the cytosol (100 nM) and in mitochondria (200 nM). Treatment of *Arabidopsis* seedlings floating in water, with cold, osmotic (mannitol), touch and oxidative (hydrogen peroxide 10 mM) stresses showed almost the same calcium signature (kinetic pattern) in both cytosol and mitochondria. However, except for hydrogen peroxide addition, the amplitude was much higher in cytosolic than in mitochondrial calcium increase. Touch stress induced an immediate elevation, followed by a return to near-resting concentration within 20 s in the cytosol, but the return to resting level was much slower in mitochondria. The addition of hydrogen peroxide caused almost the same reaction in both compartments and hence indicated that mitochondria could be more sensitive to oxidative stress than to other stresses. Calcium and palmitic acid (Pal) induced a stable and prolonged partial depolarization of the mitochondrial membrane, pore opening, release of calcium and swelling of mitochondria (Mironova et al. 2007). Addition of inhibitors of Ca^{2+} uniporters, like ruthenium red and La^{3+}, as well as EGTA, added 10 min after Pal/Ca^{2+}-activated pore opening, prevented the release of Ca^{2+} and re-polarized the membrane to initial level. The authors also found similar effects in mitochondria accumulating high strontium, Sr^{2+}, concentration in the absence of exogenous Pal, leading to activation of phospholipase A2 and formation of endogenous fatty acids. They concluded that Ca^{2+} is taken up into mitochondria by a uniporter and that Ca^{2+} efflux is mediated by a short-living Pal/Ca^{2+}-activated pore. Under oxidative stress, an increase of electron transport in mitochondria triggers H_2O_2 production leading to depletion of ATP, opening of permeability transition pores (PTP) and finally causes death of the cell (Tiwari et al. 2002).

4.2.4 The Nucleus Can Generate and Regulate Calcium Signals on Its own

The nucleus can be considered as two compartments: the nucleoplasm and the nuclear envelope, in which calcium is stored, and possibly both have a calcium buffering capacity (Briere et al. 2006). The envelope lumen is connected with the lumen of the ER (Bach et al. 1992). The envelope also possesses pores that allow molecules up to 40 kDa to penetrate (Brandizzi et al. 2004). It is well known that calcium-dependent processes take place in the nuclei (Xiong et al. 2006). For instance, an increase in free calcium level is necessary for activation of nuclear sensing kinases, phosphatases in the nucleus and other steps in the signalling pathways both in animals (Carafoli 2002), as well as in plants (Harper et al. 2004). It has been suggested that the nucleus can be described as "a cell within the cell" (Bkaily 2006; Gomes et al. 2006).

Since 1999 it was possible to detect free calcium concentration changes in the nucleus, $[Ca^{2+}]n$. van der Luit et al. (1999) succeeded to fuse aequorin to nucleoplasmin, a nuclear protein. They also investigated the effects by wind and cold shock on both cytosolic and nuclear changes of calcium in tobacco seedlings, and found different dynamics in $[Ca^{2+}]n$ and $[Ca^{2+}]$ cyt. A simultaneous rapid increase of both $[Ca^{2+}]$ cyt and $[Ca^{2+}]n$ was obtained, but the increase in $[Ca^{2+}]n$ was delayed with respect to the cytosolic changes. Wind and cold also induced the expression

of a CaM gene (NgCAM-1) and a comparison between calcium dynamics with gene expression indicated that wind-induced expression depends on nuclear calcium signalling, while cold shock-induced expression is mediated by cytosolic calcium elevation. When tobacco seedlings were subjected to decreased osmolarity of the culture medium, an increase of $[Ca^{2+}]$cyt was followed by an increase of $[Ca^{2+}]n$ but the $[Ca^{2+}]n$ remained for longer time than $[Ca^{2+}]$cyt (Pauly et al. 2001). On the other hand, increase of the osmolarity of the culture medium elicited a smaller change in $[Ca^{2+}]$cyt, but did not modify the biphasic shape of the cytosolic response, and did not affect the $[Ca^{2+}]n$.

Bimodal long-lasting changes in $[Ca^{2+}]$cyt were also found when proteinaceous elicitors were used (Lecourieux et al. 2005). Cryptogein, a polypeptidic elicitor induced a transient peak in $[Ca^{2+}]$cyt after 5 min followed by a sustained increase for at least 2 h. On the other hand, a weak increase in $[Ca^{2+}]n$ was followed by a substantial increase for 1 h, with a maximum at 23 min, and thereby, peaked much later than the $[Ca^{2+}]$cyt. It was suggested that a 1-s delay between the cytosolic and nuclear responses is enough to exclude the possibility of just a diffusion of Ca^{2+} from the cytosol to the nucleus of the cell (Meyer et al. 1995). Xiong et al. (2008) showed that sphingolipid metabolites selectively elicited an increase in nuclear calcium concentrations in a dose-dependent manner both in cell suspensions and in isolated nuclei of tobacco BY-2 cells. Thus, the nuclear calcium changes were independent of the cytosolic compartment.

In pollen tubes, calcium gradients in the tip were oscillating in the cytosol during normal polarized growth, but no measurable changes in $[Ca^{2+}]n$ were found (Watashiki et al. 2004). Thus, the available results so far demonstrate that different calcium signals proceed in different cell compartment and that the $[Ca^{2+}]n$ is not directly linked by diffusion to a change in $[Ca^{2+}]$cyt (Mazars et al. 2009). Proteins that are important for the amplification or diminishing of calcium signals are found to shuttle to and from the nucleus. Rodriguez-Concepcion et al. (1999) showed that CaM 53 could be localized reversible to the nucleus and plasma membranes. When localized to the plasma membrane it was isoprenylated at the C-terminus domain, but inhibition of isoprenoid biosynthesis caused an accumulation of CaM to the nucleus. They also showed that CaM was associated with the plasma membrane after light exposure, then transferred to the nucleus in darkness. It has been suggested that other calcium-binding or CAM-binding proteins, as well as calcium-dependent enzymes and different transcription factors are present in the nucleus and regulate the activity (Mazars et al. 2009). Calcium signals may also be generated via G-coupled receptors localized to the nucleus without involvement by the plasma membrane. It is suggested that the nucleus can produce inositol 1,4,5 triphosphate (IP3) and IP3 receptor-mediated Ca^{2+} release (Gomes et al. 2006). Nuclear activities are probably regulated by a cross-talk between ROS and calcium (Mazars et al. 2010).

5 Calcium Signalling Under Anoxia

Higher plants are strict aerobic organisms, and thus, directly depend on molecular oxygen for their respiration and other metabolic processes. Nevertheless, very often they suffer from oxygen shortage in different agricultural, horticultural and industrial areas. Availability of oxygen has a strong influence on distribution of plant species in ecosystems and severe economical impact. Yield loss due to deficiency of oxygen could reach up to 50% (Dennis et al. 2000). The lack of oxygen usually results from excess of water, ice crust and soil compaction. Waterlogging of rhizosphere and partial flooding of the aboveground parts of plants lead to gradual hypoxia (deficiency of oxygen) and complete submergence brings about anoxia (total absence of this gas).

5.1 Anoxic Injury in Plants

The lack of molecular oxygen leads to inhibition of aerobic respiration, which in turn ultimately causes energy starvation. After the switch from

aerobic condition, ATP level in the cell exhausts within 1–2 min (Drew 1997). To provide ATP for energizing cellular metabolism, glycolysis is passed into fermentation by activation. Overconsumption of respirable sugars by these processes aggravates energy starvation, particularly in hypoxia-susceptible species (Vartapetian and Jackson 1997). On the other hand, stimulation of lactic fermentation and lack of ATP to energize transport ATPases account for cytosol acidification (Drew 1997). Alcoholic fermentation generates a toxic intermediate (acetic aldehyde) and end-products (ethanol) that together with acidosis self-poison plant under oxygen deficiency (Vartapetian and Jackson 1997; Gibbs and Greenway 2003; Bailey-Serres and Voesenek 2008). In natural habitats flooding leads to low soil-redox potential and production of reduced substances including Mn^{2+}, Fe^{2+}, H_2S, NO_2^- along with the intermediates of carbon metabolism such as methane, ethane, ethylene, acetylene, acetic and butyric acid (Drew 1997 and references wherein). Moreover, reestablishment of normoxic conditions triggers oxidation of these substances and synthesis of reactive oxygen species (ROS) resulting in post-anoxic injury.

5.2 Oxygen Deficiency Tolerance Mechanisms in Plants

Capacity to survive under oxygen deprivation depends on a number of developmental, morphological and metabolic adaptations in plants. Imposition of hypoxia accelerates growth of shoot axial organs, stimulates formation of adventitious roots and aerenchyma particularly in tolerant plant species, as a result, the shoot actively transports oxygen to a flooded root (for review, see Crawford and Braendle 1996; Drew 1997; Vartapetian and Jackson 1997; Kende et al. 1998; Sauter 2000; Gibbs and Greenway 2003; Bailey-Serres and Voesenek 2008). Simultaneous shifts occur in the metabolism, particularly underneath lack of oxygen. Metabolic adaptations mainly include avoidance of energy starvation, prevention of toxicity of anaerobic intermediate and end products, besides, post-anoxic injury, disposal of cytosol acidification and use of alternative electron acceptors (like nitrate, nitrite, unsaturated lipids, etc.) (Crawford and Braendle 1996; Drew 1997; Vartapetian and Jackson 1997; Gibbs and Greenway 2003; Greenway and Gibbs 2003; Bailey-Serres and Voesenek 2008). Hypoxia-tolerant plants are notable for maintenance of their cell ultra structure (Vartapetian and Jackson 1997), membrane stability (Crawford and Braendle 1996) and synthesis of anaerobic stress proteins (Vartapetian and Jackson 1997; Greenway and Gibbs 2003). Most of the anaerobic stress proteins belong to enzymes of the glycolytic or fermentative pathways, carbohydrate mobilization and nitrogen metabolism (Vartapetian and Jackson 1997; Greenway and Gibbs 2003). All these metabolic adaptations mutually allow tolerant plants to generate sufficient amount of energy, maintain mineral uptake and even to grow in total absence of oxygen.

5.3 Perception of Anoxic Stress in Plants

The mechanism(s) of oxygen sensing by plant cell and sensor itself are still to be elucidated. Oxygen depletion could be detected directly by binding molecular oxygen (direct sensing) or recognized by altered cellular metabolism, i.e. indirect sensing (Bailey-Serres and Chang 2005; Licausi and Perata 2009). Only prokaryotes have direct oxygen sensors, including heme-containing protein kinases, Fe/S cluster- and SH-containing transcription factors, which induce anaerobic gene expression involved in aerotaxis (Green et al. 2009). None of the eukaryotes are reported to possess direct mechanism of oxygen sensing. In fungi and animals, anaerobic genes are regulated at transcriptional level. Hap-transcription factors involved in oxygen sensing (yeast) and hypoxia-inducible factor (HIF) are widespread throughout the animal kingdom (Bailey-Serres and Chang 2005; Bailey-Serres and Voesenek 2008; Licausi and Perata 2009). Activity of these factors is regulated by the oxygen level, production of ROS and redox state of the cell. Furthermore, intracellular pH, ATP and sugar level are good candidates for indirect sensing signal.

5.4 Cytosolic Calcium Signalling in Plants Under Anoxia

The main effect of anoxia sensing in eukaryotic cells is an increase in [Ca^{2+}]cyt. There are two major hypotheses to explain this reaction in animal physiology (Lahiri et al. 2006; Fähling 2008) (Fig. 15.1). The "mitochondrial" hypothesis states that mitochondrial electron-transport chain is retarded by depletion of oxygen, produces ROS and leads to a Ca^{2+} release into the cytosol from the mitochondria and other intracellular compartments. While as the "plasma membrane" or "NAD(P)H-oxidase" hypothesis concerns involvement of ROS generated by NAD(P)H-oxidase leading to suppression of outward plasmalemma K^+ channels, a depolarization of the membrane and a Ca^{2+} influx from extracellular Ca^{2+} stores.

Subbaiah et al. (1994a) first reported an elevation of [Ca^{2+}]cyt in maize suspension-cultured cells, which was completely reversible after a few seconds of reoxygenation. Treatment with Ca^{2+}-channel blockers prevented the anoxic induction of ADH1 and SuS1 genes in plant cell and post-anoxic seedling survival (Subbaiah et al. 1994b; Subbaiah 2009). In maize cells, the elevation of [Ca^{2+}]cyt within the first minute of anoxia derived from efflux of Ca^{2+} from intracellular stores, as it was significantly inhibited by ruthenium red (RR, inhibitor of intracellular membrane Ca^{2+}/H^+-antiporter), but not affected by

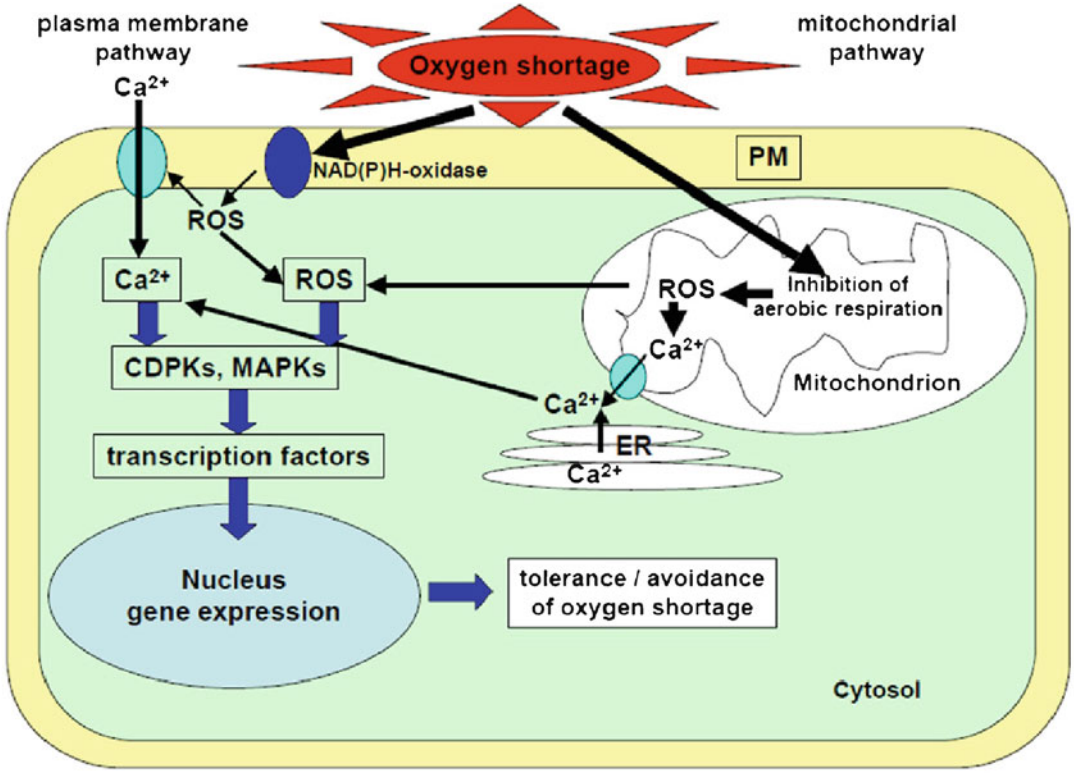

Fig. 15.1 A proposed model for Ca^{2+} signalling under anoxia. The "mitochondrial" pathway starts with retardation of the mitochondrial electron-transport chain by depletion of oxygen. It stimulates production of ROS that leads to a Ca^{2+} release into the cytosol from the mitochondria and other intracellular compartments. The "plasma membrane" or "NAD(P)H-oxidase" pathway is switched on by NAD(P)H-oxidase-dependent synthesis of ROS under oxygen shortage. This leads to a Ca^{2+} influx from extracellular Ca^{2+} stores. Increase of cytosolic Ca^{2+} concentration activates CDPKs, and ROS production stimulates a MAPK cascade, leading to activation of transcription factors and gene regulation

EGTA (Ca^{2+} chelator) and various inhibitors of plasma membrane channels (Subbaiah et al. 1994a). Moreover, subsequent research showed that elevation of Ca^{2+} in the cytosol of maize cell originates from mitochondria (Subbaiah et al. 1998) and could be induced by production of ROS by the mitochondrial electron transport chain under lack of oxygen, according to "mitochondrial" hypothesis (Rhoads et al. 2006).

On the other hand, a transient spike and prolonged elevation of [Ca^{2+}]cyt in transgenic apoaequorin-expressing *Arabidopsis* seedling upon imposition of anoxia depends on external as well as internal stores, since the downstream anaerobic gene expression is partially inhibited by EGTA, Gd^{3+}, La^{3+} (non-specific inhibitors of plasma membrane Ca^{2+} channels) and RR (Sedbrook et al. 1996) supporting both the hypothesis. Our results showed an ultimate importance of both external and internal Ca^{2+} stores for anoxic signalling in rice, whereas the hypoxia-intolerant wheat does not require external sources for that purpose (Yemelyanov et al. 2011). Ca^{2+} was shown to be important for anoxic induction of ADH in *Arabidopsis* and rice seedlings (Dolferus et al. 1997; Tsuji et al. 2000). Aurisano et al. (1995, 1996) demonstrated involvement of Ca^{2+}cyt, plasma membrane Ca^{2+} channels, CDPKs, CaM and G-proteins in anoxic signal transduction leading to accumulation of GABA and other amino acids in rice. Influx of Ca^{2+} via plasma membrane channels in *Arabidopsis* and rice is closely related with production of ROS on the cell surface. Blokhina et al. (2001) revealed production of ROS in the apoplast of different monocot species under anoxia. In *Arabidopsis* seedlings, induction of ADH gene depends on H_2O_2. Moreover, treatment of seedlings with diphenyleneiodonium, an inhibitor of ROS production by NAD(P)H-oxidase, blocked hypoxia-induced ADH activation (Baxter-Burrell et al. 2002). This links ROS production by NAD(P)H-oxidase, ADH induction and Ca^{2+} signalling. A scheme of Ca^{2+} signalling in plants under anoxia is shown in Fig. 15.1. In maize and wheat plants, accumulation of [Ca^{2+}]cyt occurs mainly via "mitochondrial" pathway, whereas in *Arabidopsis* and rice it passes through both "mitochondrial" and "plasma membrane" pathways.

6 Cold Temperature Stress Signalling

Many plant species have the possibility to enhance freeze tolerance after exposure to non-freezing temperatures. Cold acclimation is associated with alterations in plasma membrane lipid composition, increase in proline and sugar contents, synthesis of new polypeptides and changes in the mRNA populations (Steponkus and Lynch 1989; Guy 1990; Lin et al. 1990; Monroy et al. 1993; Uemura et al. 1995).

6.1 Cold-Induced Changes in Plasma Membrane Lipid Composition

The plasma membrane lipid composition is changed under cold stress in order to stabilize the membranes against freeze injury. Acclimation results in more unsaturated fatty acids causes a drop in the transition temperature. In *Arabidopsis*, maximal freeze tolerance was induced after 1 week at 2°C (Uemura et al. 1995). During that time, the proportions of phospholipids in the plasma membranes increased, while cerebrosides and free sterols decreased. Moreover, the di-unsaturated species of phosphatidylcholine and phosphatidylethanolamine increased. Some proteins, like dehydrins and lipocalin may help the plant to prevent damage to the plasma membrane during freezing (Uemura et al. 2006).

6.2 Calcium Is Involved in Acclimatization to Cold Temperature

Calcium has a role in cold-induced changes in protein phosphorylation, gene expression and development of freeze tolerance (Monroy et al. 1993; Knight et al. 1996; Tähtiharju et al. 1997; Viswanathan et al. 2006). In the freeze-tolerant

alpine plant, *Chorispora bungeana*, chilling induction at 0°C increased the calcium contents (Fu et al. 2006) and these Ca^{2+} levels were different in various tissues and organs. Knight et al. (1996) reported that inhibition of calcium influx caused a partial inhibition of cold-dependent kin1 expression in *Arabidopsis*. It was also shown that inhibitors of calcium channels, CaM action or protein kinases, inhibited development of freezing tolerance. In *alfalfa* plants, calcium elevation caused by low temperature induced expression of two cas (cold acclimation-specific) genes: CAS15 and CAS18 (Monroy and Dhindsa 1995). In *Arabidopsis*, cold stress induced the KIN1 and KIN2 genes but KIN2 mRNA accumulated to a higher level than KIN1 mRNA under acclimation (Kurkela and Franck 1990). Many cold-regulated genes have one or several copies of the DRE/CRT *cis*-element (dehydration responsive element/C repeat) in their promoters. Other transcription factors bind to this element and activate transcription of downstream genes (Zhu 2001). The CBF/DREB1 genes are also induced directly by a low temperature and the induction precedes that of genes containing the DRE/CRT *cis*-element. Thus, a network of multiple signalling pathways is involved in cold stress response in plants, some of them are also induced by salt and drought stresses in a complicated way. It was proposed by Zhu (2001) that low temperature specifically induces the transcription of the CBF/DREB1-based cascade. Calcium is required for full expression of the CRT/DRE controlled COR6 and KIN1 genes of *Arabidopsis* (Monroy and Dhindsa 1995; Knight et al. 1996). In the regulation of cold-responsive genes and freezing tolerance, also a CBF-independent pathway exists (Viswanathan et al. 2006). The transcription factors, HOS9 (a homeodomain type) and HOS10 (a myeloblastosis type), play an important role in this system.

Calcium is important not only for gene expression during cold acclimation, but also has an effect on resealing the membranes after cold stress (Yamazaki et al. 2008). In experiments with *Arabidopsis* protoplasts, extracellular calcium increased tolerance to electroporation that otherwise punctures the membrane. An antibody against a homolog of synaptotagmin, SYT1, inhibited the calcium-dependent freezing tolerance in *Arabidopsis* leaf protoplasts and the substance is a calcium sensor that causes exocytosis. Moreover, this inhibition indicates that the puncture allowing the antibody to enter into the cytoplasm occurs during freeze/thawing. The authors suggested that calcium-dependent freezing tolerance results from membrane resealing and that SYT1 is involved in this mechanism.

6.3 Calcium Signalling Induced by Cold or Chilling Stresses

In transgene *Arabidopsis*, wherein aequorin was targeted to different types of root cells, as well as to the whole plant, cold water addition caused a transient rise in [Ca^{2+}cyt] (Kiegle et al. 2000). The maximum peak occurred at approximately 15–20 s in all cell types. However, the increase in [Ca^{2+}cyt] was lower in the root cells, compared with shoot cells. The highest elevation was obtained in the deepest cell type, the pericycle, and the lowest peak level in the elongation zone. Hence, concluded, the size of the perceived agonist does not decide the magnitude of the calcium response, instead, there is a cell-specific response.

Calcium dynamics under chilling stress were also investigated in single mesophyll protoplasts from tomato plants loaded with the calcium specific dye Fura2, AM (Sebastiani et al. 1999). The protoplasts subjected to chilling stress were kept in a temperature-controlled (10–15°C) perfusion chamber. The results showed that different kinetics in [Ca^{2+}cyt] occurred, depending on different resting calcium levels. In 84% of the investigated protoplasts, there was an increase in [Ca^{2+}cyt] followed by a maximum increase obtained after 10–20 s. In 21% of the reacting protoplasts, the maximum [Ca^{2+}cyt] was also obtained after 10–20 s, thus corroborating results from root cells of *Arabidopsis* (Kiegle et al. 2000); in 11% of the protoplasts both the increase and decrease in [Ca^{2+}cyt] were slower; and in 32% a constant increase of [Ca^{2+}cyt] was obtained 1 min after start of temperature decrease. When the resting calcium concentration was higher

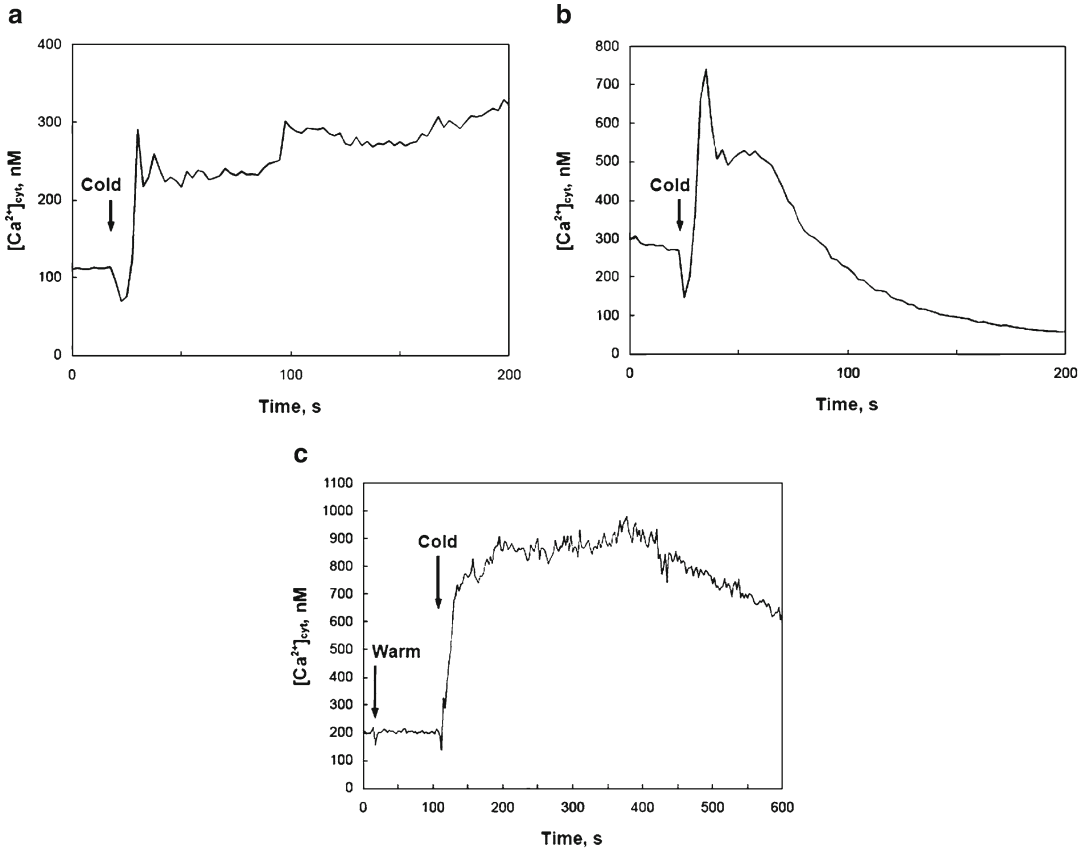

Fig. 15.2 Cytosolic calcium changes, detected by Fura 2-AM and fluorescence intensity ratio 340/380 nm, in a single wheat protoplast upon cold shock. (**a**) First addition of cold (5°C) solution. (**b**) Second addition of cold solution to the same protoplasts. (**c**) Reaction in the presence of erythrosine B (Lindberg and Sebastiani, unpublished results)

than normal, the increase in [Ca^{2+}cyt] was constant. In these experiments, the cooling rate was constant, as the perfusion system was used. Therefore, it is likely that different cells have different competence and ability to maintain calcium homeostasis. A sustained high [Ca^{2+}cyt] is implicated in apoptosis and in hypersensitive responses to pathogens (Levine et al. 1996).

When a cold solution (5°C) was added to mesophyll protoplasts of wheat leaves (Fig. 15.2), much larger changes in [Ca^{2+}cyt] were obtained compared to chilling sensitive tomato protoplasts, in which a perfusion system was used (Sebastiani et al. 1999). A second addition of cold solution to the same wheat protoplast induced different [Ca^{2+}cyt] kinetics. The first addition of cold solution gave a transient prolonged increase of [Ca^{2+}cyt], much smaller than that induced by a second addition (Fig. 15.2a). The second addition gave a peak response and thereafter; a prolonged calcium increase was observed (Fig. 15.2b). These results cannot be compared with results shown by Knight et al. (1996), since these authors report a mean of 3 or more cold additions at time zero. When the wheat protoplasts were treated with erythrosine B, an inhibitor of Ca^{2+} ATPase in the ER and plasma membrane, the calcium transient was prolonged and the magnitude was much higher than without the inhibitor (Fig. 15.2c). Thus, it is likely that the Ca^{2+} ATPase is involved in the signalling by pumping Ca^{2+} out from the cytosol. It has been proposed that [Ca^{2+}cyt] signatures are modified by previous experience, indicating that the plant has a calcium "memory"

(Knight et al. 1996). The magnitude of [Ca^{2+}cyt] elevation elicited by wind becomes progressively smaller upon repeated stimulation and for some stimulus several hours are needed for a second reaction to take place (Tuteja and Mahajan 2007). The calcium signature can also be modified during a second exposure to a stress as shown in Fig. 15.2. Moreover, the magnitude of [Ca^{2+}cyt] increase can be changed by prior in vivo exposure to a contrasting stress. These observations imply a cross talk between the signalling cascades.

In response to cold shock, an immediate rise in [Ca^{2+}cyt] was obtained in both chilling-sensitive tobacco as well as in chilling-tolerant *Arabidopsis* (Knight et al. 1996). In *Arabidopsis,* both EGTA and lanthanum caused a partial inhibition of [Ca^{2+}cyt] and of cold-dependent KIN1 gene expression. To investigate if the vacuole was involved in the calcium signalling, aequorin was targeted to the cytosolic face of the tonoplast. As a result, a higher peak of calcium elevation was obtained in the cytosol than in tonoplast microdomain. In addition, the elevation in the microdomain, [Ca^{2+}mic], was maintained for a longer time than [Ca^{2+}cyt]. Pre-treatment with neomycin or lithium, which interferes with phosphoinositide cycling, diminished the calcium reactions, showing that some efflux of calcium occurred from the vacuoles. The magnitude of the cold-shock induced [Ca^{2+}cyt] response was similar in *Arabidopsis* and tobacco, although it was more prolonged in tobacco. Upon repetitive addition of cold solution, the response after 3 and 10 min was weaker in both species. However, tobacco was able to recover its ability to respond fully to cold shock 30 min after the initial shock, whereas *Arabidopsis* was not. Another difference between the species was that they responded in different ways to cold shock after acclimation. Only in chilling-tolerant *Arabidopsis*, cold acclimation altered the signature of [Ca^{2+}]cyt, so that cold shock caused a reduced peak but a prolonged profile. The different mechanisms in stress response could depend on the different sensitivity of *Arabidopsis* and tobacco to chilling stress. Cold shocks of different amplitude applied to protoplasts of freeze-sensitive olive tree caused transient increases in [Ca^{2+}cyt] that differed in non-acclimated and acclimated protoplasts (D'Angeli et al. 2003). Upon repeated cold-shock treatment and by using non-severe rate changes in temperature, the transient increases in [Ca^{2+}]cyt fall, while in the acclimated protoplasts, the [Ca^{2+}]cyt elevations were further reduced.

Temperature sensing in *Arabidopsis* depends both on the cooling rate (Plieth et al. 1999) and the final temperature to which cooling occurs (Knight 2002). Plieth (1999) presented a mathematical model of how cold is sensed by a plant based on a passive influx across the plasma membrane and an active efflux by a pump. A single peak in [Ca^{2+}cyt] obtained with a cold shock, specifically decreases, at a very fast temperature change but at low cooling rate, the response lacks the initial peak more or less. The pump slows down at low temperature leading to a second slow phase of the increase in [Ca^{2+}cyt]. A biphasic response to a single cooling step is thus obtained, when the sensitizing action by the temperature change on the pumps and channels is of equal magnitude (Plieth 1999). By applying patch clamp technique to mesophyll cells of *Arabidopsis*, Carpaneto et al. (2007) showed that cold induced a rapid increase in [Ca^{2+}cyt], and that the influx of calcium could occur through non-selective cation channels.

6.4 Cold Shock Induces Change in the Membrane Potential

A drop in temperature can change the transmembrane potential. Krol et al. (2004) showed that the obtained transient depolarization of membrane potential in mesophyll cells of *Arabidopsis*, *Helianthus* and *Vicia*, induced by temperature decreases, depended on calcium influx both from apoplast and internal stores. It was verified later that the cold-induced depolarizations depended on calcium influx (Carpaneto et al. 2007). Verapamil, a calcium channel blocker, caused significant suppression of the cold-induced potential changes. Since the presence of CaM antagonists prolonged the repolarization, this could be attributed to activation of CaM-dependent Ca^{2+}-ATPases (Krol et al. 2004). It was reported that

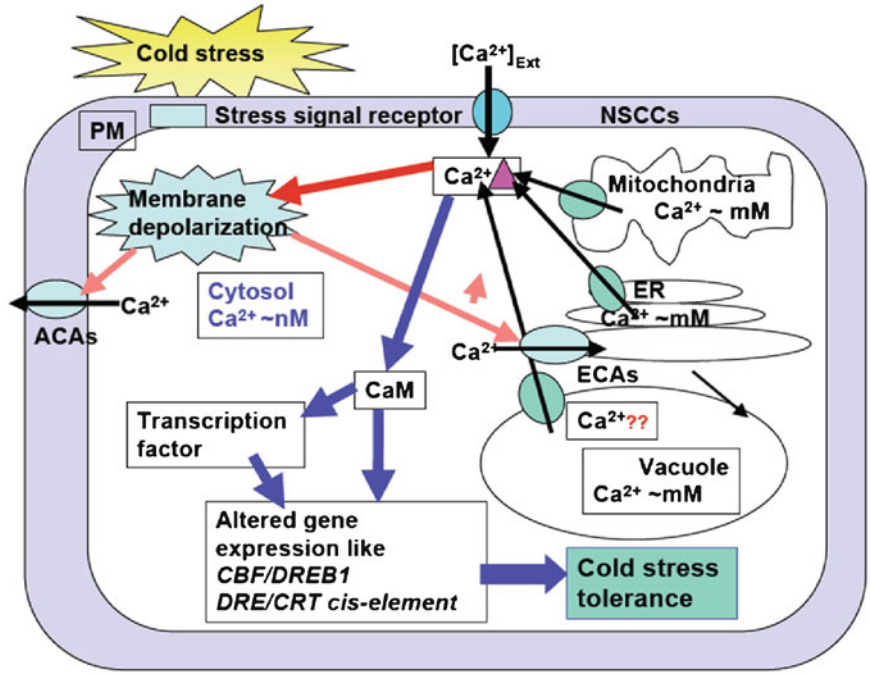

Fig. 15.3 A proposed model for cold-stress signalling in plants. Cold and chilling stress induce a transient or prolonged increase in [Ca_{cyt}^{2+}]. Calcium influx is mainly mediated by non-selective-cation channels in the plasma membrane (PM) (Carpaneto et al. 2007), but also by channels in the tonoplast or other endomembranes (Knight et al. 1996; Krol et al. 2004). Calcium influx causes a depolarization of the PM potential, and then a re-polarization (Krol et al. 2004). Erythosine B is supposed to inhibit the PIIA ATPases (ECAs) channels in the ER, but may also inhibit PIIB ATPases (ACAs) in the PM. *ACA8* and *ACA10* were differently regulated by cold stress (Schiott and Palmgren 2005). The ACA type channels contain a Ca–CaM binding site and a serine-residue phosphorylation site. Their activity can be modulated by Ca_{cyt}^{2+}, either through activation upon binding CaM, or by inhibition upon phosphorylation by CDPK (Hwang et al. 2000). The signals from CaM also induce mRNA and gene expressions, either directly, or by CaM-binding to specific transcription factors (Tuteja and Mahajan 2007). The *CBF/DREB1* genes are induced by low temperature and the induction precedes that of genes containing the *DRE/CRT cis*-element (Zhu 2001; Zhu et al. 2007)

this enzyme is involved in low temperature response in rice (Martin and Busconi 2001). A suggested model for cold signalling in plants is shown in Fig. 15.3.

7 Salinity; Sodium and Osmotic Stress Signalling

Soil salinity is a major environmental hazard worldwide as more than 40% of the earth is arid or semi-arid and most of the planet's water is saline. Currently, more than 6% of the world's land which exceeds 20% if only the irrigated area is considered, is affected by varying degrees of soil salinity (Flowers and Yeo 1995; Munns 2002; Kader and Lindberg 2008). However, nearly 50% of the irrigated land in the arid and semi-arid regions of the world is facing most serious salinity problems.

7.1 Salinity Stress Has an Impact on Agricultural Productivity

Salinity stress adversely affects agricultural productivity by decreasing the crop yield in many ways. Furthermore, the saline area increases day by day due to sea-level rise and, thus, have profound harmful effects on agricultural productivity in

many countries of the world. Long time ago, Buringh (1979) estimated that among 10 hectares, 3 hectares of arable land are lost in every minute due to soil salinization. In 2002, FAO reported that about 20–30 million hectares of irrigated land are seriously damaged by the increased levels of salts, moreover, 0.25–0.50 million hectares are lost from production every year (Martínez-Beltran and Manzur 2005). The disastrous consequence of this increasing salinity stress together with the growing world population is certainly threatening the future stable global food availability. Agricultural productivity in future will depend mostly on our ability to identify or develop salt-tolerant crop plants and to grow them in rapidly increasing salt-affected lands.

7.2 Salinity Stress Injury in Plants

The salinity in nature imposes two primary harmful effects on plants: one is osmotic stress and the other, ionic toxicity. Due to the presence of high salt, salinity stress increases the osmotic pressure in the soil solution over the osmotic pressure in plant cells. As a result, plant loses its ability for uptake of water and minerals, especially the uptake of K^+ and Ca^{2+} (Glenn et al. 1997; Munns et al. 2006). Inhibition of plant growth by high amounts of Na^+ and Cl^- is one of the main deleterious effects of salinity stress. When present in excess amount, Na^+ and Cl^- ions enter into the plant cells and can exert toxic effects on cell membranes and metabolic activities in the cytosolic part of the cell (Greenway and Munns 1980; Hasegawa et al. 2000; Zhu 2001). The resultant effect of osmotic stress and ionic toxicity may lead to secondary effects in plants such as decreased cell expansion, production of assimilate and membrane functions, decreased cytosolic metabolism with raised production of ROS.

7.3 Salinity Stress Tolerance Mechanisms in Plants

As paleontological and molecular evidence suggest that some 500 million years ago, the embryophytes (terrestrial plants that are not algae) were evolved from the Streptophyta (Raven and Edwards 2001). The evolution of salinity tolerance mechanisms in halophytic plants has recently been reviewed by Flowers et al. (2010a). Though physiological foundations of salinity tolerance are present in all plants and show a very wide range of adaptability to salinity stress. For example, glycophytes like chickpea is very sensitive and suffer toxicity at 25 mM NaCl (Flowers et al. 2010b), whereas halophytes can tolerate salinity concentration as high as 1,000 mM (Khan et al. 2005). The recent advancement in molecular biology research is uncovering the mechanisms of salinity stress tolerance including the key genes involved in the molecular networks and the signalling cascade that mediates plant responses to salinity stress. Plants need different tolerance mechanisms to be adopted, since salinity stress elicits two different adverse effects like osmotic stress and ionic toxicity. To deal with the ionic toxicity, under salinity stress, the key mechanisms for tolerance are a diminished toxic ion uptake into the cytosol, the ability to limit the entry of these toxic ions into the transpiration stream, the ability to regulate transpiration in the presence of these toxic ions and compartmentalization of Na^+ and Cl^- ions into the apoplast/vacuole (Blumwald 2000; Tester and Davenport 2003; Kader and Lindberg 2005, 2008; Munns and Tester 2008; Flowers and Colmer 2008; Flowers et al. 2010a). Compartmentalization of toxic Na^+ into the vacuole is advantageous, since it is no more toxic for the cell, and also a benefit for growth and adjustment of the osmotic potential (Flowers and Läuchli 1983; Zhu 2003; Subbarao et al. 2003; Rodríguez-Navarro and Rubio 2006). Jou et al. (2006) showed that excess Na^+ also can be compartmentalized in ER and Golgi bodies. An important tolerance mechanism is also a plant's capability to increase and decrease the uptake of potassium (K^+), and Na^+ into the cytosol, respectively, under high sodium concentration. Expression analyses of transporter genes for K^+ and Na^+ transporters OsHKT1 and OsHKT2 showed that they were differently expressed in tolerant and sensitive rice cultivars (Kader et al. 2006).

To obtain osmotic homeostasis, the synthesis of compatible organic solutes, such as glycine betaine, mannitol, pinitol, proline, sorbitol, sucrose and trehalose in the cytosol, is of great importance (Bohnert and Jensen 1996; Chen and Murata 2002; Zhu 2002; Zhang et al. 2004; Chinnusamy et al. 2005; Taiz and Zeiger 2006; Liang et al. 2009).

7.4 Perception of Salinity Stress in Plants

Cellular perception of salinity stress by plants is prerequisite to start the activation of the whole cell-signalling cascade. This begins with an elevation of $[Ca^{2+}]$cyt and ends with different tolerance mechanisms activated in the plant. Like other stresses, salinity stress (both osmotic stress and ionic toxicity) is perceived in plants at the cell membrane, either extracellular or intracellular by a protein spanning the plasma membrane and/or an enzyme within the cytosol (Zhu 2003). Under salinity stress, a low K^+ level in the cytosol may also lead to cytosolic calcium signals (Luan et al. 2009). For sensing the osmotic component of salinity stress, probably several sensors are involved (Urao et al. 1999; Reiser et al. 2003; Tamura et al. 2003; Boudsocq and Lauriere 2005; Tran et al. 2007; Wohlbach et al. 2008). A substantial progress in understanding the signal transduction under Na^+ toxicity was made by identification of the SOS pathway in *Arabidopsis* (Zhu 2002). In a recent review, Luan et al. (2009) suggested that CBL (calcineurin B-like proteins)–CIPK (CBL-interacting protein kinase) pathways regulate Na^+ transport in plants and thus confer salinity tolerance. The CBLs also appear to be an important group for conferring salinity tolerance through enhanced K^+ uptake under salinity stress (Luan et al. 2009). As shown in Fig. 15.4, the salinity tolerance mechanisms in plants entails SOS3, a Ca^{2+} sensor in the cytosol, that reads the changes in $[Ca^{2+}]$cyt under salinity stress and specifically binds Ca^{2+} followed by protein interaction through SOS2 protein kinase. Thereafter, the SOS3–SOS2 complex in turn activates the plasma membrane Na^+/H^+ antiporter, the SOS1 protein, and re-establishes the Na^+ homeostasis of the cells. An elevation of $[Ca^{2+}]$cyt is also detected by CBL10, interaction with SOS2 might trigger tonoplast Na^+/H^+ antiporter to transport Na^+ from cytosol to vacuole. Furthermore, the increase in $[Ca^{2+}]$cyt can also be perceived by CBL1 and CBL9, which then bind to CIPK23 and interact with the C terminus of AKT1. In this way the AKT1 channel is activated causing an increased K^+ uptake into the cell, hence confer salinity tolerance. However, it is still necessary to clarify how Na^+ toxicity is sensed by the plant cell. It was suggested that SOS1 protein, with its long C-terminal tail in the cytosol, might sense Na^+ (Zhu 2003; Zhang et al. 2004; Shabala et al. 2005). Kader et al. (2007) showed that, for Na^+ sensing in rice protoplasts, Na^+ primarily must enter into the cytosol. The results corroborate with the earlier suggestion that the cytosolic tail of the SOS1 protein might sense Na^+. Conversely, in the halophytic plant quince, Na^+ entry in to the cell was not necessary for the shift in cytosolic Ca^{2+} (D'Onofrio and Lindberg 2009). Therefore, the question still remains to be answered concerning the sensors for Na^+ toxicity in plants, and if they differ in different species, and/or in salinity sensitive as well as salinity tolerant cultivars.

7.5 Cytosolic Calcium Signalling in Plants Under Salinity Stress

It is clear, the salinity stress perception, irrespective of how the stress is perceived, triggers an intracellular signalling cascade starting with the elevation of secondary messenger molecules like calcium in the plant cytosol $[Ca^{2+}]$cyt. Many studies were done to measure the $[Ca^{2+}]$cyt changes in cells, organs or intact plants under salinity stress by use of different techniques. Fluorescence microscopy measurements were performed in root hairs (Halperin et al. 2003) and in individual mesophyll protoplasts (Kader et al. 2007; D'Onofrio and Lindberg 2009) Measurements in intact whole plants were made by aequorin luminescence detection (Knight et al. 1997; Gao et al. 2004; Henriksson and Henriksson 2005, Tracy et al. 2008). These studies suggest that the "signature" of $[Ca^{2+}]$cyt

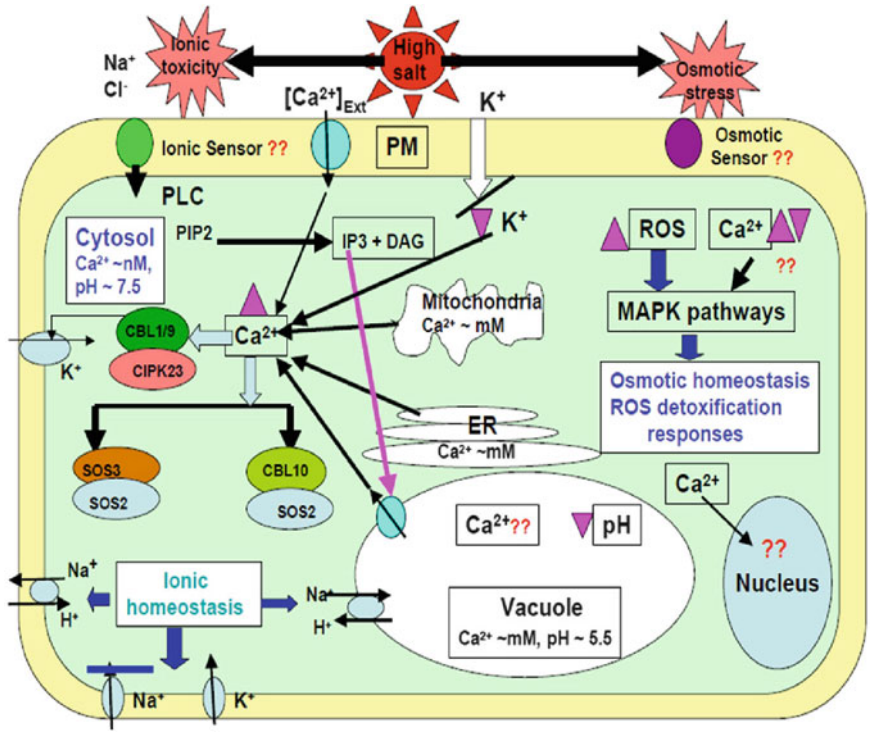

Fig. 15.4 A proposed model for calcium signalling under salinity stress. Salinity stress (both osmotic stress and ionic toxicity) is perceived in plants at the cell membrane, either extracellular or intracellular by a transmembrane protein, or within the cytosol by enzymes (Zhu 2003). Several osmo-sensors are involved in sensing the osmotic stress (Urao et al. 1999; Reiser et al. 2003; Tamura et al. 2003; Boudsocq and Lauriere 2005; Tran et al. 2007; Wohlbach et al. 2008). Na^+ stress is sensed by the SOS pathway (Zhu 2002). The CBL (calcineurin B-like proteins)–CIPK (CBL-interacting protein kinase) pathways regulate Na^+ transport in plants and thus confer salinity tolerance (Luan et al. 2009). The SOS3, Ca^{2+} sensor in the cytosol, reads the changes in calcium level and binds Ca^{2+}. This protein interacts with a SOS2 protein kinase, and then the SOS3–SOS2 complex activates the SOS1 protein, a plasma membrane Na^+/H^+-antiporter, thereby re-establishing the Na^+ homeostasis in cells. The elevation of $[Ca^{2+}]_{cyt}$ is also detected by CBL10, which in interaction with SOS2 might trigger tonoplast Na^+/H^+ antiporter to transport Na^+ from the cytosol into the vacuole. The increase in $[Ca^{2+}]_{cyt}$ can also be perceived by CBL1 and CBL9, which then bind to CIPK23 and activate the AKT1. This leads to increased K^+ uptake into the cell

change, e.g. the amplitude, duration and frequency, is very important for transferring of specific downstream reactions leading to stress tolerance.

7.6 The Signature of Cytosolic Calcium Differs

The change in $[Ca^{2+}]$cyt varies with species, cell type or tissue type and also with different techniques used (Cramer and Jones 1996; Kiegle et al. 2000; Kader et al. 2007; Tracy et al. 2008; D'Onofrio and Lindberg 2009). The change in $[Ca^{2+}]$cyt activates different downstream reactions, such as up-regulation or down-regulation of different genes. The reactions may also change with the particular stress (Kiegle et al. 2000), the stress development rate (Plieth et al. 1999; Tracy et al. 2008; D'Onofrio and Lindberg 2009), pre-exposure to the stress (Knight et al. 1997) and the tissue type (Kiegle et al. 2000; Tracy et al. 2008). Upon application of 100 mM NaCl to root cells of *Arabidopsis* (Cramer and Jones 1996; Halperin et al. 2003), or to corn root protoplast (Lynch and Läuchli 1988), a decrease in $[Ca^{2+}]$cyt was obtained. On the other hand, most studies show an

increase in $[Ca^{2+}]$cyt upon salinity stress (Bittisnich et al. 1989; Lynch et al. 1989; Knight et al. 1997; Halfter et al. 2000; Kiegle et al. 2000; Knight 2000; Zhu 2001; Gao et al. 2004; Henriksson and Henriksson 2005; Kader et al. 2007; D'Onofrio and Lindberg 2009; Kader and Lindberg 2010).

7.7 Plant Roots and Shoots Signal Differentially Under Sodium Toxicity and Osmotic Stress

Contrasting results are reported whether osmotic stress increases or decreases $[Ca^{2+}]$cyt (Cramer and Jones 1996; Knight et al. 1997; Kiegle et al. 2000; Kader et al. 2007). In *Arabidopsis* root cells, osmotic and ionic stresses induced different shifts in $[Ca^{2+}]$cyt and the heterogenous $[Ca^{2+}]$cyt changes were found only in the root (Tracy et al. 2008). Also in experiments with rice and quince protoplasts, different $[Ca^{2+}]$cyt changes were induced under sodium and osmotic stresses (Kader et al. 2007; D'Onofrio and Lindberg 2009). A proposed model for calcium- and pH-signalling under salinity stress is reviewed in Kader and Lindberg (2008, 2010). A simplified model is shown in Fig. 15.4.

8 Aluminium and Heavy Metal Stress Signalling in Plants

8.1 Aluminium Toxicity to Plants

Aluminium (Al) toxicity in plants is a serious factor, limiting crop production in acidic soils and affecting up to 40% of the world's arable soils (Haug 1984; Foy 1984). When the soil pH decreases below 5, Al^{3+} is dissolved in the soil, causing harmful effects on plant roots. At pH 4, the dominating species is $Al(H_2O)^{3+}$ while at higher pH, $Al(OH)^{2+}$, $Al(OH)_2^{+}$, $Al(OH)_3^{0}$ and $Al(OH)_4^{-}$ are present, besides sulphate complexes and polynuclear species (Lindsay 1979).When roots are subjected to Al, they become stunted and damaged with poor root hair development (Clarkson 1965). The uptake of water and minerals is severely inhibited. At a low pH, Al mainly binds to the root apex and inhibits root elongation (Ryan et al. 1993; Kochian 1995; Matsumoto 2000; Barcelo and Poschenrieder 2002). Aluminium affects the transmembrane potential of root cells and inhibits ATPase activities. After cultivation of sugar beets in the presence of low pH and/or $AlCl_3$, the transmembrane potential, PD, between the vacuole and external medium, PDv, of root cells was largely depolarized (Lindberg et al. 1991). Since the effect of dinitrophenol was negligible, it was suggested that Al interacts with the active component of the PD. This was confirmed by experiments showing that Al inhibits the plasma membrane ATPase activity (Lindberg and Griffiths 1993) as well as proton transport (Matsumoto 1988). Lipid analysis of sugar beet plasma membranes showed that Al treatment during cultivation caused an increase in the ratio of phosphatidylcholine: phosphatidylethanolamine (Lindberg and Griffiths 1993). The lipid changes correlated with the observed change in the Km for the Mg ATPase, and, hence, it was concluded that Al could bind to the membrane-bound enzyme and/or modify the lipid environment. The inhibition of the ATPase activity causes a reduced uptake of minerals. In the soil, precipitation of phosphate with Al causes phosphate deficiency in plants (Horst et al. 1982). Addition of low concentrations of Al (10–50 μM) to plant roots cultivated without Al caused a fast hyperpolarization of PDv, and of PDc, the membrane potential across the plasma membrane. A depolarization of PDc was obtained at pH 6.5 (Lindberg et al. 1991). At the latter pH, the dominant species $Al(OH)_3^{0}$ can easily penetrate membranes. Using artificial liposome vesicles it was shown that Al uptake was facilitated at neutral pH compared with pH 4 and 5 (Shi and Haug 1988). Therefore, toxic effects of Al on plants can also occur at a neutral pH. Most of the Al binds to the cell walls of root epidermal and cortical cells (Delhaize et al. 1993) and to the plasma membranes. It can also penetrate the plasma membrane (Lazof et al. 1994). When Al enters into a cytosol, it may inhibit cell division in the meristem and cell elongation in the elongation zone (Baluska et al. 1993) probably by binding to nucleic acids (Matsumoto et al. 1976).

8.2 Aluminium Interferes with Calcium Homeostasis

A disruption of cytosolic calcium homeostasis is a primary trigger of Al toxicity. Calcium plays an important role in cell division and cell expansion. For instance, transient changes in the cytosolic calcium concentration, [Ca^{2+}cyt] have been observed to accompany the mitotic mechanism (Hepler 1994). Calcium promotes elongation in many plant cells (Takahashi et al. 1992; Levina et al. 1995) and calcium antagonists can block elongation (Cho and Hong 1995). Sustained gradients in Ca^{2+}cyt are necessary for expansion of tip-growing plant cells, such as pollen tubes (Clarkson et al. 1988; Felle and Hepler 1997; Wymer et al. 1997). A maintained homeostatic control is, thus, necessary for cell viability (Bush 1995). Aluminium affects the calcium homeostasis in cell by inhibition of calcium uptake or efflux (Lindberg 1990). In 1-h experiments with intact sugar beet plants, the metabolic influx of $^{45}Ca^{2+}$ and K^+($^{86}Rb^+$), and efflux of $^{45}Ca^{2+}$ were inhibited in the presence of Al (Lindberg 1990). Aluminium at a low concentration and low pH can elevate the [Ca^{2+}cyt] (Lindberg and Strid 1997). Aluminium may also interact with the phosphoinositide signalling pathway. Both $AlCl_3$ and Al-citrate inhibited the phospholipase C (PLC) action in a dose-dependent manner (Jones and Kochian 1995).

8.3 Aluminium Induces Cytosolic Calcium Elevation

In protoplasts of wheat roots, $AlCl_3$ (80 μM) induced immediate and transient oscillations in [Ca^{2+}cyt], during 1–2 min in the presence of 0.1, 0.2 and 0.5 mM external calcium concentration (Lindberg and Strid 1997). No difference in the transients was obtained in the tolerant and sensitive wheat cultivar. Different cells may behave differently, since the reaction was found in only 60% of the protoplasts. Jones et al. (1998) reported an opposite effect of Al on [Ca^{2+}cyt], using cultured cells of tobacco, BY-2. Upon addition of 200 μM Al, the [Ca^{2+}cyt] decreased, suggesting a blockage of calcium channels. Addition of $LaCl_3$ or EGTA also decreased the resting calcium level, and thus inhibited the cell growth. The different results from wheat protoplasts and tobacco cells depend on the different concentrations of Al used, or that different plant material reacts differently. However, most investigations using other suspension cells, intact root tips, root hairs, excised whole roots and protoplasts showed an increase in [Ca^{2+}cyt] (Rengel and Zhang 2003). The source of Ca^{2+} for increase in [Ca^{2+}cyt] under Al exposure is partly extracellular, likely due to flux through DACC and through Ca^{2+}-permeable non-selective cation channels in the plasma membrane (Rengel and Zhang 2003 and references therein). Lin et al. (2005) proposed that in *Arabidopsis*, the AtTPC1 is the only channel, sensitive to Al. This channel also is responsive to ROS and cryptogein, a fungal elicitor protein. It is believed that intracellular channels in the tonoplast and ER participate in the signalling. The latter channels could be activated by the increase in [Ca^{2+}cyt]. An Al-related inhibition of the plasma membrane, endo-membrane Ca^{2+}-ATPases and Ca^{2+} exchangers (CaX) are likely to exist. Kawano et al. (2003) showed that addition of $AlCl_3$ to tobacco cells triggers the generation of superoxide (O_2^-) and that NADPH oxidase was involved in the production of $O_2^-$and was produced in a dose-dependent way concerning Al concentration. A fast spike in O_2^- generated a gradual increase in Ca^{2+}cyt. A scavenger of O_2^- and a calcium chelator significantly lowered the level of [Ca^{2+}cyt] increase, indicating, that O_2^- stimulates the influx of Ca^{2+}. Moreover, high concentrations of Al inhibited the [Ca^{2+}cyt] increase. A change in [Ca^{2+}cyt] is often connected with a change in cytosolic pH (pHcyt) (Kader and Lindberg 2010). In wheat-root protoplasts, a decrease in pHcyt, upon $AlCl_3$ additions was observed (Lindberg and Strid 1997). This pH decrease was most obvious in the Al-sensitive cultivar. Membrane potential measurements with intact roots of the same wheat cultivars showed similar results as with sugar beet roots (Lindberg et al. 1991). Additions of $AlCl_3$ both at pH 4 and 5 caused hyperpolarizations, and upon removal of Al depolarizations, the final depolarization was found larger in the

sensitive cultivar (Lindberg and Strid 1997). As concluded, the toxic effect of Al on the sensitive cultivar should be related to the larger change in pH, probably as a result of inhibition of the plasma membrane H^+ ATPase.

8.4 Heavy Metal Toxicity and Uptake in Plants

Increased anthropogenic activities have caused rapid release of toxic compounds, such as trace elements (including heavy metals and metalloids) into the environment. Within the European community, the 11 trace elements of highest concern are arsenic, cadmium, cobalt, chromium, copper, mercury, manganese, nickel, lead, tin and thallium. Some of them, usually called "heavy metals", e.g. cadmium, mercury and lead, pose serious threat to microorganisms, plants, animals and humans, while other trace elements in low concentrations like zinc, copper and iron are essential for normal growth and functioning of organisms. Their toxicity depends on their available concentration, efficiency in uptake and translocation within the plant (Lindberg and Greger 2002). The uptake depends on pH, other ions and chelating agents present, as well as root microbes. Different trace elements are taken up and translocated in different ways depending on species and type of trace element. Cadmium is highly mobile in the soil and is easily taken up by the plant roots via active and passive pathways (Greger and Lindberg 1986; Hart et al. 1998; Lindberg et al. 2004). Most of the cadmium ions (Cd^{2+}) are bound to the cell wall, but some Cd^{2+} enters into the cytosol by unspecific cation channels (Perfus-Barbeoch et al. 2002; Lindberg et al. 2004).

Cadmium, mercury and lead are considered as non-essential and are potentially toxic because of their reactivity with S and N atoms in amino acid chains of proteins (Chiang et al. 2006). Heavy metals also bind to oxygen atoms of membranes, or to histidine, tryptophan and tyrosine groups of polypeptides (Maksymiek 1997), interfere with several metabolic processes and alter the physiological functions of different enzymes. Cadmium and other trace metals strongly bind to thiol groups and thus to cystein-rich proteins (Vallee and Ulmer 1972; De Filippis 1979). Cadmium binding to plasma membrane sulfhydryl (SH) groups could be the reason for cadmium inhibition of plasma membrane ATPase activity and mineral uptake in sugar beet roots (Greger and Lindberg 1987; Lindberg and Wingstrand 1985; Greger et al. 1991). Cadmium and copper also inhibited the plasma membrane ATPase in root cells of cucumber and changed the lipid composition of the membrane (Kabata et al. 2008). Heavy metals inhibit photosynthesis and growth (Foy et al. 1978; Vangronsveld and Clijsters 1994; Maksymiec et al. 1994; Maksymiec and Baszynski 1999; Vinit-Dunand et al. 2002; Alaoui-Sossé et al. 2004; Rahman et al. 2005). Besides chlorophyll degradation, cadmium along with lead and mercury has been reported to interact with light-harvesting proteins of chloroplast's thylakoid membranes (Ahmed and Tajmir-Riahi 1993). Root growth inhibition in barley was suggested to depend on cadmium-induced water and oxidative stress (Tamas et al. 2008). Heavy metals can cause lipid peroxidation of photosynthetic membranes and affect the lipid composition of the plasma membranes (Chaoui et al. 1997; Quartacci et al. 2001; Nouairi et al. 2006).

8.5 Tolerance Mechanisms to Heavy Metals

Plant metal homeostasis must be tightly regulated in order to prevent toxic concentrations of non-essential metals and also to ensure sufficient micronutrients like Zn, Cu, Fe, etc. (Clemens 2001; Hall 2002). Plants have mechanisms that enable them to extrude cadmium and other heavy metals from the cytosol. Different transporters have been identified such as ATP-binding cassette (ABC) and Zinc–Iron-Like Proteins (ZIP) (Colangelo and Guerinot 2006; Clemens 2006). The P-ATPase HMA (heavy metal associated) also plays an important role in allocation and detoxification of heavy metals (Williams and Mills 2005). In *Arabidopsis*, eight genes belong to HMAs (Baxter et al. 2003). Among them the

HMA1-4 specifically may transport $Zn^{2+}/Cd^{2+}/Pb^{2+}/Co^{2+}$, while HMA5-8 transports Cu^{2+}, and probably Ag^{+}. In *planta*, AtHMA1 is expressed at the inner envelope membrane of the chloroplast (Seigneurin-Berny et al. 2006), while AtHMA4 is expressed in the plasma membrane (Hussein et al. 2004) involved in Zn and Cd xylem loading. The AtHMA3 was found to be a vacuolar transporter whose overexpression increased the Cd, Pb and Zn tolerance (Morel et al. 2009). Besides HMAs, CAX and metal chelators like phytochelatins, metallothioneins and organic acids could be important in plant tolerance to heavy metals, transporting metals into the vacuole (Clemens 2006; Morel et al. 2009). It was found that wheat protoplasts took up more cadmium when added to control than protoplasts from wheat seedlings pretreated 1 week with 1 μM $CaCl_3$ (Lindberg et al. 2007). In the control protoplasts, a H^{+}/Cd^{2+} antiport was suggested at the plasma membrane, but in the Cd-pretreated protoplasts, a phytochelatins-Cd transport was more likely. An ABC transporter AtPDR8 is also supposed to be involved in Cd^{2+} efflux at the plasma membrane (Kim et al. 2007).

8.6 Heavy Metal Signalling

Toxic effects of heavy metals on plants depend on direct effects on membranes, including photosynthetic membranes, as well as indirect effects caused by signalling pathways (Maksymiek 2007). Jasmonate, ethylene and H_2O_2 were reported to be involved in the signalling pathways (Maksymiek 2007, and references therein). Investigations showed that trace elements are able to induce oxidative stress in plants (Clijsters et al. 1999; Unyayar et al. 2006; Sharma and Dietz 2009). During oxidative stress, increased concentrations of superoxide anion (O_2^{-}), hydrogen peroxide (H_2O_2) and its radical (OH) are produced and damage the cellular components. After the first hours of stress, H_2O_2 can be synthesized as a result of activation of the NADPH oxidase (Foreman et al. 2003) or indirectly, by increased lipid peroxidation and increased level of jasmonate (Maksymiec and Krupa 2002, 2006, Metwally et al. 2003). At prolonged exposure of cadmium to barley seedlings, lipid peroxides were formed (Metwally et al. 2003). It was suggested that Cd toxicity might be alleviated by salicylic acid (SA), a known factor that can block the jasmonate pathways (Gupta et al. 2000). Although many investigations deal with induction of different genes and proteins by heavy metals (see references in Maksymiek 2007) little information is available about calcium signalling in heavy metal transduction pathways. An increased level of H_2O_2 can alert plant cells against environmental stresses (Lamb and Dixon 1997; Foyer and Nocter 2003) and may increase the antioxidant response by expression of glutathione transferase gene through calcium signalling (Rentel and Knight 2004). Calcium and calcium channels may be involved in Cu^{2+} toxicity in bean plants (Maksymiec and Baszynski 1999). Recent results with protoplasts from *Elodea canadensis* showed that addition of low concentration of $CdCl_2$ induced an increase in both cytosolic and vacuolar pH, followed by a cytosolic calcium increase, and was found to be dose dependent (T. Javed, S. Lindberg, M. Greger 2011, unpublished results). Rodriguez-Serrano et al. (2009) reported that cadmium reduced the superoxide dismutase (SOD) iso-enzyme activity in pea plants. They suggested that the down-regulation of SOD could be due to cadmium-induced calcium deficiency. Production of superoxide radicals was also prevented by external calcium. On the other hand, nitric oxide (NO) production was strongly reduced by cadmium and treatment with calcium prevented this effect. Therefore, they proposed a complicated cross talk between ROS, NO and calcium.

9 Calcium Interactions with Hormones in Plants Under Stress

9.1 Abscisic Acid

ABA is a prime mediator of plant responses to cold, drought and salt stresses (Zhu 2002). It has many different functions, including regulation of

stomatal closure and activation of gene expression. Many second messengers, such as Ca^{2+}, InsP3 and others are involved in ABA-mediated signalling. Both ABA-dependent and ABA-independent pathways are interacting in a complex way. Under drought stress, drought-responsive factors, such as DREBA2A and ERD1 were identified (Shinozaki and Yamaguchi-Shinozaki 2000, 2007; Yamaguchi-Shinozaki and Shinozaki 2006). InsP3 may act as a negative regulator in the DREB2A drought-signalling pathway (Perera et al. 2008). ABA-responsive elements, ARBREs, are present in the promoter of the transcription factors C-repeat/DRE binding factors (CBF or DREB1), that function as main regulators of abiotic stress responses (Finkler et al. 2007). These findings suggest a direct connection between Ca^{2+} regulation of transcription and abiotic stress responses. CAMTAs are Ca^{2+}-dependent CaM-binding transcription factors, which may mediate the binding of CAMTAs to DNA. CAMTA gene expression in *Arabidopsis* responds rapidly to cold, salinity and hormones, such as ABA and jasmonic acid (Yang and Poovaiah 2002). ABA effects on plant-guard cells have been intensively studied. When maize seedlings were subjected to drought stress, ABA induced calcium increase in the mesophyll and guard cells of the leaves (Ma et al. 2009). The increase in calcium concentration occurred in cytosol, chloroplasts and nucleus under PEG treatment, while it decreased in vacuoles and intercellular spaces. The calcium elevation in the cytosol was ABA dependent and originated from extracellular Ca^{2+}. CaM also participated in ABA signal transduction, since ABA induced changes in Ca^{2+}/CaM concentration. In *Arabidopsis* guard cells, ABA induced an increase in ROS level (Pei et al. 2000). This increase in ROS would stimulate ABA-regulated HACC both in *Arabidopsis* and *Vicia faba* guard cells and regulate stomatal closure. It is suggested that ROS also activate other Ca^{2+} permeable channels (Mori and Schroeder 2004). Staxen et al. (1999) have shown that phosphoinositide-specific phospholipase C can be involved in ABA-induced calcium oscillations in guard cells. ABA can affect the transport of sucrose from mesophyll cells during apoplastic phloem loading. In protoplasts of *Pisum sativum*, sucrose efflux was maximally increased at 10^{-7} M ABA. ABA also increased the pHcyt, independent of sucrose concentration (Opaskornkul et al. 1999). Not only calcium changes, but also pH changes are also induced in the cytosol of plant cells subjected to different stresses (Kader and Lindberg 2010).

9.2 Jasmonic Acid

Jasmonic acid (JA) plays an important role in protecting plants from pathogens or insect attacks, as well as from abiotic stress (Hu et al. 2009). Calcium influx, ROS and NO are often involved in JA signalling (Besson-Bard et al. 2008; Beligni and Lamattina 2008; Hu et al. 2009). The influx of calcium seemed to be necessary for elicitor-induced JA synthesis and secondary metabolite accumulation in cultured ginseng cells (Hu et al. 2009). Addition of JA to *Arabidopsis* leaves could induce an increase in [Ca^{2+}cyt] (Sun et al. 2006). Moreover, JA induced gene expression could be mimicked by high concentration of extracellular Ca^{2+}. It was proposed that the influx of Ca^{2+} through a Nif-sensitive channel in the plasmalemma may be responsible for the elevation of Ca^{2+} and the expression of genes encoding molecules downstream to JA. Increased concentrations of magnesium (Mg) and Ca in the apoplast of *V. faba* leaves significantly enhanced the biological activity of systemin (Liu et al. 2005). Liu et al. (2005) also showed that addition of Ca ionophores, which cause influx of calcium, also induced accumulation of proteinase inhibitors.

9.3 Ethylene

Ethylene concentration increases in plants under stress. Heavy metals, such as Cd, Cu, Fe and Zn can induce ethylene production (Gora and Clijsters 1989; Maksymiek 2007). Ethylene induces stomatal closure by a signalling system involving Ca^{2+} and NO. Treatment of *V. faba* with ethephon, an ethylene-released compound, caused a time- and dose-dependent stomatal closure. Ethylene is also involved in epinastic movements.

The ethylene-induced epinasty of tomato petioles was further stimulated by Ca^{2+} addition (Lee et al. 2008). Pre-treatment with NPA, an auxin-transport inhibitor prevented the effect by calcium ions on the petiolar epinasty. Ethylene caused accumulation of Ca^{2+} towards the lower (abaxial) side of the petioles, opposite to the ^{14C}IAA redistribution. These results suggest that the gravity-insensitive ethylene-induced Ca^{2+} redistribution and accumulation towards the abaxial side are closely coupled to the upper (adaxial) auxin redistribution and accumulation and, hereby, to the epinastic curvature. Addition of ethephon and ACC (precursor of ethylene), to tobacco protoplasts, activated the plasma membrane channels permeable to Ca^{2+}, Mg^{2+} and Ba^{2+} (Zhao et al. 2007). Ethephon also modified the calcium signalling in the cytoplasm induced by herbivores (Arimura et al. 2008). This was necessary for both JA and terpene biosynthesis. Thus, elevation of [Ca^{2+}cyt] is supposed to be an important component in ethylene signalling in plants. Ludwig et al. (2005) found an ethylene-mediated crosstalk between CDPKs and mitogen-activated protein kinases (MAPKs) in response to stress in tobacco.

9.4 Auxin and Calcium Signalling

Auxin, an important plant hormone, regulates the growth of plant and development. Early investigations showed that auxin induces elongation growth and causes oscillating kinetics of the transmembrane potential, starting with an immediate depolarization, and then changing to a hyperpolarization (Cleland et al. 1977; Poovaiah and Reddy 1987; Cho and Hong 1996; Polevoi et al. 1996 and others). Auxin addition to maize-coleoptile cells showed a rapid elevation in [Ca^{2+}cyt] within the first 3–5 min (Felle 1988). Later on, similar reactions were found in maize roots, parsley hypocotyls, wheat and maize protoplasts (Gehring et al. 1990; Shishova and Lindberg 2004, 2010; Shishova et al. 2007). In wheat protoplasts, calcium elevation occurred within 5–10 s after addition of 1-naphthyl acetic acid (1-NAA), a synthetic and physiologically active auxin (Shishova and Lindberg 2004). Inhibitor analyses showed that the shift in [Ca^{2+}cyt] could depend on the activation of calcium channels, i.e. both in the plasma membrane and tonoplast. External calcium also increased the sensitivity to auxin. In several investigations, a simultaneous shift in pHcyt was obtained (Felle 1988; Irving et al. 1992; Shishova and Lindberg 1999). Shishova and Lindberg (1999) showed that verapamil, an inhibitor of calcium channels, blocked the cytosolic acidification, indicating the cell response to auxin depends on Ca^{2+}-channel activation. Receptors for auxin are supposed to be located both at the plasma membrane and intracellular. A soluble receptor, the F-box transport inhibitor resistant 1 (TIR1) protein in the nucleus was discovered (Dharmasiri et al. 2005; Kepinski and Leyser 2005). For the plasma membrane receptor, two models have been proposed: either the receptor includes a protein kinase or it is connected with a G-protein. Based on the found auxin-induced reactions, such as changes in membrane potential, shifts in cytosolic concentrations of Ca^{2+} and pH as well as increase in cell sensitivity to auxin by external calcium, a third model was also proposed (Shishova and Lindberg 2010). This model suggests that an associative domain of the ABP1 protein is closely connected with the calcium-permeable ion channel. Little is known about the mechanisms of integrating auxin effects on growth and stress responses. However, it was recently shown that the calmodulin-binding transcription activator 1 (CAMTA 1) is involved in auxin signalling and responds to stress (Galon et al. 2010a). The expression pattern of CAMTA1 changed significantly and differentially on exposure of *Arabidopsis* to high salt concentration and heat.

9.5 Gibberellins

Calcium is also involved in gibberellin signalling. The homeostasis of gibberellins (GAs) in plant cells is maintained by a negative-feedback regulation. A transcriptional activator (RSG, Repression of Shoot Growth) is suggested to take part in the feedback mechanism causing RSG

and regulating genes coding GA biosynthetic enzymes (Nakata et al. 2009). A 14-3-3 signalling protein negatively regulates RSG by moving into the cytoplasm in response to GAs. A phosphorylation is necessary for binding of 14-3-3 to RSG. It was found that a CDPK promoted the binding. This CDPK decodes the Ca^{2+} signal produced by GAs.

10 Biotic Stress Signalling

Pathogens, as well as symbiotic microorganisms induce Ca^{2+} signals in plant cells; however, the defence responses obtained can be both activated and suppressed by the signals. This suggests that Ca^{2+}-responsive, but antagonistic, signalling mechanisms are present (Dodd et al. 2010).

Molecules with microbe-associated molecular patterns, called MAMPs, can cause both cytosolic and nuclear calcium increases, but the signals may be more prolonged in the nucleus. Lecourieux et al. (2005) found the Ca^{2+} elevations induced by harpin, in cell cultures from tobacco cells, continued for 5 min in the cytosol and for 150 min in the nucleus. Calcium was mobilized from both extracellular (apoplast) and intracellular (vacuole or ER) stores. It was reported that MAMP-induced Ca^{2+} signals caused activation of SA and mitogen- and wound-activated protein kinases (Lecourieux et al. 2005; Ma and Berkowitz 2007). Usually Ca^{2+} increase encodes stimulus-specific information, but MAMP-induced signals cause similar defence responses irrespective of the type of elicitor (Ma and Berkowitz 2007 and references within). Moreover, Du et al. (2009) found the Ca^{2+} signals may also suppress the SA-mediated gain of systemic acquired resistance. When the Ca^{2+}/CaM complex binds to the transcription activator CAMTA, EDS1 (enhanced disease susceptibility 1) is repressed. As EDS1 functions as a positive regulator of resistance and SA levels, Ca^{2+} signals in this case repress the resistance mechanisms. Calcium also plays an important role in the symbiosis between a plant and a microorganism. Since atmospheric nitrogen, N_2, cannot be used by the plant, it needs to be converted to ammonia by certain prokaryotic bacteria in a process called nitrogen fixation. The microsymbiont is hosted in special organs, called root nodules, and are developed on the plant roots upon infection (reviewed by Mylona et al. 1995).

Two types of root-nodule symbiosis are known: symbiosis between legumes and gram-negative, unicellular soil bacteria, called rhizobia, and between actinorhizal plants (of the order Fagales and Cucurbitales) and gram-positive actinomycetes of the genus Frankia (reviewed by Sprent 2006). Less information is available on the actinorhizal, than on legume–rhizobia symbiosis. In the legume–rhizobia interaction, the plant secretes flavonoid compounds from the roots and induces the expression of rhizobial nodulation (nod) genes. The Nod genes encode proteins involved in the rhizobial signal transduction, such as Nod factors, which induce deformation of plant roots, plant gene expression and nodule development. Most terrestrial plants (80%) can also establish a symbiosis with a fungus of the phylum Glomeromycota (Parniske 2008). Such symbiosis is an advantage to the plant, since the mychorrhiza increases the availability and uptake of soil nitrogen and phosphorus into the plant. For the establishment of mycorrhiza, diffusible fungal signal factors, so-called Myc factors must be recognized by the plant. Both Myc and Nod factors are representatives of lipochito-oligosaccharides (Denarie et al. 2010) and play a great role of the signalling system for development of nodules and mycorrhizal infection (Kosuta et al. 2008; Oldroyd and Downie 2008). Kosuta et al. (2008) suggested that in legume plants, at least seven common proteins mediate infection by both mycorrhizal fungi and rhizobial bacteria. For instance in *Medicago truncatula*, the proteins DMI1, DMI2 and DMI3 are necessary for both types of symbiosis signalling. In symbiotic signalling, cytosolic calcium changes are supposed to be linked to nuclear calcium changes by a complicated system (Oldroyd and Downie 2006). Two receptor-like kinases at the plasma membrane, NFR1 and NFR5, are required for Nod-factor perception (Madsen et al. 2003) and an equivalent receptor-like kinase is supposed to exist for Myc factors. A third kinase

could also function in a common symbiotic pathway (Endre et al. 2002; Limpens et al. 2003). The phosphorylations at the plasma membrane following Nod factor recognition must be linked to the induction of calcium changes associated with the nucleus (Oldroyd and Downie 2006). The secondary messenger involved so far is unknown, but could be a product of phospholipase C and D (PLC and PLD). The perception of Nod and Myc factors at receptors in legume roots induces calcium oscillations, but the frequencies of the spikes differ depending on the elicitor factor (Kosuta et al. 2008). Kosuta and co-workers showed that Nod factors induced calcium spiking during longer periods and with higher amplitude than the mycorrhizal spiking. It is interesting that in both cases, the oscillation was chaotic in nature, that is, deterministic and predictable (Casdagli 1992), as shown by the Lyapunov method and other mathematical analyses (Kantz and Schreiber 1997). Chaos is not common in biological systems, but may provide a mechanism for flexibility (Kosuta et al. 2008). Chaotic systems can produce variable responses with very little energetic input.

Arbuscular mycorrhizal fungi were shown to induce calcium signalling in the host plant (Navazio et al. 2007). A difference in amplitude and frequency of calcium oscillations has been linked to differences in gene expression and enzymatic activities in several systems (Kummer et al. 2005). For nodule development and mycorrhizal association, an activation of CCaMK (calcium–calmodulin activated kinase) is necessary and the different outcome could depend on the difference in calcium oscillations (Kosuta et al. 2008). A sustained high cytosolic calcium concentration leads to apoptosis during the normal development and in hypersensitive responses to pathogens (Levine et al. 1996; Tuteja and Mahajan 2007).

11 Conclusion and Future Perspective

Calcium plays an important role in stress signalling. A change in the cytosolic calcium concentration is induced by all types of abiotic and biotic stresses investigated. Calcium signalling is very complex and takes place not only in the cytosol, but also in the nucleus, mitochondrion and ER. The specific "signature" of calcium, such as duration, frequency and amplitude of calcium concentration changes is important for the downstream reactions. Plants have many different calcium sensor proteins, such as CaM, CaM-binding proteins, CDPKs and phosphatases, annexins, as well as CaM-like and calcineurin-like proteins. They exist in the cell as free proteins or bound to membranes. Some of these proteins are "buffer" protein, that is, they regulate the free calcium concentration, others are so-called trigger proteins, which are activated by calcium binding and in turn activate other proteins. Calcium and CAM can also activate transcription factors and are supposed to be involved in stress-related gene expression.

In most stress reactions investigated so far, the change in calcium concentration is connected with a change in pHcyt. The pH change differs in salt-tolerant and -sensitive cultivars or species, but the reason for this is unknown. More information is needed about pH changes in plant cells under various stresses since the proteins and enzymes in the downstream reactions are pH sensitive.

Even if there is considerable knowledge about the tolerance mechanisms and gene expression under different types of stress, the nature of the sensors for different stresses is still enigmatic and needs clarification. Further research should also focus on the complex signalling system in different compartments of a cell and how they interact with each other.

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Role of H_2O_2 as Signaling Molecule in Plants

16

M.A. Matilla-Vázquez and A.J. Matilla

Abstract

Hydrogen peroxide (H_2O_2) is continuously produced as a by-product of oxidative plant aerobic metabolism. It plays a dual role in cells. Under normal conditions, H_2O_2 acts as a key regulator of many biological processes because H_2O_2 have been identified as an important second messenger in signal transduction networks. On the other hand, H_2O_2 is a sharp oxidant triggering cell damages or even death during oxidative stress. Therefore, cellular "redox homeostasis" is tightly regulated by H_2O_2 production and scavenging. In these processes a great number of genes are involved. Organelles with a high oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria, or peroxisomes are major sources of H_2O_2 production. However, its notable apoplastic production by means of a plasmalemma NADPH-oxidase must be taken in account. H_2O_2, interacting with thiol-containing proteins, can modulate the activities of many components in signaling, such as protein kinases, protein phosphatases, or transcription factors. Moreover, the H_2O_2 cascade can result in the downstream activation of Ca^{2+} channels, which may be the central step in many H_2O_2-mediated processes. All these summarized alterations trigger the regulation of important processes like gene expression, elongation growth, ABA-mediated stomatal closure, programmed cell death, defense against pathogens, seed dormancy, and germination. The molecular mechanisms by which these processes are affected by H_2O_2 signaling have not been completely clarified. Here, we describe several aspects of H_2O_2 production, scavenging and gene regulation, and cross-talk with ABA and ethylene during plant growth and development.

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H_2O_2 • Production • Scavenging • Signaling • Transcription factors • Plant interactions • Plant growth

1 Introduction

The evolution of oxygen (O_2) metabolism in higher plants led to the production of reactive oxygen species (ROS) in the organelles involved in this aerobic process (mitochondria, chloroplasts, and peroxisomes) (Apel and Hirt 2004; Slesak et al. 2007; Corpas et al. 2009). ROS appear under physiological conditions and they are highly reactive molecules that can disturb membranes and other cellular components. Hence, it is important to remove these toxic molecules from cells to prevent stress-induced injuries. The plants possess a complex battery of antioxidant defense systems to regulate ROS production with beneficial effects. ROS are generated in the cells by several enzymatic reactions and electron transport systems (Fig. 16.1). The ROS includes superoxide ($O_2^{\bullet-}$), hydroxyl (HO) radicals, singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). HO• has indiscriminate reactivity towards biological molecules, whereas $O_2^{\bullet-}$ and H_2O_2 have preferential biological targets. Plants have an abundance of antioxidant defense systems to regulate their production with beneficial

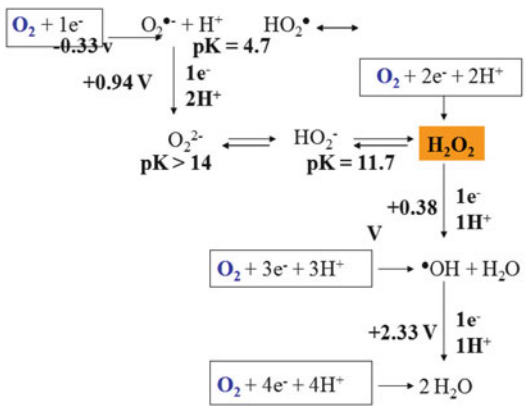

Fig. 16.1 Sequence of electron transfer reactions at the O_2 molecule

effects. Under abiotic (e.g., drought, salinity, flooding, heat, and cold) and biotic stresses (Gechev et al. 2006; Gadjev et al. 2008; Miller et al. 2008), the overproduction of ROS can overcome the antioxidant systems (scavenging mechanisms) resulting in oxidative damage of lipids, sugars, proteins, and nucleic acids. In some cases, the inactivation of specific targets loss of physiological functions takes place, thereby leading to cell death (Gadjev et al. 2008). It became clear that ROS play a dual role in plants as toxic compounds as well as key regulators of many biological processes such as growth, cell cycle, hormone signaling, biotic and abiotic cell responses, programmed cell death (PCD), and plant development (Apel and Hirt 2004; Miller et al. 2008; Corpas et al. 2009). In the last decade, H_2O_2 has been identified as an important second messenger in signal transduction network of plants. Here, the latest data related to H_2O_2 signaling are described and summarized.

2 Chemical Properties of H_2O_2

In aqueous solution, $O_2^{\bullet-}$ is moderately reactive, but it can generate H_2O_2 by dismutation. The H_2O_2 belongs to non-radical ROS, carries no net charge, is the only ROS species that is stable in solution (cellular half-life ~1 ms, steady-state levels ~10^{-7} M), and it can diffuse across biological membranes making H_2O_2 fit for signaling. Diffusion of H_2O_2 might be modulated by changes in lipid membrane permeability or by transport through aquaporins (Bienert et al. 2006). Its toxicity is essentially the consequence of its reduction to HO• by metal-catalyzed Fenton chemistry. H_2O_2 is actually a poor oxidant that reacts mildly with [Fe-S] (rate constant of 10^2–10^3 $M^{-1}\,s^{-1}$), it loosely binds metals (10^3–10^4 $M^{-1}\,s^{-1}$), and it also reacts with methionine residues (10^2 $M^{-1}\,s^{-1}$). In contrast, its reactivity

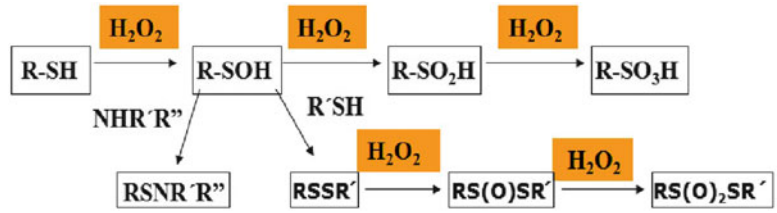

Fig. 16.2 Oxidation by H_2O_2 of containing Cys products

towards Cys residues can significantly increase to 10–10^6 M^{-1} s^{-1} depending on the protein environment (D'Autréaux and Toledano 2007). Sulfur-mediated nucleophilic attack of the peroxide O–O bond by the Cys thiol group (–SH) leads to H_2O_2 release and formation of sulphenic acid (–SOH). The –SOH is highly reactive, its stability is influenced by the availability of a proximal nitrogen to form a sulphenamide or by the presence of H_2O_2, which further oxidizes it to form sulphinic ($-SO_2H$) or sulphonic ($-SO_3H$) acids (Spadaro et al. 2010) (Fig. 16.2).

3 Production of H_2O_2 by Plants

Close to 1% of O_2 consumed by plants is diverted to produce ROS in various subcellular sites (Bhattachrjee 2005). The multiple sites and sources of ROS production increase its complexity. The endogenous concentration of H_2O_2 is reported in a wide margin, ranging from nmol to several hundred μmol/gFW. Plant tissues can tolerate high concentrations of H_2O_2 in the range of 10^2–10^5 μM. Normally, H_2O_2 generation is often maintained at a constant basal level in healthy cells, but their levels increase transiently or persistently in response to stress (Desikan et al. 2003; Apel and Hirt 2004). Although it was previously hypothesized that H_2O_2 produced intracellularly diffuses to other cells, it now appears more probable that intracellularly produced H_2O_2 is consumed quickly and locally, and that extracellular metabolism uses H_2O_2 produced extracellularly. Transmembrane movements of H_2O_2 (e.g., from the apoplast to the cytosol) probably involve aquaporins (Bienert et al. 2007). The H_2O_2 production requires a continuous Ca^{2+} entry to activate the plasmalemma-localized NADPH-oxidase, leading to Ca^{2+}-dependent cellular responses resulting from anion and K^+ exit. This fate was also confirmed by the administration of Ca^{2+} inhibitors which prevent increases of cytosolic Ca^{2+} concentration. Under these conditions, the accumulation of endogenous H_2O_2 was prevented.

H_2O_2 is produced by means of the following reactions:

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

$$O_2^{\bullet -} + H^+ + XH_2 \rightarrow H_2O_2 + XH$$

(X ≡ reductant molecule)

That is, H_2O_2 production is related to the synthesis of $O_2^{\bullet -}$. However, glycolate, glucose, amino-acid, and sulfite oxidases also release H_2O_2 following the oxidation of their respective substrates. Moreover, cell wall (CW)-bound peroxidases and oxalate, amine and plasmalemma NADPH-oxidases are other enzymatic sources of $O_2^{\bullet -}$ and H_2O_2. Peroxisomes, mitochondria, and chloroplasts are cellular compartments involved in H_2O_2 production (Mittler et al. 2004). It is estimated that the chloroplast/peroxisome system generates about 90% of the total H_2O_2 in the photosynthetically active plant cell. Excess H_2O_2 leads to oxidative stress and it is capable to cause injury to cells. For that reason, during the course of evolution plants were able to achieve a high degree of control over H_2O_2 accumulation. It is suggested that the tolerance of plants to high H_2O_2 levels is due to the fact that plant antioxidant response systems are designed more for the control of the cellular redox state than for complete elimination of H_2O_2.

Table 16.1 H_2O_2 (nmol mg^{-1} protein) production in different plant organelles

Chloroplast	Mitochondria	Peroxisomes
1.79	1.30	2.81

For more details see Corpas et al. (2009)

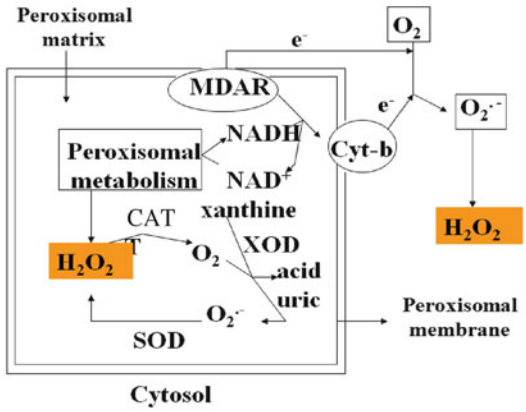

Fig. 16.3 Generation of H_2O_2 as a consequence of plant peroxisomal metabolism. *CA* catalase, *Cyt-b* cytochrome-b, *MDAR* mono-dehydro-ascorbate reductase, *SOD* superoxide dismutase, *XOD* xanthine oxidase. For details see Corpas et al. (2009)

Table 16.2 Peroxisomal enzymes involved in the H_2O_2 production

Fatty acid degredation
Short chain acyl-CoA oxidase
Medium chain acyl-CoA oxidase
Long chain acyl-CoA oxidase
Photorespiration
Glycolate oxidase
Purine metabolism
Urate oxidase
Xanthine oxidoreductase
Catalase (CAT)
Ascorbate–glutathione cycle
Ascorbate peroxidase (APX)
Peroxiredoxin
Others
Sarcosine oxidase
Sulfite oxidase

With the exception of APX (membrane-bound), all enzymes have been found in the peroxisomal matrix

3.1 Peroxisomes (Glyoxisomes)

Peroxisomes are one of the main sites for the generation of H_2O_2 due to an essentially oxidative type of metabolism (Table 16.1). The H_2O_2 production is mainly through the photorespiration and fatty acid β-oxidation pathways. At least two sites of $O_2^{\bullet-}$ generation were demonstrated in the peroxisome (Fig. 16.3): (1) in the matrix by means of xanthine oxidase (XOD), which catalyzes the oxidation of xanthine and hypoxanthine to uric acid and it is a well-known producer of $O_2^{\bullet-}$; and (2) in the organelle membranes being dependent on NAD(P)H (Corpas et al. 2009). The superoxide dismutase (SOD) is a metalloenzyme that catalyzes the synthesis of H_2O_2 following the reaction: $O_2^{\bullet-} + O_2 + 2H^+ \rightarrow H_2O_2 + O_2$. The SOD is an important enzymatic component of a defense mechanism that protects cells from the damaging action of $O_2^{\bullet-}$ radicals. The various SOD forms are induced with different kinetics during sustained stress conditions. The presence in peroxisomes of Cu/Zn-SOD was unequivocably demonstrated. However, the genes encoding this peroxisome Cu/Zn-SOD have not yet been identified. Other peroxisome enzymes involved in the H_2O_2 production are included in Table 16.2 (Corpas et al. 2009). On the other hand, the uricase or urate oxidase, essential in the plant catabolism of purines, catalyzes the oxidation of uric acid to allantoin with the concomitant generation of H_2O_2. Under physiological conditions, the peroxisomal level of H_2O_2 and $O_2^{\bullet-}$ are controlled by catalase (CAT) and ascorbate peroxidase (APX) and by SOD, respectively. In peroxisomes/glyoxisomes, CAT predominates, but its properties suggest that the enzyme is inefficient to remove low concentrations of H_2O_2. Taken together all the disposable data on the H_2O_2-producing peroxisomal β-oxidation of fatty acids, they strongly suggest that any type of plant stress inducing the peroxisomal production of $O_2^{\bullet-}$ and $^{\bullet}NO$ radicals inhibits the CAT and APX activities and, perhaps, increases the level of H_2O_2 by enhancing the peroxisomal fatty acid β-oxidation.

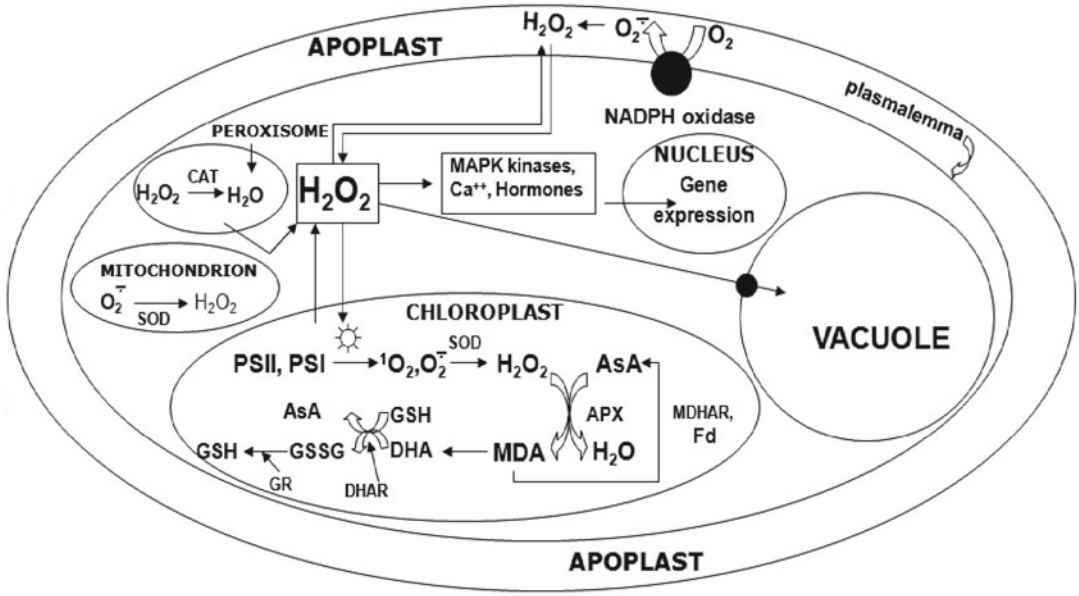

Fig. 16.4 Generation and scavenging of H_2O_2 in plant cells. *APX* ascorbate (AsA) peroxidase, *CAT* catalase, *Fd* ferredoxine, *GR* glutathione reductase, *GSSG*-oxidized glutathione, *GSH*-reduced glutathione, *MDA* monodehydroascorbate, *MDHAR* MDA-reductase, *SOD* superoxide dismutase. Chloroplastic H_2O_2 is detoxified by APX with the AsA as an electron donor and transformed in GSH by means of the intervention of GR and DHAR activities, respectively. During photorespiration and fatty acid oxidation, peroxisomes and glyoxisomes, respectively, produce large amounts of H_2O_2 which is rapidly scavenged by CAT. The movement of H_2O_2 between different cellular compartments is facilitated by peroxoporins (specialized aquaporins). The excess of H_2O_2 leaking into cytosol from different compartments is metabolized by various peroxidases or may eventually be transported and detoxified into the vacuole. Adapted from Gechev et al. (2006)

3.2 Mitochondria

In the dark or in non-photosynthetic tissues, mitochondria can be the major source of ROS comparing with chloroplasts and peroxisomes (Rhoads et al. 2006). The mitochondrial contribution in H_2O_2 generation in plant cells under light conditions requires further study since photosynthesis and respiration in the light are strongly interdependent. About 1–5% of mitochondrial O_2 consumption leads to H_2O_2 production (Moller 2001). The plant mitochondria have five H_2O_2-generating enzymes that exist only in plants (i.e., one alternative oxidase and four NADPH dehydrogenases assembled to flavoproteins). During respiration, H_2O_2 production is carried out in the NADH dehydrogenase complex (CI) and in the ubiquinol-cytochrome bc1 complex (CIII), respectively. In CIII, which is inhibited by KCN, it takes place the conversion of $O_2 \rightarrow O_2^{\bullet-}$ and it appears to be the major site of mitochondrial H_2O_2 production. A plant mitochondrial-specific Mn-SOD is involved in the formation of H_2O_2 from $O_2^{\bullet-}$ produced in two complex of the electron transport chain (ETC), NAD(P)H dehydrogenase and cytochrome bc_1, respectively.

3.3 Chloroplasts

Much of the ROS generated in photosynthetic plant cells is produced in chloroplasts which are notable sites of H_2O_2 production as a by-product of the reduction of O_2 (Fig. 16.4). Chloroplasts produce singlet O_2 from the excited triplet state of chlophyll (photosystem II) and $O_2^{\bullet-}$ in the Mehler reaction (photosystem I). As in the mitochondria, H_2O_2 generation in chloroplasts is also linked to the ETC (pseudocyclic electron flow). In PSI, under limiting conditions the availability

of NADP or in situations of overloading of the ETC, a part of the electron flow is diverted from ferredoxin to O_2, and the $O_2^{\bullet-}$ can be produced via a Mehler reaction. There seems to be a consensus that the Mehler reaction acts as an alternative sink for an excess of electrons, generated during excess excitation energy stress. Currently, this reaction is considered as the primary and the most powerful source of H_2O_2/ROS in chloroplasts (Logan et al. 2006). In the stroma side of the membrane, $O_2^{\bullet-}$ is spontaneously dismutated to H_2O_2 by Cu/Zn-SOD. On the other hand, is known that the acceptor side of PSII also provides sides (A_A, Q_B) with electron leakage to O_2 producing $O_2^{\bullet-}$ (Quan et al. 2008). Besides exercising its signaling function in the chloroplast, H_2O_2 can be secreted from it and elicit signaling in the cytosol, elsewhere in the cell or even systematically. Thus, H_2O_2 may play an important role as initiator or transducer of signals from chloroplast to the nucleus in response to environmental factors (Gálvez-Valdivieso and Mullineaux 2010).

3.4 Other Sites Producing H_2O_2

3.4.1 Plasmalemma and Cell-Wall Apoplast

CW-associated peroxidases as well as plasmalemma-bound NADPH-oxidases are the main $O_2^{\bullet-}$ and H_2O_2 producing apoplastic enzymes which are regulated by various developmental and environmental stimuli. The NADPH-dependent oxidase system (*rboh*, *r*espiratory *b*urst *o*xidase *h*omologue) has received further attention. It catalyzes the production of $O_2^{\bullet-}$ by one-electron reduction of O_2 using NADPH as the electron donor (Apel and Hirt 2004). Genes of *rboh* have been cloned from several plant species (Desikan et al. 2003). The $O_2^{\bullet-}$ is most likely located in the CW apoplastic space and is converted either spontaneously or by extracellular SOD to H_2O_2. The role of H_2O_2 and other ROS in CW apoplast should not only be limited to defense responses, but their primary role is in the regulation of the synthesis of CW components (e.g., lignin). On the other hand, $O_2^{\bullet-}$ and H_2O_2 are also produced by XOD during purine catabolism, ribonucleotide reductase during deoxyribonucleotide synthesis and various other oxidases induced by biotic and abiotic stresses (Foreman et al. 2003; Gechev et al. 2006). Interestingly, Rops (Rho-like small G-proteins) signaling have an important role in regulating H_2O_2 production, potentially via NADPH-oxidase (Neill et al. 2002). The O_2 deprivation was found to activate Rop signaling that in turn activates NADPH-oxidase. This fate was attenuated by H_2O_2-induction of *RopGAP* gene expression that leads to the deactivation of Rop (Neill et al. 2002).

3.4.2 Cytoplasm

The cytosol cannot be regarded as the major source of H_2O_2 in plant cells, but it may act as a sink for H_2O_2 leaking from other cellular compartments. The function of cytosol-generated H_2O_2 is not understood. The ETC associated with the endoplasmic reticulum is the main source of H_2O_2/ROS, where reduced forms of cytochrome P450 and cytochrome P450 reductase that are involved in oxidation and hydroxylation processes. Likewise, cytochrome b_5 and cytochrome b_5 reductase that are engaged in fatty acid desaturation, donate electrons to O_2 producing $O_2^{\bullet-}$, and a cytosolic form of SOD can convert $O_2^{\bullet-}$ to H_2O_2.

4 Scavenging of H_2O_2

In plant cells, enzymes and redox metabolites act in synergy to carry out H_2O_2 scavenging. Plant antioxidant enzymes together with ROS-producing enzymes constitute a highly sophisticated and redundant network, which in *Arabidopsis thaliana* consist of at least 289 genes (Gechev et al. 2006). This network maintains ROS homeostasis in all cellular compartments and regulates the adjustment of ROS levels according to the cellular need at a particular time (Fig. 16.4). SODs are the only plant enzymes capable of scavenging $O_2^{\bullet-}$, whereas high concentrations of H_2O_2 can be catabolized directly by CATs or with the help of various reductants like APXs, glutathione peroxidases (GPXs), and guaicol peroxidases. The balance between SOD and the different H_2O_2-scavenging enzymes in cells is considered

to be crucial in determining the steady-state level of $O_2^{\bullet-}$ and H_2O_2. APX has a high affinity for H_2O_2 and it is probably the most important H_2O_2-scavenging enzyme produced in chloroplasts; this enzyme is also present in cytoplasm, peroxisomes, and mitochondria (Narendra et al. 2006). Likewise, by combining APX with glutathione reductase (GR), H_2O_2 can also be removed by redox reactions promoted by glutathione (GSH), hence preventing cell damage. Lipid-soluble antioxidants like ascorbate (AsA; vitamin C), GSH, tocopherol (vitamin E), and carotenoids are non-enzymatic antioxidants that contribute to ROS homeostasis in plants (Dellapenna and Pogson 2006; Gechev et al. 2006). Since GSH appears in plant cells in millimolar concentrations, it is regarded as the key determinant of the cellular redox state. GSH levels, redox status, and biosynthesis can regulate the expression of a large number of genes including the antioxidant defense system and the pathogenesis-related 1 (*PR1*) gene (Ball et al. 2004). Taking it all together, all cellular compartments are well equipped with antioxidant enzymes and antioxidants. However, when this local antioxidant capacity cannot cope with ROS production, H_2O_2 can leak into cytosol and diffuse to other compartments, for example, vacuoles (Bienert et al. 2006). Vacuoles are enriched in flavonoids, AsA, and GSH, whereas the peroxidases are localized at the tonoplast inner surface. The main scavenging enzymes are described below.

1. *Catalase* was the first antioxidant enzyme found in peroxisomes ($2H_2O_2 \rightarrow 2H_2O + O_2$). It degrades H_2O_2 without any reducing power and it is active only at relatively high H_2O_2 concentrations. Thus, lower H_2O_2 levels are eliminated by APX and other peroxidases with the aid of various reductants like AsA and GSH. CAT is of enormous importance for regulating H_2O_2 homeostasis, as it can function as a cellular sink for H_2O_2. CAT is tightly regulated at transcriptional and translational levels and in plants CAT is encoded by a multigene family. Therefore, two genes in cotton, three in maize, and five in pea were described (Gechev et al. 2006; Corpas et al. 2009). In *Arabidopsis*, CAT is encoded by a multigene family consisting of three genes (*CAT1*, *CAT2*, *CAT3*) encoding individual subunits that associate to form at least six isoenzymes. Furthermore, peroxisomal CAT2 and CAT3 show circadian regulation (Du et al. 2008). On the other hand, a genetic system that controls H_2O_2 levels with the involvement of CAT has been identified and described in *Arabidopsis* (Mateo et al. 2004, 2006). A network of at least 152 genes is involved in managing the level of H_2O_2 (Mittler et al. 2004). Transcriptome analysis of CAT-deficient plants demonstrated that perturbation of the H_2O_2-scavenging capacity significantly affects nuclear gene expression (Queval et al. 2007). Likewise, in response to H_2O_2, 349 transcripts were significantly induced (upregulated) and 88 were repressed (downregulated) in the above CAT-deficient plants (Vanderauwera et al. 2005). A detailed description on photorespiratory H_2O_2-dependent gene expression is reported by Vanderauwera et al. (2009).
2. *Ascorbate peroxidase* catalyzes in distinct cell compartments the electron transfer from AsA to H_2O_2 to render dehydroascorbate and H_2O. APX is the main enzyme responsible for H_2O_2 removal in the chloroplast, peroxisome, and mitochondria. Expression of genes for the cytosolic APX1 and APX2 is controlled, at least in part, at the chloroplast level. The activity of APXs is thought to form a second barrier of defense against H_2O_2/ROS produced in the chloroplasts (Chang et al. 2004). Recently, it has been reported that H_2O_2-mediated inactivation of two chloroplastic peroxidases, APX and 2-cys peroxiredoxin, and it is discussed how the chloroplast become susceptible to H_2O_2 damage (Kiitajima 2008; Tripathi et al. 2009). It has also been described that the overexpression of APX in barley peroxisomal membrane increases the tolerance to heat stress, whereas the *Arabidopsis* peroxisomal APX is essential for plant growth and development (Narendra et al. 2006).
3. *Glutathione reductase (GR)* catalyzes the reduction of oxidized glutathione (GSSG) to glutathione (GSH). This ubiquitous enzyme is a component of the AsA–GSH cycle (AGC)

that controls the cellular level of H_2O_2 (Foyer and Noctor 2005). The peroxisomal isoform of GR has been characterized, but the gene encoding it has not yet been identified (Romero-Puertas et al. 2006). Other component of AGC is the monodehydroascorbate reductase (MDAR) which catalyzes the reaction: monodehydroAsA + NAD(P)H → AsA + $NAD(P)^+$. In *Arabidopsis* it has been shown that the membrane-bound MDAR4, component of the AsA-dependent electron transfer system, is necessary to detoxify H_2O_2 (Eastmond 2007). In relation to GR, the GSH peroxidase (GPX) uses GSH to reduce H_2O_2, thereby protecting cells against oxidative damage. In plants, GPX exist in the cytosol to reduce H_2O_2 to H_2O. However, its ability to scavenge H_2O_2 decrease due to its Cys residue without selenium. Therefore, the major functions of GPX are lignin biosynthesis, resistance to pathogens, and degradation of indole-3-acetic acid (IAA).

5 H_2O_2 Signaling

Oxidants utilized in cellular signaling must exhibit specificity for their target substrate. Furthermore, the given redox-based modification must be reversible to ensure signal transduction and is transient rather than constitutive. H_2O_2, O_2^-, and NO execute Cys-based modifications that are substrates-specific and generally reversible (D'Autréaux and Toledano 2007). The relative stability, rapid turnover, and higher concentrations of H_2O_2 in plant cells could point to the fact that H_2O_2 plays a key role as a signal transduction factor. Nowadays, there are many reports indicating that H_2O_2 is a cellular component at physiological conditions. However, signal molecules are usually present in very low concentrations. Therefore, it is thought that H_2O_2 is not only a signaling molecule, but also plays a key role in primary plant metabolism. Although H_2O_2 is a signal molecule affecting transcriptome, it is not clear whether H_2O_2 is actually the signal per se, or whether oxidation of other molecules by H_2O_2 is needed to generate an intracellular signal. In order to H_2O_2 as a specific signaling molecule, its cellular alterations must be tightly perceived. To start the H_2O_2 signaling process it is necessary that a concrete protein specifically recognize H_2O_2, or alternatively a direct chemical interaction exist to propagate the H_2O_2 signal. The first possibility is unlikely due to the small size of H_2O_2. One likely mechanism for cells to perceive H_2O_2 was proposed by Hancock et al. (2006). In this sense, mechanism should involve H_2O_2-dependent protein modifications. H_2O_2 can directly interact with Cys residues within proteins. This modification of –SH groups is the key to H_2O_2 perception since it could alter protein conformation, affecting their activity and therefore initiating subsequent cellular responses (Foyer and Noctor 2005; Hancock et al. 2006). If two –SH groups are present in a specific protein, the formation of a S–S bridge could be a possible mechanism for protein conformational changes involving functional alterations. Alternatively, single –SH groups can be oxidized and reversal oxidation carried out under reducing conditions, for example, in the presence of GSH or thioredoxin (TRXH). Many cellular and extracellular proteins are potential targets of such oxidative modification.

5.1 H_2O_2 Signal Perception

The first genes involved in ROS perception and signal transduction have been identified (Lee et al. 2007; Vanderauwera et al. 2009). However, it still remains to be identified a cell surface protein that acts as H_2O_2 sensor. In contrast, two possible H_2O_2 sensor candidates exist in the cytosol (e.g., glyceraldehide-3-P-dehydrogenase) and the endoplasmic reticulum (ethylene receptor), respectively. Miller and Mittler (2006) have argued that heat-shock transcription factors are the molecular sensors of ROS and their complexity, flexibility, and specialty allow them to control the expression of a wide range of stress-response genes, not only those involving in heat shock. But confirmation of this possibility has not been carried out.

1. *Glyceraldehide-3-P-dehydrogenase (GAPDH)*: using the 5-iodoacetamide fluorescein (IAF)

methodology is possible to mark a number of cytosolic proteins. Pretreatment with H_2O_2 severely reduced the IAF binding to one particular protein (Mw≈40 kDa) which corresponded to the glycolytic enzyme GAPDH (Hancock et al. 2005). GADPH inhibition with low concentrations of H_2O_2 (<250 μM) can be restored with GSH or dithiothreitol (DTT), suggesting that inhibition is indeed reversible. GADPH has also been inhibited by NO, and this inhibition could be reversed with DTT (Lindermayr et al. 2005). At present it is unknown whether NO and H_2O_2 are targeting the same –SH groups in GADPH. On the other hand, GAPDH has been shown in other different systems to plant being S-thiolated by the addition of GSH to the thiol side group. It is unknown whether this modification also occurs in plants.

2. *Ethylene receptor (ETR1)*: in yeast, histidine kinases (HKs) of two-component signaling systems have been reported to function as sensors of oxidative stress. In *Arabidopsis*, the HK of the ETR1 appears to be essential for H_2O_2 perception during stomatal movement. Thus, mutation of a Cys residue in the N-terminal region of ETR1 disrupted the perception, indicating that this thiol group is important for the H_2O_2 signaling. As the ETR1 kinase domain is not required for H_2O_2 perception, the H_2O_2-provoked signaling through ETR1 was seemingly unrelated to its well-described role as an Et receptor. This fate suggests that the signaling through ETR1 provoked by H_2O_2 is different from Et induced. The *S. cerevisiae* mutant TM219 has not SLN1-SSK1, a functional two-component system and therefore it has enhanced susceptibility to H_2O_2 and shows a great growth inhibition. The transformation of SLN1-SSK1 with AtETR1 restored the yeast tolerance to H_2O_2 (Desikan et al. 2005). Likewise, *etr1-1* which contains the Cys65Tyr mutation, has reduced stomatal closure in response to H_2O_2, suggesting that the –SH group of Cys65 is important for H_2O_2 signaling. On the other hand, the HK AtHK5 plays a crucial role in mediating H_2O_2-dependent processes in stomatal cells that are induced by environmental and hormonal signals (Desikan et al. 2008). It appears that modification of –SH groups belonging to ETR1 may be the key for the H_2O_2 signaling. However, it is not currently known whether this modification is a direct effect of H_2O_2 or it is conducted by another H_2O_2-sensing protein.

5.2 Transduction of H_2O_2 Signal

H_2O_2 acts as a second messenger, mediating the acquisition of tolerance to biotic and abiotic stresses. One of the earliest events that follow elevation levels of H_2O_2 is alteration of K^+ and Ca^{2+} ion fluxes. The transient Ca^{2+} oscillations are stress-specific and can lead to various downstream effects through the numerous Ca^{2+}-interacting proteins (e.g., calmodulins, CaM) and Ca^{2+}-dependent protein kinases (PKs) or/and over-amplification of the H_2O_2 signal. Changes in plasmalemma permeability leading to Ca^{2+} and H^+ influx appear to be necessary and sufficient to induce H_2O_2 production. Exogenous H_2O_2 could activate Ca^{2+} channels to concomitantly elevate the cytosolic Ca^{+2} (Ca_{cyt}^{2+}) content. The activation of these channels by endogenous H_2O_2 production could be an autocatalytic process in which NADPH-oxidase is also involved. This fact is a positive regulation of H_2O_2 level. The Ca^{2+}/CaM complex has been supposed to increase H_2O_2 production through Ca^{2+}/CaM-dependent NAD-kinase which affects the concentration of available NADPH during activation of NADPH-oxidase. On the other hand, it can also effect a negative regulation of H_2O_2 level in plants. That is, the Ca_{cyt}^{2+} elevation activates the CaM and subsequently the signal is transmitted to a downstream target protein (i.e., CAT). These findings suggest that Ca^{2+}/CaM complex and CAT activation downregulates the H_2O_2 levels. Likewise, there are evidences indicating that Ca^{2+} has dual functions in the regulation of H_2O_2 homeostasis, influencing the redox signaling in response to environmental signals (Yang and Poovaiah 2002; Hung et al. 2005). CaM activated some plant CAT in the presence of Ca^{2+}, thus, AtCAT3 can bind CaM in a Ca^{2+}-dependent way (Du et al. 2008).

Recently, it was demonstrated that intraperoxisomal Ca^{2+} rise increases CAT activity, which in turn increases peroxisomal H_2O_2 scavenging efficiency (Costa et al. 2010). On the other hand, H_2O_2 increases when the plants are under deficit of K^+. Therefore, H_2O_2 might play a role in cellular signaling of K^+ deprivation (Shin and Schachtman 2004). During stomatal closure, in addition to regulate Ca^{2+} channels, H_2O_2 also inhibits K^+ channel activity and induces cytosolic alkalinization in guard-cells. The overall effect of exogenous H_2O_2 on the cellular redox state may mean that a multitude of proteins can be involved (Foyer and Noctor 2005). A variety of forward and reverse genetics studies revealed a number of components in the H_2O_2 signaling network, including PKs, protein phosphatases (PPs), and ROS-responsive transcription factors (Cheng and Song 2006). The activity of PKs has been a particular focus. Thus, studies of PKs have shown that the mitogen-activated protein kinases (MAPK) cascade in plants are activated by H_2O_2, in particular the *Arabidopsis* AtMPK3 and AtMPK6, thereby regulating the cellular redox state (Hancock et al. 2006). Whether H_2O_2 has a direct effect on MAPKs or activates upstream effectors is unclear. Interestingly, the stabilization of MKK4 and MKK5-MAPKKs by H_2O_2 was demonstrated and the authors hypothesized that H_2O_2 might have a general stabilizing effect on MAPKKs (Dòczi et al. 2007). In addition, the ANP1-MAPKKK and the Ser/Thr-PK OXI1 (*ox*idative signal-*i*nducible), have also been shown to be induced specifically by H_2O_2, being important for H_2O_2 sensing and the activation of MAPK cascade in *Arabidopsis* (Rentel et al. 2004). ANP1 activates two downstream MAPKs, AtMPK3 and AtMPK6 and eventually regulates gene expression of specific H_2O_2-inducible transcripts. OXI1 activity is required for full activation of AtMPK3 and AtMPK6 (Rentel et al. 2004). Another H_2O_2-inducible PK is OMTK1 (*ox*idative stress-activated *M*APK *t*riple *k*inase), isolated from alfalfa. In contrast to OXI1, OMTK1 is H_2O_2-specific and not activated by phytohormones (Nakagami et al. 2004, 2005). In contrast, OMTK1 activated the downstream MAPK-MMK3 which can be activated by ethylene (Et). Summarizing, all existing data suggest that both environmental and cellular stress induce the generation of cytosolic H_2O_2, which in turn induces a MAPKs cascade that subsequently stimulates expression of antioxidants genes, reducing H_2O_2 levels and restoring cellular homeostasis. Besides everything described, PPs can be involved in H_2O_2 signaling. However, H_2O_2 inhibits PPs activities, probably by the direct oxidation of Cys in the active site of these enzymes. The *Arabidopsis* PP2C enzymes ABI1 and ABI2, negative regulators of ABA signaling, and the Tyr-PP AtPTP1 have been suggested to play a role (Gupta and Luan 2003). ABI1 and ABI2 could be receptors for the H_2O_2 signal in higher plans (Hung et al. 2005). Interestingly, AtPTP1 regulates the activity of MAPKs, suggesting a tight relationship among H_2O_2, PKs, and PPs. All these described effects provoked by H_2O_2 in plants raise the question: if H_2O_2 is involved in so many diverse responses, how can it be specific?

6 Expression of Transcription Factor Genes H_2O_2-Dependent

The control of gene expression is one of the most important changes induced by H_2O_2. In plants, the first report on a genome-wide expression analysis was provided by Desikan et al. (2001). By using a cDNA microarray from *Arabidopsis*, at least 113 and 62 transcripts were induced and repressed, respectively, in the presence of H_2O_2. The predicted function of all these genes was: cell signaling, transcription, cell death, and defense response. Among these genes are transcription factors (TFs) due to their capacity of activating the expression of downstream target genes. Several redox-controlled TFs have also been identified. In *Arabidopsis*, a TF called CBF1 (*C*-repeat *b*inding *f*actor) belonging to the APETALA/EREBP-family was described. CBF seems to be able to indirectly regulate the redox status of cytosol by activating downstream genes that encode antioxidants enzymes (e.g., CAT). Nevertheless, there is still no evidence that join the H_2O_2 signaling with CBF accumulation or activation. However, the overexpression in tomato

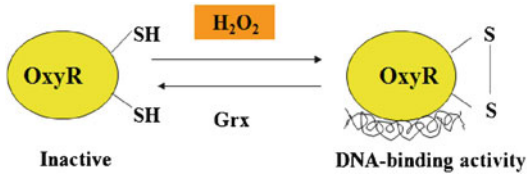

Fig. 16.5 The OxyR transcription factor regulates the H_2O_2 *E. coli* response. H_2O_2 oxidizes OxyR Cys199 to an R-SOH that reacts with Cys208 in an intramolecular disulphide bond. The resulting allosteric change activates OxyR DNA binding. OxyR is reduced by glutaredoxin-1 (Grx)

of *AtCBF1* induced a high tolerance for water deficit stress, CAT activity increased and H_2O_2 concentration decreased comparing with wild type (wt) plants (Hsieh et al. 2002). The activity of TFs can be directly regulated by oxidative modifications as exemplified by the *S. cerevisiae* oxidative stress TF Yap1 (Herrero et al. 2008). However, oxidation of Yap1 is not directly by H_2O_2, but mediated by GSH peroxidase like Gpx3 enzyme. In this process, Cys36 of Gpx3 forms an intermolecular disulphide with Cys598 of Yap1 (a TF that activates the expression of genes for antioxidants), forming a disulphide which is rearranged forming a disulphide in Yap1 itself and activating it (Hancock et al. 2006). Yap1 is a functional homologue of OxyR which is an *E. coli* (Fig. 16.5) transcriptional regulator with six Cys residues, being their Cys199 pivotal in the response of OxyR to redox cues. A Cys-rich domain of Yap1, that influenced in the nuclear localization of Yap1 when it was activated, it was also necessary for H_2O_2 tolerance. Therefore, alteration of key Cys residues of Yap1 provides a direct and powerful means to influence nuclear gene expression (Delaunay et al. 2002). In summary, Gpx3 acts as a protein that perceives H_2O_2 and transduces their signal to Yap1. On the other hand, the H_2O_2 gene network eventually transmits the signal to ROS-specific TFs. Some of these are LSD1 and LOL1 (negative and positive regulators of ROS-induced cell death), the senescence-specific WRKY53, and the ROS-inducible WRKY75 and some heat-shock TFs. Two other zinc finger TFs, Zat11 and Zat12, are also induced by H_2O_2 (Gadjev et al. 2008). More alterations in TFs caused by H_2O_2 will be described later in the stomatal closure section (Sect. 7.1).

7 H_2O_2-Mediated Physiological Processes

7.1 H_2O_2-Signaling in Stomatal Movements

The exposure of guard-cells to H_2O_2 induced elevations of (Ca_{cyt}^{2+}) and stomatal closure. There is considerable interest in the study of the relationship of ABA/H_2O_2. The discovery that ABA induces H_2O_2 production in guard-cells was a significant finding. It is well established that H_2O_2 production is required to initiate the ABA-induced stomatal closure and that chloroplasts are the principal sources of H_2O_2 in guard-cells (Wang and Song 2008). It has been clearly demonstrated that: (1) stomatal closure induced by ABA is a different process that inhibition of stomatal opening induced by ABA; and (2) H_2O_2 is involved in inhibition of stomatal opening induced by ABA (Yang et al. 2007). Moreover, the mediation of H_2O_2 in ABA-induced stomatal closure by targeting inward K^+ channels in plasmalemma was also suggested. Experiments with abi1, abi2, and abi2-1 suggest that the last double mutation impairs ABA signaling downstream of H_2O_2 production (Wang and Song 2008). An overlap exists between ABA- and H_2O_2-induced transcription genes, and both signaling molecules regulate many dowstream genes in a coordinated manner (Yang et al. 2007). Several TFs that are redox-controlled have also been identified during guard-cell signaling for H_2O_2 and ABA. Thus, the beforehand described Yap1 (Delaunay et al. 2002), AtMYB60, and AtMYB61 have been described to be specifically expressed in guard-cells. However, the relationship between AtMYB60 and AtMYB61 with H_2O_2 signaling is under study (Liang et al. 2005). A cross-talk between ABA and H_2O_2-signaling cascades seem to be clear. On the other hand, various common components of ABA and H_2O_2-signaling cascades (e.g., cytosolic-free Ca^{2+} levels, MAPKs,

and NADPH-oxidase) are involved during guard-cell movements. Two *CDPK* (i.e., *CPK3* and *CPK6*) genes expressed in *Arabidopsis* guard-cells have remarkable functions in the regulation of guard-cells ion channels, and provide genetic evidence for the existence of Ca^{2+} sensors that transduce stomatal ABA signaling (Mori et al. 2006). The results of Chen et al. (2004) strongly suggest that extracellular CaM activates a signaling pathway which involves the activation of G-protein, H_2O_2 production, and changes in (Ca_{cyt}^{2+}), thus regulating the stomatal movements. Likewise, H_2O_2 and ABA can activate the same MAPK which mediates both ABA- and H_2O_2-induced stomatal closure (Wang and Song 2008). Time-course analysis of both MAPK activation and ROS production indicated that the increase of H_2O_2 preceded the MAPK activation and subsequently takes place the activation of the ABA-signaling pathway. That is, the rise of H_2O_2 induced by ABA activates MAPK, which in turn leads to the upregulation of antioxidant defense systems. Recently, the MAPK-MPK3 was shown to be essential in *Arabidopsis* for ABA and H_2O_2-inhibition of stomatal opening (Gudesblat et al. 2007). On the other hand, the PK-OST1 also regulates the production of H_2O_2 in guard-cells by means of signaling pathways requiring AtrbohD and AtrbohF (Kwak et al. 2003). As previously described in Sect. 5.1, one member of the *Arabidopsis* histidine kinase (HK) family (De la Torre et al. 2006) has a dual function in guard-cells: (1) it is a possible target for H_2O_2 during stomatal closure, ETR1-Cys65 being essential for this function (Desikan et al. 2005, 2008); and (2) it perceives the hormone Et. Therefore, Et induces stomatal closure that is dependent on H_2O_2 production in guard-cells, generated by NADPH-oxidase AtrbohF. Moreover, Et and H_2O_2 signaling in guard-cells are mediated by ETR1 via EIN2 and ARR2-dependent pathways(s) (Desikan et al. 2006). Recently, AHK5, another member of the *Arabidopsis* HK family, was studied (Desikan et al. 2008). In addition to the predicted cytoplasmic localization of AHK5, it also appears to co-localize to the plasmalemma. Mutants lacking AHK5 show a reduced stomatal closure in response to H_2O_2. The AHK5 over-expression results in a constitutive lower stomatal closure than wt plants. Interestingly, treatment with flagellin (flg 22) also exhibited reduced stomatal closure and H_2O_2 production in ahk5 mutants (Desikan et al. 2008). Finally, the two *Arabidopsis* NADPH-oxidase catalytic subunit genes *AtrbohD* and *AtbohF* function in the signal transduction of ABA in guard-cells (Kwak et al. 2003). In AtrbohD/F, the exogenous H_2O_2 restored the ability of the Ca^{2+} channel activation and stomatal closure in response to ABA stimulation. The rboh transposon-insertion mutants of *Arabidopsis* have been used to provide unequivocal evidence that NADPH-oxidase-mediated H_2O_2 production is required for ABA-induced stomatal closure (Kwak et al. 2003). Likewise, treatment with ABA increased the expression of AtrbohD in guard-cells. The activation of MAPKs can amplify H_2O_2 signals directly regulating NADPH-oxidase activity, or activating also TFs to increase the expression of NADPH-oxidase genes in the ROS signal transduction (Mittler et al. 2004). ABA can also enhance the gene expression (Kwak et al. 2003) and NADPH-oxidase activity. Therefore, the MAPK activated by ABA might increase H_2O_2 signals via NADPH-oxidase activity.

7.2 H_2O_2-Signaling in Germinating Seeds

Likely, the seeds have high levels of stress and accumulate free radicals in their desiccated state and show high levels of CAT and SOD (Bailly et al. 2008). This detoxification occurs during dormancy, after-ripening (AR) (Iglesias-Fernández et al. 2011), and germination to prevent irreversible alterations in proteins, membranes, and DNA (Kibinza et al. 2006). The H_2O_2 production during seed storage in the dry state was initially documented by Pukacka and Ratajezak (2006). More broadly, $O_2^{\bullet-}$ and other ROS appear to play roles during seed germination and dormancy (Oracz et al. 2007; Müller et al. 2009). In a recent update, it was proposed that ROS play a key role in the completion of germination and that they should be considered

as messenger or transmitters of environmental cues during seed germination (Bailly et al. 2008). Thus, rice germination is inhibited by inhibiting NADPH-oxidase, and exogenous H_2O_2 stimulates the germination of dormant seeds belonging to several species. Likewise, exogenous H_2O_2 results in a decrease of ABA in dormant seeds and alleviation of dormancy by HCN induces an increase in H_2O_2 level and a decrease in ABA content (Bailly et al. 2008). On the other hand, Oracz et al. (2007) show that AR is associated with accumulation of H_2O_2 in the embryonic axes of sunflower seeds. In summary, H_2O_2 acts directly, or as a messenger of hormonal networks, signaling molecules involved in the transition from dormant to non-dormant state. But the H_2O_2 mechanism at the cellular level during the regulation of dormancy is far from being resolved. Data recently obtained in *Arabidopsis* seem to indicate that H_2O_2 could regulate seed dormancy by triggering ABA catabolism and GA synthesis (Liu et al. 2010). Thus, exogenous H_2O_2 decreased seed dormancy and rose transcription of genes involved in ABA conjugation (*ZYP707A*) and GAs synthesis (*GA3ox* and *GA20ox*), respectively. These data also support the possibility that H_2O_2 downregulates genes responsible of GA biosynthesis and ABA catabolism.

On the other hand, ROS can alter the function of seed-specific proteins through its carbonylation (i.e., introduction of CO into a molecule), thereby relieving dormancy (El-Maarouf-Bouteau and Bailly 2008; Oracz et al. 2007). The carbonylation is the most commonly occurring oxidative protein modification. Protein carbonylation inhibits or alters protein activities and intensifies their susceptibility to proteolytic attack. There are no indications that carbonylation is reversible. The identification of the factors causing the carbonylation is very complex. Several main possibilities include: (1) a decrease in the antioxidant defense system, (2) an increase in the ROS production, (3) a decreased capacity to remove oxidized proteins, or (4) an augment in the proteic sensitivity to oxidative attack. These four possibilities are not mutually exclusives. In sunflower seeds, ROS accumulation leads to targeted protein carbonylation not only during dry AR but also during artificial breaking of dormancy by cyanide application (HCN) (compound that triggers ROS accumulation because it is an inhibitor of SOD, CAT, and carbonylation) or methylviologen (ROS-generating compound) (Oracz et al. 2007; El-Maarouf-Bouteau and Bailly 2008). It was hypothesized that the action of HCN in sunflower seed dormancy alleviation does not involve Et production (Oracz et al. 2008). However, the hypothesis that HCN interacts with ROS-producing pathways have been supported by data on intracellular ROS production in response to HCN treatment (Oracz et al. 2009).

It has been proposed that NADPH-oxidases are involved in H_2O_2 production in seed germination (Liu et al. 2007) and dormancy breaking (Oracz et al. 2009). Recently, Müller et al. (2009) have demonstrated that dry AR in *Arabidopsis* is impaired in the NADPH-oxidase (*AtrbohB*) mutant which also shows reduced protein oxidation. The ABA sensitivity was not affected in dry AR *AtrbohB* mutant seeds. It mentions that: (1) *AtrbohB* gene is expressed in the embryo but not in the endosperm, (2) AtrbohB pre-mRNA is spliced in seeds depending on ABA and the seed dry AR status, and (3) *AtrbohD* gene is more abundant in the endosperm than in the embryo of ABA-treated seeds. On the other hand, Oracz et al. (2009) have demonstrated that in sunflower the expression of genes related to ROS production (i.e., *NADPHox*, NADPH-oxidase, and *POX*, peroxidase) is differentially affected during dry AR or by HCN treatment, and the effect of HCN is likely to be mediated by ROS.

Bailly et al. (2008) propose a model to account for the dual role of ROS in seed physiology. Seed germination is only possible when the ROS level is in an oxidative threshold. Below this level (i.e., dormancy), the amount of ROS during imbibition is too low for allowing germination. Alleviation of dormancy (i.e., AR) leads to an increase of the cellular level of ROS during seed imbibition thus ensuring germination completion, owing to the ROS signaling role. Above this threshold, ROS content is too high, because seeds are aged or placed in inappropriate environmental conditions

during their imbibition, and ROS become deleterious and cause cellular oxidative damages that prevent delay of germination.

7.3 Role of H_2O_2 in Plant–Microorganism Interaction

Plants have developed well-organized defense mechanisms against a wide range of pathogens. Therefore, plants can react actively to attack of pathogenic microorganisms with an array of inducible responses that lead to local and systemic expression of a broad spectrum of antimicrobial defenses. These defensive systems can be triggered by recognition, via cell surface receptors, of pathogens- or microbial-associated molecular patterns (PAMPs or MAMPs), such as flagellin, lipopolysaccharides, peptidoglycanes, or siderophores from bacteria or glucan and chitosan from fungi (Bolwell and Daudi 2009; Jones and Dangl 2006). Since Doke reported the production of $O_2^{\bullet-}$ during incompatible interactions between potato and the pathogen *Phytophthora infestans* (Doke 1983), the generation of ROS, especially H_2O_2, have been repeatedly associated with successful resistance responses (Torres 2010). This transient production of ROS is termed "oxidative burst" (Shetty et al. 2008) and it has been also observed through the direct application of purified siderophores (De Vleesschauwer et al. 2008), mycotoxis (Desmond et al. 2008), or cyclic lipopeptides (Jourdan et al. 2009) to the plant or plant cells inducing a timely and highly localized H_2O_2 production.

Although three phases have been observed in specific interactions (Shetty et al. 2008) and H_2O_2 production occurs in a biphasic manner during incompatible interactions (Apel and Hirt 2004). First, an unspecific and transitory phase usually takes place within minutes after the interaction followed by a second and prolongated production of H_2O_2 that occurs between 3 and 6 h after pathogen attack (Lamb and Dixon 1997). This second phase is usually associated with the establishment of the defenses and the hypersensitive response (HR), further characterized by a rapid cell death at the site of infection to restrict pathogen growth and which is mainly effective against biotrophic pathogens (Apel and Hirt 2004; Shetty et al. 2008). In contrast, during compatible interactions only the first transient phase is induced (Jourdan et al. 2009). During these responses, H_2O_2 is produced by enhanced apoplastic CW-peroxidases or plasmalemma NADPH-oxidases (Almagro et al. 2009; Bolwell and Daudi 2009). Additionally, H_2O_2 can be directly generated by diamine and oxalate oxidases.

Several functions are attributed to H_2O_2 during plant–pathogen interactions (Shetty et al. 2008; Torres 2010). H_2O_2 could kill directly the pathogen functioning as an antimicrobial agent. However, this toxicity depends on the sensitivity of the pathogen to the concentration of H_2O_2 present because many pathogens can grow in high concentrations of H_2O_2 (mM) or possess effective detoxification mechanisms (Aguirre et al. 2005; Matilla et al. 2007; Molina and Kahmann 2007; Shetty et al. 2008). Interestingly, H_2O_2 production has been detected during the rhizobium–legume interaction, suggesting that symbiotic bacteria are initially recognized as pathogens by the host plant (Chang et al. 2009). Additionally, H_2O_2 production has been reported to benefit infection by necrotrophic pathogens because these usually kill host cells and extract nutrients from them (Shetty et al. 2008). Alternatively, H_2O_2 production has been associated with establishment of physical defensive barriers by reinforcing the CW to prevent plant invasion by pathogens, through cross-linking of CW structural proteins and by deposition of callose-rich papillae, lignin, and suberin in the penetration sites (Almagro et al. 2009; Hückelhoven 2007; Shetty et al. 2008). However, experimental data also suggest that H_2O_2 could have a signaling role mediating changes in gene expression (Shetty et al. 2008; Torres 2010). The fast production of H_2O_2 in response to pathogen attack and its capacity to diffuse across membranes suggest that it can exert this function directly through redox control of TFs or indirectly by interacting with other signaling components like phosphorylation cascades (Torres 2010). Furthermore, H_2O_2 and other ROS, in association with salicylic acid (SA), have been

related with the establishment of SAR (Conrath 2006; Vlot et al. 2009), an inducible plant defense response triggered by necrotizing microbes and SA-dependent (Conrath 2006). Redox regulation has been reported for the *n*onexpressor of *p*athogenesis-*r*elated 1 (NPR1) and the leucine zipper TF TGA1 that are important mediators of *s*ystemic *a*cquired *r*esistance (SAR) during plant–pathogen interactions. Reduction of key Cys residues in these proteins relocates NRP1 to the nucleus and modulates the DNA-binding activity of the NPR1/TGA1 protein complex, thereby affecting decisively downstream gene expression (Mou et al. 2003). Interestingly, the *oxi1*-null mutant has abnormal root hair growth and enhanced susceptibility to pathogen infection, two processess mediated by H_2O_2 (Rentel et al. 2004). Collectively, the modifications of ROS levels in the host might be a strategy that is extended among the pathogens to increase host susceptibility.

Following with the study on pathogenesis and oxidative stress, nitric oxide (NO) is often produced at the same time and in the same locations in plants as H_2O_2 and it is also involved in a plethora of responses and functions (Neill et al. 2003). S-nitrosylation, the covalent attachment of an NO group to a reactive Cys thiol to form an *S*-nitrosothiol (SNO), has emerged as a prototypic redox-based posttranslational modification (Spadaro et al. 2010). NO seems to collaborate with H_2O_2 in plant disease resistance. During plant–pathogen interactions: (1) NO can act as an antioxidant, scavenging excess H_2O_2, ending radical-mediated lipid peroxidation and inhibiting H_2O_2 signaling pathways, which leads to cell death; and (2) NO can act synergistically with H_2O_2 to induce SAR (Quan et al. 2008). NO may also react with thiol groups on proteins to yield a –S-NO group. Many of the proteins modified in this way by NO are also potentially modified by H_2O_2 (Lindermayr et al. 2005).

7.4 Plant Growth H_2O_2-Mediated

The root hair growth is one of the most important studied effects of H_2O_2 as developmental regulator. The initiation of root hair growth process is known to be a physiologically and genetically separate program from tip growth, suggesting that the lesion in *rhd2* (*r*oot *h*air *d*efective 2) mutant is selectively affecting the tip growth machinery. In the *rhd2* background, root hairs are able to initiate their developmental program. When various alleles of the *rhd2* mutation were cloned, they were found to reside in a gene encoding AtrbohC of *Arabidopsis*, a homolog of the gp91phox domain of the neutrophyll respiratory burst NADPH-oxidase. At difference of mammals, plant Rbohs are not glycosilated. Rbohs NADPH-oxidase proteins are localized into the plasmalemma, where they oxidize cytosolic NADPH, transferring an electron across the membrane to generate apoplastic $O_2^{\bullet -}$. This $O_2^{\bullet -}$ dismutates to H_2O_2 which is thought to diffuse back into the cell, providing a possible cytosolic regulator (Swanson and Gilroy 2010). The gene family has ten known members in *Arabidopsis*; *AtrbohA-J* and its homologues have also been found in other angiospermic species. Each member of Rboh family differ in their expression pattern across plant and have been shown to be involved in a range of processes as root elongation and root hair development (Foreman et al. 2003; Swanson and Gilroy 2010). Thus, *AtrbohC* is present in root epidermal cells of the elongation zone but its expression becomes limited to those capable of making root hairs. In the *rhd2* mutant lacking AtrbohC, the hair roots fail to extend. Further evidence for the role of H_2O_2 in hair roots growth came from the studies of the *atrbohC* mutant which has low ROS levels in root hairs and is defective in activation of Ca^{2+} channels required for formation of Ca^{2+} gradient necessary for root hair growth (Foreman et al. 2003). According to these findings, the *atoxi1*-null mutant has reduced root hair growth (Rentel et al. 2004). AtOXI1 has been implicated in root hair development and AtOXI1 kinase is induced by H_2O_2. *Oxi1* null mutants are impaired in the activation of MAPKs, MPK3 and MPK6, upon oxidative stress, suggesting that OXI1 functions downstream of ROS but upstream of the MAPK module.

7.5 H_2O_2 in PCD

Cell death is essential for plant growth and development and responses to the environment. PCD can be initiated by all types of ROS. H_2O_2 is the key factor in PCD (Vanderauwera et al. 2009). However, the mechanisms by which H_2O_2 induces PCD are not yet established, although several studies point to effects on mitochondrial function. Hence, exogenous H_2O_2 increased subsequent H_2O_2 production in this organelle, altering mitochondrial function and inducing PCD. On the other hand, if MAPKs are long time activated, H_2O_2 production will increase and PCD will be induced. It is already known that H_2O_2 activates MAPK pathways, and it is tempting to speculate that increased H_2O_2 can stimulate further mitochondrial H_2O_2 production, perhaps via MAPK activation, in an amplifying oxidative death cycle. During endospermic seed germination, α-amylase GA-induced degredation of carbohydrates from aleurone layer and the PCD appears. This cell death is dependent on glyoxysomal production of H_2O_2 and there are evidences that CAT, APX, and SOD are downregulated by GA_3 to ensure sufficient accumulation of H_2O_2 prior to onset of cell death (Gechev et al. 2006; Gechev and Hille 2005). The application of H_2O_2 have confirmed the role of H_2O_2 as a triggering of cell death and also showed that high concentrations of H_2O_2 can cause necrosis instead of PCD. In agreement with these observations, overexpression of *APX* can suppress the cell death induced by H_2O_2 or NO (Murgia et al. 2004). In addition, CAT deficiency leads to the increase of H_2O_2 levels and the triggering of PCD (Vandenabeele et al. 2004; Gechev et al. 2006). Following pathogen infection, transient H_2O_2 overproduction and accumulation can promote a local defense response connected with NADPH-oxidase activation. The increase of H_2O_2 levels causes the HR as well, leading to rapid localized cell death at infection sites or can induce SAR (Slesak et al. 2007). Although, in many plant–pathogen interactions PCD is a welcome event for the plant host, there are examples of pathogen-triggered cell death that are mortal for the plant. On the other hand, ROS can be involved in allelopatic plant–plant interactions. This is the case for *Centaurea maculosa* whose roots secrete the phytotoxin catechin that triggers ROS accumulation in root meristems of neighboring species and subsequent Ca^{2+}-dependent cell death (Gechev et al. 2006; Gechev and Hille 2005).

8 Conclusion and Future Perspective

As can be inferred from this review, a wealth of information about H_2O_2 was generated in the last ten years. The strategy used to study the mechanism and mode of action of H_2O_2 was similar to the one for all the signaling molecules known in plants. The H_2O_2 concentration in different subcellular compartments is not yet known and H_2O_2 accumulation is maintained at a very low level due to the existence of an antioxidant system that eliminates excess of H_2O_2 production. Sensitive intracellular imaging will be required to visualize H_2O_2 in cells. The NADPH-oxidase (*rboh* gene family) was reported to be the pivotal enzyme involved in apoplastic H_2O_2 production. H_2O_2, as a signaling molecule, must possess some cellular metabolic molecule(s) that recognize(s) it and form(s) either a complex or a derived product that triggers the transduction cascade. However, data on the existence of H_2O_2 receptor are still unclear. The interaction of H_2O_2 with proteins having Cys-groups seems beyond doubt. The discovery of specific biological system with high sensitivity and rapid response to H_2O_2 is key to all above investigations. Tip-growing cells and stomatal cells-guard movements have proven to be powerful tools in elucidation of physiological process under H_2O_2. It is very interesting to study in detail these biological systems. Biochemical evidences indicated that MAPK cascades are responsible for transmitting the H_2O_2 signal. The recent identification in *Arabidopsis* of the Ser/Thre kinase OXI1, as an essential component of the H_2O_2 signaling, provided new insights into the H_2O_2-transmiting kinase network. In addition to MAPK cascade, the H_2O_2 signal can also be transmitted through changes in Ca^{2+} ion fluxes and cellular redox state. In addition, H_2O_2 seems to work

together with other molecules (e.g., fitohormones and NO) to control a variety of processes in plants. The use of transgenic plants with non-H_2O_2, hormones, and NO products, together with the isolation of H_2O_2-signaling mutants will be helpful in elucidating the biological roles of H_2O_2 in specific cells and in response to various stimuli. Finally, we must not forget different -omics approaches to facilitate further insights into H_2O_2 cellular response and to provide more clues to how plants sense and respond to environmental stress conditions.

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Role of Phytohormone Signaling During Stress

17

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Abstract

Plant hormones are among the most important plant components regulating different plant functions, which can equip the plant to survive under stress conditions. There has been extensive research work regarding the effects of plant hormones on plant growth and development. The notable phytohormones include auxin, abscisic acid, ethylene, cytokinins, gibberellins, jasmonates, salicylic acid, brassinosteroids, and strigolactones. Role of phytohormones may be useful for the production of transgenic plants, which are tolerant to stress. Selected plant hormone biosynthesis, their role on plant growth under stress, signal transduction pathways, and interactions are reviewed.

Keywords

Auxins • ABA • Gibberellins • Jasmonates • Brassinosteroids

1 Introduction

Plant hormones are a group of biochemical products handling different functions in plant. Plant hormones include auxin, abscisic acid (ABA), ethylene, cytokinins, gibberellins, jasmonates, salicylic acid, brassinosteroids, and strigolactones. They can regulate plant growth and development under different conditions including stress (Table 17.1). There are different physiological alterations made by plant hormones at cellular and molecular level. At the time of hormonal activity plant genes are activated resulting in different morphological and physiological responses in plant (Table 17.1). Fluctuations in hormones can influence plant growth (Kagale et al. 2007; Jackson 2008; Hirayama and Shinozaki 2010).

The functions of plant hormones include: tissue organogenesis and development by affecting cell cycling, fruit ripening, controlling water behavior in plant by adjusting the stomata activity, and enhanced plant resistance to stress by activating different signaling pathways (Van der Knaap et al. 1999; Wang et al. 2007; Tuteja 2007).

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Table 17.1 Plant hormones and their activities

Hormone	Activities	Reference
Auxin	(1) Regulation of phototropism, (2) regulation of embryo and fruit development, (3) organogenesis, formation and differentiation of vascular bundle, (4) root growth and development, (5) apical formation and dominance	Han et al. (2009), Tomasz and Jiri (2006)
ABA	(1) Dormancy and development, (2) stomatal activity, (3) morphogenesis of embryo, (4) protein and lipid production, (5) senescence of different tissues, and (6) tolerance to pathogens	Tuteja (2007), Tripathi and Tuteja (2007)
Cytokinins	(1) Cell division and differentiation, (2) formation of membrane components, (3) carbon cycle in photosynthesis, (4) chlorophyll formation, (5) delaying leaf and chlorophyll senescence by decreasing the rate of protein and RNA degradation, (6) development of seed, (7) differentiation of vesicular bundle, (8) root and shoot growth, (9) balance of nutrients, and (10) stress resistance	Yordanov et al. (2000), Kulaeva and Prokoptseva (2004), Muller and Sheen (2007)
Ethylene	(1) Seed germination, (2) tissue senescence and abscission, and (3) tissue elongation under submerged conditions	Arteca and Arteca (2008)
Gibberellins	(1) Inducing plant systemic resistance, (2) seed germination, and (3) mediating plant response to environmental stresses	Ueguchi-Tanaka et al. (2005), Achard et al. (2006), Griffiths et al. (2006), Miransari and Smith (2009a, b)
Brassinosteroids	(1) Enhancing plant resistance to environmental stresses	Nunez et al. (2003), Vardhini and Rao (2003)
Jasmonates	(1) Enhancing plant resistance versus different environmental stresses and pathogen infection, (2) nodule morphogenesis	Wasternack (2007), Murray et al. (2007), Balbi and Devoto (2008), Hu et al. (2009)
Salicylic acid	Inducing plant systemic resistance	Lian et al. (2000), Sun et al. (2006), Chen et al. (2009)
Strigolactones	(1) Hyphal branching in AM fungi, (2) shoot branching in the host plant as well as the parasitic plant, *Striga*	Akiyama et al. (2005), López-Ráez et al. (2008), Miransari (2011)

Plant hormones regulate plant growth and development through a set of complex interaction between their signaling pathways. A signaling pathway is defined by a collection of elements, which eventually results in plant response to different parameters including stress (Schwartz and Baron 1999; Klipp and Liebermeister 2006). Research has indicated the importance of plant hormones during stress. During stress the activation of different signaling pathways mediated by different plant hormones and their interaction can enhance plant resistance to stress (Nakamura et al. 2006; Rolland et al. 2006; Truman et al. 2007). Accordingly, some of the most important findings regarding the effects of plant hormones through their signaling pathways on different stresses are presented.

2 Auxins

Auxin is among the most important plant hormones affecting plant growth and development. Auxin can modulate plant growth by affecting the process of phototropism in plant (Darwin 1880; Tomasz and Jiri 2006). In addition, auxin can regulate the development of embryo and fruit, organogenesis, formation and differentiation of vascular bundle, root growth and development, and apical formation and dominance (Han et al. 2009). The place of auxin synthesis is stem tip and young leaf, and it is then translocated to the site of action (Ljung et al. 2001).

Among the most important functions of auxin in plant is the formation of lateral roots

(Han et al. 2009), which is of special significance to plant growth under different conditions including stress. For example, root growth under compaction is adversely affected, decreasing plant growth as a result of reduction in the uptake of water and nutrients (Miransari et al. 2007, 2008, 2009a, b). Therefore, role of auxin under such conditions can be important in the alleviation of stress by stimulating root growth.

The expression of different auxin-responsive genes indicates that there is a cross-talk between auxin and signaling pathways (Jain and Khurana 2009). Auxin can rapidly induce the accumulation of a significant number of transcript factors related to different plant genes under different conditions including stress. Such plant genes include *Aux/IAA*, *GH3*, and small auxin-up RNA (*SAUR*) genes (Guilfoyle 1999). Molecular genetics and biochemical research have suggested that the *Aux/IAA* genes are related to auxin signaling (Leyser 2002; Woodward and Bartel 2005). These genes can activate the proteins, which can suppress the transcriptional activities regulated by auxin (Tiwari et al. 2004; Woodward and Bartel 2005).

The *GH3* genes are responsible for the production of enzymes, which produce amino acid-related products by adenylation of indole 3-acetic acid and hence inhibit the production of extra-free auxin resulting in auxin homeostasis (Staswick et al. 2005). The *GH3* genes can also turn the produced amino acid-related products into salicylic acid and jasmonates (Staswick et al. 2002). The *SAUR* genes can result in the production of proteins, which may influence cell elongation regulated by auxin (Hagen and Guilfoyle 2002).

Auxin signaling pathway is related to the expression of different genes, which are mostly induced by two transcriptional factors including auxin response factors (ARFs) and the Aux/IAA repressors. The auxin response promoter elements in the responsive auxin genes are bound by ARFs. Reduction of auxin concentration to the amounts less than the threshold level results in the combined activation of ARFs and Aux/IAA repressors and hence the inhibition of the responsive genes. However, high concentration of auxin would adversely affect the Aux/IAA repressors and hence restores the activity of such responsive genes (Han et al. 2009; Jain and Khurana 2009; Ghanashyam and Jain 2009).

3 Abscisic Acid

There are a wide range of functions controlled and affected by ABA in plant including seed dormancy and development, stomatal activity, morphogenesis of embryo, protein and lipid production, senescence of different tissues, and tolerance to pathogens (Tuteja 2007; Tripathi and Tuteja 2007). Different stresses result in cell desiccation and osmotic imbalance and hence there may be similar signaling pathways and genes, expressed during the stress (Tuteja 2007).

Stresses such as drought and salinity result in the production of ABA in the roots and its eventual translocation to the shoots affecting stomata activities and cellular growth. In addition, ABA can also be produced in plant leaf and translocated to the other parts of plant (Wilkinson and Davies 2002; Chaves et al. 2009). The other parameter controlling ABA localization is the xylem/apoplastic pH. For example, when plant is subjected to drought stress the higher xylem/apoplastic pH prohibits the movement of ABA from the xylem/apoplastic to the symplastic space resulting in the enhancement of ABA concentration in the guard cells. Different stresses including drought, light, salinity, and nitrate can increase xylem sap pH and hence affect stomata activities (Jia and Davies 2007). ABA can also influence plant growth under stress by affecting gene expression. Furthermore, under drought and high light stress, the production of sugars and their translocation in the xylem can affect stomata response to ABA (Wilkinson and Davies 2002).

Chaves and Oliveira (2004) indicated that under stress the production of soluble sugars, which can also act as signal molecules in plant, is altered. In addition, such sugar molecules can also be interactive with plant hormones (Rolland et al. 2006). It has been indicated that nitrous oxide is also a signal molecule influencing the effects of plant hormones and other signal

molecules in response to environmental parameters by enhancing the sensitivity of plant cells to such signal molecules. In addition, nitrous oxide can also influence ABA effects on the activity of stomata (Neill et al. 2003).

Under water stress the amount of ABA increases (Kulkarni et al. 2000; Liu et al. 2005). ABA can primarily control water transpiration from the leaf and then can alleviate the stress and enhance plant tolerance by activating the expression of different stress genes (Bray 2004; Zhang et al. 2006). Genes, which are expressed under drought stress, include functional and regulatory ones. Functional genes can perform some specific functions related to the alleviation of stress by inducing different transporters including detoxifying enzymes, enzymes related to the production of osmolyte, and different proteases. However, regulatory genes including transcription factors, phosphatases and protein kinases, and the ones related to the production of ABA, can regulate the activity of functional genes (Aroca et al. 2007).

With respect to the functions mentioned for ABA, it is the most important plant signal activated during stress (Zhang et al. 2006). Interestingly and similarly, the soil fungi, arbuscular mycorrhiza (AM), can alleviate drought stress in their host plants. AM fungi can establish symbiosis with their host plant and enhance its water and nutrient uptake by its extensive hyphal network in exchange for carbon. Mycorrhizal plants are able to regulate their ABA level more efficiently (Auge 2001; Aroca et al. 2007).

The *nced* genes, which are expressed in plant under drought stress (Wan and Li 2006), are induced by ABA, if available at a minimum amount (Cohen et al. 1999). The effects of small RNA pathways on ABA were evaluated by Zhang et al. (2008) using mutants. They found that adverse effects on small RNA pathways can increase the production of ABA indicating that there is a link between small RNA pathways and signaling pathways resulting in the production of ABA in plant cells.

Under submerged conditions there is some kind of interesting interactions between different plant hormones. The accumulated amounts of ethylene down regulate the production of ABA through inhibiting the expression of 9-*cis*-epoxycarotenoid dioxygenase and by turning ABA into pahseic acid. The 9-*cis*-epoxycarotenoid dioxygenase are among the genes, responsible for the production of ABA through the pathway of carotenoid cleavage dioxygenases (Kende et al. 1998; Benschop et al. 2005). Prevented production of endogenous ABA results in the activation of the enzyme, gibberellin 3-oxidase, which catalyses the pathway related to the production of gibberellins (Benschop et al. 2006), and in submerged rice the production of gibberellins by the internodes (Kende et al. 1998). Down regulation of gibberellins-related genes can induce the elongation of rice roots under submerged conditions. The activities of such genes include the loosening of the cell wall, cell cycle, and starch turnover (Van der Knaap et al. 1999, 2000).

4 Cytokinins

The plant hormone cytokinins can regulate the following important functions in plant: (1) cell division and differentiation, (2) formation of membrane components, (3) carbon cycle in photosynthesis, (4) chlorophyll formation, (5) delaying leaf and chlorophyll senescence by decreasing the rate of protein and RNA degradation, which is related to the controlling effects of hormone on the production of protease and activity of RNase, (6) development of seed, (7) differentiation of vesicular bundle, (8) root and shoot growth, (9) balance of nutrients, and (10) stress resistance (Yordanov et al. 2000; Muller and Sheen 2007). The receptors perceiving cytokinins were found in the cellular membrane. Cytokinins are able to act multifunctionally by affecting different physiological processes in plant and controlling stresses (Kulaeva and Prokoptseva 2004).

Under nonstressed or stressed conditions, decreased level of cytokinins result in leaf senescence (Naqvi 1995). Exogenous application of cytokinins delays the process of leaf senescence (Okamoto et al. 2010). The process of leaf senescence can also be delayed by regulating the

related genes pathways, which are under the influence of cytokinins level in plant (Naqvi 1995). The gene, which is responsible for the production of cytokinins is *ipt*, which produces the enzyme isopentyl transferase, promoting the formation of isopentenyladenosine-5′-monophosphate (McGraw 1987). The *ipt* genes are expressed at low levels under controlled and drought-stress conditions, being highly specific in the cellular tissue (Vyroubalova et al. 2009).

Cytokinins are able to protect the process of photosynthesis in plants under stress. The related mechanism includes the interaction of cytokinins with receptor proteins resulting in the production of the signal pathway. Consequently, the genes are expressed and produce mRNAs, photosynthesis-related proteins, the enzyme ribulose bisphosphate carboxylase/oxygenase, electrons, and carbon (Chernyadev 2009; Bianco et al. 2009).

It is likely to enhance plant tolerance to drought by genetically modifying the plant using the *ipt* gene regulated by the related promoter, which delays the process of leaf senescence. In addition, the rate of photosynthesis and production of antioxidant enzymes increase under such genetic modification. It must be mentioned that there is a kind of regulatory interactions between leaf transpiration and leaf photosynthesis while the leaf senescence takes place in the plant (Rivero et al. 2007, 2009).

While leaf senescence can increase plant tolerance to drought stress by significantly reducing the rate of leaf transpiration, the presence of old leaf in plant can contribute to the enhanced photosynthesis process in plant (Chaves et al. 2003; Rivero et al. 2007, 2009). Under stresses such as drought while the production and transport of cytokinins are prohibited, its degradation is encouraged resulting in a decrease in plant growth as well as plant-reduced tolerance to stress (Yang et al. 2002; Kudoyarova et al. 2006).

The two-component pathways related to cytokinins regulation may affect ABA activity and can alleviate the effects of osmotic stress. The three histidine kinases as cytokinins receptors can adversely regulate ABA activity, as well as the stress. Accordingly, it can be mentioned that during the stress control in plant, there is a cross-talk between cytokinin, ABA, and the osmotic stress indicating that cytokinin pathway and metabolism is of particular importance to plant growth and development, especially under stress (Tran et al. 2009).

5 Ethylene

Ethylene is a gaseous plant hormone with important functioning in plant. Although ethylene is among plant hormones with the simplest structures, it can regulate some of the most important functioning in plant including seed germination, tissue senescence, and abscission (Arteca and Arteca 2008). There are some complex pathways and signaling related to ethylene, stimulated by other plant hormones and parameters such as plant growth, pathogens, and sugars (Abeles et al. 2004; Stepanova and Alonso 2005).

There is a set of interactions between ethylene and ethylene receptors in the plasma membrane indicating the pathway of ethylene signaling, although such a pathway is adversely affected by the ethylene receptors. The membrane receptors are two-component histidine protein kinases (Mount and Chang 2002). The degradation of ethylene receptors after ethylene binding can further increase ethylene sensitivity, determined by the presence of a novel protein. The degradation of transcription factors in the nucleus is also controlled (Kendrick and Chang 2008).

In the recent years the signaling pathway of ethylene is among the most known pathways. The important transcription factor is ETHYLENE INSENSITIVE3 (EIN3). However, what has to be yet investigated is the biochemical mechanism by which the ethylene receptor signaling is performed (Kendrick and Chang 2008). A key process in the functioning of ethylene is the degradation of proteins, which control both ethylene biosynthesis and ethylene perceiving by the receptors. The other important component related to ethylene signaling is the degradation of EIN3 by the proteins EIN-BINDING F-BOXES (McClellan and Chang 2008).

Importantly, the elevated production of ethylene under stress adversely affects plant growth and development. If there is any way by which the degradation of ethylene is performed, the stress of ethylene on plant growth and development can be controlled. Plant growth promoting rhizobacteria (PGPR) have the ability to produce the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can degrade the precursor for ethylene production by the ACC oxidase pathway. There has been extensive research work regarding such alleviating effects of ACC-deaminase on plant growth and development under stress, especially by professor Glick and his research team (Glick et al. 2007; Yang et al. 2008; Jalili et al. 2009).

The other important functioning regarding ethylene is its important role under submerged condition, which is considered stressful to most plant species. Under submerged conditions, the plant must own some unique abilities to be able to grow under such conditions. Increased rate of adventitious roots as well as formation of new leaf and hence increased photosynthesis under submerged conditions is among such abilities regulated by ethylene. Ethylene concentration can increase up to 100 times higher in plants under submerged compared with nonsubmerged conditions resulting in tissue elongation. Such kind of elongation is a result of processes, which would loosen the cell wall, extend and divide the cell, and is also under the influence of ethylene interaction with other plant hormones (Jackson 2008).

Under submerged, due to the efficiency of oxygen and high concentration of CO_2 the synthesis of ethylene can increase up to four times higher in the base of rice stem. The increased ethylene concentration is a result of higher biosynthesis of ethylene prerequisite 1-aminocyclopropane-1-carboxylic acid (ACC). Such alterations are accompanied with some other genetic processes including mRNA control on the activation of ACC oxidase genes. These activities would collectively result in the enhanced production of ethylene in rice under submerged conditions (Mekhedov and Kende 1996; Vriezen et al. 1999; Zhou et al. 2001).

6 Gibberellins

Gibberellins are plant hormones performing different functions in plant. The three enzymes of cyclases, monooxygenases, and dioxygenases catalyze the production of gibberellins, which are tetracyclic diterpenoids, from geranylgeranyl diphosphate (Sponsel and Hedden 2004). Gibberellins are able to enhance plant growth by degrading the negative growth regulators DELLA proteins (Ueguchi-Tanaka et al. 2005; Griffiths et al. 2006). Gibberellins can induce plant systemic resistance and result in seed germination. Fungi and bacteria are also able to produce gibberellins as secondary metabolites resulting in the signaling interaction with their host plant (MacMillan 2001; Miransari and Smith 2009a, b).

It has been recently indicated that *Arabidopsis* DELLA proteins, as negative regulator of gibberellins signaling, can influence plant systemic resistance by affecting jasmonates and salicylic acid signaling pathways (Navarro et al. 2008). The *Arabidopsis* mutant which does not have the DELLA genes is very susceptible to pathogenic fungi (Navarro et al. 2008). DELLA proteins are able to activate plant systemic resistance to pathogenic fungi by affecting the jasmonates/ethylene pathways. Accordingly, DELLA proteins can influence plant systemic resistance by affecting the combined response of salicylic acid, jasmonates, and ethylene pathways to the fungal infection.

It has also been indicated that the response of plant to the environmental stresses is also mediated by DELLA proteins affecting the combined response of plant hormonal pathways to the stress (Achard et al. 2006). Mutant, which do not have gibberellins receptors in their cellular membrane, accumulate higher rate of gibberellins enhancing plant response to pathogenic fungi relative to the wild types (Tanaka et al. 2006). In addition to fungi, viral infection can also affect gibberellins pathway in plant. For example, rice dwarf virus suppressed the expression of the enzyme *ent*-kaurene oxidase, which results in the production of gibberellins in rice plants (Zhu et al. 2005).

7 Brassinosteroids

Brassinosteroids are plant hormones found in different parts of the plant including seed, pollen, flower, fruit, leaf, vesicular bundle, root, and shoot. Such steroidal compounds can be found in association with sugars and fatty acids. So far about 70 different plant brassinosteroids have been recognized and isolated, affecting plant growth and development (Sasse 2003; Yu et al. 2008). Under biotic (pathogen infection) and abiotic stresses the level of brassinosteroids in plant may increase. However, the related mechanisms are not known yet (Krishna 2003).

Different stresses such as salinity, drought, heavy metals, high or low temperature, etc., usually result in the similar cellular pathways and responses including the regulation of antioxidants, production of stress protein, and increased concentration of solutes altering the production of hormones in plant (Smirnoff 1995; Sajedi et al. 2011). There is a cross-talk between different hormones affecting plant growth and development. Under stress, the production of kinase protein in plant and the related responses by plant hormones is altered resulting in the induction of signals related to the production of reactive oxygen species, which is of high significance in the alleviation of stress. In addition, there is also some sort of interaction between different plant hormones under stress. For example, among the mechanisms by which brassinosteroids can affect plant response to stress is the production of jasmonates (Mussig et al. 2000; Schaller et al. 2000; Miller et al. 2010).

Under stress the production of reactive oxygen species can adversely affect cellular growth and development (Sajedi et al. 2011). The role of brassinosteroids in different plant physiological mechanisms, which results in the regulation of plant growth and development, has been indicated. However, there is not much known about the controlling effects of brassinosteroids on oxidative stress.

Under stress, use of exogenous brassinosteroids results in the modification of antioxidant enzymes including gluthatione peroxidase, ascorbate peroxidase, superoxide dismutase, and catalase as well as the non-enzymatic antioxidants including gluthatione, cartenoids, tocopherols, ascorbic acid, etc. (Nunez et al. 2003; Vardhini and Rao 2003). In sorghum (*Sorghum vulagre* L.) subjected to osmotic stress while brassinosteroids increased the activity of catalase, they decreased the activities of ascorbic acid oxidase and peroxidase (Vardhini and Rao 2003).

Under salinity stress, treating rice seedlings with brassinosteroids significantly enhanced the activity of catalase, superoxide dismutase, and glutathione reductase and slightly increased the activity of ascorbate peroxidase (Nunez et al. 2003). The fact that molecular oxygen is necessary for the production of brassinosteroids at different stages indicates that this hormone can mediate the effects of hypoxia on plant growth and development. Upreti and Murti (2004) indicated that brassinosteroids can increase root nodulation in *Phaseolus vulgaris* as well as ABA contents and cytokinin transzeatin riboside.

Brassinosteroids can also enhance root nodulation and crop yield under nonstressed and water-stressed conditions by affecting the content of cytokinin in the nodulated roots of *Phaseolus vulgaris*. Seedling growth of sorghum and sugar beet was increased by brassinosteroids under osmotic and water stress, respectively. Such alleviating effects were attributed to the enhanced level of acid invertase in the plant young leaf (Schilling et al. 1991). As a result of osmotic stress, the content of protein in three sorghum varieties decreased, which was completely restored and stimulated by brassinosteroids. The stress also increased the proline level (Vardhini and Rao 2003).

Use of brassinosteroids alleviated the stress of cadmium on the performance of chickpea (Hasan et al. 2008) and mustard (Hayat et al. 2007) and also the stresses of aluminum and nickel on the growth of mung bean (Ali et al. 2008) and mustard (Alam et al. 2007), respectively. Such alleviating effects may be attributed to the enhanced activities of antioxidant enzymes such as catalase, superoxidase, and peroxidase by brassinosteroids (Hasan et al. 2008).

In case of salinity stress, brassinosteroids alleviated the stress of salinity on rice (*Oryza sativa*),

seed germination, and seedling growth. The hormone was also able to restore plant chlorophylls and enhance the activity of nitrate reductase under salinity. Brassinosteroids increased the cellular growth of rice seedlings under suboptimal temperature (15°C) as well as the germination of rice and corn seeds (He et al. 1991; Fujii and Saka 2001). Although brassinosteroids and ABA may affect plant systemic resistance at the time of pathogen infection, the induction of plant systemic resistance is mostly related to the combining effects of signaling pathways induced by salicylic acid, jasmonic acid, and ethylene.

8 Jasmonates

Jasmonates are lipid plant hormones affecting plant systemic resistance as well as plant growth and development and elasticity by their signaling pathways. The lipase enzymes synthesize jasmonates as oxylipins (oxygenated fatty acids). The enzymes, which are located in the chloroplast membrane, result in the release of linolenic acid, which is then oxygenated by lipoxygenases and produces hydroperoxide derivatives (Wasternack 2007; Schaller and Stintzi 2009).

Jasmonates are able to affect gene expression in plant positively or negatively while interacting with other plant hormones including salicylic acid, auxin, ABA, and ethylene (Wasternack 2007). Jasmonates can enhance plant resistance versus different environmental stresses and pathogen infection. There is a high rate of positive or negative cross-talk and interactions between jasmonates and the other plant hormone salicylic acid determining the ultimate response of plant to stress (Wasternack 2007; Balbi and Devoto 2008).

Plant mutants lacking the ability to synthesize jasmonates have been used to investigate the signaling pathways, which result in the production of jasmonates (Devoto and Turner 2005; Lorenzo and Solano 2005; Schilmiller et al. 2007). Jasmonates can also mediate plant response to stress by affecting the production of reactive oxygen species, nitrous oxide (NO), influx of calcium as well as activation of nitrogen protein kinase (Hu et al. 2009).

The important role of jasmonates in nodule organogenesis has also been indicated by different researchers. Nodules are root organs developed during the symbiosis between the soil bacteria *Rhizobium* and their specific host plant from the leguminous family. Nodules are the place of rhizobium residence, for the fixation of atmospheric N by the production of rhizobium nitrogenase (Miransari and Smith 2007, 2008, 2009a, b). It has been recently indicated that for the onset of nodule development, the cytokinins signaling pathway is necessary (Murray et al. 2007).

The other plant hormones including auxin, ABA, ethylene, gibberellins, and brassinosteroids are also required for nodule development (Oldroyd et al. 2001; Ferguson et al. 2005; van Noorden et al. 2006). However, more research must be performed to indicate the other important details regarding the complex effects of hormonal signaling pathways on nodule organogenesis.

It has also been indicated that jasmonates can also influence nodule formation. For example, the antagonistic effects of jasmonates on the process of nodulation in *Medicago truncatula* and *Lotus japonicus* have been indicated (Sun et al. 2006; Nakagawa and Kawaguchi 2006). There is also some kind of positive and negative interactions between jasmonates and salicylic acid during the process of nodule formation (Sun et al. 2006). Jasmonates are able to alleviate the stress of salinity on barley growth. In plants treated with jasmonates, lower amounts of Na^+ were found in plant shoot. Such alleviating effects were attributed to the performance of the following three genes including apoplastic invertase, arginine decarboxylase, and Rubisco, regulated by jasmonates (Tuteja and Sopory 2008).

9 Salicylic Acid

Salicylic acid is also another important plant hormone affecting plant systemic resistance to pathogen infection (Lian et al. 2000). During the activation of plant systemic resistance, transcriptional factors are activated and transcriptional

repressors are inhibited (Dong 2001). It can adversely affect gene expression when affected by the stress hormone jasmonates. Borsani et al. (2001) indicated that salicylic acid can affect the production of reactive oxygen species as a result of osmotic stress by NaCl in *Arabidopsis* seedlings. According to Ndamukong et al. (2007), glutaredoxin is the protein regulating the pathways related to salicylic signaling. It has been indicated that the adverse effects of salicylic acid on pathogen growth in plants is by the suppressing effects of salicylic acid on the auxin signaling pathway (Wang et al. 2007). There are also positive and negative interactions between salicylic acid and jasmonates during the process of nodule organogenesis (Sun et al. 2006).

There are two different pathways by which salicylic acid is synthesized. The cinnamate pathway in which cinnamate is synthesized from the phenylalanine ammonia lyase (PAL) resulting in the production of salicylic acid. Silencing the PAL genes inhibits the production of salicylic acid in plant and inhibiting the activity of PAL genes chemically reduces the production of salicylic acid in plant. Salicylic acid is also produced in the isochorismate pathway catalyzed by isochorismate synthase (Chen et al. 2009).

In brief, it can be stated that plant parameters such as salicylic acid can enhance plant systemic resistance to stresses such as pathogen invasion by the following mechanisms: (1) expression of PAL genes, (2) expression of priming genes, (3) activation of pathways resulting in phytoalexin production, (4) deposition of callose, (5) oxidative burst, (6) phenolic compounds deposition, and (7) deposition of hydroxycinnamoyltyramine products (Goellner and Conrath 2008).

10 Strigolactones

Strigolactones are new classes of plant hormones produced from carotenoids, probably by carotenoid cleavage deoxygenase or 9-*cis* epoxycarotenoid deoxygenase. They affect the process of symbiosis between the soil fungi AM and the host plant as hyphal branching factors, shoot branching, and seed germination of parasitic weeds such as *Striga*. Their production by plants roots is significantly enhanced by phosphate starvation (Akiyama et al. 2005; López-Ráez et al. 2008; Miransari 2011).

During the process of symbiotic association between AM fungi and the host plant, an extensive network of hypha is developed, substantially increasing the uptake of water and nutrients by the host plant (Smith and Read 2008). Among different nutrients, phosphorous is more affected by AM fungi symbiosis with the host plant. Interestingly, strigolactones are more produced under phosphate starvation, which mediate the activation of strigolactones producing genes (López-Ráez and Bouwmeester 2008). However, other than P starvation, there is no any other details regarding the effects of strigolactones on stresses affecting plant growth.

11 Conclusion and Future Perspectives

Some of the most important details regarding the production of plant hormones, their signal pathways under different conditions including stress and the interactions between plant hormones were reviewed. Accordingly, plant hormones are among the most important plant components which can make the plant survive under different conditions including stress. Under stress plant hormones mediate plant genes, which can alleviate the stress by the production of stress proteins. There are yet more details that must be known regarding the functions of plant hormones under different conditions including stress. Elucidation of such details may result in the production of transgenic plants, which are more tolerant under stress.

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Ethylene and Abiotic Stress Tolerance in Plants

18

Elisa Gamalero and Bernard R. Glick

Abstract

Plants are commonly exposed to large number of different environmental stresses including extremes of pH and temperature, flooding, drought, high salt, both organic and inorganic contaminants, and a variety of pathogenic organisms. As a consequence of these environmental stresses, plants typically synthesize increased levels of the phytohormone ethylene and are often unable to grow and proliferate to any great extent, at least until the stress is removed and the ethylene level is lowered. To reduce the deleterious effects of ethylene stress, plant growth-promoting bacteria (PGPB) that facilitate the proliferation of plants under stress conditions may be added to the system. These bacteria lower the level of growth inhibiting stress ethylene within the plant through the action of the enzyme ACC deaminase, and are also able to directly promote plant growth, usually by providing the plant with the phytohormone indoleacetic. The net result of adding PGPB to plants is a significant increase in both the number of seeds that germinate and the amount of biomass that the plants are able to attain under otherwise stressful and inhibitory conditions. In this chapter we provide a detailed overview regarding the functioning, the biochemistry and the regulation of ACC deaminase, with emphasis on application of PGPB synthesizing this enzyme and supporting plant growth under abiotic stress such as salinity, flooding, drought, organic, and inorganic pollution. Finally, recent developments on the exploitation of transgenic plants expressing ACC deaminase are discussed.

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Ethylene • Plant growth-promoting bacteria • Salt • Flood • Drought • Metal stress • Transgenic plants

1 Introduction

Plants are characterized by a high level of physiological plasticity, which ensures a wide range of protective responses and enables them to survive a variety of different environmental stresses (Lusk et al. 2008; Schmidt 2008; Potters et al. 2009). The exposure of plants to mild chronic stress causes stress-induced morphogenic responses (SIMRs), with auxin, reactive oxygen species (ROS), and ethylene playing the role of response mediators. The visible consequences of the SIMRs are the inhibition of root and/or shoot elongation and the simultaneous enhancement of root and axillary branching.

Ethylene is a gaseous phytohormone that is involved in several phases of plant growth (e.g., fruit ripening, flower senescence, leaf and petal abscission) as well as in plant responses to biotic and abiotic stresses (Abeles et al. 1992). In fact, the term "stress ethylene" (Abeles 1973) indicates the increase in ethylene synthesis associated with environmental stresses such as extremes of temperature, high light, flooding, drought, heavy metal and organic pollution, radiation, wounding, insect predation, high salt, and various pathogens including viruses, bacteria, and fungi (Morgan and Drew 1997).

In higher plants, the synthesis of ethylene involves three enzymes: (1) *S*-adenosyl-l-methionine (SAM) synthetase, which catalyzes the conversion of methionine to SAM (Giovanelli et al. 1980), (2) 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which mediates the hydrolysis of SAM to ACC and 5′-methylthioadenosine (Yang and Hoffman 1984; Kende 1989), and (3) ACC oxidase which catalyzes the conversion of ACC to ethylene, carbon dioxide, and cyanide (John 1991).

Ethylene synthesized in response to environmental stresses can either exacerbate the symptoms of stress or it can lead to responses that enhance plant survival under adverse conditions. Thus, alleviation or exacerbation of the stress-induced effects of the stress depends on the plant species, its age and the nature of the stress (VanLoon and Glick 2004). This behavior has been explained by the two-phase model proposed by Glick et al. (2007a). A few hours after the onset of a stress, an initial small peak of ethylene occurs. This beneficial peak of ethylene initiates a protective response by the plant, such as transcription of pathogenesis-related genes and induction of acquired resistance (Ciardi et al. 2000; VanLoon and Glick 2004). Then, if the stress becomes chronic or more intense, 1–3 days later, the ACC synthase genes are transcribed; as a consequence of the synthesis of additional ACC, a second much larger ethylene peak is observed. This second ethylene peak is considered to be deleterious to the plant in that it induces processes such as senescence, chlorosis, and abscission that may lead to a significant inhibition of plant growth and survival.

2 Plant Growth-Promoting Bacteria

Due to the release of photosynthate by the plant roots, the rhizosphere (the zone of soil surrounding the roots of plants) is a microhabitat relatively rich in nutrients which favors the establishment of complex multitrophic interactions among the plant, the microorganisms, and the soil itself (Lynch and Whipps 1990). Consequently, the rhizosphere is considered as a "hot spot" that attracts large and active microbial populations able to affect plant growth by exerting beneficial, detrimental, or no effects. "Plant growth-promoting rhizobacteria" (PGPR) (Kloepper and Schroth 1978) are soil bacteria able to efficiently colonize

plant roots after their inoculation onto seeds. Once the colonization of the roots by PGPR is well established plant biomass by direct or indirect stimulation of plant health are frequently achieved (Cook 2002). In order to discuss the modes of action of a wide range of plant beneficial bacteria, the term "plant growth-promoting bacteria" (PGPB) was proposed (Bashan and Holguin 1998). In agreement with this proposal, in this chapter we will use the term PGPB instead of PGPR.

Enhancement of plant development by PGPB has been demonstrated on a wide range of different crops under both controlled conditions and in the field, and a large number of papers have been published on this topic (Reed and Glick 2004). As an initial requirement for effective use of PGPB, efficient root colonization by the bacteria is required, in particular in open field conditions, where competition with autochtonous microorganisms is high (Gamalero et al. 2004, 2005; Pivato et al. 2008).

2.1 Mechanisms Used by PGPB

PGPB can promote plant growth in two different ways: either indirectly by plant disease suppression or through direct stimulation (Glick 1995). While plant disease suppression leads to improvement of plant health, the direct effects of many soil bacteria on plants is mediated by a range of mechanisms including improvement of mineral nutrition (P, N, Fe), enhancement of plant tolerance to biotic and abiotic stresses, and modification of root development (Glick 1995; Glick et al. 1999; Kloepper et al. 1989; Gamalero et al. 2002, 2004). The bacterial traits involved in these activities include nitrogen fixation, phosphate solubilization, iron sequestration, synthesis of phytohormones, and modulation of plant ethylene levels (Gamalero and Glick in press).

2.2 ACC Deaminase

In 1978, an enzyme capable of degrading the ethylene precursor, ACC, to ammonia and α-ketobutyrate was isolated from the bacterium *Pseudomonas* sp. strain ACP (Honma and Shimomura 1978). Since then, numerous studies have demonstrated ACC deaminase activity in a wide range of soil microorganisms including the fungus *Penicillium citrinum* (Honma 1993) and various bacteria (Jacobson et al. 1994; Glick et al. 1995; Burd et al. 1998; Belimov et al. 2001; Ghosh et al. 2003; Ma et al. 2003a; Sessitsch et al. 2005; Blaha et al. 2006; Madhaiyan et al. 2007; Kuffner et al. 2008; Chinnadurai et al. 2009). Bacteria that have ACC deaminase facilitate plant growth under different ethylene-producing environmental stresses, including flooding (Grichko and Glick 2001a, b; Farwell et al. 2007), pollution caused by organic toxicants such as polycyclicaromatic hydrocarbons (PAHs), polycyclic biphenyls (PCBs) and total petroleum hydrocarbons (TPHs) (Huang et al. 2004; Saleh et al. 2004; Reed and Glick 2005) and by heavy metals including nickel, lead, zinc, copper, cadmium, cobalt and arsenic (Burd et al. 1998, 2000; Belimov et al. 2001, 2005; Nie et al. 2002; Glick 2003; Reed and Glick 2005; Farwell et al. 2006; Rodriguez et al. 2008; Ma et al. 2009), salinity (Mayak et al. 2004b; Saravanakumar and Samiyappan 2006; Cheng et al. 2007; Gamalero et al. 2010), drought (Mayak et al. 2004a; Arshad et al. 2008; Belimov et al. 2009), and phytopathogen attack (Wang et al. 2000; Hao et al. 2007; Toklikishvili et al. 2010).

In addition to the above-mentioned stresses, other natural processes such as nodulation of legumes and mycorrhizal establishment in the host plant induce local increases in ethylene content which has the potential to decrease various aspects of plant growth and development. In this context, ACC deaminase-producing bacteria, lowering the ethylene content in the plants, can increase nodulation in legumes such as pea and alfalfa (Ma et al. 2003a, 2004) and mycorrhizal colonization in cucumber (Gamalero et al. 2008).

2.2.1 Model

In a model that was previously reported by Glick et al. (1998), PGPB that synthesize ACC deaminase begin seed or rootlet colonization following the release of tryptophan and other small molecules by seed or root exudates (Bayliss et al. 1997; Penrose and Glick 2001). Thereafter, the bacteria

synthesize and secrete IAA (Patten and Glick 1996, 2002) that, together with the IAA synthesized by the plant, can either stimulate plant growth or induce the transcription of the gene encoding the plant enzyme ACC synthase, which converts SAM to ACC. A portion of the ACC that is produced by this reaction has been shown to be exuded from seeds or plant roots, taken up by the bacteria, and subsequently converted by the enzyme ACC deaminase to ammonia and α-ketobutyrate (Bayliss et al. 1997; Penrose and Glick 2001). As a result of this activity, the level of ethylene produced by the plant is lowered and does not reach levels which are inhibitory for plant growth. In addition, ethylene inhibition of IAA signal tranduction is decreased thereby facilitating IAA stimulation of plant growth (Glick et al. 2007a). The main short-term visible effect of seed inoculation with ACC deaminase-producing bacteria, under gnotobiotic conditions, is the enhancement of root elongation (Glick et al. 1995; Hall et al. 1996; Shah et al. 1997).

2.2.2 Protein Biochemistry

ACC deaminase is a multimeric enzyme (either a dimer or a trimer) that utilizes the coenzyme pyridoxal phosphate as a tightly bound cofactor, with an approximate subunit mass of 35,000–42,000 and a native size estimated to be 100–112 kDa (Jacobson et al. 1994; Hontzeas et al. 2004; Karthikeyan et al. 2004; Klee et al. 1991; Sheehy et al. 1991). In all instances examined, the K_m indicated that the enzyme did not have a particularly high affinity for ACC with Km values typically around 1.5–6.0 mM. While most organisms with ACC deaminase contain a basal level of enzyme activity, ACC is induced by ACC, at levels as low as 100 nM (Jacobson et al. 1994) with full induction requiring up to 10 h. The amino acids l-alanine, dl-alanine and dl-valine can also induce enzyme activity to a small extent, and α-aminoisobutyric acid can induce activity to almost the same level as ACC (Honma 1983). ACC deaminase activity is optimum at a temperature of 30°C and at pH 8.5 (Hontzeas et al. 2006). The affinity of the substrate ACC and the competitive inhibitors l-alanine and l-serine for the enzyme is also highest at pH 8.5.

2.2.3 Occurrence in Nature

Bacterial ACC deaminase activity is relatively common. Genes coding for ACC deaminase have been found in gram-negative (Wang et al. 2000; Belimov et al. 2001; Hontzeas et al. 2005) and -positive (Belimov et al. 2001; Ghosh et al. 2003; Raddadi et al. 2008) bacteria, rhizobia (Ma et al. 2003a, b; Shaharoona et al. 2006; Duan et al. 2009), methylobacteria (Chinnadurai et al. 2009), endophytes (Sessitsch et al. 2005; Rothballer et al. 2008), and fungi (Jia et al. 1999; Viterbo et al. 2010). In one study, 12% of isolated *Rhizobium* spp. from various sites in southern and central Saskatchewan possessed this enzyme (Duan et al. 2009). In another study, ACC deaminase activity/genes were found in a wide range of bacterial isolates including *Azospirillum*, *Rhizobium*, *Agrobacterium*, *Achromobacter*, *Burkholderia*, *Ralstonia*, *Pseudomonas*, and *Enterobacter* (Blaha et al. 2006).

Although, based on sequence similarity, many organisms have putative ACC deaminase genes (Kaneko et al. 2010; Taghavi et al. 2010), sequence homology by itself does not suffice to define these open reading frames as ACC deaminase encoding sequences (Glick 2005) since other pyridoxal phosphate enzymes have sequences similar to ACC deaminase (Todorovic and Glick 2008).

2.2.4 Regulation

Analysis of DNA sequence data for the region upstream of the ACC deaminase structural gene (i.e., *acdS*) from the PGPB *Pseudomonas putida* UW4 indicated that this DNA segment contained a CRP (cyclic AMP receptor protein) binding site, an FNR (fumarate-nitrate reduction regulatory protein) binding site (which is a known anaerobic transcriptional regulator), an Lrp (leucine-responsive regulatory protein) binding site, and a gene encoding an Lrp protein (Grichko and Glick 2000; Li and Glick 2001). All of these features were previously shown to be components of the transcriptional regulatory mechanism of the ACC deaminase gene from this bacterium, with the *acdS* promoter under the transcriptional control of the regulatory Lrp protein (encoded by *acdR*). Subsequent studies have indicated that in

a large number of bacteria the *acdS* gene is under the transcriptional control of *acdR* (Ma et al. 2003b; Blaha et al. 2006; Prigent-Combaret et al. 2008; Duan et al. 2009; Sun et al. 2009). Moreover, in strain *P. putida* UW4, a protein that specifically interacts with ACC, the Lrp protein and the region of DNA upstream of *acdS* has been identified and characterized (Cheng et al. 2008; Glick et al. 2007b).While transcriptional regulation of *acdS* by *acdR* is a key feature of the expression of many bacterial ACC deaminases, in some instances *acdS* is not under the transcriptional control of *acdR*. For example, an acdS gene from a strain of *Mesorhizobium loti* has been shown to be transcriptionally regulated by the *nifA* promoter (Kaneko et al. 2000), and to be expressed exclusively within legume nodules (Uchiumi et al. 2004). It is possible that the expression of ACC deaminase genes within nitrogen-fixing nodules might decrease the rate of nodule senescence – as nitrogen fixation with its high energy demand could activate stress ethylene synthesis – and thereby effectively increase the amount of fixed nitrogen.

3 Decreasing Plant Stress with PGPB

3.1 Salt

Salinity is an enormous worldwide problem for agriculture, especially for crops that are grown under irrigation. The amount of salt-affected land worldwide is >900 million ha which is approximately 6% of the total global land mass, or about 20% of the world's cultivated area. Moreover, it has been estimated that around half of the land devoted to the growth of irrigated crops is adversely affected by salinity (Flowers 2004).

Plants exposed to excessive salinity are inhibited in their growth and development: seed germination, seedling growth and vigor, flowering and fruit set are negatively affected by salt stress which induces osmotic stress, Na^+ and Cl^- toxicity, plasmolysis, nutrient imbalance, production of reactive oxygen species, and interference with photosynthesis, in a wide range of plants. Notwithstanding other effects, salinity-induced stress in plants is partly ascribed to ethylene production (O'Donnell et al. 1996; Cuartero and Fernandez-Munoz 1999; Blumwald 2000; Mayak et al. 2004a, b; Shibli et al. 2007). For instance, ethylene synthesis was enhanced from two- to about tenfold in tomato (*Lycopersicon esculentum*) and *Arabidopsis* plants exposed to salinity stress (Richard and El-Abd 1989; Hall and Smith 1995). In addition, in chickpea (*Cicer arietinum*), salinity induced increases of ethylene, ACC content, and in the activity of the enzyme ACC oxidase (Kukreja et al. 2005). One of the major mechanisms by which PGPB facilitate plant growth under stressful conditions is the reduction of ethylene levels through the enzyme ACC deaminase (Glick 1995, 2004; Glick et al. 1998, 2007a, b).

PGPB isolated from soil samples derived from dried river beds in the arid and salty Arava region in the southern part of the Negev desert in Israel were selected to assess their ability to produce ACC deaminase and, on the basis of preliminary testing, one bacterium, *Achrobacter piechaudii* ARV8, was further characterized (Mayak et al. 2004a). Tomato plants inoculated with this strain and exposed to salt concentrations up to 200 mM showed increased root and shoot biomass, this effect being related to the reduction of ethylene levels in plants. This pioneering work served as the basis for a spate of experimental work on several different plants by scientists from all over the world. Since then, labs from India, Pakistan, China, Italy and Canada have reported successfully employing ACC deaminase-expressing bacteria to promote plant growth in presence of high concentrations of salt (Saravanakumar and Samiyappan 2006; Cheng et al. 2007; Nadeem et al. 2007; Yue et al. 2007). These studies have included groundnut (Saravanakumar and Samiyappan 2006), maize (Nadeem et al. 2007), cotton (Yue et al. 2007), canola (Cheng et al. 2007), wheat (Nadeem et al. 2010), and cucumber (Gamalero et al. 2010) as experimental systems. A very important point is that the efficacy of the ACC deaminase-producing strains in protecting plants against growth inhibition induced by salinity is evident in the field as well as in a

laboratory or greenhouse setting (Saravanakumar and Samiyappan 2006). In the experiments reported by Cheng et al. (2007) and Gamalero et al. (2010), only wild-type *Pseudomonas putida* UW4 and not an ACC deaminase minus mutant of this bacterium protected canola and cucumber plants, respectively, against growth inhibition by salt. These results provide a clear demonstration of the role of ethylene in growth inhibition by high salt.

3.2 Flooding

The main consequence of flooding is oxygen deprivation to the plant roots (Kozlowski 1984; Jackson 1985) which respond with reduced root permeability, water absorption and mineral uptake; closure of stomata followed by a decrease in photosynthesis, alterations in hormone balance, inhibition of stem and root growth, hypertrophy of lenticels, development of aerenchyma and adventitious roots, epinasty, chlorosis, leaf abscission and premature fruit drop (Vartapetian and Jackson 1997). It has been calculated that under normal conditions, about 230 mmol m^{-3} of oxygen is dissolved in water, and when the oxygen level is reduced to 50 mmol m^{-3}, hypoxia occurs (Nilsen and Orcutt 1997; Vartapetian and Jackson 1997). In this condition, the remaining available oxygen is quickly consumed by soil microorganisms leading to a worsening of the stress (Frankenberger and Arshad 1995). In addition, hypoxia induces transcription of ACC synthase genes in flooded roots (Drew 1997); however, the oxidation of ACC to ethylene (by ACC oxidase) is thwarted by the lack of oxygen (Abeles et al. 1992; Shiu et al. 1998). Therefore, some of the ACC synthesized by the plant is exuded into the surrounding soil and becomes available to the soil bacteria expressing ACC deaminase (Else et al. 1995) that in turn could support the growth of the plants exposed to flooding. In plants whose roots are not associated with bacteria that produce ACC deaminase, the excess ACC moves up from the plant roots to the shoots where more oxygen is available and ethylene synthesis is thereby enabled. This idea has been demonstrated by the experiments performed by Grichko and Glick (2001a). Tomato plants inoculated with bacteria able to produce ACC deaminase and subjected to 9 days of flooding were taller, healthier, and greener (due to increased levels of chlorophyll) compared to noninoculated ones. In addition, an increased development of adventitious roots and stem aerenchyma was observed in flooded plants treated with ACC deaminase-expressing bacteria compared to flooded plants inoculated with bacteria which did not contain this enzyme or plants that were flooded but not inoculated with bacteria.

3.3 Drought

Soil aridity is one of the greatest limitations being faced by present-day agriculture leading to serious reduction of crop yield. When exposed to drought, plant roots sense the soil drying condition. In order to maintain cellular water status, the roots send chemical signals, including abscisic acid (ABA), via the xylem to the shoots to restrict water use. In addition, drought stress has been extensively associated with other long-distance signals such as ACC. As a consequence of this raised level of ACC, high amount of endogenous ethylene is synthesized by the plant (Graves and Gladon 1985; Kalantari et al. 2000; Mayak et al. 2004a; Sobeih et al. 2004) leading to growth inhibition, premature senescence and abscission, and reduction of crop yield (Davies and Zhang 1991; Dodd 2005). Therefore, together with the promotion of root growth which is beneficial for improving plant water uptake (Reynolds and Tuberosa 2008), the lowering of the ethylene levels could be a strategy to improve the development and health of plants grown under drought stress.

The first demonstration of the efficacy of ACC deaminase-expressing bacteria in supporting the growth of plants cultivated in water scarcity came from Mayak et al. (2004a). In this work, tomato and pepper plant tolerance to water deficit was conferred by the bacterial strain *Achromobacter piechaudi* ARV8, able to synthesize ACC deaminase, resulting in significant biomass increases

when plants were rewatered following several days of drought. Although the relative water contents were unaffected by seed inoculation with the bacterial strain, ethylene synthesis was reduced in inoculated plants compared to controls, with a simultaneous dramatic improved recovery from water deficiency (Mayak et al. 2004a).

In a subsequent study, two pseudomonads, showing ACC deaminase activity, significantly decreased the "drought stress-imposed effects" on the growth and yield of peas (*Pisum sativum* L.) subjected to drought stress (Arshad et al. 2008). Exposure of plants to drought stress at the vegetative growth stage reduced shoot biomass by 41% in uninoculated plants, and by only 18% in inoculated plants. While plants exposed to drought stress at the flowering and pod formation stages showed a lower grain yield, bacterial inoculation resulted in better grain yields (up to 62% and 40% higher at these two stages, respectively) than the uninoculated ones. Finally, delay of pod ripening observed in inoculated plants was associated with reduction of the endogenous ethylene concentration (Arshad et al. 2008).

A conclusive demonstration of the involvement of ACC deaminase in supporting plant growth under drought stress comes from the recent work of Belimov et al. (2009). Inoculation with *Variovorax paradoxus* 5C-2, but not with a transposon mutant of this bacterium with decreased ACC deaminase activity, improved growth, yield and water-use efficiency of drought-stressed peas. At a local level (within the root), the bacterial strain enhanced nodulation by symbiotic nitrogen-fixing bacteria, thereby preventing a drought-induced decrease in nodulation and seed nitrogen content. Systemic (within the shoot) inoculation with strain *V. paradoxus* 5C-2 induced an increased ABA concentration in the xylem, and a reduction of the xylem ACC concentration. The exploitation of ACC deaminase-expressing bacteria combined with deficit irrigation may be an effective way of more efficiently using water in agriculture while reducing the yield loss induced by soil drying (Belimov et al. 2009).

3.4 Metals

Plants exposed to toxic levels of heavy metals show several macroscopic consequences such as reduced growth (of both roots and above ground parts), leaf chlorosis and necrosis, loss of turgor, a decreased rate of seed germination, cell and plant death (Bingham et al. 1986; Foy et al. 1978). These effects are typically ascribed to ultrastructural, biochemical, and molecular changes in plant tissues and cells caused by the presence of heavy metals. At a biochemical level, the protein profile of plants exposed to heavy metals is modified as a consequence of the reestablishment of the unstressed plant's cellular and redox homeostasis and is an indication of the plant's adaptation to chronic stress (Bona et al. 2007). Other plant reactions are the synthesis of polyamines, accumulation of reactive oxygen species (Mithöfer et al. 2004; Rodrìguez-Serrano et al. 2006), changes in plant nutrient levels (Sandalio et al. 2001) and in water status (Perfus-Barbeoch et al. 2002), a decrease in plasma membrane H^+-ATPase activity, and a reduction in photosynthesis (Krupa 1988; Hsu and Kao 2004; Bačkor et al. 2007). In addition, independent of the varying amounts of heavy metal mobilization and accumulation, the presence of heavy metals raises plant ethylene levels which lead to subsequent inhibition of root elongation and a stress/senescence response (Deikman 1997). For example, ethylene can: speed up senescence of plants exposed to high concentrations of Cu for a prolonged period of time (Maksymiec et al. 1995; Maksymiec and Baszyński 1996), inhibit cell growth, and increase cell wall rigidity through deposition of lignin (Enyedi et al. 1992).

Despite the fact that heavy metals adversely affect soil microflora (Bååth 1989; Van Beelen and Doelman 1997; Giller et al. 1998; Regvar et al. 2001; Rajapaksha et al. 2004), some beneficial microorganisms typically found living on plant roots can relieve some of the metal toxicity to plants. This may occur as a result of the microorganisms modifying the metal bioavailability (via volatilization, or the sequestering/accumulation of the metal), through the release of chelators, acidification, or redox changes (Abou-Shanab

et al. 2008), or by increasing the tolerance to the presence of the metals through the synthesis of the enzyme ACC deaminase (Glick 1995, 2004; Glick et al. 1998, 2007a, b). Several studies have demonstrated the involvement of this bacterial activity in improving the survival and health of plants grown in heavy metal-polluted soils through reduction of the endogenous ethylene levels.

Kluyvera ascorbata SUD165 is a nickel-resistant siderophore-producing bacterial strain that can grow at cold temperatures (5–10°C) and synthesize ACC deaminase (Burd et al. 1998). This organism was originally isolated from nickel-contaminated soil from northern Ontario, Canada. It promoted the growth of Indian mustard, tomato, and canola in the presence of high concentrations of nickel (and other metals) under a range of different experimental conditions (Burd et al. 1998, 2000; Ma et al. 2001) by increasing plant tolerance to the metal through an observed three- to fourfold reduction of the ethylene level. Moreover, the siderophores produced by this bacterium helped the plants to accumulate sufficient iron so that they were no longer chlorotic (and iron-stressed, which also can cause the synthesis of stress ethylene).

Several cadmium-tolerant bacterial strains of *Pseudomonas*, *Alcaligenes*, *Variovorax*, *Bacillus*, and *Rhodococcus* spp. that express the enzyme ACC deaminase have been isolated from the rhizoplane of pea and Indian mustard plant that were cultivated in the presence of heavy metal-polluted soil or sewage sludge (Belimov et al. 2001). These strains stimulated the root elongation of canola and Indian mustard cultivated in presence of 300 μM $CdCl_2$. In addition, a positive correlation was observed between the measured in vitro ACC deaminase activity and the effect of the added bacteria on plant root elongation (Belimov et al. 2005).

In addition to stimulation of the growth of plants cultivated on heavy metal-polluted soils, in some instances ACC deaminase-expressing bacteria can improve phytoremediation efficacy. In that situation, the plant can accumulate higher levels of heavy metals and/or there may be an enhanced mobilization of metals in the soil due to an increase in the exudation of a variety of organic compounds by plant roots. Recently, the ability of *Pseudomonas tolaasii* strain ACC23 to increase the biomass of canola plants in the presence of Cd stress (83% for roots and 94% for shoots) and the cadmium uptake per plant (107%), compared to uninoculated plants, was demonstrated (Dell'Amico et al. 2008). Since the specific uptake of Cd into shoots and roots did not change following bacterial inoculation, strain ACC23 did not affect metal availability or mobility; however, plant biomass was increased following the addition of this strain and the total amount of Cd accumulated was also increased (Dell'Amico et al. 2008). Detailed physiological characterization of this bacterium indicated that it had the ability to produce IAA, siderophores as well as the enzyme ACC deaminase. While this study did not show direct involvement of any of these plant beneficial traits in the promotion of plant growth in the presence of cadmium stress, it is nevertheless probable that the success of this bacterial inoculant depended on one or more of these bacterial traits.

3.5 Organic Compounds

Many soil bacteria are able to degrade anthropogenic organic chemicals such as herbicides (Sorensen et al. 2008), pesticides (Wackett et al. 2002; Kim et al. 2004), refrigerants (Parnell et al. 2006; Field and Sierra-Alvarez 2008; Pieper and Seeger 2008), solvents (Coleman et al. 2002; Park et al. 2002; Lee et al. 2010), and other organic xenobiotics. As a consequence of their versatile catabolic properties, bacteria belonging to the pseudomonad group are often well equipped to carry out the biodegradation of complex organic compounds, a process that typically requires the concerted efforts of several enzymes. The genes that code for the enzymes of these biodegradative pathways are often located on large (~50–200 kb) "biodegradative" plasmids (Ghosal et al. 1985; Cork and Krueger 1991). Nonhalogenated aromatic compounds can be enzymatically converted by degradative bacteria to either catechol or protocatechuate, compounds that are readily metabolized by almost all organisms. Halogenated

aromatic compounds, which are the main components of most pesticides and herbicides, may also be degraded by these plasmid-encoded enzymes, but with a much slower rate of degradation (Glick et al. 2010). Therefore, microbial degradation might provide a reasonable and effective means of disposing of toxic chemical wastes and inoculation of bacteria with biodegradative capabilities in polluted soils can facilitate the breakdown of the contaminants. While this is especially true under laboratory conditions, successful bioremediation is often more difficult to achieve when bacteria are inoculated directly in environment.

On the other hand, the use of plants to clean up contaminated soils (i.e., phytoremediation) is a viable remediation strategy for environmentally persistent organic compounds, but several limitations hamper its widespread application in the field (Salt et al. 1998; Pilon-Smits 2005; Glick 2003). Thus, plant photosynthesis, respiration, and metabolism may be impaired by organic contaminants leading to low plant biomass and, subsequently, low remediation efficacy (Medina et al. 2004; Pilon-Smits 2005). In addition, plants showing some degree of tolerance to soil contaminants are often characterized by a reduced biomass that does not allow for the efficient and timely degradation of the contaminant. One strategy to overcome these problems is rhizoremediation, that is, the combined use of plants tolerant to the organic pollutant together with biodegradative bacteria having plant growth-promoting activity (Gerhardt et al. 2009). In this case, degradation of organic compounds to nontoxic, or less-toxic, chemicals results from complex interactions involving roots, root exudates, rhizosphere soil, and microbes. Depending on the plant species, age and environmental conditions, root exudation can account for up to 40% of net fixed carbon (Lynch and Whipps 1990). On average, 15–20% of net fixed carbon is released by the roots (Nguyen 2003) into the soil as sugars, amino acids, organic acids, and larger organic compounds (Kumar et al. 2006). These compounds attract a dense and diverse microbial population which can bind efficiently to plant roots and colonize the root surface and often the interior of the plant as well. This provides various benefits to plants, including the synthesis of compounds that increase plant tolerance to stress by lowering plant stress hormone levels and the degradation of contaminants before they can negatively impact the plants (Gerhardt et al. 2009). Besides degrading polycyclic aromatic hydrocarbons, bacteria with hydrophobic external surfaces may make these relatively insoluble compounds more bioavailable (Johnsen and Karlson 2004) through the formation of biofilm on the surface of some crystal-like polycyclic aromatic hydrocarbons.

Several studies have reported the efficacy of improving phytoremediation by the combined use of plants and biodegradative bacteria for removing: petroleum products (Escalante-Espinosa et al. 2005; Huang et al. 2005; Radwan et al. 2005; Alarcón et al. 2008; Al-Awadhi et al. 2009), polycyclic and other aromatic hydrocarbons (Daane et al. 2001; Huang et al. 2004; Sheng and Gong 2006; Sheng et al. 2008; Germaine et al. 2009), and halogenated compounds (Sicilano and Germida 1997; Yee et al. 1998; Leigh et al. 2006; Liu et al. 2007; Uhlik et al. 2009) from contaminated soils. Although most of these studies have been performed under controlled laboratory, growth chamber or greenhouse conditions and some field scale phytoremediation studies have also been reported (Gurska et al. 2009; Kamath et al. 2004).

In general, plants inoculated with PGPB having degradative capabilities withstand the stress of growing in the polluted soils better than noninoculated ones. Gurska et al. (2009) performed a 3-year field test at a Southern Ontario site (~130 $g\,kg^{-1}$ of total petroleum hydrocarbons, TPH) used for land farming of refinery hydrocarbon waste for many years. The low molecular weight TPH fractions were removed through land farming and bioremediation; the high molecular weight, recalcitrant fractions remained at high levels in the soil and were remediated by combining plants (annual ryegrass, tall fescue, barley, and fall rye) and the ACC deaminase-expressing strains *P. putida* UW3 and *P. putida* UW4. Using this approach, TPH levels in soil decreased from 129.3 to 46.4 $g\,kg^{-1}$ over a 3-year period. In addition,

the biomass of the plants grown in these polluted soil was enhanced by PGPB by 40%.

Although phytoremediation represents a promising alternative to other, more expensive, remedial biotechnologies, the efficacy of this system may be limited when toxic levels of contaminants hinder plant growth and depress microbial populations. Inoculation of seeds or seedlings with PGPB addresses these problems directly by increasing the plant's tolerance to a variety of xenobiotic compounds.

4 Decreasing Plant Stress with Transgenic Plants

The impact of abiotic stress on plant development represents an advantage both in agriculture and horticulture as well as in more recent developed environmental biotechnologies such as phytoremediation of contaminated soils. Although several transgene products altering ethylene synthesis have been reported, laboratory studies of transgenic plants expressing the bacterial gene encoding ACC deaminase are characterized by an enhanced tolerance to abiotic stresses such as flooding (Grichko and Glick 2001a), salinity (Mayak et al. 2004b; Sergeeva et al. 2006), and metal contamination (Stearns et al. 2005; Grichko et al. 2000; Nie et al. 2002). To our knowledge, no report regarding the growth of transgenic plants expressing ACC deaminase exposed to either drought stress or organic pollutant contamination has yet been published.

The main advantage of this approach is that with genetic manipulation (i.e., lowered level of stress ethylene), plant tolerance to a wide range of different abiotic stresses may be gained. But PGPB are typically more effective in protecting plants against the deleterious effects of various abiotic stresses than the transgenic plants expressing ACC deaminase. This is because PGPB may contribute to plant growth under stressed or natural environments by a number of different mechanisms, including the synthesis of IAA and siderophores, and not solely by reducing plant ethylene levels.

4.1 Salt

A variety of genetic engineering approaches to the improvement of salt tolerance in plants have been reported (Karuna Stree et al. 2000; Shi et al. 2000; Tabaei-Aghdaei et al. 2000; Zhang and Blumwald 2001; Zhang et al. 2001; Zhu 2002), but only one paper dealt with the salt-stress tolerance by transgenic plants expressing the bacterial gene encoding for ACC deaminase (Sergeeva et al. 2006). In this work, the transformed and nontransformed canola plants were cultivated in the presence of 0–200 mM NaCl and several parameters of plant growth and physiology were assessed.

The data are consistent with the possibility that the presence of ACC deaminase confers tolerance to transgenic canola lines under salt stress as compared to the nontransformed canola plants. This protection occurs due to the decreased synthesis of ethylene. Nevertheless, the protection given by the insertion of the ACC deaminase gene in canola plants was not significantly different from that provided by seed inoculation with PGPB able to synthesize ACC deaminase (Mayak et al. 2004b).

4.2 Flooding

Experiments involving ACC deaminase transgenic plants exposed to flooding were conducted on tomato (Grichko and Glick 2001a) and canola (Farwell et al. 2007) as model plants. Preliminary results obtained by Grichko and Glick (2001a) showed that the transgenic tomato plants expressing ACC deaminase gene showed some increased tolerance to flooding stress and were less affected by deleterious effects of root hypoxia than nontransformed plants. Recently, Farwell et al. (2007) compared the growth of transgenic canola expressing ACC deaminase to that of nontransformed canola, inoculated or not with the bacterial strain *P. putida* UW4, able to synthesize ACC deaminase, and simultaneously exposed to two major stresses, i.e., flooding and nickel contamination. The biomass of transgenic canola, especially when inoculated with *P. putida* UW4, was significantly

higher than that of nontransformed canola in metal-contaminated soil under low flood-stress conditions. However, as observed earlier, using either transgenic canola or a bacterial strain able to express ACC deaminase, a similar level of tolerance under low flood-stress conditions in the presence of nickel was observed.

4.3 Metals

The use of transgenic plants in the phytoremediation of contaminated environments may provide the plant with some significant growth and survival advantages. Transgenic plants increase the plant's capacity to tolerate, accumulate, and/or metabolize pollutants thereby resulting in the production of large biomass (Krämer and Chardonnens 2001; Pilon-Smits and Pilon 2002). Previously, transgenic tobacco, canola, and tomato plants expressing the bacterial enzyme ACC deaminase have been constructed and tested (Grichko et al. 2000; Nie et al. 2002; Stearns et al. 2005; Farwell et al. 2006). While the use of transgenic plants may appear to be advantageous in comparison with the use of plant growth-promoting bacteria, especially in soils that might inhibit or prevent the proliferation of added bacteria, in practice, when they are tested, these transgenic plants are found to perform quite similarly to plants treated with ACC deaminase-expressing rhizobacteria.

The fitness of transgenic tomato plants expressing ACC deaminase versus nontransgenic tomato plants was compared in presence of heavy metals (Cd, Co, Cu, Mg, Ni, Pb, or Zn) and measured as metal concentration and ACC deaminase activity in both plant shoots and roots; root and shoot development; and leaf chlorophyll content (Grichko et al. 2000). The transgenic tomato plants accumulated a greater amount of metal within their tissues and, at the same time, were less subject to the deleterious effects of the metals on plant growth than the nontransgenic plants. Similarly, transgenic tobacco plants co-expressing ACC deaminase and tryptophan monooxygenase showed faster growth with larger biomass with a more developed root system, and accumulated a greater amount of Cu^{2+} and Co^{2+} than nontransformed plants (Zhang et al. 2008). Finally, inoculation of transgenic plants expressing ACC deaminase with bacteria able to synthesize this enzyme also led to better plant performance, highlighted by improved growth and higher metal accumulation. Thus, inoculation of transgenic canola plants expressing ACC deaminase in the roots, with *Pseudomonas putida* Biovar B strain HS-2, which is also able to synthesize this enzyme, induced an increase in plant biomass and nickel (Ni) uptake by shoots and roots (Rodriguez et al. 2008).

5 Conclusion and Future Perspective

Soil-based plant–bacterial interactions have likely existed for millions of years. However, it was not until the middle of the twentieth century that scientists selected and tested a wide range of bacteria that actively facilitated plant growth. Many early experiments with PGPB suffered from a lack of reproducibility, probably reflecting the lack of a detailed understanding of the key bacterial traits essential to make this relationship work effectively. However, modern biochemical and genetic techniques have allowed scientists to develop a deeper understanding of the mechanisms used by many bacteria to promote plant growth. Thus, there are a large number of reports of successful field trials of bacteria that were initially selected and tested in the laboratory to produce ACC deaminase, IAA, and siderophores. Notwithstanding the involvement of other bacterial traits, especially in specific circumstances, selection of bacteria for the three above-mentioned traits appears to be one way to short circuit the otherwise long and laborious search for effective and reproducible PGPB. In addition, while all of the evidence is not yet in, it appears that more efficacious and reproducible PGPB may be found amongst bacterial endophytes than amongst rhizospheric bacteria. This is probably a reflection of the fact that endophytes are somewhat protected from the sometimes hostile soil environment.

Finally, given the increasing understanding of the mechanisms used by PGPB that has developed over the past 20 years or so, it would appear that this is a technology whose time has come. Over the next 20 years, commercial use of PGPB is likely to increase dramatically, first in the developing world, where the cost of chemical fertilizers and pesticides is often prohibitive, and then in more developed countries with organic farmers possibly being in the vanguard.

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New Approaches to Study Metal-Induced Stress in Plants

19

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Abstract

The contamination of water, soil, and sediments with toxic metals has been and will continue to be a major environmental problem that needs to be dealt with. In more recent years, the studies with metals have concentrated on the antioxidant stress response characterization in a wide range of plant species. The effects of many different metals have been published, but all have consistently addressed very similar problems and investigated basic parameters. These kinds of studies are important as they allow the verification of the sensitivities of different plant species to distinct metals, eventually indicating specific biomarkers to stressful situations. However, metal-induced stress studies need new approaches that are likely to increase our understanding as how these elements affect plant metabolism and to identify the modifications that are needed to improve plant adaptation and tolerance. New techniques that have greatly improved the identification, localization, and quantification of metals within plant tissues have led to the science of metallomics. This advancement in knowledge should eventually allow the characterization of plants used in the process of phytoremediation of soils contaminated with toxic metals. In this chapter, we discuss the use of new techniques and approaches to study the effects of toxic metals and we propose a more integrated action among distinct areas in the field of metallomics and other "omics."

Keywords

Heavy metals stress • Signaling • Proteomics • Grafting • Cytogenetics • Metallomics

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1 Introduction

There is abundant evidence of shifts in temperature, rainfall pattern, species distribution, and biogeochemical cycles in response to global climate change (Parmesan 2006; Allan and Soden 2008; Campbell et al. 2009). This change will probably affect the behavior of contaminants present in the environment. High temperatures can lead to changes in organic matter and increase the availability of the bound toxic metals (Porcal et al. 2009). An increase in the frequency of precipitation can transport toxic metals from a contaminated area to a noncontaminated one (Harmon and Wyatt 2008). Moreover, toxic metals may be continuously introduced into the environment by mining, metalliferous industries, application of fertilizers, sewage sludge, metal-based pesticides, and other anthropogenic activities. Thus, contamination of water, soil, and sediments with toxic metals has been and will continue to be a major environmental problem that needs to be dealt with (Gratão et al. 2005).

Some metals have been classified as toxic, persistent, and bioaccumulative elements. According to the Agency for Toxic Substances and Disease Registry (ATSDR 2007), among the ten most hazardous substances to human health, four are toxic metals: lead (Pb), mercury (Hg), arsenic (As), and cadmium (Cd). Interestingly, Pb and Hg rank first among the most harmful metals to plants, followed by copper (Cu), cadmium (Cd)/arsenic (As), cobalt (Co)/nickel (Ni)/zinc (Zn), and manganese (Mn) (Kopittke et al. 2010). Pb and Hg have been reported as mutagenic agents in plants (Patra et al. 2004). Due to its chemical similarity to phosphorus, As may interfere with several physiological and biochemical processes (Patra et al. 2004). Cd does not appear to have any physiological function, with the exception of the marine diatom *Thalassiosira weissflogii*, that possesses a carbonic anhydrase with Cd as its metal center (Lane et al. 2005). Cu, Ni, Co, Zn, and Mn are all plant micronutrients. They participate in prosthetic groups and as cofactors of many proteins and are therefore essential for growth and development, however at high concentrations cause oxidative stress (Hansch and Mendel 2009). Aluminum (Al) is another toxic element with significant implications for agriculture, because 30% of the world's land areas consist of acid soils (Horst et al. 2010). Several other metals have been investigated for their effects on plant metabolism, toxicity, and crop productivity (Becana et al. 1998; Panda and Choudhury 2005; Gomes-Junior et al. 2007).

Exposure to a toxic metal can result in inhibition of seed germination, photosynthesis, plant growth, and consequently causes yield losses. These symptoms are normally related with overproduction of reactive oxygen species (ROS), changes in the permeability and structure of cell membranes, imbalance of mineral nutrients, incorporation of the metal into S-containing molecules, and cell cycle disruption (Schutzendubel and Polle 2002; Benavides et al. 2005). However, the toxicity of metals varies among plant species (Tolrà et al. 2006), cultivars (Liu et al. 2007), according to the characteristic of the soils (Bradl 2004; Li et al. 2009a), and the dose and time of metal exposure (Gratão et al. 2008). Moreover, metals can be found in different oxidation states and form various species. The different oxidation states of a particular metal ion possess different physicochemical properties and vary in their toxicity (Shanker et al. 2004).

The majority of toxic metals are presented as cations under physiological conditions, with a high redox potential. Thus, when exposed to toxic metals, cells can suffer oxidative damage caused either directly by redox-active metals or indirectly by metal-induced metabolic disturbance (Valko et al. 2005). Any current literature search will reveal that a lot of attention has been given to the responses of antioxidant defense systems to exposure to toxic metals (Azevedo and Azevedo 2006). Although these studies have led to a considerable increase in our understanding of the responses of plants to abiotic stress, new methodologies have emerged (Arruda and Azevedo 2009) and will be discussed in this chapter.

2 Studies on Metal-Induced Stress in Plants

Plants respond to metal toxicity in many different ways, according to species sensitivity, metal speciation, mobility within the living organism, intensity and duration of the exposure. A metal can cause the accumulation of an array of metabolites, such as proline and other amino acids (Sharma and Dietz 2006), anthocyanins and other phenolic compounds (Posmyk et al. 2008), phenylpropanoids, terpenoids, alkaloids, and many others secondary metabolites (Mithofer et al. 2004). It is known that metals influence plant development at all level of organization (Prasad and Strzalka 2002).

Metal-induced stress increases the generation of ROS by imbalance of cellular redox status, leading to a condition of oxidative stress and increased cellular damage (Møller et al. 2007). A relationship between redox homeostasis, metal stress, and antioxidant capacity was indicated recently through a link attributed to salicylic acid (Sharma and Dietz 2009). Reactive nitrogen species (RNS) are also important endogenous signals in plant stress; for instance, nitric oxide (NO) can mediate responses to several stimuli. Exogenous NO can protect plants against oxidative stress, acting also in stomatal closure (Neill et al. 2008). The data suggest that NO can induce cGMP production and mitogen-activated protein kinase (MAPK) activity, which are required for abscisic acid (ABA)-induced stomatal closure (Neill et al. 2008).

ROS such as the superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), and oxygen singlet ($^1O_2$) are common products of aerobic metabolism. Their production often leads to a nonspecific oxidation of membrane lipids, carbonylation of proteins, DNA and carbohydrate damage, blocking essential groups, and displacement of essential ions from biomolecules (Schutzendubel and Polle 2002; Mithofer et al. 2004). In fact, during stress situations, ROS production is an essential component of the signals (Gechev et al. 2006; Møller et al. 2007) that plants use to make adjustments in gene expression, cell structure (Shao et al. 2008), and modulation of antioxidant response and programmed cell death (Gechev et al. 2006).

The most important sites of ROS generation and scavenging in plants are the chloroplasts, due to the interaction between electron escape from the photosynthetic electron transfer system and molecular oxygen (Asada 2006; Wu et al. 2008) and mitochondria (Navrot et al. 2007; Wu et al. 2008). In chloroplasts, Cd can induce alterations in organization and shape of the thylakoid membranes with deposition of electron-dense material in double membranes (Ouzounidou et al. 1997; Vitória et al. 2006; Lage-Pinto et al. 2008). Moreover, Cd can cause structural changes in mitochondria; breakdown of the nuclear envelope (Vitória et al. 2006); disintegration of the epidermis; changes in root diameter, cell size, and intercellular spaces (Gratão et al. 2009). These effects may also be found when plants are exposed to other metals such as Cr (Choudhury and Panda 2004; Panda 2007), Ni, Zn (Jin et al. 2008), Cu (Wójcik et al. 2009; Upadhyay and Panda 2009), Pb (Choudhury and Panda 2005; Islam et al. 2008), and Tb (Wang et al. 2009b).

In the last few years, a number of reports have focused on the oxidative stress characterization and antioxidant enzyme response of a wide range of plant species. The effects of many different metals have been published, but all have consistently addressed very similar problems and investigated basic parameters. These kinds of studies are important, as they allow the verification of the sensitivities of different plant species to distinct metals, eventually indicating specific biomarkers to stress situations. However, metal-induced stress studies need new approaches that are likely to increase our understanding of how these elements affect plant metabolism and to identify the modifications that are needed to improve plant adaptation and tolerance (Arruda and Azevedo 2009).

Acquired tolerance can in some cases be observed by growing the plant under increasing metal concentrations, thus indicating the role of antioxidant enzymes and compounds in the adaptation process (Gratão et al. 2008).

Chronic exposure of plants is interesting because it can indicate tolerant or bioaccumulator species that will be useful for phytoremediation and rhizoremediation programs to degrade, stabilize, and/or remove soil contaminants. Plant tolerance to metals can occur through a high capacity for sequestration, immobilization or chelation and by exclusion mechanisms (Mora et al. 2009; Válega et al. 2009; Yang et al. 2010). Genes involved in glutathione and phytochelatin biosynthesis have been identified (Sung et al. 2009) and also the contribution of metallothioneins (Guo et al. 2008) and vacuolar membrane transporters (Kawachi et al. 2009) in increasing the response/tolerance to metal stress (Shim et al. 2009). Critical parameters for the use of phytoremediation such as environmental and climatic characteristics, nutrient sustainability, feasible ecosystems, and pollutant characteristics influence the level of technical success for such an approach (McCutcheon and Jorgensen 2008). The rhizosphere plays an important role in phytoremediation, because microorganisms can decisively interfere with and alter metal mobility and availability to plants (Li et al. 2009b; Martínez-Alcalá et al. 2009; Yang et al. 2010). However, more field experiments are necessary to validate the results obtained in the laboratory and greenhouse.

In the field, plants are often exposed to a combination of different stress-causing agents and the response of individual plants species may be unique and should be one of the focuses of future studies (Moffat 2002; Mittler 2006). Molecular and genetical approaches, genome response, transcriptome and proteomic analyses are essential tools to be used more often from now on for the generation of a wide range of information on plant responses to metal stress or any other environmental contaminant.

3 Hormonal Modulation of Stress Signaling

It is not surprising that some of the major components believed to be involved in the responses to stress signaling are the plant hormones. Since these substances regulate almost every step of plant development, it is expected and it has been confirmed that they play an important role in the biochemical program during stress, also influencing the adaptive response.

Although virtually every hormone may be involved in plant defense responses against various biotic and abiotic stresses, the work with brassinosteroid (BR), jasmonic acid (JA), and salicylic acid (SA) has presented new perspectives on stress tolerance studies. For instance, it has been reported that there is an ameliorative influence of BR on drought stress (Behnamnia et al. 2009; Fariduddin et al. 2009), low (Kamuro and Takaysuto 1991) and high (Dhaubhadel et al. 1999) temperature, and salinity (Hayat et al. 2007a; El-Khallal et al. 2009). BR may also reduce the inhibitory effect of metals such as Cd (Anuradha and Rao 2007; Hasan et al. 2008) and Al (Ali et al. 2008), and more recently to have an ameliorative effect on excess manganese-induced oxidative stress in maize (Wang et al. 2009a). JA has been correlated with pathogen defense, but recently it has been shown to be an important component in the mediation of abiotic stresses, such as toxic metals (Maksymiec et al. 2005) and drought (Shan and Liang 2010). SA can also modulate the plant response to several stresses, such as ultraviolet light (Yalpani et al. 1994), toxic metal (Mishra and Chordhuri 1999), chilling (Janda et al. 1999; Kang et al. 2003), heat (Dat et al. 1998), salt (Taşgın et al. 2006), and pathogens (Khaosaad et al. 2007). Recently, Belkhadi et al. (2010) showed that the presoaking of dry flax grains in SA-containing solutions partially protected seedlings from Cd toxicity during the following growth period.

One of the points that had been approached in the above-cited works is that the mutual modifications induced by hormones result in an antioxidant burst, which can explain an adaptive system (Mazorra et al. 2002; Behnamnia et al. 2009). For instance, the activities of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione peroxidases (GPX) were increased in the seedlings of *Raphanus sativus* treated with BR (Anuradha and Rao 2007). In maize (El-Khallal et al. 2009) and tomato

(El-Khallal 2007) plants, an increase in the activities of SOD and CAT after pretreatment with BR was also observed. Recently, BR was shown to lead to an increase in the activity of the SOD, CAT, APX, and guaiacol peroxidase (GPOX) in tomato (Ogweno et al. 2008; Behnamnia et al. 2009) as well as the activity of CAT and SOD in *Brassica juncea* (Hayat et al. 2007a, b; Fariduddin et al. 2009). It has been shown that JA can induce an increase in the activities of a wide range of antioxidant enzymes. APX, CAT, glutathione reductase (GR), and SOD were increased with JA treatments in the leaves of *Phaseolus vulgaris* L. var. Top Crop (Clarke et al. 2002). JA was also reported to enhance the antioxidant response of *Brassica napus* roots and shoots, particularly by a higher activity of SOD when compared to the control (Comparot et al. 2002). In other studies, the activities of SOD and CAT have also been shown to increase in JA-treated plants (Hung and Kao 2004; Kumari et al. 2006), as well as in recent reports in which enhanced GR, GPX, APX, and CAT and SOD activities were observed (Cao et al. 2009; Parra-Lobato et al. 2009; Shan and Liang 2010). Equally, activation of antioxidant enzymes induced by the treatment with SA may contribute to its ability to protect plants against stress. SA pretreatment of *Zea mays* L. caused an increase in GR, SOD, CAT, and APX activities (Janda et al. 1999, 2000; Kang et al. 2003), besides other recent observations in which SOD, CAT, and APX activities were shown to be altered (El-Khallal et al. 2009; Mutlu et al. 2009). Hormonal applications have also been exploited to enhance production of antioxidants in fruits (Kubicka and Zadernowski 2007; Cao et al. 2009), an interesting approach toward the improvement of food quality.

Although in the examples cited earlier it is proposed that exogenous hormones increased the activity of some antioxidant enzymes causing mitigation of oxidative stress, the details of the biochemical and molecular mechanisms involving BR, JA, and SA in these amelioration responses are still unclear. The linear concept in which an antioxidant burst appears as a result of hormone-induced ROS could explain the defense response, although the biochemical pathway from hormone to response seems to be a very complex phenomenon. This complexity is due to four main issues: (1) it has been assumed that hormones act as secondary messengers for the induction of an antioxidant defense system in stressed plants and thereby could effectively scavenge ROS in plants under stress leading to reduced levels (Ogweno et al. 2008; Zhang and Xing 2008); (2) although there are enhanced activities of antioxidant enzymes in plants treated with exogenous hormones, high ROS levels can still be maintained (Hung and Kao 2004; Cao et al. 2009; Parra-Lobato et al. 2009); (3) a wide range of stress-associated genes can be induced by hormones (O'Donnell et al. 2003; Uquillas et al. 2004; Blanco et al. 2005), and therefore become a more acute issue, primarily because the activation of these genes can result in the induction of ROS which can induce stress genes resulting in a complex biochemical network (Moon et al. 2003; Torres et al. 2002; Seo et al. 2007); (4) signaling stress can be accompanied by different forms of biochemical regulation due to exogenous hormones. Thus, when applied at physiological concentrations, these substances may cause a temporary low level of oxidative stress in plants, which acts as a hardening process. This low stress can improve the antioxidative capacity of the plants and help to induce the synthesis of protective compounds. On the other hand, overdosage, repeated or prolonged application can cause irreversible disturbance of plant metabolism. Certainly, these issues fall into the question of how one hormone and the various components of oxidative stress work together interacting with others hormones. The final response to one hormone depends on the crosstalk between the different signaling routes, involving perhaps synergetic or antagonistic interactions inducing a wide spectrum of antistress reactions in plants (Campos et al. 2009).

Considerable attention has been focused on the use of new molecular and genomics tools, which have provided useful information for improving our understanding of the complex interplay between hormones and components of oxidative stress. Over the past decade, several

mutants with changed hormone signal perception and transduction have been isolated and well characterized with some of them being useful in helping to improve our understanding of the modulation of ROS signaling by the reactive oxygen gene network of plants. In addition, the cellular localization and coordination of the ROS scavenging pathways of plants, including components involved in mediating the cross-talk between these pathways and hormones have been studied. These often operate synergistically to activate the expression of some defense-related genes after stress, including NO and peptide hormones, such as systemin and hydroxyproline-rich glycopeptides systemins (Glazebrook 2005; Miao and Zentgraf 2007). Additionally, key components of the reactive oxygen gene network have been identified by reverse genetics, and microarray analysis of defense-related genes, which have revealed a significant overlap in the number of genes induced by ROS and hormones (Schenk et al. 2000). Finally, the recent identification of oxidative stress-specific transcription factors (Dietz et al. 2010) represents an additional effort in the elucidation of stress signaling in plants. However, a better understanding of phytohormone-mediated plant defense responses is important in designing effective strategies for engineering crops that will be resistant to biotic and abiotic stresses (Overmyer et al. 2000; Mittler et al. 2004; Bari and Jones 2009).

4 The Proteome in the Context of Stress

Dramatic biochemical changes during a wide range of stresses have been shown to be closely related to gene expression (Mousavi and Hotta 2005). Progress in obtaining complete plant genome sequences has not only updated our knowledge but has also provided a substantial platform from which to study plant stress physiology (Cui et al. 2005). Microarray analysis has provided a global examination of gene expression corresponding to stress (Perez-Torres et al. 2009; Soren et al. 2010). It has been shown that a wide range of genes appear to be induced under stress, and the gene expression level for several genes reached a maximum, for example, after cold treatment for 24 h (Rabbani et al. 2003). On the other hand, 18 cold-responsive rice MicroRNAs (miRNAs) were downregulated (Lv et al. 2010). Current data of microarray analysis showed that the RPK1 (receptor-like protein kinase 1), which act as a regulator of ABA, enhances the expression of stress-responsive genes, such as *Cor15a*, *Cor15b*, and *rd29A*, in addition to H_2O_2-responsive genes (Osakabe et al. 2010). Although this gene expression profiling under stress has increased our understanding a great deal, it is still important to know how the transcriptional changes are reflected at the translational level (Cui et al. 2005).

In recent years, proteomic profiling has been used to study the effects of stresses on plants under different conditions. Changes in proteomic profiles at the organelle, cell, organ and tissue levels, induced by several stress have been described in a wide range of plant species and compared to proteomes affected by different physiological conditions (Porubleva et al. 2001). Moreover, the new generation of proteomic techniques facilitates the investigation of the global protein expression profile using efficient protein extraction and identification methods (Cui et al. 2005). This has helped in the identification of specific proteins, elucidation of protein structures and identification of protein functions, protein–protein interactions, and localization of proteins during stress-signaling cascades.

Today proteomics is boosted by new technologies and equipments/instruments, which have made it a key research field (Agrawal et al. 2006). Oxidative stress proteomic studies have employed the use of 2D-polyacrylamide gel electrophoresis (PAGE) associated with a mass spectrometry (MS) (Agrawal et al. 2006; Rinalducci et al. 2008). The use of these techniques has allowed the recognition of proteins involved in ROS reactions triggered by stresses (Seggara et al. 2007; Ahsan et al. 2008a). This approach has allowed the identification, for example, of predominant proteins related to ROS, and how, what, and why the proteins are modified during stress. Comparative proteomics of salt tolerance revealed

a total of 79 and 32 proteins involved in photosynthesis, energy metabolism, and stress response in *Arabidopsis thaliana* and *Thellungiella halophila*, respectively, showing distinct patterns of protein changes in the two species (Pang et al. 2010).

The answer to these questions had also allowed the understanding of the importance of proteins in ROS and oxidative damage for maintenance of plant function under stress in field condition as well as controlled environments (Salekdeh and Komatsu 2007). Thus, using a proteomic approach, a wide range of reports demonstrated that the expression of various cell compartment proteins was up- or downregulated following exposure to ROS (Shulaev and Oliver 2006). However, ROS can affect the proteome not just by increasing or decreasing the levels of proteins directly or indirectly, but also by modification of protein activity. For example, Irar et al. (2010) compared several shock proteins, enzyme metabolism, and radical scavengers in mature embryos from wheat genotypes, *Mahmoudi* (salt and drought sensitive) and Om Rabia3 (salt and drought tolerant), suggesting that the protein differential accumulating could be used for the screening of tolerance/sensitivity to drought and salt stress.

Recently, several efforts have been made to generate proteome maps and to investigate the overall response at the proteome level of plants under toxic metal stresses. The results of these studies have shown that plants operate various mechanisms in which proteins play central roles in coping with metal stress (Ahsan et al. 2008a). Thus, a large set of differentially expressed proteins is involved in several cellular functions during metal toxicity. These functions have been analyzed in different species, from algae (Gillet et al. 2006) to trees (Kieffer et al. 2008), and showed that metals, for example, Cd (Rodríguez-Celma et al. 2010), Al (Yang et al. 2007), Cu (Li et al. 2009c), and As (Ahsan et al. 2008a), have a severe effect on proteins involved in photosynthesis and respiration. For example, Visioli et al. (2010) showed the importance of mitochondria and chloroplasts in the response to metal stress, verifying that the proteins which were more abundant in response to Cd were located in these organelles. Certainly, these stress tolerance mechanisms involve antioxidant molecules, which, based on recent proteomic data, indicate the involvement of enzymes of the ascorbate–glutathione pathway (Lee et al. 2010).

Future progress in proteomics tools will allow the identification and complete characterization of protein markers in a given stressed physiological condition. These findings in association with genomics and metabolomics will result on global cellular responses to oxidative stress, providing data for the biochemical networks involved in functional and/or structural alterations to proteins (Rinalducci et al. 2008).

5 Grafting

Stress signaling is an aspect that still demands more attention for future research. For this purpose, grafting can be a powerful tool to improve our understanding of the stress signaling and specific reponses between plant organs during stress. The grafting technique relies on an old principle and has been extensively used in the horticultural industry for woody species.

Analyses of such plants and their controls may indicate a complex array of long distance or systemic signals, including abiotic stress responses (Turnbull et al. 2002). Recently, the production of grafted plants has been shown to be a useful strategy in increasing the salinity tolerance of *Cucurbita focifolia* (Huang et al. 2010), *Cucumis sativus* L. (Zhen et al. 2010), and salinity resistance in tobacco plants (Ruiz et al. 2005).

However, the use of the grafting technique on plants subjected to toxic metals is rare and only very few studies have been reported. Thus, grafting studies focusing on stress signaling by toxic metals need to be more firmly addressed and explored. For instance, Sugiyama et al. (2007) reported that the seed Cd concentration can be influenced by differences in the translocation of Cd to the seed and in the Cd accumulation capacity of roots among soybean cultivars. In another study, *Solanum lycopersicum* L. cv. *Belladona* F-1 plants were either self-grafted or grafted onto the rootstock "He-Man" showing that cv. *Belladona*

was more tolerant to excess Mn than to Mn deficiency in terms of vegetative growth and fruit yield (Savvas et al. 2009). The message is very clear: there is plenty of room for research using this approach which appears to be quite powerful.

6 Cytogenetic

As already discussed, ROS can affect macromolecules and cause severe damage to nucleic acids leading to alterations in the number and structure of chromosomes. This aspect has been poorly explored in plants subjected to metal stress, but the interest in the genetical and cytological effects of toxic compounds was greatly stimulated by the results obtained with cyanide which indicated that hydrogen peroxide was involved in the production of mutations and chromosome aberrations (Kihlman 1957). Furthermore, studies on genotoxicity can be correlated with tissue concentration and plant availability of the toxic metal, having ecotoxicological implications.

A variety of cytogenetic tests have been applied to plants species as bioindicators, evaluating the toxicity and mutagenicity of environmental contaminants such as cyanide, fluoride, organics, and metals (Andrade et al. 2010). Cytotoxic evaluation using root length, weight gain, mitotic index (MI), frequency of micronuclei (MN) and chromosomal aberrations (CAs) was investigated on *Cicer arietinum* L. root tip cells submitted to different concentrations of Pb and Hg ions (Cavusoglu et al. 2009). A high frequency of interphase aberrations was found in *Armeria maritima* grown in soils with high concentrations of toxic metals or increased salinity (Coulaud et al. 1999). In root tip cells of *Helianthus annuus* submitted to copper chloride, condensed and sticky chromosomes, scattered chromosomes, and chromatid bridges were observed (Ünceer et al. 2003).

It is now clear that cytogenetical alterations can also be detected in plants submitted to metal toxicity. Cu decreased mitotic index, inhibited meristematic cell proliferation, resulting in changes in morphology of the chromosomes (Posmyk et al. 2008). Cd also caused chromosome aberrations and micronuclei formation in *Allium cepa* (Seth et al. 2008). Finally, it has also been demonstrated that exogenous antioxidants such as anthocyanins limit and prevent the cytogenetical damage induced by Cu in *Vicia faba* (Posmyk et al. 2009).

7 Metallomics

Metal ions are used by biological systems in fundamental processes such as signaling, gene expression, and catalysis and the absence of some metals can result in cellular disorders, whereas the presence of others has frequently been evoked in the context of toxicity (Szpunar 2004). The aim of metallomics is to achieve a better understanding of the function and regulation of metals in biological systems, allowing a comprehensive look at the role of essential and toxic metals. Several original papers have been published on total element determination and, only recently, the research has moved towards elemental speciation analysis, characterizing metals or metalloids in a cell or sample (Muñoz et al. 2009).

The metallomics techniques require the development of versatile measurement tools involved with detection, mapping, and/or quantification of metals associated with proteins in biological systems (Mounicou et al. 2009). Thus, the metallome can give us the information about how the element (metal or metalloid) is distributed among the cellular compartments, in which biomolecule it is incorporated, and the concentrations of the individual metal species present (Szpunar 2004).

Metallomics can elucidate the metal speciation and biological functions of biometals in toxic metal accumulating and hyperaccumulating plant species (Peng and Yang 2006). In contrast to traditional approaches, metallomics allows the investigation of global and multielement interactions in a protein or enzyme system. Although well established, the sample preparation has been subject to change and new strategies have been adopted (Arruda 2007). It is important that the stability of the linkage between the metal and the organic species or the biomolecule is considered. However, the more common methods used for total

metal extraction and determination are not able to maintain metal-organic linkages (Magalhães and Arruda 2007). Others methods, such as microwave-assisted extraction (Sussulini et al. 2007), microwave water-assisted extraction (Salgado et al. 2006), and molecularly imprinted solid phase extraction (Xu et al. 2009) have been shown to be efficient.

Two dimensional (2D) electrophoresis such as PAGE, or more recently fluorescence difference gel electrophoresis (DIGE) are particularly useful techniques for a high resolution separation (Timms and Cramer 2008), as well as two dimensional liquid chromatography (Pedrero et al. 2007). After the separation of the proteins/enzymes, the next step is related to their identification and some techniques like MALDI-QTOF-MS (matrix assisted laser desorption ionization-quadrupole time of flight-mass spectrometry) (Garcia et al. 2009), and ESI-MS (electrospray ionization mass spectrometry) can be applied (Aki and Yanagisawa 2009).

At the last step, some techniques are used for metal identification, as well as sensitivity for metal quantification. Inductively coupled plasma mass spectrometry (ICP-MS) can be used for ultra-sensitive quantification of metal-containing proteins or peptides which are determined down to the low attomole range (Bettmer et al. 2009). ICP-MS can provide higher sensitivity, selectivity, and reliability in molecule tracing (González-Fernández et al. 2008).

Metallomics can be successfully used in traditional biomarker analyses, providing a quantitative analytical approach in proteomics (metalloproteomics), which converts this relatively recent technology to a valuable alternative for biomolecule analysis (González-Fernández et al. 2008).

8 Conclusion and Future Perspective

It has been observed that the majority of the published reports related to metal-induced oxidative stress have shown signs of repetition, by applying similar methods and analysis (Arruda and Azevedo 2009). The measurements of antioxidants as stress markers will remain an essential aspect in assessing the stress response in plants, but other aspects related to an interdisciplinary view should be taken into account, particularly when considering the complexity of the studies that are now required (Arruda and Azevedo 2009).

The information available in this chapter is just an overview of aspects that have not received the attention that they deserve. At the same time, the text provides some new points to the emerging techniques that are available or are being developed, which, eventually may be powerful tools to aid our understanding of the different molecular and cellular mechanisms involved in cell stress responses. Thus, a more comprehensive view has to be considered and must necessarily include studies on gene expression, protein translation, enzyme activity, and metabolite concentrations, all considered simultaneously, wherever possible.

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Sulfur in the Alleviation of Cadmium-Induced Oxidative Stress in Plants

20

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Abstracts

The toxicity of cadmium (Cd) is an emerging environmental problem that has attracted the attention of plant scientists all over the world. It deteriorates soil, plant and human health. Researchers have focused their attention in evolving strategies to reduce its toxicity at cellular, molecular and/or whole plant level. Sulfur is an important plant nutrient that takes part in plant metabolism and provides vigor to plants under stressful environments. This nutrient element could be used in agricultural system for reducing Cd toxicity and increasing sustainability. Sulfur uptake results in the formation of the first stable product cysteine through a cascade of enzymatic reactions. The formation of cysteine leads to the synthesis of glutathione, a nonenzymatic antioxidant known to be involved in Cd detoxification either through quenching reactive oxygen species or formation of phytochelatin that binds Cd and sequester it into vacuole. Manipulation of sulfur-assimilating enzymes, cysteine, glutathione and/or phytochelatin content could possibly lead to Cd detoxification. The present work gives insight into the role of sulfur in the alleviation of Cd stress.

Keywords

Sulfur • Cadmium toxicity • Cadmium tolerance • ROS • Antioxidants • Nutrient availability • Sulfur sink

1 Introduction

Plants experience a range of abiotic stress factors such as drought, cold, salinity, temperature and heavy metals during their life time, which cause extensive loss to agricultural productivity worldwide (Boyer 1982; Bray et al. 2000; Peters et al. 2004). Heavy metal toxicity in particular is one of the major abiotic stresses that lead to reduction

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in health and vigor of plants, and also of animals through food chain. The growth of anthropogenic activities associated with development has added high concentrations of heavy metals in agricultural soils and large magnitude to environmental pollution (Sanita di Toppi and Gabbrielli 1999; Rao and Reddy 2008). Heavy metals such as arsenic, cadmium, cobalt, copper, nickel, zinc, and chromium are phytotoxic either at all concentrations or above certain threshold levels. Some of these heavy metals are essential for plant growth when they are present at optimal level, but become toxic at high concentration, and may cause toxic effects on plant growth and yield or even death of plants. Heavy metal toxicity primarily interferes biochemical reactions of photosynthetic apparatus leading to adverse effects on biomass production and leads to progressive senescence (Sobkowiak and Deckert 2003; Alaoui-Sossé et al. 2004; Lin et al. 2005). Of all toxic heavy metals, cadmium (Cd) is of major environmental concern because the discharged rate of Cd in soil is of the order of 22,000 tons per year globally (Nriagu and Pacyna 1988) and it ranks highest in terms of damage to plant growth and human health. The conscious or unconscious addition of Cd in the soil disturbs the plants biological functions as the residence time of Cd in soil is more than thousand years (Alloway 1995). Excess Cd induces complex changes in plants at genetical, biochemical and physiological levels, leading to phytotoxicity. Plants are also able to load part of the Cd taken up from soil into the xylem and transport it to leaves (Chardonnens et al. 1998). Cadmium can interfere with photosynthetic and respiratory activities, mineral nutrition, enzymatic activities, membrane functions, and hormone balance (Chien and Kao 2000).

In fact, the inhibition of Cd stress effects on plant physiological processes is related to its effect on nutrient uptake and distribution. The interactions of Cd and metal nutrients, such as Fe, Zn, Cu, and Mn have been reported in some plant species (Zhang et al. 2002; Wu and Zhang 2002). Most plants are sensitive to low Cd concentrations and show inhibition in plant growth as a consequence of alterations in the photosynthesis rate and the uptake and distribution of macronutrients and micronutrients (Lozano-Rodriguez et al. 1997; Sandalio et al. 2001; Benavides et al. 2005; Dube et al. 2009).

As heavy metals cause deficiency of essential mineral nutrient elements by restricting their uptake and distribution in plant tissues (Siedlecka 1995), better understanding of how the supply of these nutrients may reverse metals toxicity is needed (Pankovic et al. 2000). Optimization of mineral nutrients may probably reduce some of the metal-induced negative effect on plants as mineral nutrients affect the activity and bioavailability of Cd in the soil–plant environment. The study of interaction of Cd with plant nutrients in soil–plant systems is, therefore, of great importance and may be used to minimize Cd accumulation in edible plant parts (Dheri et al. 2007). Among the various macronutrients, sulfur (S) serves a distinguished role in Cd detoxification because it is an integral part of most of the defense compounds involved in Cd detoxification. Optimum S nutrition is helpful in reducing Cd translocation within the plant system (Sarwar et al. 2010). Cadmium induces generation of reactive oxygen species (ROS) that causes oxidative stress in plants (Skórzyńska-Polit et al. 2003/04; Mobin and Khan 2007; Anjum et al. 2008; Khan et al. 2009; Iqbal et al. 2010). These excess ROS cause damage to proteins, lipids, carbohydrates, DNA and ultimately may result in cell death (Foyer and Noctor 2005; Djebali et al. 2008). Cadmium-induced ROS leads to reduction in photosynthesis, plant growth and yield. Iqbal and Khan (2010) reported that Cd leads to reduction in photosynthetic pigments, growth and yield. The present review focuses mainly to gain detailed insight on the effects of Cd toxicity in plants and elucidates the potential of sulfur in modulating Cd-induced oxidative stress.

2 Updates on the Cadmium Toxicity in Plants

Cadmium is a highly toxic heavy metal that negatively affects physiological processes in plants, growth and development, and eventually plant death. The critical concentration at which Cd

causes injuries is in the range of 3–10 mg/kg dry mass (Balsberg-Pahlsson 1989). Its accumulation in plants adversely affects photosynthetic processes and diminishes water and nutrient uptake (Sanita di Toppi and Gabbrielli 1999; Maksimović et al. 2007), root and shoots growth and disturbs cellular redox control (Clemens 2001; Schützendübel and Polle 2002).

Cadmium induces significant disturbances in the structural organization and functional activity of the photosynthetic apparatus (Baszynski 1986; Vassilev et al. 1995; Dahlin et al. 2000). The main targets of toxic Cd effects are the pigment apparatus and photosynthetic gas exchange system (Clijsters and Van Assche 1985; Tukiendorf and Baszynski 1991; Lang et al. 1995). The most characteristic symptoms of Cd stress are brown and short roots, chlorosis, fewer tillers, senescence and reduced plant growth and biomass (Arduini et al. 1994; Wu and Zhang 2002; Wu et al. 2003; Cosio et al. 2006). Moussa and El-Gamal (2010) reported that at higher concentrations of Cd (400–1,000 μM Cd), the toxic symptoms of root cells are mainly continued to disintegration of cell organelles, disruption of membranes, withdrawal of plasma membrane from cell walls, and formation of multivesiculate bodies in the cytoplasm. The uptake of Cd from soil seems to occur mainly via Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} transporters (Clemens 2006). The best-studied nonspecific transporter is the ZIP IRT1, which is the major transporter responsible for high-affinity iron uptake from the soil. Cadmium exposure rapidly induces apparent Zn deficiency that may be through binding to a Zn sensor protein (Weber et al. 2006; Roth et al. 2006). Cadmium stress inactivates macromolecules and cellular structure (Stroi ski et al. 1990; Jiang et al. 2009) resulting in altered physiology processes and biochemical mechanisms.

The Cd-induced toxicity in plants is because of altered physiological phenomena (Demirevska-Kepova et al. 2006). Cadmium exerts its toxic effect in plants by inducing oxidative stress generated due to overproduction of ROS (Sandalio et al. 2001; Ali et al. 2002; Ranieri et al. 2005; Smeets et al. 2005; Singh et al. 2008). Oxidative stress occurs when there is a serious imbalance in any cell compartment between the production of ROS and antioxidant defense, leading to significant physiological challenges (Foyer and Noctor 2000). These excess ROS cause damage to proteins, lipids, carbohydrates, DNA and ultimately result in cell death (Mittler et al. 2004; Foyer and Noctor 2005; Shulaev and Oliver 2006; Djebali et al. 2008). Evidence that Cd causes the production of ROS in plants arises from the observations that new isozymes of peroxidases were detectable in both root and leaves of *Phaseolus vulgaris* (Van Assche and Clijsters 1990). Besides, this fact is also confirmed from the detection of lipid peroxidation, increased lipoxygenase activity, chlorophyll degradation and inhibition or stimulation of the activity of several antioxidant enzymes under Cd stress (Dixit et al. 2001; Leon et al. 2002; Skórzyńska-Polit et al. 2003/04; Mobin and Khan 2007; Anjum et al. 2008; Agrawal and Mishra 2009). Cadmium-induced increase in ROS production acts as a cellular signal triggering the stress response (Verbruggen et al. 2009). Stress-responsive MAP kinases seem to be involved in transcriptional responses to Cd as they are activated possibly by ROS under Cd^{2+} excess (Jonak et al. 2004). Another putative player in Cd-induced oxidative stress signaling is AtOS1, a member of the Abc1 family localized in the chloroplasts. AtOS1 does not transport Cd but seems to be crucial for Cd tolerance, possibly through a putative kinase activity (Jasinski et al. 2008). An important source of Cd toxicity is its chemical similarity with essential elements, in particular Zn, but also Ca and Fe, deregulating the homeostasis of the latter elements or causing their displacement from proteins (Verbruggen et al. 2009; Sarwar et al. 2010).

The principal mechanisms of plant response to Cd stress include phytochelatin (PC)-based sequestration and compartmentalization, and additional defense mechanisms based on cell wall immobilization, plasma membrane exclusion and induction of stress proteins (Draži et al. 2006). Cadmium stress leads to the activation of antioxidant defense system. The effects of Cd on antioxidative capacity are dual: on one hand, Cd can induce oxidative stress via the inhibition of antioxidants, but on the other hand it also activate

several antioxidative components as a result of a disturbed redox balance and a consecutively induced signal transduction cascade.

A putative chromatin remodeling factor, named OXS3, was recently identified in a screen for Cd tolerance of a *Brassica juncea* cDNA library in *Schizosaccharomyces pombe* (Blanvillain et al. 2008). An oxs3 mutant was hypersensitive to Cd and its overexpression improved Cd tolerance. Verbruggen et al. (2009) postulated that OXS3 might protect DNA or alter its transcriptional selectivity. While most Cd are chelated before its transport to the vacuole, Cd^{2+} can be directly transported into the vacuoles by Cd^{2+}/proton antiporters such as CAX2 and CAX4 and possibly also by MHX (Korenkov et al. 2007; Berezin et al. 2008). Korenkov et al. (2007) have reported that the overexpression of AtCAX2 or AtCAX4 in tobacco enhances Cd and Zn transport into root tonoplast vesicles and enhances Cd accumulation in roots of plants exposed to Cd. NRAMP3 and NRAMP4 are responsible for Cd^{2+}efflux from the vacuole (Thomine et al. 2003). Their overexpression increased Cd sensitivity in *Arabidopsis* as a result of the impairment of Fe homeostasis, as NRAMP3 and NRAMP4 are responsible for the release of vacuolar Fe^{2+}. Plant cells contain a range of protective and repair systems, which under normal circumstances minimize the occurrence of oxidative damage. There are systems which either react with ROS and keep them at a low level or with antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidases (POD), ascorbate peroxidase (APX), glutathione reductase (GR) that quench ROS. The quenching of ROS is supported by nonenzymatic antioxidants, glutathione, ascorbic acid, α-tocopherol and carotenoids (Sairam et al. 2000; Shah et al. 2001) or systems that regenerate oxidized antioxidants (glutathione, mono- and dehydroascorbate) (Markovska et al. 2009).

Genes that are involved in Cd detoxification notably include ATP-binding cassette (ABC) proteins (Hall and Williams 2003; Plaza and Bovet 2008). Members of the ABC transporter family that are known to confer Cd tolerance to plants include MRP3 (multidrug-resistance-related protein) (Kolukisaoglu et al. 2002), ATM3 (ABC transporter of the mitochondria) (Kim et al. 2006) and PDR8 (pleiotropic drug resistance) (Kim et al. 2007). The strong upregulation effects in the roots on genes that are putatively involved in Cd detoxification indicate that the ATPase and the ABC transporters might have been activated by both the increased Cd concentration and the increased availability of sulfur (Fässler et al. 2011). Nicotianamines (NA) are involved in Cd chelation, transport and detoxification in plants. Nicotianamines are ubiquitously present in plants and synthesized from three molecules of methionine by nicotianamine synthase (NAS) (Sharma and Dietz 2006).

Responses to Cd also include the induction of genes involved in sulfur and glutathione metabolism (van de Mortel et al. 2008). Cadmium shows a high affinity for thiols, and therefore the major thiol antioxidant, reduced glutathione (GSH), that is highly abundant in cells is a primary target for free Cd ions. The Cd-induced depletion of the reduced GSH pool (Lopez et al. 2006) results in a disturbance of the redox balance leading to an oxidative environment.

3 Nutrients Availability and Cadmium Tolerance

Plant metabolism is affected by optimal composition of mineral nutrients. Cations such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+} compete with Cd for uptake by plants (Tlustos et al. 2006) and for exchange site in soils (Degryse et al. 2004). Studies have shown that appropriate application of plant nutrients increases plant biomass and grain yield, and decreases Cd concentration in grain and other edible plant parts by dilution effect (Sarwar et al. 2010). Plant nutrients also help to sequester Cd in vegetative parts by production of PCs and avoid Cd accumulation in grains. They help to alleviate physiological stress caused due to an excess of Cd. However, antagonistic interaction between Cd and content of these nutrients has been reported (Uraguchi et al. 2006). In fact, the toxicity symptoms due to Cd are correlated with disturbances in the uptake and distribution of macro

and micronutrients in plants (Gussarson et al. 1996). Cadmium is taken up by plants via cation transport systems normally involved in the uptake of essential elements, such as members of ZIP and Nramp families or Ca^{2+} channels and transporters (Pence et al. 2000; Perfus-Barbeoch et al. 2002). Zhao et al. (2002) reported that Cd is taken up by the roots via the Ca channels or via Zn and Mn transporters. Its entry through the Ca channel in the leaves disturbs the plant–water relationship (Perfus-Barbeoch et al. 2002) causing stomatal closure in many plants, leading to lower transpiration rate and inhibition of photosynthesis through an adverse effect on chlorophyll metabolism. This subsequently leads to growth inhibition and imbalance in the nutrient level (Sandalio et al. 2001; Chaffei et al. 2004). The effect on growth reduction may also be due to the rate of interference of toxic metals and nutrients with sensitive metabolic reactions (Gupta et al. 1995, 1998; Haag-Kerwer et al. 1999). The decrease of Mn, Fe, Mg, S and P concentrations in Cd-sensitive cultivar's (*Brassica oleracea* L.) leaves under Cd stress may be the key reason for the restraint of leaf photosynthesis, and the decrease of plant growth (Sun and Shen 2007).

The increase or decrease in plant nutrients under Cd stress may vary in root and shoot. Inhibition may occur at the uptake level or in translocation of nutrients. Different transporters are involved in the translocation of nutrients into the aerial part of the plant at different levels and Cd can inhibit these transporters. Morphological changes of the conducting xylem tissue may also contribute to a limited translocation of nutrients from the roots (Barceló et al. 1988). Zornoza et al. (2002) have reported that cadmium addition reduces P, K, Fe, Mn and Zn concentrations in the shoot and Mn in the root of Cd-treated plant. However, the effect of Cd on the nutrient content depends on the plant species and the experimental conditions. A number of factors, viz. concentrations of ions, pH, abundance of chelators, etc. govern these interactions and thus various types of responses may take place in different experiments using cadmium. It is of general concern that cadmium in certain concentration may cause deficiency of other elements essential for normal growth of plants. Birch plants grown in the presence of Cd showed a reduction in the concentration of Fe, Mn and Cu in the shoot, while in roots no significant changes were observed, and the content of Zn did not change either in shoots or roots (Gussarson et al. 1996). Thus, the presence of Cd in the plants caused a general decrease in the nutrient content of plants.

Cd stress leads to a decrease in nutrients content, and the severity of Cd toxicity can be reduced through the optimization of these nutrients. Sufficient amount of nutrients may reduce the accumulation of the metal in plants thereby reducing its toxicity. Pankovic et al. (2000) have shown that optimal N supply decreases the inhibitory effects of Cd on photosynthesis of sunflower plants probably by increasing ribulose 1,5-bisphosphate carboxylase (rubisco) activity. They found that it was only at optimum N that the adverse affect of Cd was reduced and thus N nutrition could be manipulated as a means of decreasing Cd phytotoxicity. Potassium may protect plants from Cd-toxicity-induced oxidative damage by reducing the Cd availability to the plants thereby depressing the contents of H_2O_2 and TBARS in the mustard leaves and increasing the content of antioxidative enzymes (Umar et al. 2008). He et al. (2005) suggests that Ca^{2+} can enhance Cd tolerance by increasing the expression of PC synthase gene under Cd^{2+} stress. The deficiency of Mg enhances Cd-induced oxidative stress resulting in damage to chlorophyll, photosynthesis and plant growth. Abul Kashem and Kawai (2007) have reported that Mg decreases the content of Cd in the shoots because of the enhancement of shoot dry weight induced by the detoxifying effect of higher Mg concentration in Japanese mustard spinach.

4 Sulfur as Essential Factor for Cd Tolerance

Among all the nutrients involved in Cd detoxification, the role of S in Cd tolerance is important because of its presence in most of the defense compounds. Sulfur has been accepted as an outstanding factor for the improvement in production and

quality of crops in several plant species. The negative relation between Cd and NPK could be mitigated with S nutrition as several studies have shown the synergistic interaction effects of S with N and K in influencing the yield, quality (oil, protein, amino acid, and fatty acid synthesis) and uptake of nutrients by different crops (Tandon 1991; Aulakh and Chhibba 1992). Probably, the improved S nutrition allows a more adequate plant defense response by synthesis of sulfur defense compounds, including PC and GSH. The importance of S in Cd tolerance is discussed emphasizing on the upregulation of various S-assimilating enzymes under Cd stress, which ultimately lead to synthesis of S containing defense compounds.

Sulfur is an essential nutrient, taken up as sulfate from soil, reduced and incorporated into bioorganic compounds in plant cells. The pathway of sulfate assimilation is highly regulated in a demand-driven manner in seed plants. The importance of S as a plant nutrient is becoming more imminent due to its effect on crop productivity and quality. Sulfur is present in amino acids, proteins, peptides, coenzymes and vitamins. Plants and many microorganisms are able to utilize inorganic sulfate and assimilate it into these compounds (Leustek et al. 2000; Kopriva 2006). A large number of studies have reported a marked influence of applied S on the yields of several cereals, pulses, oilseeds, vegetables, forages and other crops (Pasricha et al. 1987; Tandon 1991; Ahmad et al. 1998; Aulakh and Pasricha 1998). Oilseed rape has a high requirement for S and is particularly sensitive to any shortfall in S supply (Ahmad et al. 2005). Yield responses of oilseed rape to S supply have been reported (Walker and Booth 1992). In fact, S is an important nutrient for oilseed rape due to its association with yield and also a range of quality factors. It is required by *Brassicas* for the synthesis of the S-bearing compound, glucosinolates. Seeds from many plants, especially of legume crop, contain low concentrations of small 2S proteins that are relatively rich in cysteine and methionine (Shewry and Pandya 1999). *Glycine max* also produces low molecular weight polypeptides that contain disproportionately high methionine content (Paek et al. 2000). In fact, the availability of reduced S (i.e., cysteine and methionine) is the rate-limiting factor for the regulation of β-conglycinin chains that usually synthesized only during late seed development (Meinke et al. 1981). Sulfur deficiency is an environmental condition that highly upregulates sulfate assimilation in seed plants (Hirai et al. 2003; Maruyama-Nakashita et al. 2003; Nikiforova et al. 2003).

Sulfur is found in soil in the form of sulfate and, through a set of reaction, is converted to sulfide and into an N/C-skeleton forms cysteine or its homologues (Droux 2004). The assimilation of sulfate could be summarized in four steps: (1) uptake of sulfate, (2) activation of sulfate, (3) reduction of sulfate and (4) synthesis of cysteine. Sulfate uptake is facilitated by sulfate transporters; once sulfate is within the cells, it can either be stored or enter the metabolic stream. Metabolism of sulfate is initiated by its activation by the reaction of adenylation catalyzed by ATP-sulfurylase. The reaction product adenosine 5′-phosphosulfate (APS) is a branch point intermediate, which can be channeled toward reduction or sulfation (Leustek et al. 2000). The key enzyme of plant sulfate assimilation is the adenosine 5′-phosphosulfate reductase (APR), which reduces activated sulfate as demonstrated by control flux analysis (Vauclare et al. 2002). The sulfate assimilation pathway and APR are highly regulated in a demand-driven manner (Lappartient and Touraine 1996; Leustek et al. 2000; Vauclare et al. 2002; Kopriva 2006; Davidian and Kopriva 2010). In periods of low sulfate availability, plants increase sulfate transport and rate of reduction. Activation of sulfate reduction is the dominant route for assimilation and is carried out in plastids (Leustek et al. 2000; Saito 2000). APS is reduced to sulfite by APS-reductase (APR) (Leustek and Saito 1999; Kopriva and Koprivova 2003), and finally sulfite is reduced to sulfide by sulfite reductase (SiR). Sulfide is then transferred to activated serine by *O*-acetylserine(thiol)lyase (OAS-TL) to form cysteine. The formation of cysteine is a direct coupling step between S and N assimilation in plants (Brunold 1990, 1993; Brunold et al. 2003). Cysteine is the precursor or S-donor for most other organic S-compounds in plants. In addition, cysteine is the precursor of

GSH, a low molecular weight water soluble non-protein thiol compound which functions in protection of plants against varied environmental stresses (De Kok et al. 2005).

Cysteine synthesis in plants represents the final step of assimilatory sulfate reduction and the almost exclusive entry reaction of reduced sulfur into plant metabolism (Wirtz and Hell 2006). The importance of reduced sulfur is further illustrated by the multitude of functions that are directly or indirectly mediated by the major sulfur metabolites cysteine, methionine and GSH. This step also marks the convergence of S and N metabolism giving rise to regulatory interactions (Kopriva et al. 2002). Further, increased rate of synthesis and accumulation of cysteine occur in order to form GSH during the abiotic defense response of plants to heavy metals or xenobiotics (Rüegsegger and Brunold 1992; Farago et al. 1994).

Sulfur assimilation has an important effect on Cd accumulation. A significant induction in S assimilation has been reported in heavy metal exposed higher plants (Tukiendorf and Rauser 1990). Exposure of plants to Cd induces enzymes involved in the sulfate assimilation pathway (Herbette et al. 2006; Khan et al. 2007). Cadmium stress induces intracellular sulfur sink because of increased PC synthesis. In response, the plant may adapt the expression of genes involved in S assimilation and GSH biosynthesis, allowing an increased flux through the entire pathway when sulfate is not limiting. In *S. cerevisiae*, very high Cd concentrations (1 mM) led to upregulation of the MET3 gene (ATPS, Fauchon et al. 2002) and concomitantly, ATPS protein levels increased sixfold (Vido et al. 2001). In addition, a microarray analysis of *S. cerevisiae* under yet another Cd concentration (300 μM CdCl) showed that MET14 (APK) and MET 16 (PAPSR) had increased gene expression of 21- or 6-fold (Momose and Iwahashi 2001).

It has been noted that genes involved in S assimilation pathway are rapidly upregulated such as Sultr1;1 and Sultr2;1 encoding two sulfate transporters which are upregulated after 2 or 6 h of Cd treatment and 12–24 h after sulfate depletion (Takahashi et al. 2000; Herbette et al. 2006; Sarry et al. 2006). Heiss et al. (1999) reported the upregulation of sulfate transporter(s), ATPS and APR under Cd stress. Hermsen et al. (2010) reported that mRNA levels of APR, SiR2 and SULTR4;1 were increased while ATPS2, SULTR1;1, -1;2 and -1;3 were significantly reduced upon Cd treatment in *P. patens*.

Nussbaum et al. (1988) reported that Cd accumulation increased ATPS and APR activities in *Zea mays* seedlings. Rüegsegger et al. (1990) have shown that APR activity is induced coordinately with glutathione synthetase in Cd-treated *Pisum sativum* plants. ATPS is regulated as demand-driven homeostatic mechanisms in order to match the rate of cysteine and GSH biosynthesis with the total sulfur needs of the whole plant (Lappartient and Touraine 1996; Lappartient et al. 1999).

ATPS and serine acetyl transferase (SAT) play important roles in heavy metal tolerance and accumulation (Hawkesford 2003; Freeman et al. 2004). Harada et al. (2002) reported the expression of three sulfur assimilation pathway enzyme genes including ATPS increased significantly, and that the total thiol compounds increased threefold under Cd stress in *Arabidopsis*. These may be due to the need of thiol for increased chelation. Rother et al. (2006) have reported that in heavy metal dependent induction of genes involved in cysteine and GSH biosynthesis, the increased activity of the corresponding proteins and thiol metabolites are the bases for elevated intracellular GSH concentrations. Treatment with Cd^{2+} influenced the transcriptions of genes coding for enzymes involved in sulfate reduction and GSH biosynthesis in time- and concentration-dependent manner. However, transcription of the SAT gene, encoding the OAS-generating enzyme, was hardly affected upon Cd^{2+} stress in *P. patens*.

Krishnan (2005) reported increased OASTL in Cd-treated soybean. Manipulation of critical enzymes in the sulfur assimilation pathway is one of the most promising approaches for increasing the amounts of sulfur containing amino acids in soybean. In fact, the relatively higher expression of GmOASTL4 gene occurred in transgenic plants treated with $CdCl_2$ than that in untreated transgenic plants, which could explain the clear induction of OASTL activity. Ning et al. (2010) reported that GmOASTL4 overexpression stimulates the

antioxidant enzyme system. Howarth et al. (2003) observed an increase in the expression of all SAT genes in response to cadmium in *A. thaliana*.

In *A. thaliana*, OASTL gene Atcys-3A and the SAT gene Sat-5 are induced in leaf lamina, root and stem cortex and trichomes in response to Cd treatment (Domínguez-Solís et al. 2001; Howarth et al. 2003). Additionally, the overexpression of Atcys-3A in *A. thaliana* resulted in increased Cd tolerance (Domínguez-Solís et al. 2001). These results suggest that specific OASTL and SAT isoforms may play a role in increasing cysteine production under conditions of heavy metal stress when increased biosynthesis of GSH is required. Plants overexpressing SAT were found to have elevated levels of cysteine and glutathione when compared to the wild type (Blaszczyk et al. 1999; Harms et al. 2000). Thus, the overexpression of S-assimilating enzymes led to the production of GSH and subsequently PC which are involved in Cd detoxification.

5 Sulfur Sinks and Cd Tolerance

Glutathione is a tripeptide thiol (Glu-Cys-Gly) and is a major water soluble antioxidant and redox buffer in plants, performing critical functions in cell cycle regulation, development, sulfur transport and storage, stress responses, and heavy metal detoxification (Maughan and Foyer 2006). It is synthesized from its immediate precursor cysteine formed through reductive sulfur assimilation pathway in a cascade of enzymatic reactions (Kopriva 2006). The synthesis of GSH is accomplished in two sequential ATP-dependent reactions catalyzed by c-glutamylcysteine synthetase (cECS) and glutathione synthetase (GSHS) (Becana et al. 2010). Sulfur supplementation might further help the plants to improve the content of GSH by enhancing γ-ECS enzymes (Schneider and Bergmann 1995; Strohm et al. 1995). Hassan et al. (2008) reported that addition of $(NH_4)_2SO_4$ to Cd-fed plants showed increased glutathione content, which reduced oxidative stress in *Oryza sativa* plants that were related to more S supply for this N form. As discussed above, the entry of Cd into the cytosol promptly activates the sulfur metabolism resulting in the production of GSH. Glutathione, besides performing critical functions in regulating plant growth and adaptation to abiotic stresses, acts as an important S sink in the plant system (Leustek et al. 2000; Maughan and Foyer 2006).

Glutathione occurs in reduced form (GSH) in plant tissues and is localized in all cell compartments such as cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, peroxisomes as well as in apoplast (Jimenez et al. 1998). It is necessary to maintain the normal reduced state of cells so as to counteract the inhibitory effects of ROS-induced oxidative stress (Meyer 2008) and is a potential scavenger of $^1O_2$ and H_2O_2 (Briviba et al. 1997; Noctor and Foyer 1998). In addition, GSH is a substrate for glutathione peroxidase (GPX) and glutathione-S-transferases (GST), which are also involved in the removal of ROS (Noctor et al. 2002). Glutathione-S-transferases can reduce peroxides with the help of GSH and produce scavengers of cytotoxic and genotoxic compounds. An increased GST activity was found in leaves and roots of Cd-exposed *Pisum sativum* plants by Dixit et al. (2001) and in roots of *Oryza sativa* and *Phragmites australis* plants (Iannelli et al. 2002; Moons 2003). Besides, GSH plays a key role in the antioxidative defense system by regenerating another potential water soluble antioxidants such as Ascorbate (AsA), via the Ascorbate–glutathione (AsA–GSH) cycle (Foyer and Halliwell 1976). GSH is a constituent of the AsA–GSH cycle that is a major pathway for the conversion of H_2O_2 and is stimulated under Cd stress (Smeets et al. 2008; Smeets et al. 2009).

Further, Chen et al. (2010) reported that GSH promotes acclimative and adaptive responses in antioxidant systems of barley seedlings to cope with Cd stress. It not only acts as a directly preformed antioxidant to scavenge ROS but might also act indirectly in modifying the redox balance by pro- or con-activating such antioxidant responses depending on its endogenous levels to alleviate Cd toxicity. They reported that GSH-induced alleviation of Cd stress by stimulating APX (especially cAPX, sAPX and tAPX iosenzymes) and CAT activities. It upregulated root cAPX and leaf cAPX, CAT1 and CAT2 expression

Table 20.1 Role of thiols in reducing cadmium (Cd)-induced oxidative stress. (+) sign indicate increase and (–) sign indicate decrease

Thiols	Increase/ decrease	Plants	Parameters	Response	Reference
Glutathione (added)	Increase	*Brassica juncea*	Cd content	–	Zhu et al. (1999a)
Glutathione (added)	Increase	*Glycine max*	$OH^{\bullet}$	–	El-Shintinawy (1999)
Phytochelatin (added)	Increase	Transgenic *Nicotiana tobacum*	Cd content	–	Harada et al. (2001)
Glutathione	Increase	*Phragmites australis*	Antioxidative enzymes	+	Pietrini et al. (2003) and Ederli et al. (2004)
Phytochelatin	Increase	Bread wheat	H_2O_2	–	Ranieri et al. (2005)
Glutathione	Increase	*Oryza sativa*	MDA	–	Hassan et al. (2005)
Glutathione	Decrease	*Helianthus annuus*	TBARS	+	Gallego et al. (2005)
Glutathione	Increase	*Brassica chinensis* and *Brassica pekinensis*	Cd content	–	Liu et al. (2007)
Glutathione	Increase	*Brassica campestris*	Cd content, TBARS	–	Anjum et al. (2008)
Glutathione and total sulfur	Increase	*Sedum alfredii*	H_2O_2	–	Chao et al. (2008)
Phytochelatin	Increase	*Lycopersicon esculentum*	Cd content	–	Ammar et al. (2008)
Cysteine and glutathione (added)	Increase	*Tagetes erecta* L.	Cd content	–	Feng et al. (2009)
Glutathione	Increase	*Brassica juncea*	H_2O_2, TBARS, e-leakage	–	Khan et al. (2009) and Iqbal et al. (2010)
Glutathione (added)	Increase	*Solanum nigrum*	TBARS, H_2O_2, superoxides	–	Deng et al. (2010)
Glutathione	Increase	*Cucurbita pepo*	Lipid peroxidation	+	Kolb et al. (2010)
Glutathione (added)	Increase	*Solanum tuberosum*	Oxidative stress	–	El-Tayeb et al. (2010)

in the sensitive genotype of barley to achieve stimulation. Cuypers et al. (2010) reported that glutathione metabolism played a crucial role in controlling the gene regulation of the antioxidative defense mechanism under Cd stress in the roots of *Arabidopsis thaliana*.

The role of GSH in the antioxidant defense system provides a strong basis for its use as a stress marker (Tausz et al. 2004). Pietrini et al. (2003) reported that the antioxidant activity in the leaves and chloroplast of *Phragmites australis* Trin. (cav.) ex Steudel was associated with a large pool of GSH which resulted in protecting the activity of many photosynthetic enzymes against the thiophilic bursting of Cd. GSH is a precursor of PCs, which plays an important role in controlling cellular heavy metal concentration (Grill et al. 1985). Increased GSH levels are connected with enhanced plant tolerance to stress (Stancheva et al. 2010). Xiang et al. (2001) observed that plants with low levels of GSH were highly sensitive to even low levels of Cd^{2+} due to limited PC synthesis.

Therefore, sulfur plays an important role in Cd detoxification through the synthesis of GSH and PC. Fan et al. (2010) reported that sulfur could alleviate the Cd stress in rice due to S-induced increase of GSH for synthesis of PCs related to Cd tolerance. Studies have shown that thiols reduce Cd-induced oxidative stress (Zhu et al. 1999a, b; Ammar et al. 2008; Feng et al. 2009; Khan et al. 2009; Iqbal et al. 2010; Kolb et al. 2010, Table 20.1).

Phytochelatins are peptides that consist of repetitions of the γ-glutamylcysteine (γ-GluCys)

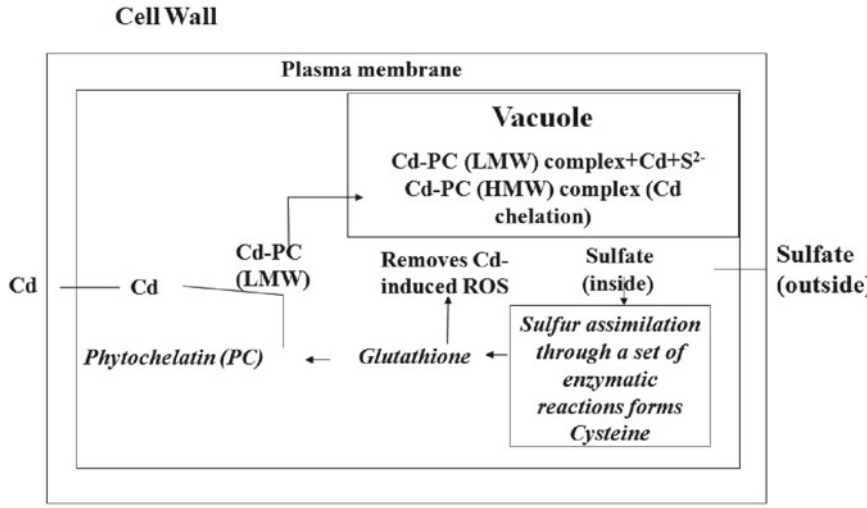

Fig. 20.1 Representation of sulfur assimilation and cadmium tolerance. *ROS* reactive oxygen species, *LMW* low molecular weight complex, *HMW* high molecular weight complex

dipeptide followed by a terminal glycine. The basic structure being (γ-Glu-Cys)-Gly, where n is generally in the range 2–7. These peptides are synthesized from glutathione and related thiols by PC synthases (PCS, a γ-glutamylcysteinyl transpeptidase (EC 2.3.2.15)). PCS mediates a dipeptidyl transfer reaction in the presence of heavy metals, leading to the formation of PCn with a greater number of γ-glutamylcysteinyl units, which has increased the affinity for heavy metals and the capacity to sequester them more effectively (Löffler et al. 1989; Clemens 2006).

Phytochelatins have been involved in metal detoxification, and used as potential biomarkers for an evaluation of metal toxicity (Wang et al. 2008). The levels of PCs were positively correlated with external metal concentrations (Keltjens and van Beusichem 1998; Sneller et al. 1999; Sun et al. 2005). The increase in PCs has been reported to increase Cd tolerance in plants (Martínez et al. 2006; Pomponi et al. 2006; Gasic and Korban 2007). It is an important intracellular Cd chelating molecule that mediates the sequestration of the heavy metal-PC conjugant in the vacuole (Siripornadulsil et al. 2002; Mendoza-Cozatl and Moreno-Sanchez 2006). Overproduction of PCS from wheat in *S. cerevisiae* enhances tolerance to and accumulation of cadmium (Clemens et al. 1999).

Cadmium leads to activation of sulfur metabolism leading to the production of PC (Sanita di Toppi and Gabbrielli 1999) since biosynthesis of PC is closely dependent on sulfur metabolism (Leustek et al. 2000). An increased expression of genes involved in sulfur assimilation and GSH and PC syntheses in response to Cd treatment has been shown in *Arabidopsis* (Harada et al. 2002), and *Brassica juncea* (Heiss et al. 1999). Among the mechanism utilized by the plants to detoxify ROS, production of GSH appears to be more important as this is a substrate for the biosynthesis of PCs, which are involved in heavy metal detoxification (Inouhe 2005). Sulfur assimilation results in GSH synthesis that detoxifies ROS either directly or through the synthesis of PCs. Phytochelatins are involved in Cd removal by chelation. They form low molecular weight Cd-PC complex (LMW) that enters the vacuole and combines there with more Cd and sulfide to form high molecular weight Cd-PC complex (HMW), thus, binding maximum Cd and storing it into vacuole. This keeps the cell free from Cd toxicity (Fig. 20.1).

6 Conclusion and Future Perspective

Cadmium toxicity can be alleviated by modulating various enzymes involved in S assimilation and manipulating the pathway of S metabolism. In fact, the nutritional and environmental conditions may act as molecular signals in the regulation of S assimilation under stress (Nazar et al. 2011). Chen and Huerta (1997) have shown that S is a critical nutritional factor for alleviating Cd toxicity. A positive effect of S on Cd detoxification has also been reported by Popovic et al. (1996). It is most likely that plant type with higher S accumulation capacity is expected to show more tolerance to Cd stress. Overexpression of sulfate assimilating enzymes increases tolerance to heavy metals (Pilon-Smits et al. 1999; Domínguez-Solís et al. 2001). The general alteration of the sulfur metabolic pathways induced by Cd is a possible consequence of an increase in the GSH demand driven by PC biosynthesis. In other words, exposure to Cd would induce an "additional sink" increasing the need for thiol compounds by cells (Tukiendorf and Rauser 1990; Heiss et al. 1999) which could be compensated by application of S.

The mechanisms of alleviation of Cd stress by sulfur may also include synthesis of phytohormones in addition to reduced S compounds. Phytohormones are believed to influence biochemical and molecular mechanisms under optimal and stressful environment. Cadmium stress affects the signaling of phytohormones and its toxicity can also be reverted by phytohormones (Hsu et al. 2006; Çelik et al. 2008; Dalcorso et al. 2008; Popova et al. 2009; Stroiński et al. 2010). In this context, it may be said that S could alleviate Cd toxicity by affecting ethylene evolution because *S*-adenosyl methionine (SAM), an ethylene precursor, contains sulfur. Cysteine is the central precursor of all organic molecules containing reduced sulfur including SAM (a precursor of ethylene), which acts as a signaling molecule in the control of plant developmental processes under optimal and stress conditions (Höfgen et al. 2001). Sulfur nutrition thus may provide a novel strategy to reduce Cd toxicity through various pathways that are accelerated by its application.

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Role of Salicylic Acid in Alleviating Heavy Metal Stress

21

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Abstract

Both plant breeders and crop producers have an interest in finding crops capable of tolerating environmental changes with damage as little as possible. In order to develop such crops, the knowledge of plant defense mechanisms and regulatory processes is essential. The study presented in this chapter was performed to analyze the role of salicylic acid (SA) in regulation of plant growth and development, flowering, ion uptake, stomatal regulation and photosynthesis. The role of SA in development of plant resistance to different environmental stresses is described. Besides the physiological functions of SA, the general properties, biosynthesis and metabolism of this plant growth regulator are discussed. The present chapter focuses on the mechanisms of the beneficial effect of SA on maize plants exposed to toxic Cd concentrations.

Exposure of plants to Cd (10, 15 and 25 μM) caused a gradual decrease in the dry weight accumulation of shoots and roots. Pretreatment of seeds with 500-μM SA for 6 h alleviated the negative effect of Cd on plant growth parameters. The same tendency was observed for the chlorophyll level. The rate of CO2 fixation was lower in Cd-treated plants, and the inhibition was partially overcome in SA-pretreated plants. A drop in the activities of carboxylating enzymes ribulose-1,5-bisphosphate carboxylase (RuBPCase) and phosphoenolpyruvate carboxylase (PEPCase) was observed for Cd-treated plants. Pretreatment with SA alleviated the inhibitory effect of Cd on the enzymes activity. In vivo the excess of Cd-induced alterations in the redox cycling of oxygen-evolving centers and the assimilatory capacity of maize leaves as revealed by changes in the termoluminescence emission. Pretreatment with SA before imposition of high

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concentration of Cd has a stabilizing effect on photochemical reactions. Changes in the activity of several important antioxidative enzymes, namely superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (POD), glutathione reductase (GR) and glutathione-*S*-transferase (G-S-Tr) were measured. The presence of Cd in the nutrient solution led to disturbances in the activity of the antioxidant enzymes. Pretreatment with SA alleviated the negative effect of Cd on the studied enzymes. Our results suggest that the phytotoxicity of Cd is mainly induced by oxidative stress and SA is involved in the defense responses of maize plants to Cd exposure. This suggestion was consistent with the observed protective role of SA on the lipid membranes of Cd-treated maize plants.

Keywords
Maize • Oxidative stress • Photosynthesis • Thermoluminescence • Salicylic acid

1 Introduction

Salicylic acid (SA) is a widespread phenolic compound, and many of its physiological and biochemical effects have been known for a long time (Raskin 1992a). As a potent signaling molecule in plants it is involved in eliciting specific responses to biotic and abiotic stresses. It has been shown that SA provides protection in maize plants against different stresses, e.g., low-temperature stress (Janda et al. 1999; Tasgin et al. 2003) induces thermotolerance in mustard seedlings (Chen et al. 1997; Dat et al. 1998a, b) or modulates plant responses to salt and osmotic stresses (Borsani et al. 2001), ozone or UV light (Sharma et al. 1996), drought (Senaratna et al. 2000; Singh and Usha 2003), herbicides (Ananieva et al. 2004) or pathogens (Malamy et al. 1990; Durner et al. 1997). Furthermore, SA is also known to be involved in plant protection to heavy metals. SA pretreatment alleviates Pb- and Hg-induced membrane damage in rice (Mishra and Chudhuri 1999) and Cd toxicity in barley (Metwally et al. 2003) and maize plants (Pal et al. 2002).

Cadmium (Cd^{2+}) is a highly toxic trace element which enters the environment mainly from industrial processes and phosphate fertilizers. It can reach high levels in agricultural soils and is easily assimilated by plants. Taken up in excess by plants it induces various visible symptoms of phytotoxicity, for example, leaf roll, chlorosis and growth reduction of root and shoot, browning of root tips and finally death (Kahle 1993). A large number of studies have demonstrated the toxic effect of Cd on of photosynthesis through effects on the chlorophyll metabolism and chloroplasts structure (Gadallah 1995), the activity of both photosystem II and the enzymes of photosynthetic carbon metabolism (Krantev et al. 2008; Popova et al. 2009). Cadmium also produces alterations in the functionality of membranes by inducing changes in their lipid composition (Hernandez and Cooke 1997), and this can affect some enzymatic activities associated with membranes such as H^+-ATPase (Fodor et al. 1995). Once Cd enters the cytosol, other detoxification mechanisms are induced, primarily the formation of complexes between Cd and phytochelatins (PCs) and their subsequent compartmentalization (Vazquez et al. 2006). Other mechanisms that plants have developed to cope with damages caused by cadmium are related with some stress signaling molecules, such as SA, jasmonic acid and ethylene. All these compounds were induced by Cd treatment, which suggest that they are involved in cell response to Cd toxicity (Rodriguez-Serrano et al. 2006).

Although there have been many reports on the photochemical and biochemical events occurring

in photosynthesis during Cd toxicity, a lot of contradictory data can be found in the literature. Probably this is because of the very heterogeneous experimental approaches, including both laboratory-grown conditions and field experiments. Only a limited number of studies have been carried out during the germinating stage of plants. We have attempted to study the effect of exposure of maize plants to Cd during early stages of their establishment, on the physiological and biochemical properties of maize leaves. Our main purpose was to determine the physiological and biochemical changes in maize plants treated by SA during Cd-induced stress, to investigate whether this plant regulator is involved in the induction of defense response and to test the hypothesis that the observed protection of SA on photosynthesis against Cd stress is mediated by its effect on antioxidant defense system.

2 SA: Properties, Structure and Biosynthesis

SA or ortho-hydroxybenzoic acid and related compounds belong to a diverse group of plant phenolics. Salicylates from plant sources have been used in medicines since antiquity. In 1828, in Munich, was isolated for the first time a small amount of salicin, the glucoside of salicyl alcohol, from willow bar. Ten years latter Raffaele Piria named it SA, from the Latin word Salix for willow tree. Aspirin, a trade name for acetylsalicylic acid (ASA), undergoes spontaneous hydrolysis to SA. It is rapidly converted to SA when applied exogenously. Despite the fact that aspirin was not identified as a natural product, it is widely used by many plant scientists in their experiments. The reason is the similarity in their physiological effects (Popova et al. 1997; Hayat et al. 2010).

SA could be actively transported, metabolized or conjugated, and it could also translocate rapidly from the point of initial application to different plant tissues. Salicylates are distributed in many important agricultural plant species. In many plants, such as rice, crabgrass, barley, soybean, etc., the levels of SA has been found to be approximately 1 μg g^{-1} fresh weight. A survey of SA in leaves and reproductive structures of nonthermogenic angiosperms confirmed the ubiquitous distribution of this compound in plants. Levels of SA varied substantially in the floral parts of seven nonthermogenic species and in the leaves of 27 nonthermogenic plants. The highest levels of SA were determined in the inflorescence with necrotizing pathogens (Raskin et al. 1990).

In the early 1960s it was suggested that in plants SA is synthesized from cinnamic acid by two possible pathways: one involves side-chain decarboxylation of cinnamic acid to benzoic acid followed by 2-hydroxylation to SA. Alternatively, cinnamic acid could be first 2-hydroxylated to an *o*-coumaric acid and then decarboxylated to SA. These pathways differ in the order of ß-oxidation and ortho-hydroxylation reactions and could operate independently in plants. Two key enzymes are involved in SA biosynthesis and metabolism: benzoic acid 2-hydroxylase, which converts benzoic acid to SA, and UDP glucose: SA glucosyltransferase, which catalyzes conversion of SA to SA glucoside. It was shown that the cinnamic acid → benzoic acid → SA pathway functions in rice seedlings (Sillverman et al. 1995) and tobacco plants (Yalpani et al. 1993). However, recently, genetic studies in *Arabidopsis* have shown that SA was also produced via chorismic acid – an important intermediate of shikimic acid pathways, and the rate of SA biosynthesis and excretion can be substantial (Wildermuth et al. 2001).

Some publications have suggested that the rate-limiting step in SA biosynthesis is the conversion of *trans*-cinnamic acid to benzoic acid, and that this involves a ß-oxidation pathway in which *trans*-cinnamoyl CoA is the first of four intermediates. However, evidence is increasing for an alternative, as yet undefined, rout to SA that does not involve benzoic acid (Hayat et al. 2010).

Many different types of SA conjugates have been found in plant species. They were mainly formed by glucosylation and less frequently by esterification. Large amounts of SA glucosides were detected in sunflower, oat and bean roots (Yalpani et al. 1992a, b). Methyl salicylate was found in the leaves of oats, red clover, tobacco and in the volatile fractions of fruits (plum, strawberry, black cherry and tomato). Large amounts

of volatile methyl salicylate are released from tobacco mosaic virus (TMV)-inoculated tobacco indicating that this compound may be a major metabolite of SA. It is tempting to speculate that methylsalicylate may function as an airborne signal for both intra- and inter-plant communication. SA forms also conjugates with aminoacids. Salicyloyl aspartic acid was identified in wild grapes, but its physiological function is not known (Sillverman et al. 1995).

3 Physiological Functions of Salicylates

The first indication for a physiological effect of SA was the discovery of flower-inducing action and bud formation in tobacco cell cultures (Eberhard et al. 1989). The stimulatory effect of SA on flowering was later demonstrated in other plant species, and this was ground for suggesting that SA functions as an endogenous regulator of flowering. The SA effect was not specific and it promoted flowering in combination with other plant regulators (e.g., gibberellins).

The ability of certain *Arum lilies* to generate heat in the inflorescences during blooming has provoked studies on the role of SA in heat production. When SA was applied to the inflorescence exogenously, it elicited heat production in spadix. On the day proceeding blooming, the levels of endogenous SA in the spadix increased 100-fold to 1 μg g^{-1} fresh weight and returned to control levels at the end of the thermogenic period. By this way SA appears to be calorigen. The heating is believed to be associated with a large increase in the cyanide-insensitive, non-phosphorylating electron transport pathway. It was also shown that SA caused the induction of the alternative oxidase gene and the alternative oxidase protein with molecular mass of 38.9 kDa was isolated and characterized (Rhoads and McIntosh 1991). The effects of SA on the alternative pathway respiration in slices and isolated mitochondria of dormant and dormancy-breaking potato tubers were compared (Wen and Liang 1994). It was found that treatment with 20-μM SA increased the capacity of cyanide-resistant respiration in both model systems. The involvement of the alternative pathway was enhanced by SA to a greater extent in dormancy-breaking potato tubers. The thermal effect of SA was abolished with inhibitors of the alternative pathway, such as salicylhydroxamic acid and propyl gallate. It was suggested that, as in thermogenic species, SA increased both the activity of the total respiration and the cyanide-resistant pathway in tobacco leaves, leading to an elevation in surface temperature (Van der Straeten et al. 1995).

There are experimental data indicating participation of SA in signal regulation of gene expression in the course of leaf senescence in *Arabidopsis* (Morris et al. 2000). Moreover, SA might serve as a regulator of gravitropism, and the inhibition of fruit ripening (Srivastava and Dwivedi 2000). SA is an effective inhibitor of ethylene biosynthesis, the effect being pH-dependent.

More widespread interest in SA was generated when it was closely linked to the hypersensitive response, a disease-resistant mechanism in which plants restrict the spread of fungal, bacterial or viral pathogens by producing necrotic lesions around the initial point of penetration. Treatment of tobacco plants with aspirin solution showed that plants have enhanced resistance to TMV and reduced number and size of necrotic lesions. Treatment of tobacco genotypes with SA resulted in the coordinate expression of pathogenesis-related (PR) genes. Several lines of evidence suggest that SA is the endogenous signal involved in induction of PR protein synthesis and systemic acquired resistance (SAR) in tobacco and cucumber. It has been established that during infection by pathogens, a huge number of proteins are induced, including PR protein families, which are thought to play an active role in defense processes.

Resistance to pathogens and the production of some PR proteins in plants can be induced by SA or aspirin, even in the absence of pathogenic organisms. Exogenously applied SA-induced PR proteins mainly at the side of application, in contrast to pathogens that induced PR proteins systemically (Hayat et al. 2010). The levels of SA increased dramatically following inoculation of tobacco or cucumber plants with pathogens.

Increases in SA levels have been correlated to changes in gene expression. The production of active oxygen species such as superoxide ions is a rapid response that precedes cell death. It has been proposed that oxygen radicals play a direct role in cell death during the hypersensitive response. H_2O_2 formed during the oxidative burst may also trigger cell death and serve as a diffusible signal for induction of defense-related genes in surrounding cells (Levine et al. 1994). Leon et al. (1995) have demonstrated that treatment of tobacco leaves with H_2O_2 induced accumulation of free benzoic acid and SA. They have suggested that H_2O_2 activated SA biosynthesis.

Wounding the leaves by chewing insects or other mechanical damage induces the synthesis of defensive proteinase inhibitor proteins in both wounded leaves and distal unwounded leaves. Stomatal behavior and regulation are a very important factor for photosynthetic ability. The established effects of SA on stomatal function, chlorophyll content, transpiration rate and respiratory pathways lead to the assumption that SA might possess another physiological function, most probably involved in regulation of some photosynthetic reactions. Since the past 15 years our team had studied the role of SA in regulation of photosynthesis. Pancheva et al. (1996) demonstrated that long-term treatment (7 days) of barley seedlings with SA decreased the rate of photosynthesis and the activity of RuBP carboxylase, and increased both CO_2-compensation point and stomatal resistance. The short-term treatment with SA (from minutes to 2 h) did not affect either the rate of photosynthesis or the capacity of biochemical machinery as compared with untreated control plants. An explanation for these changes could in part be because of stomatal closure and reduced supply of CO_2. However, the Ci-values were not declined in SA-treated plants. This implies that stomatal closure did not restrict CO_2 entry into the leaf enough to reduce internal CO_2 level and the reduction in photosynthesis probably was nonstomatal. It was suggested that one very possible reason for the observed inhibition of photosynthetic ability and RuBP carboxylase activity could be the effect of SA on protein synthesis, including ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) synthesis. It was also found that treatment of barley plants with SA caused decrease in the level of total soluble protein, in particular in the level of RubisCO. The percentage of inhibition on the small subunit was higher, as a result of which the small/large subunit ratio was lower for the experimental variants (Pancheva and Popova 1998). It was suggested that SA like some other stress factors diminished chloroplast photosynthetic activity as a result of effects on the thylakoid membranes and light-induced reactions connected with them, and in this way it may indirectly participate in regulating the activity of RubisCO. Additional data showed that treatment of barley seedlings with SA caused alterations in leaf anatomy and chloroplast ultrastructure (Uzunova and Popova 2000). The suggestion was that the observed low rate of growth and photosynthesis might be associated with disorders of leaf anatomy and plastid ultrastructure. At the same experimental conditions Maslenkova and Toncheva (1998) observed the inhibitory effect of SA on PSII oxygen-evolving reactions. The effect of SA depended on the time of treatment duration – no changes in these parameters were observed when barley seedlings were treated with SA for 2 h, the inhibition appeared 6 h after the start of treatment. These data confirm the suggestion that SA plays different roles based on its endogenous levels in particular plant species under specific developmental and environmental conditions (Pancheva et al. 1996).

The ability of SA and ASA to induce a protective effect on plants under stress received considerable interest during the past few years. It has been shown that SA provides protection in maize (Janda et al. 1999) and winter wheat plants (Tasgin et al. 2003) against low-temperature stress, induces thermotolerance in mustard seedlings (Chen et al. 1997; Dat et al. 1998b) or modulates plant responses to salt and osmotic stresses (Borsani et al. 2001), ozone or UV light (Sharma et al. 1996), drought (Senaratna et al. 2000) and herbicides (Ananieva et al. 2004). SA has been shown to accumulate in plants in response to various oxidizing stresses, such as H_2O_2 (Leon et al. 1995), ozone (Sharma et al. 1996), heat

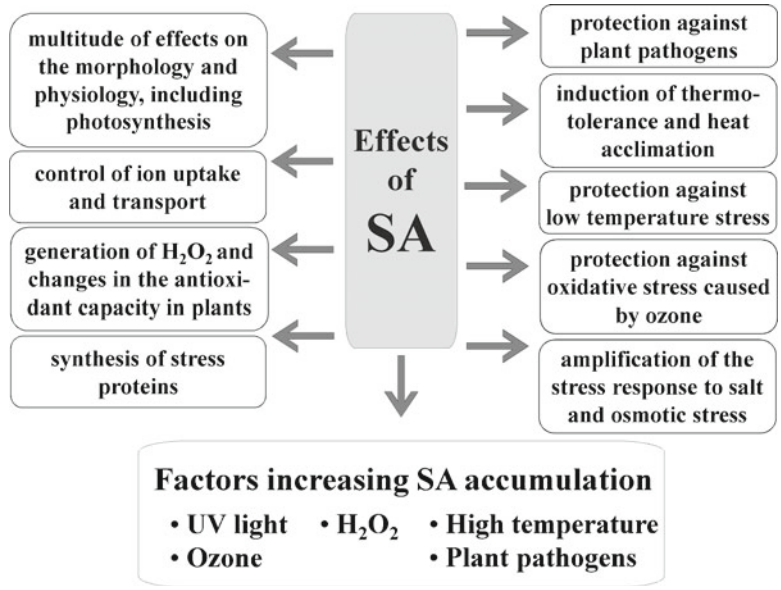

Fig. 21.1 Physiological effects of SA and factor affecting changes in its endogenous accumulation

(Dat et al. 1998b), and it has been suggested that it is directly involved in signaling different antioxidant responses (Larkindale and Knight 2002). These relationships are presented in Fig. 21.1.

4 SA and Heavy Metal Stress

The environmental quality is endangered by many adverse effects. The most important problem for plant ecosystems is a consequence of the contamination by toxic wastes, biotic stress factors and abnormal climate changes. In addition to industrial pollution (heavy metals, pesticides, oil residues, etc.), the global warming and dehydration as well as pathogen infection are recognized as environmental constraints that the vegetation endures.

Heavy metals are used in various industries and are consequently discharged into the environment. At least 20 metals are known to be toxic, and fully half of these, including cadmium (Cd), copper (Cu), mercury (Hg), nickel (Ni), silver (Ag) and zinc (Zn), are released into the environment in sufficient quantities to present a risk to human health. Soil heavy metal contamination is increasingly severe worldwide and the global average annual emission of Hg, Cu, Pb, Mn and Ni are 15,000 tons, 3.4 million tons, 5 million tons, 15 million tons and 1 million tons, respectively (Popova and Lu 2010).

Cadmium (Cd) is a trace metal, which may accumulate to potential harmful levels in certain crops. It is one of the most toxic pollutants found in air, water and soil. It is highly toxic to plants and animals. Accumulation of cadmium in plant tissue is influenced by the levels of cadmium available in the soil in which the plants are grown. It should be mentioned that phosphate fertilizers additionally increase the level of Cd in soil. Cadmium is easily taken up by plant roots and can be loaded into the xylem for its transport into leaves. Cd induces genetic and biochemical changes in plant metabolism related to general and Cd-specific stress responses. A large number of studies have demonstrated the toxic effect of Cd on plant metabolism, such as decreased uptake of nutrient elements (Sandalio et al. 2001), changes in nitrogen metabolism (Boussama et al. 1999), inhibition of photosynthesis through effects on the chlorophyll metabolism and chloroplasts

structure (Gadallah 1995; Stoyanova and Tchakalova 1997; Stoyanova and Merakchiiska-Nikolova 1992), inhibition of stomatal opening (Barcelo and Poschenrieder 1990) and activity of both photosystem II and the enzymes of photosynthetic carbon metabolism (Krupa and Baszynski 1995; Krantev et al. 2008; Popova et al. 2009). The main targets of the influence of Cd are two key enzymes of CO_2 fixation, RubisCO and phosphoenolpyruvate carboxylase (PEPCase). It has been shown that Cd^{2+} ions lower the activity of ribulose-1,5-bisphosphate carboxylase (RuBPCase) and damage its structure by substituting for Mg^{2+} ions, which are important cofactors of carboxylation reactions and also Cd can shift RuBPCase activity toward oxygenation reactions (Siedlecka et al. 1998). Stiborova (1988) and Malik et al. (1992a, b) demonstrated that Cd caused an irreversible dissociation of the large and small subunits of RuBPCase thus leading to total inhibition of the enzyme. The early studies indicated that Cd ions affect the oxidizing side of PS2 and lead to uncoupling of electron transport in chloroplasts (Atal et al. 1993; Mohantly and Mohanty 1988). With regard to site and mechanism of inhibition of Cd, it is generally accepted that the water-oxidizing complex (OEC) of PS2 is affected by Cd by replacing the Ca^{2+} in Ca/Mn clusters that constitutes the oxygen-evolving centers (Sigfridsson et al. 2004) or by some modifications in Qb-binding site (Geiken et al. 1998).

Cadmium ions are known to cause alterations in the functionality of membranes by affecting the lipid composition (Quariti et al. 1997) and some enzymatic activities associated with membranes, such as H^+-ATPase (Fodor et al. 1995). Evidences suggest that cadmium toxicity induces oxidative stress, as a result of stimulation of free oxygen radical production (Sanita di Toppi and Gabrielli 1999) and by modified activity of various antioxidant enzymes (Hagedus et al. 2001). To avoid Cd toxicity, plants adopt various defense strategies, including phytochelation and sequestration as well as induction of antioxidant machinery and stress proteins (Cobbett and Goldsbrough 2002; Vazquez et al. 2006). The prevailing part of cell Cd is bound to phytochelatins (PC) and transported into vacuole as PC–Cd–S complex through Fe-dependent transporters (Hall and Williams 2003). Survival under stressful conditions depends on the plant's ability to perceive the stimulus, generate and transmit signals, and induce biochemical changes that adjust the metabolism accordingly. Therefore, the search for signal molecules that mediate the stress tolerance is an important step in our better understanding on how plants acclimate to the adverse environment.

Convincing data have been obtained on SA-induced plant protection to heavy metals, demonstrating the involvement of SA in the induction of different antistress programs. SA pretreatment alleviates Pb-induced membrane damage in rice (Mishra and Chudhuri 1999), Hg-induced oxidative stress in *Medicago sativa* (Zhou et al. 2009), and Cd toxicity in barley (Metwally et al. 2003) and maize plants (Pal et al. 2002; Krantev et al. 2008). Recently He et al. (2010) showed that pretreatment of rice seeds with 0.1-mM SA for 24 h alleviated the negative effect of Cd on germination parameters and early seedlings growth. Kovacik et al. (2009) studying the effect of SA on Cd and Ni uptake in Chamomile (*Matricaria chamomilla*) reported that it is differentially modulated by SA. In case of Ni, it seems that SA serves as a root barrier in order to prevent Ni from reaching the above-ground organs. The exogenous application of SA conferred Al tolerance to the plants of *Cassia tora*, exposed to Al toxicity that was mediated by an increase of citrate efflux in roots of the treated plants (Yang et al. 2003). Shi and Zhu (2008) reported that exogenous SA alleviated the toxicity generated in *Cucumis sativus* by manganese exposure and the response was mediated by reduction in ROS level and lipid peroxidation.

Recent experiments revealed a clear relationship between metal stress and redox homeostasis and antioxidant capacity. It was proposed that SA serves as a link between the degree of plant tolerance to metals and the level of antioxidants (Sharma and Dietz 2009).

5 Role of SA in the Regulation of Cadmium Toxicity in Maize (*Zea Mays* L.)

Different plant species and varieties show a wide rage of plasticity in Cd tolerance, reaching from the high degree of sensitivity to the hyper accumulating phenotype of some tolerant plants. Legume plants are less tolerant to Cd toxicity than cereals and grasses (Metwally et al. 2005). Although there have been many reports on the photochemical and biochemical events occurring in photosynthesis during Cd toxicity, a lot of contradictory data can be found in the literature. Probably this is because of the very heterogeneous experimental approaches, including both laboratory-grown conditions and field experiments. Only a limited number of studies have been carried out during the germinating stage of plants. We have attempted to study the effect of exposure of maize plants to Cd during the early stages of their establishment, on their physiological and biochemical properties. The study reported here is mainly focused on the mechanisms by which SA influences the photosynthetic processes to overcome Cd toxicity in maize plants. In appropriate parts of the work we tried to compare the obtained results with maize to pea plants because they differ in their sensitivity to Cd toxicity. It has been shown that Cd produced a concentration-dependant reduction in the growth of maize and pea. Under our experimental conditions, pea plants did not tolerate Cd concentrations higher than 5 μM without showing any visible toxicity symptoms. This confirms the data by other authors (Belimov et al. 2003) that pea plants can be considered as cadmium-sensitive species.

Maize plants grown for 14 days with 10-, 15- and 25-μM $CdCl_2$ exhibited a significant inhibition of growth. After 14 days of treatment, plants grown on 25-μM Cd showed visible toxicity symptoms and the survival rate declined greatly.

The comparison of the growth injuries of both plant species confirmed that maize plants are able to tolerate higher Cd concentrations. In general, it can be said that the sensitivity of given plant species to heavy metal toxicity depends on its concentration, the treatment duration on the plant species, its age and the plant organ examined.

Next data will be focused only on physiological changes of maize plants to Cd toxicity and on the protective role of SA.

Changes in some parameters associated with oxidative stress, namely proline production, lipid peroxidation, CO_2 fixation and the activity of the carboxylating enzymes RuBPCase and PEPCase, were assayed as they are known to be most affected by Cd treatment. The other goal was to test the hypothesis that the observed protection of SA on photosynthesis against Cd stress is mediated by its effect on antioxidant defense system. also In addition we tried to explain the possible effect of SA on Cd toxicity with events leading to detoxification of cadmium.

5.1 CO_2 Assimilation Rate and Carboxylating Enzymes Activity

Here we demonstrated that growth inhibition of maize plants was accompanied by a decrease in the rate of photosynthetic CO_2 fixation expressed on both fresh weight and chlorophyll content bases because Cd treatment caused a reduction in chlorophyll level.

The activities of both carboxylating enzymes (RuBPCase and PEPCase) were also affected by Cd treatment. PEPCase activity was reduced only after exposure to 25-μM Cd, while RuBPCase activity exhibited a strong reduction at all Cd concentrations applied.

Pretreatment of maize plants with SA before exposure to Cd alleviated the inhibitory effect of Cd and led to nearly a twofold increase in PEPCase activity compared with untreated plants. A very strong protective effect of SA was observed on RuBPCase activity (Fig. 21.2).

5.2 Thermoluminescence Emission in Cd-Stressed and SA-Pretreated Maize Leaves

The illumination of unfrozen dark adapted leaves from nontreated (control) maize plants with two consecutive flashes (2Fl) induced a main TL peak

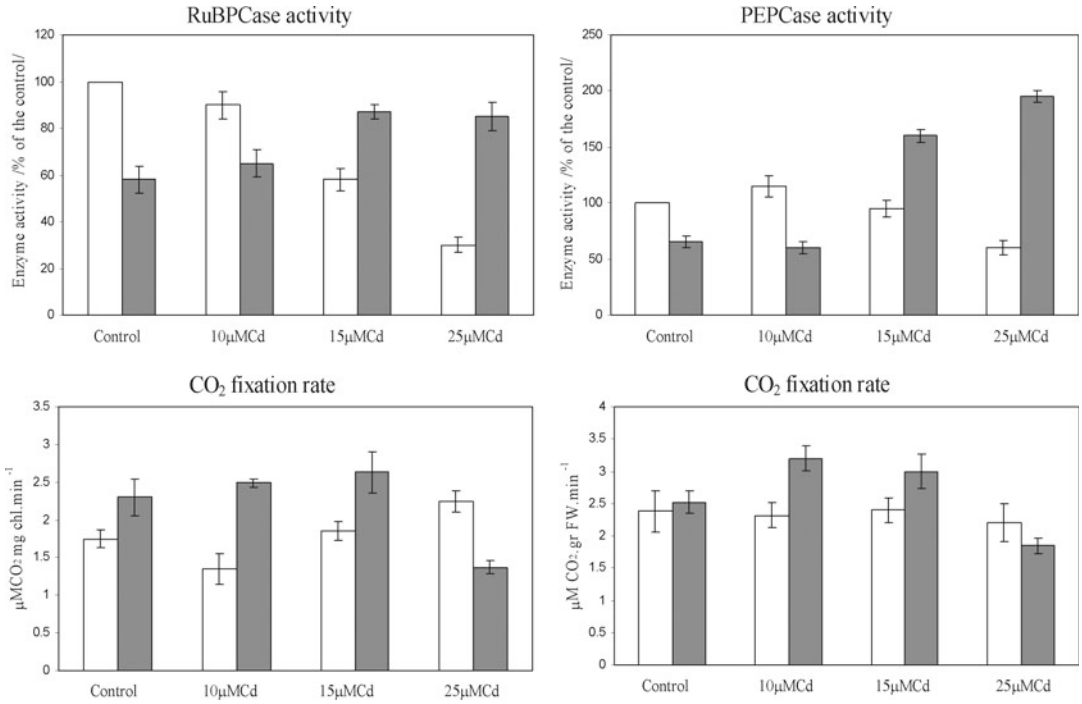

Fig. 21.2 Photosynthetic CO_2 fixation rate and activities of carboxylating enzymes RuBPCase and PEPCase in maize plants treated with Cd or pretreated with SA before exposure to Cd. Dry seeds were soaked in 500-μM SA (*black bars*) or water (*white bars*) for 6 h and were germinated for 3 days in moist filter paper. They were grown for 14 days in hydroponic medium without Cd or with Cd (in the respective concentrations). Data are means ± s.e. ($n = 3$) from three experiments

at 35°C, the so-called B-band (S_2Qb^-) and a hardly distinguishable shoulder at temperature about 42°C (Fig. 21.3), designated as afterglow (AG). The B-band results from the thermal activated recombination of the trapped electrons and positive charges on the reduced quinone acceptor (Qb^-) and the $S_2(S_3)$ oxidation state of the water-oxidizing complex of PSII, respectively (Rutherford et al. 1982). The AG TL emission corresponds to a back electron transfer toward PSII centers initially in the $S_{2(3)}QB$ state (Ducruet 2003). Increasing Cd concentrations decrease the overall TL intensity, the inhibitory effect of B-band amplitude being more expressed in comparison with AG emission (Fig. 21.3). A damping of period-four oscillation of B-band, according to exciting flash number, was also observed (not shown). The characteristic damping of oscillation of B-band intensity can be ascribed to already known effects of Cd on PSII, by increasing misses in charge separation. Short-term application of 500-mM SA to the maize seeds did not exert substantial changes in the investigated TL parameters. However, pretreatment with SA before the imposition of high concentration of heavy metal has a stabilizing effect on photochemical reactions, judging from some restoration of TL bands amplitude (Fig. 21.3) and B-band oscillation pattern (not shown).

5.3 Effect of SA on Chlorophyll Content, Proline Level and Relative Water Content

Chlorophyll content decreased only in the variant treated with 25-μM Cd and the inhibition was partially overcome in those pretreated with SA seeds. The concentration of the stress metabolite proline increased upon Cd exposure. The most

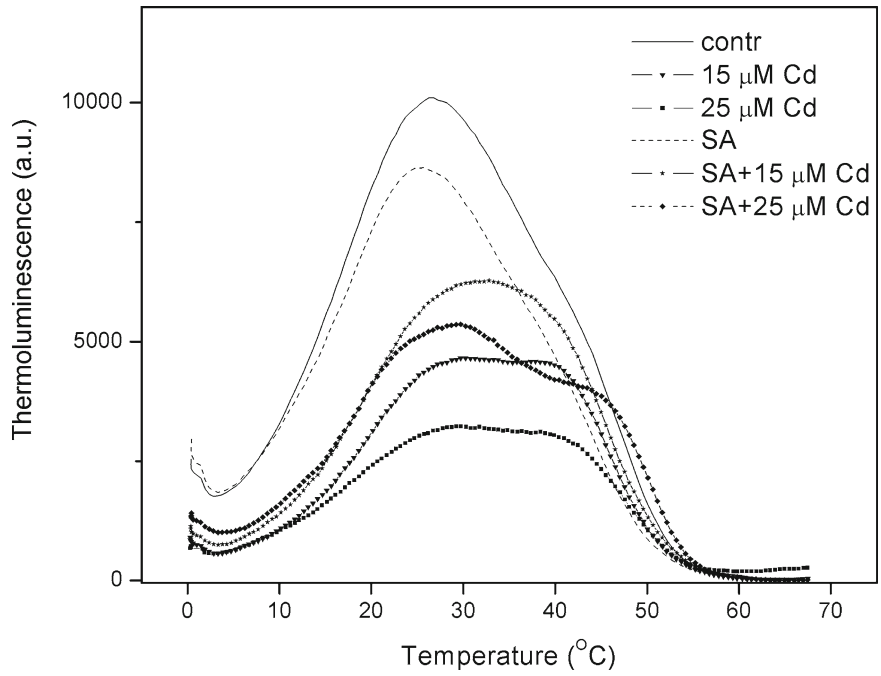

Fig. 21.3 Changes in thermoluminescense glow curves of maize plants treated with 15- and 25-mM Cd or pretreated with 500-mM SA before exposure to heavy metal. TL was recorded immediately after two flashes excitation, with a 0.5°C s^{-1} heating rate

prominent effect was observed at 15–25 μM Cd (a nearly twofold rise compared with the control). SA pretreatment counteracted the Cd-induced increase in proline levels. Exposure of maize plants to Cd led to a slight decrease in leaf RWC. The values of this parameter for the control plants were 95–97% and 92–93% for H_2O- and SA-presoaked plants, respectively. The reduction in RWC was approximately 6 and 11% for H_2O- and SA-presoaked plants, respectively, in the variants treated with 10–15 μM Cd (Table 21.1).

5.4 Effect of SA and Cd on the Amount of Biomass, Total Lipophilic Extracts and FAME

It is evident that SA treatment increased the amounts of the total lipophilic extracts and the lipid composition (expressed as FAME), but there were no statistically significant differences in the amounts of the leaf biomass (compare samples 1 and 5, Table 21.2). The treatment with Cd led to an increase of the total lipophilic extracts. The moderate Cd stress (10 μM) led to increased FAME, but the higher Cd concentration caused decreased FAME amounts (samples 3 and 4, Table 21.2). The decrease of lipids (respectively, FAME) was observed in Cd-stressed wheat (Malik et al. 1992a, b), barley (Vassilev 2004), tomato (Krupa and Baszynski 1989,1995, Quariti et al. 1997; Ben Ammar et al. 2005) and mustard (Gaur and Grupa 1994; Nouairi et al. 2006) plants. Pretreatment of maize plants with SA resulted in an increase in Cd-induced changes in lipid content by low and mild Cd stress. Only the severe Cd stress led to a decrease in FAME content, similar to those observed in non-pretreated with SA plants (compare samples 4 and 8, Table 21.2). Probably, by high Cd concentrations in the nutrient solution the SA cannot prevent the effect of Cd on lipid membranes of maize plants.

Table 21.1 Effect of Cd and SA on relative water content (RWC), chlorophyll content and proline accumulation. Means ± s.e, $n = 4$

Variants	RWC (%)	Chlorophyll $a+b$ (μg chl/g fw)	Proline (μmol/g fw)
Control	95.700	1.7 ± 0.11	1.12 ± 0.08
10-μM Cd	91.500	1.7 ± 0.09	1.60 ± 0.07
15-μM Cd	91.820	1.6 ± 0.10	2.38 ± 0.45
25-μM Cd	90.010	0.9 ± 0.08	2.26 ± 0.37
Control + SA	92.680	1.57 ± 0.10	0.82 ± 0.09
10-μM Cd	81.385	1.11 ± 0.09	0.94 ± 0.09
15-μM Cd	82.900	1.10 ± 0.10	0.91 ± 0.06
25-μM Cd	88.285	1.37 ± 0.08	1.02 ± 0.07

Table 21.2 Effect of SA and Cd on the amount of biomass, total lipophilic extracts and FAME in maize leaves

No.	Variants/samples	Dry weight (g)[a]	Total lipophilic extract (mg g^{-1} DW)[a]	FAME (mg g^{-1} DW)[b]
1	Control	0.7 ± 0.1	150 ± 6	6.3 ± 0.5
2	10-μM Cd	0.6 ± 0.1	183 ± 8	10.2 ± 0.8
3	15-μM Cd	0.6 ± 0.1	175 ± 7	1.8 ± 0.1
4	25-μM Cd	0.5 ± 0.1	170 ± 7	3.2 ± 0.2
5	Control + SA	0.8 ± 0.1	185 ± 8	10.9 ± 0.9
6	10-μM Cd	0.7 ± 0.1	214 ± 11	11.0 ± 0.9
7	15-μM Cd	0.5 ± 0.1	100 ± 4	10.4 ± 0.8
8	25-μM Cd	0.6 ± 0.1	175 ± 7	4.7 ± 0.4

[a] Results ± s.e. from three parallel experiments
[b] Results obtained from three parallel preparative thin-layer chromatographic and GC procedures

5.5 Effect of SA and Cd^{2+} on the Fatty Acid Composition in Maize Leaves

Treatment with Cd (samples 2–4, Table 21.3) led to a decrease in the content of the short-chain (C14–C15) and an increase in the long-chain fatty acids (C20–C24). Possibly the Cd treatment caused activation of enzymes, responsible for elongation of C18 – acids. The higher Cd stress (sample 4, Table 21.3) increased the content of saturated acids (16:0 and 18:0) and decreased the content of linolenic (18:3) acid. This is a typical reaction of plant lipid membranes to environmental stress, leading to decreased lipid membranes permeability. The same effect was observed in other plants too (Jemal et al. 2000). The FA changes in maize plants treated with low doses of Cd were apparently smaller. A similar effect was observed in tomato plants (Quariti et al. 1997). The more severe Cd stress led to decreased linolenic acid content, as observed in other Cd-treated plants (Krupa and Baszynski 1989; Jemal et al. 2000; Vassilev 2004; Nouairi et al. 2006). The changes in the content of the oleic (C18:1) and linoleic (C18:2) acids showed a reserve trend. The same was observed in mustard seeds treated with Cd (Gaur and Grupa 1994). The content of saturated acids increased, but the amount of hexadecanoic acids remained constant. Pretreatment of maize plants with SA seemed to have an insignificant effect on their fatty acid composition (sample 5, Table 21.3). There was a slight increase of linolenic acid and a decrease of all saturated FA.

The mild Cd stress (10 μM) applied on the pretreated with SA plants led to the same changes in their FA composition, as in the nontreated (compare samples 2 and 6, Table 21.1). The

Table 21.3 Effect of SA and Cd on the fatty acid composition in maize leaves

		Fatty acids (wt% of total)*												
No.	Variants/samples	14:0	14:1	15:0	15:1	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0	24:0
1	Control	3.3	3.2	1.2	1.9	25.1	3.7	3.1	3.8	14.8	36.3	1.0	1.4	1.3
2	10-μM Cd	0.4	2.2	0.9	1.8	23.4	3.5	2.6	3.7	16.0	42.4	0.8	1.0	1.4
3	15-μM Cd	1.2	2.7	1.0	1.5	27.2	4.0	3.2	3.7	15.8	35.6	1.0	1.5	1.7
4	25-μM Cd	1.0	3.1	1.4	2.2	33.2	3.5	4.2	5.0	14.3	26.3	1.4	2.1	2.4
5	Control + SA	1.5	2.4	0.8	1.8	22.9	3.5	2.6	4.5	16.4	40.4	0.7	1.3	1.1
6	10-μM Cd	1.7	2.2	0.8	1.4	21.5	4.1	2.2	3.4	18.5	41.1	0.7	1.1	1.2
7	15-μM Cd	0.6	1.4	0.2	1.2	20.5	3.1	2.3	4.5	18.4	46.3	0.4	0.6	0.4
8	25-μM Cd	1.8	3.5	1.1	1.6	21.9	3.5	2.4	2.9	13.7	43.9	0.8	1.4	1.6

* Values obtained from three parallel measurements; the s.e. (related to peak proportions on the chromatogram) are as follows: ±0.3 for 16:0 and 18:3; ±0.2 for 18:2 and ±0.1 for the others

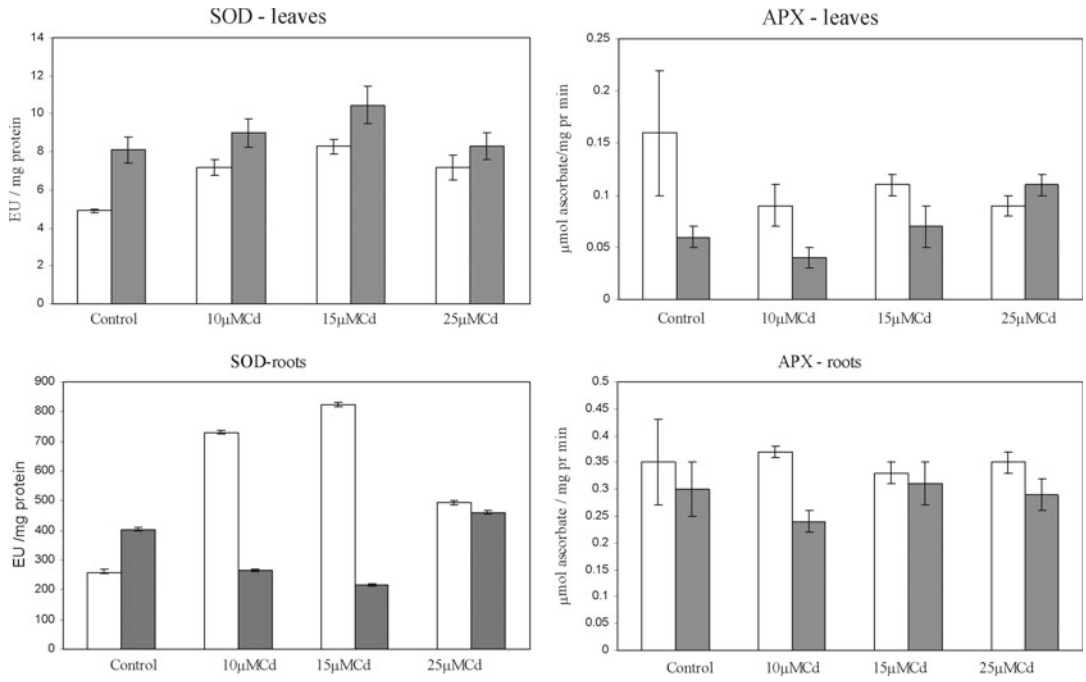

Fig. 21.4 Effect of Cd and SA on SOD and APX activity in leaves and roots of 14-day-old maize plants. For variants and treatments, see Fig. 21.2. Data are means of four independent experiments ± s.e.

amount of the saturated acids decreased, whereas the content of linoleic and linolenic acid increased. The severe Cd stress (25 μM) applied on the pretreated plants with SA led to FA profiles, similar to those of the control plants (compare samples 1 and 8, Table 21.3). In all cases, the changes in fatty acid composition after severe Cd stress in the pretreated plants with SA were opposite, as in the nontreated plants (compare samples 4 and 8, Table 21.3). Antagonistic influences of Cd and SA have been observed also in other plants (Drazic and Mihailovic 2005; Drazic et al. 2006). On the basis of the results presented in Table 21.3, it could be concluded that SA plays a protective role on the lipid membranes of Cd-treated maize plants.

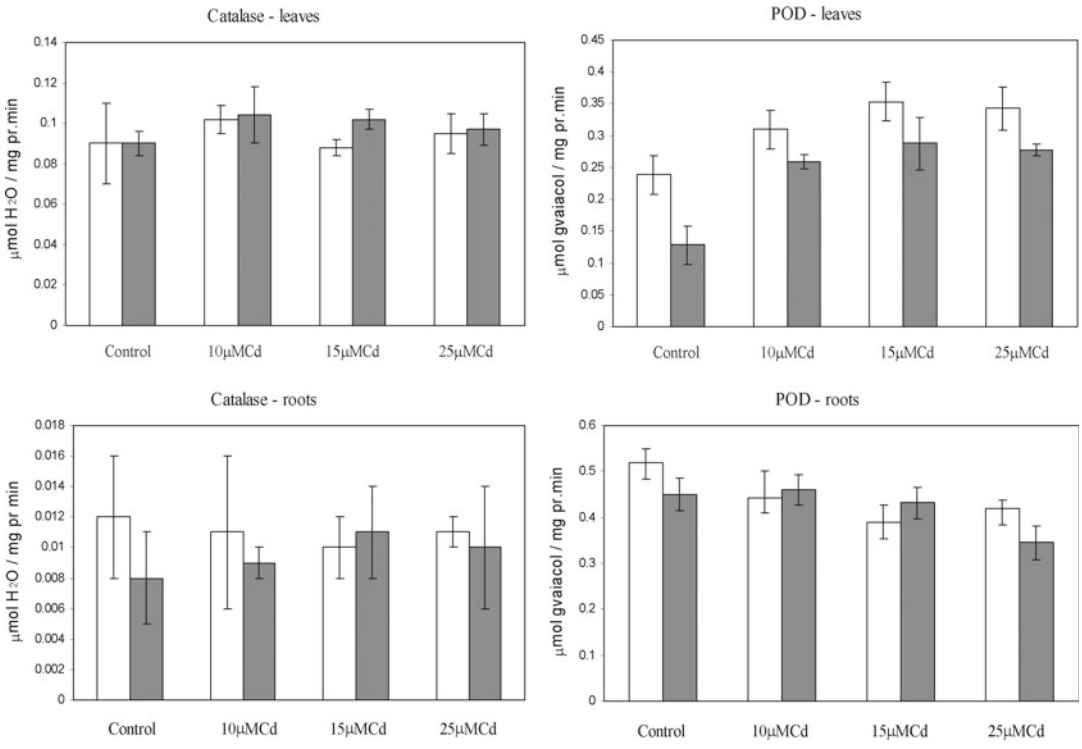

Fig. 21.5 Activity of CAT and POD in the leaves and roots of 14-day-old maize plants. For variants and treatments, see Fig. 21.2. Data are means of four independent experiments ± s.e.

5.6 Effect of SA and Cd on Antioxidant Enzymes Activity

The activity of the antioxidative enzymes was measured in leaves and roots of treated plants. The presence of Cd in the nutrient solution (10-, 15- and 25-µM Cd) led to disturbances in the activity of the antioxidative enzymes (Fig. 21.4). The superoxide dismutase (SOD) activity of leaves and roots increased at all Cd concentrations. Pretreatment with SA before Cd application kept the same tendency in leaves while SA reduced the activity of SOD of roots when compare with Cd treatment alone. In contrast to SOD, ascorbate peroxidase (APX) activity was suppressed by all Cd concentrations and SA treatment alone with smaller effect on roots.

Catalase (CAT) activity was not affected by Cd treatment both in leaves and roots. guaiacol peroxidase (POD) activity of leaves and roots increased in all Cd concentrations but dropped after SA pretreatment mainly in leaves (Fig. 21.5).

The same tendency was observed and for GR activity – nearly 50% increased in Cd-treated variants and without changes after SA pretreatment. Regarding to the last studied enzyme glutathione-*S*-transferase (G-S-Tr) we observed that Cd caused an opposite effect in leaves and roots. Pretreatment with SA alleviated the stimulating effect of Cd in leaves and had no significant effect on roots G-S-Tr (Fig. 21.6).

6 Discussion

Summarizing our results we showed that Cd produced a concentration-dependant reduction in the growth of maize plants (Photo 2). Maize plants grown for 14 days with 10-, 15- and 25-µM $CdCl_2$ produced a significant inhibition of growth. In our model system maize plants did not tolerate Cd concentrations higher than 25 µM, which suggests that maize can be considered as a relatively cadmium-sensitive species. We explain this result

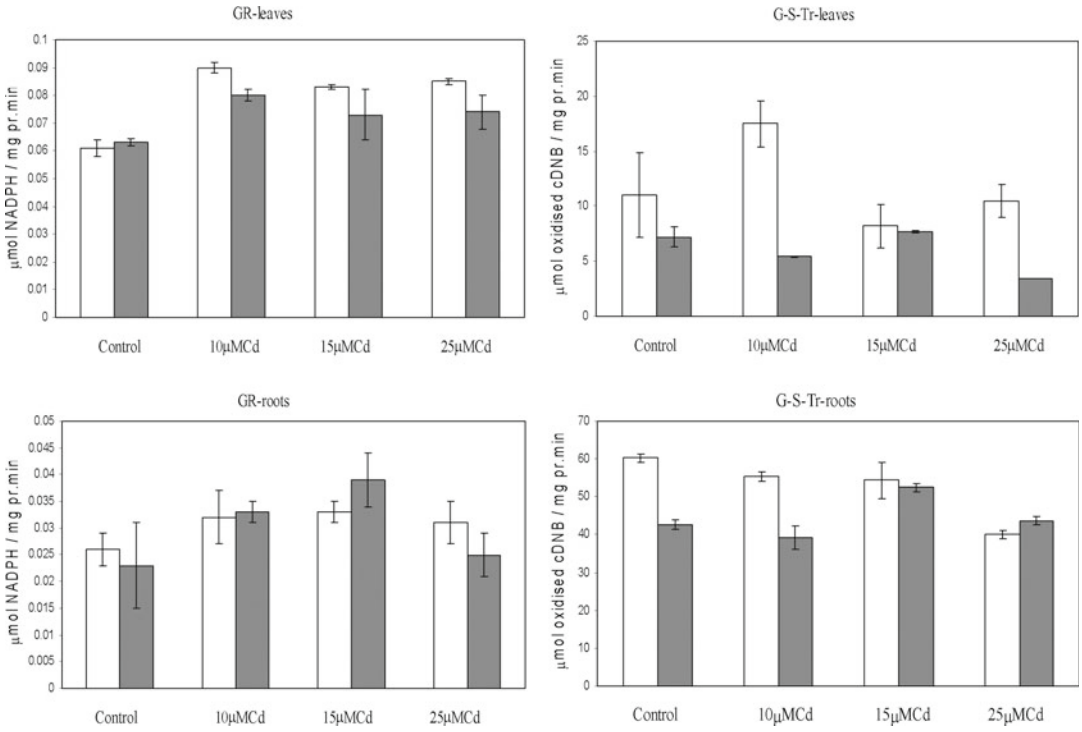

Fig. 21.6 Activity of GR and G-S-Tr in the leaves and roots of 14-day-old maize plants. For variants and treatments, see Fig. 21.2. Data are means of four independent experiments ± s.e.

with the fact that plants were exposed to Cd at the very early stage of their development. The data showed that chlorophyll content was reduced in Cd-treated plants (Table 21.1). The growth inhibition of maize plants was accompanied by a decrease in net photosynthesis measured as CO_2 assimilation (Fig. 21.2). The activity of RuBPCase (Fig. 21.2) and chlorophyll content (Table 21.1) also decreased with rising Cd concentrations.

These severe alterations on the chlorophyll level, chloroplast photochemistry and carboxylating enzyme activities are ultimately responsible for the destruction of photosynthesis caused by Cd. In addition to the negative effects of Cd on the photosynthetic carboxylation reactions PSII electron transport and especially oxygen-evolving complex were found to be very sensitive to the effect of Cd (Clijsters and van Assche 1985). Different components of electron transport chain were proposed as primary targets of Cd and different mechanisms of actions were discussed. Donor side (Clijsters and van Assche 1985; Krupa et al. 1993) or acceptor side (Atal et al. 1993) was implicated as a main site of the inhibition. In our attempt to contribute to the understanding the effects of cadmium on PSII reactions, an analysis of the TL glow curve parameters was performed in Cd-stressed pea. Our data showed that higher Cd concentrations affect the B-band oscillation pattern and decrease the TL intensity. These results showed a reduction in the number of PSII reaction centers and an increase of misses in charge separation thus suggesting that the centers cannot reach higher oxidation state S_3 and S_4. Although the B-TL band is specific for PSII charge recombination (Rutherford et al. 1982), the AG band is thought to originate from a feedback of reducing equivalents from the stroma toward PSII centers initially in TL inactive S2(S3) Qb state (Ducruet 2003). AG to reflect the [NADPH + ATP] assimilatory potential in the chloroplasts when induced by flashes has been proposed (Miranda and Ducruet 1995). The decrease of the assimilatory potential can be as a result of the reduced electron transport. In the case of high concentration of heavy metal, this

can be a result of harmful effect of reactive oxygen species on the thylakoid membrane composition and function. Our data showed that Cd toxicity, especially in sensitive pea plants, was linked to free radical processes in membrane components leading to alterations in membrane stability and increasing their permeability (Popova et al. 2009).

Presoaking of maize seeds for 6 h with 500-μM SA before exposure to Cd had a beneficial effect on growth, photosynthesis, carboxylation reactions, thermoluminescence characteristics and chlorophyll content, and it led to a decrease in oxidative injuries caused by Cd.

Recently we have published that leaves of maize plants contained both the SA-free and bound forms. Cd treatment caused accumulation of free and conjugated SAs, to a greater extent in the bound form (Krantev et al. 2008). A similar effect of cadmium on SA accumulation has been reported by Pal et al. (2005) for maize plants.

In addition, we have reported that Cd content of dry seeds and root tissue was low in the absence of Cd in the growth medium and strongly increased after treatment with Cd (Krantev et al. 2008). SA pretreatment led to an insignificant decrease in the root level of Cd in 15- and 25-μM Cd-treated plants. Our results excluded the possibility that formation of stable SA–Cd complexes has lowered Cd toxicity after SA pretreatment. Cd–SA complex formation in the hydroponic solution could not be the cause for the beneficial effect of SA because the exposure to Cd started 3 days after 6-h SA soaking of seeds. Another cause to exclude the formation of such a complex is that pretreatment with SA decreased the level of root Cd accumulation to a very lesser extent [from 27.23 ng/g fw for the control to 35.79 ng/g fw for SA-treated-alone maize plants (Krantev et al. 2008)].

Accumulation of large amounts of osmolytes (proline) is an adaptive response in plants exposed to stressful environment. Proline is known to accumulate in plants under drought, salt, hypoxia, UV radiation, etc. Proline accumulation appeared to be a suitable indicator of a heavy metal stress. The observed decrease in the level of proline in SA-pretreated seeds indicated partial relief from Cd stress (Table 21.1). Previously we found that SA pretreatment decreased MDA accumulation and altogether with the reported decline in the level of electrolyte leakage in pretreated with SA maize plants (Krantev et al. 2008) led to the suggestion that SA is involved against oxidative damage. Our data are in agreement with those reported by Metwally et al. (2003).

When plants are exposed to various environmental stresses they produce large quantities of ROS sufficient to disturb cellular and metabolic functions of the plants. These oxygen species (O^-, OH^-, H_2O_2) can convert fatty acids to toxic lipid peroxides, destroying biological membranes. Although Cd does not generate directly ROS like other heavy metals as Cu and Fe, it also generates oxidative stress via the interference with the antioxidant defense system (Sanita di Toppi and Gabrielli 1999; Somashekaraiah et al. 1992).

An important ROS-scavenging antioxidate enzyme is SOD. By catalyzing the detoxification of O_2^- (superoxide radical) to O_2, SOD blocks O_2^--driven cell damage. Our results showed that Cd treatment increased SOD activity both in roots and leaves indicating activation of the antioxidative system. The activity of SOD was found to be much higher in roots than in leaves and treatment with 10- and 15-μM Cd caused nearly threefold increase in the enzyme activity (Fig. 21.4). Among the H_2O_2 destroying enzymes it was the POD activity that was stimulated by Cd (Fig. 21.5). Pretreatment with SA lowered the activity of POD. This result fits well with the increased rate of lipid peroxidation reported recently by Krantev et al. (2008) and corresponds to other observations (Shaw 1995). The findings indicate that the activities of these enzymes (SOD and POD) are directly or indirectly regulated by SA, thereby providing protection against Cd stress. It is also known that POD participates in the lignin biosynthesis and by this way might build up a physical barrier against poisoning heavy metals. Earlier data in the literature concerning the CAT and APX response in leaves exposed to Cd stress are completely contradictory since both enzyme activation (Lee et al. 1976) and inhibition (Gallego et al. 1996) have been described. In our experiments Cd did not induce

changes in CAT activity (Fig. 21.5), while the activity of APX was decreased (Fig. 21.4). GR is known to catalyze some vital steps of the ascorbate–glutathione cycle. The enzyme maintains high ratio of GSH/GSSG, which is essential for the recovery of ascorbate so as to activate a number of enzymes involved in CO_2 fixation. Here we found that GR activity increased upon Cd treatment but suppressed upon SA treatment. Similar tendency was observed and for G-S-Tr activity and changes were well expressed both in roots' and leaves' tissues (Fig. 21.6).

Summarizing our results, we can conclude that among the H_2O_2-eliminating enzymes POD responds to Cd stress. The absence of changes in CAT activity suggests a different role of CAT in the heavy metal-induced oxidative stress. At the first glance, it may appear surprising that Cd, which is not a transient metal, may cause oxidative stress. However, Cd binds to thiol groups and thereby inactivates thiol-containing enzymes. That could be the reason for the observed inhibition of APX activity as it is known that the enzyme is sensitive to thiol reagents.

Our data suggest that endogenous SA plays an important anti-oxidant role in protecting maize plants from oxidative stress. SA is a direct scavenger of hydroxyl radical and iron-chelating compounds as well as their generation via the Fenton reaction (Dinis et al. 1994; Halliwell et al. 1995). Data have been presented for a salicylate–iron complex with SOD activity catalyzing the dismutation of superoxide radicals (Jay et al. 1999). Therefore, high levels of SA in maize plants may act directly as a preformed antioxidant to scavenge ROS and/or indirectly modulate redox balance through activation of antioxidant responses as was suggested by Yang et al. (2004) for rice plants. Rao and Davis (1999) proposed two different mechanisms to explain the role of SA in ozone-induced cell death in *Arabidopsis*. In some cases, SA potentiates the activation of antioxidant defense responses to minimize the oxidative stress induced by ozone, while in other cases, high levels of SA led to the activation of oxidative bursts and cell death. These examples demonstrate that SA is an important component in modulating stress responses and may play pro- or antioxidative roles based on its endogenous levels (Yang et al. 2004). It has been observed that not only SA but also other related compounds, such as oHCA, could provoke protection against abiotic stress (Janda et al. 2000). On the basis of ability of oHCA to quench singlet molecular oxygen, it was suggested that it may play a role in the antioxidative response (Foley et al. 1999).

Several hypothetical explanations may account for the positive effect of SA on Cd-induced stress in maize plants. SA prevented cumulative damage development in response to Cd. The suggestion is supported by the data of the lowered root level of Cd in SA-pretreated maize plants (Krantev et al. 2008). Similar data have been reported by Szalai et al. (2005) in maize and by Popova et al. (2009) in pea plants. Cd is usually accumulated in the roots, because this is the first organ exposed to heavy metals in the soil, but it is also translocated into the shoots. Obviously the lowered root level of Cd in SA-pretreated maize plants reduced the harmful effect of Cd and exerted a beneficial effect on the growth and photosynthesis. SA alleviated the oxidative damages caused by Cd. The values of MDA, electrolyte leakage (Krantev et al. 2008) and proline content of SA-pretreated plants were lower compared with the Cd-exposed plants (Table 21.1). Pretreatment with SA exerted a protective effect on the membrane stability judging by the increased total lipids' level and by changes in their FA composition.

Taken together these evidences support the conclusion that SA may indirectly attenuates Cd toxicity through a development of general antistress response of the plants which probably includes the regulation of antioxidant system and lipid metabolism leading to maintenance of membrane integrity.

7 Conclusion and Future Perspective

Phytohormones play a vital role in plant tolerance against environmental stresses. Plant resistance can be induced by adopting various strategies. One of these exogenous uses of various growth regulators and other chemicals has been proved worthwhile in producing resistance to many stresses in a

number of plants. Another important function of plant growth regulators is their agricultural use for improving various physiological parameters and crop yield. Among other regulators SA has been proved to be effective in increasing plant production and quality. Seed priming is a short-term and a very pragmatic approach for agricultural use. Another strategy is foliar application of plant growth regulators, both natural and synthetic. SA response against heavy metal stress is a new study in the field of crop physiology. Excessive use of chemical fertilizers in agriculture industries has appeared as a threat to soil health and yield. Results indicated that seed imbibitions with SA affected physiological processes related to growth and development and photosynthesis in maize plants. An important question of this study was how this short-term treatment with SA (presoaking of seeds for 6 h) affected certain physiological processes, such as plant growth, photosynthesis and antioxidant defense system. The beneficial effect of SA during the earlier growth period may help plants to avoid cumulative damage upon exposure to Cd. Alternatively, SA could be involved in the expression of specific proteins or defense-related enzymes. These results may provide a good background for strategies aimed at manipulating plants for decreased Cd content in order to develop crops capable of tolerating environmental changes with damage as little as possible.

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Bioremediation and Mitigation of Organic Contaminants in the Era of Climate Changes

22

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Abstract

In the present chapter, we addressed the possible links between climate changes and the fate, degradation, and mitigation of organic contaminants in the environment. Particular interest was devoted to techniques based on plants (phytoremediation, wetlands, and buffer strips), organic biomass residues (biobeds), as well as on bioremediation processes controlled by microorganisms. Climate change scenarios were identified, and the obtained info critically correlated on available info about the effects of climatic parameters (temperature, precipitations, soil humidity, pH, organic matter, and nutrients) on the fate, degradation, and mitigation of contaminants.

We conclude that climate change most probably has a significant effect on the fate and behavior of contaminants, and a more limited effect on bioremediation and mitigation strategies. Since fate and behavior determine the exposure of biological receptor to contaminant toxicity, it will be very important to carry out risk assessment evaluations in the context of climate change. Bioremediation and mitigation will remain as powerful tool to address the ever increasing global pollution. Attention must be devoted to adapt these tools to climatic changes, in order to maintain and, if possible, improve their efficiencies.

Keywords

Climate change • Organic contaminants • Bioremediation • Phytoremediation • Wetlands • Buffer strips • Biobeds

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1 Introduction

A large debate is ongoing about climate change: the main issues including the influence and responsibilities of anthropic activities, the possible predicted scenarios, and the effects on ecological system. Climate change is expected to have a wide range of impacts on the environment, such as direct effects from increased temperatures, changes in precipitation, more intense floods, droughts, hurricanes and storms, a lower air quality and a potential change in the environmental and human exposure to toxic environmental pollutants such as persistent organic pollutants (POPs), metals, and pesticides.

In the present chapter, we will throw light on different scenarios indicated by the scientific literature in relation to their possible effects on the main ecological parameters affecting the persistence and the bioremediation/mitigation of pollutants in the environment.

The chapter begins with a brief introduction on climate change, with description on the main possible scenarios depicted by scientists in terms of possible changes on ecological parameters (temperature, water availability, soil moisture, soil organic matter (SOM), and pesticides utilization); these scenarios are then critically discussed in order to assess their possible effects on the fate and behavior of organic contaminants in the environment and about the main strategies for their bioremediation and mitigation.

2 Climate Change Scenarios

The reconstruction of the historical climate trends of our planet demonstrates how climate is constantly changing, showing peculiar oscillations at different time scales. Climate is far from being constant, even in the short term, as the alternation of glacial and postglacial periods is accompanied by less dramatic short-term climate oscillations that nonetheless can result in significant alterations of ecosystems and living organism distribution (Dalla Valle et al. 2007).

The United Nations Intergovernmental Panel on Climate Change (IPCC 2007) has projected significant warming over Europe by the 2030s, with greater warming in winter in the North, and in summer in Southern and Central Europe. It is expected that the mean annual precipitation will increase in Northern Europe and decrease in Southern Europe. Significant changes to climate variability and extremes are also projected, although many of the studies also refer to the 2050s or 2080s.

In accordance with climate change model predictions, the IPCC has depicted different emission scenarios that are based on predictions about future demographic, economic, and technological developments and related greenhouse gases emissions (Nakicenovic et al. 2000). Six scenarios have been specifically made: A1 assumes a high demographic rise reaching its peak in the middle of the twenty-first century, but also includes the introduction of new efficient technologies. A1 is then divided into three subscenarios based on the relative future reliance on fossil fuels: A1F1 is fossil intensive, A1T is mostly based on nonfossil energy resources, and finally A1B is in between the two. A2 assumes high population growth, slow economic development, and slow technological change, B1 is assumed to be similar to A1, but with a lower technological development, and finally B2 is a scenario that takes into account the introduction of local solutions for economic, social, and environmental sustainability. For each of these scenarios, the ICPP has then made the following previsions of warming estimates at year 2090: 1.8°C for B1, 2.4°C for A1T, 2.4°C for B2, 2.8°C for A1B, 3.4°C for A2, and 4.0°C for A1F1.

The predictions at climate level have then been linked by the IPCC to the ecological level. According to IPCC (2007), it is likely that climate change and associated disturbances such as flooding, drought, and wildfires will affect negatively the resilience of many ecosystems in the world. The situation will be most probably worsened by other global change drivers such as changes in land use, pollution, fragmentation of natural systems, and resources overexploitation. It is also expected that the extinction rates for plant and

animal species will increase to approximately 20 to 30% for temperature increases ranging from 1.5 to 2.5°C.

Possible effects on crops productivity are more differentiated. Production is projected to increase slightly at mid-to-high latitudes for local mean temperature increases of up to 1 to 3°C depending on the crop, but it is then to decrease beyond that temperature. At lower latitudes the situation is instead different, since crop productivity is generally projected to decrease for even small local temperature increases (1–2°C).

One of the most important factors affected by climate change will be of course water and hydrogeological cycles. Previsions include changes in precipitation patterns with more intense and extreme events, melting of snow and ice, increases in evaporation and atmospheric water vapor, changes in soil moisture and runoff. A likely increase in typhoons and hurricanes is expected, as well as heavier precipitation and higher peak wind speed (IPCC 2007).

Another very important issue related to climate change is related to ice melting from mountain glaciers and icecaps (Raper and Braithwaite 2006): this will in turn not only raise the global sea level, but also affect the exchange rates of contaminants from air to water, and thus affect their environmental distribution.

3 Effects of Climate Change on Fate and Behavior of Organic Pollutants

Once organic contaminants are intentionally or unintentionally released into the environment, they undergo a series of distribution and fate processes related to their physicochemical properties and the properties and processes of the environment (Fig. 22.1). It is thus clear that the environmental scenarios related to climate change summarized earlier will indeed affect the fate and behavior of organic contaminants.

Chemicals may volatilize into the air, runoff or leach into surface water and groundwater. Volatilization is a major cause of losses (Kurtz and ACS 1990) and the rate of these losses often exceed that by chemical degradation, runoff, or leaching (Taylor and Spencer 1990). Loss of chemicals in the air typically range between 20 and 30% of the applied active ingredient during the application, and around 50–60% after the application and can sometimes reach up to 90% (Gregoire et al. 2009). Once volatilized, pesticides can then be easily deposited with rainfall events (Trevisan et al. 1993). A significant proportion may also remain in the soil as a persistent residue bound to the soil colloids (Calderbank 1989). In this bound state contaminants are usually difficult to extract and characterize, but they also tend to lose their biological activity.

Uptake of chemical from the soil by plants is another important route, and also a likely major source of food chain bioaccumulation and an important way of exposure to humans and animals (Paterson et al. 1990).

Water may disperse contaminants into the environment via foliar wash off, surface runoff, and leaching. Runoff may contribute to pollution of surface water, and leaching to contamination of groundwater.

Runoff may include dissolved, suspended particulate and sediment-adsorbed pollutants. Chemicals that remain at the soil surfaces for longer period of time, because they are strongly adsorbed and resistant to degradation and volatilization, will be more susceptible to runoff, whereas incorporation into the soil will reduce runoff risk (Larson et al. 1995).

Groundwater contamination is mainly due to the leaching through infiltration. The extent to which groundwater contamination occurs will depend among others on chemicals properties, soil characteristics, drainage rate, and water table depth. For many years, chemical mobility has been identified as a key characteristic in assessing groundwater potential pollution. However, mobility alone is not a good indicator of the groundwater pollution potential of a chemical, but rather the combination of mobility and persistence determines whether a compound will be degraded during its residence time in the zone above the groundwater (Jury et al. 1987; Gustafson 1989).

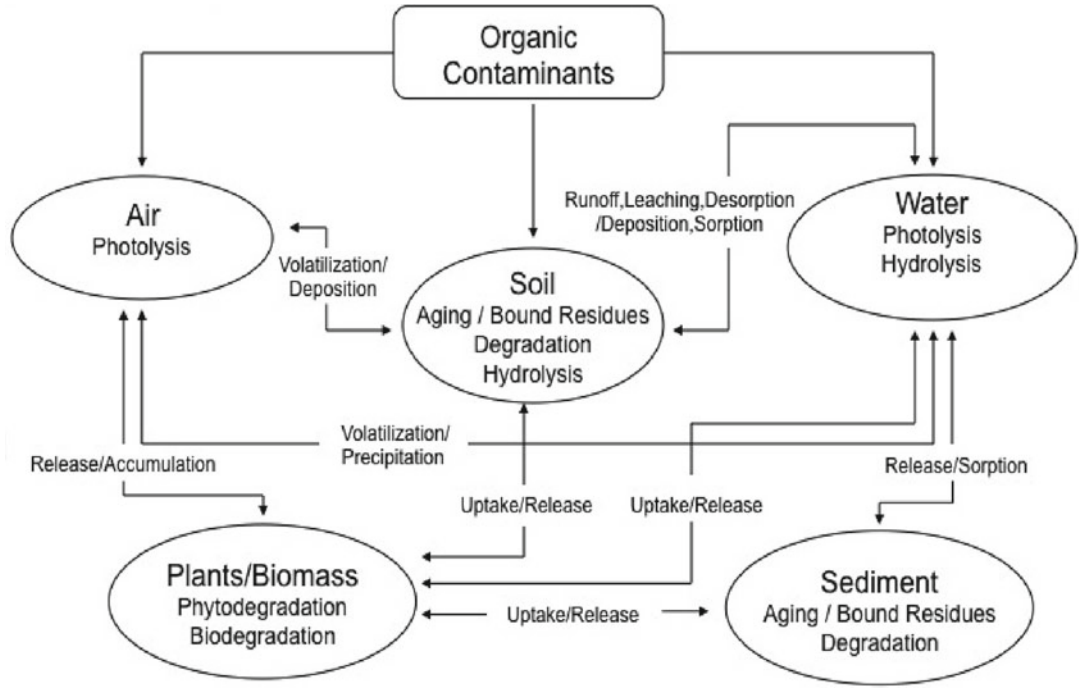

Fig. 22.1 Description of the processes affecting the fate and behavior of organic contaminants in soil, air, water, sediment, and plants/biomass

Table 22.1 Effects of increases in temperature (T) and precipitation (P) on the main processes governing the fate of organic contaminants in air, soil, and water

Effects	Environmental compartment	Expected impact on processes
↑T	Air	↑ Photodegradation
	Soil	↑ Volatilization
		↑ Microbial degradation
	Water	↑ Volatilization
		↑ Hydrolysis
		↑ Solubility
		↑ Microbial degradation
↑P	Air	↑ Deposition
	Soil	↑ Soil moisture
		↑ Runoff
		↑Leaching
		↓ Persistence
	Water	↑ Dilution

↑ increase, ↓ decrease

Even if concerns for surface water are often separated from groundwater, the hydrolytic cycle provides direct connection between these compartments (Leonard 1990). Therefore, levels of pollution in surfaces water may affect groundwater or in turn be affected by groundwater.

Climate change will affect organic contaminant fate and distribution in several ways: it is possible to distinguish between direct and indirect effects. Direct effects are those affecting the processes shown in Fig. 22.1. As discussed in detail later, it is expected that the increase in temperature and precipitation due to climate change will affect almost all processes involved in organic contaminants environmental fate. As outlined in Table 22.1, most of these processes will be increased, with different effects on contaminant distribution.

Indirect effects are instead related to shifts in agriculture systems because of climatic changes. Climate change is indeed likely to affect agriculture by shifting the locations and type of crops grown and the range and magnitude of crop: pesticide use will thus shift in response to these altered cropping patterns and crop pest distribution (Noyes et al. 2009).

A study was carried out in order to assess the possible effects of climate change on the range and severity of the plant disease "phoma stem canker," an important disease of *Brassica* crops caused by *Leptosphaeria maculans*, a fungal pathogen (Evans et al. 2008). In this study, scenarios for the future severity of epidemics in the UK were generated by joining weather-based models predicting the development of phoma stem canker epidemics with climate change models. Different parameters such as total rainfall, mean maximum temperature, start of leaf spotting and thermal canker, as well as different climate models were used to develop the multiparametric model. Results showed that increase in temperature as well in changes in the intensity of rainfall events will most probably affect not only the severity of the disease but also spread its geographical distribution, at least in the UK. This type of phenomena will surely affect the global costs and the use of pesticides. Chen and McCarl (2001) carried out a statistical study to assess how the costs of pesticide usage are influenced by temperature and precipitation. The investigation was carried out using pesticide usage and weather data, and considering corn, cotton, potato, and soybean cultivations in the USA. For all four crops, the authors found that increase in precipitation significantly increases pesticide usage cost. Regarding temperature, the results showed mixed effects: corn, cotton, and soybeans costs also increased with temperature but wheat costs decrease. In a following study, Reilly et al. (2003) predicted for US agriculture yield reduction for crops such as corn, potato, soybean, and cotton, and subsequent increase in pesticide production and use.

Climate change is also expected to affect the distribution of pollutants. Dalla Valle et al. (2007) conducted a semiquantitative assessment of the possible influence of climate change on the distribution and fate of some selected chemicals (namely, polychlorinated biphenyls (PCBs) and PCDDs) in a temperate region, the Venice Lagoon area, which is quite polluted by industrial activities. An environmental fate model was then applied, using experimental data about chemical properties and sources of pollution as model inputs. Three scenarios were then assumed. In scenario A, defined as the "control scenario," environmental conditions have been considered constant along the 50 y of the simulation, with constant temperature (15°C, the annual mean temperature in Venice), precipitation (900 mm/y), solar radiance, and degradation rate. Scenario B assumed a modest and gradual evolution of the initial conditions along the simulation run, with temperature increasing from 15 to 16°C, precipitations decreasing by 5% of the degradation rate increasing by 10%. In the last scenario C, more dramatic variations were assumed, with the mean annual temperature reaching 18°C, precipitations decreasing by 10%, and degradation rate increasing by 30%. Four PCB and PCDF congeners were considered: PCB 118, PCB 180, 1,2,3,4,7,8-HCDF, and 2,3,7,8- TCDF. The net result of the tested scenarios pointed towards a faster reduction of the overall contaminants budget, but at the same time an enhancement of their mobility. This may result in a greater transfer to polar regions, where degradation and removal from the environment is more difficult for such chemicals, causing an increase in bioaccumulation and biomagnification.

Lamon et al. (2009) reviewed a number of environmental factors potentially affected by climate change in relation to their possible effects on POPs environmental behavior. Wind and precipitation patterns can alter the way chemical redistribution in the environment, temperature is going to affect the degradation rate as well as the emission rate from soils, changes in duration and intensity in seasonal precipitation may lead to changes in the spatial and temporal distribution of POPs wet deposition and their degradation products, floods may result in POPs environmental dissemination as contaminated sediments may be redistributed on large uncontaminated soil areas, increase in salinity may lead to a decrease in POPs solubility. The situation is of course very

uncertain, because of the variability of the foreseen scenario and the lack of enough data, but the authors confirm the importance of taking into account climate changes in the monitoring, modeling, and risk assessment of pollutants fate.

Changes in climatic conditions can also have consequences on the ecotoxicity of pesticides. The effect of increase in salinity and temperature on the toxicity towards grass shrimp (*Palaemonetes pugio*) of two common pesticides, chlorothalonil and scourge (a mixture of resmethrin and piperonyl) has been assessed (deLorenzo et al. 2009). Toxicity of chlorothalonil increased with both temperature (10°C increase) and salinity (10 ppt increase), while the toxicity of the fungicide source was also increased by temperature, but reduced by salinity. These results suggest that standard toxicity bioassays may not be predictive of actual pesticide toxicity under variable environmental conditions, and testing under a wider range of exposure conditions is important in order to improve the accuracy of chemical risk assessments.

Assessment of the effects of increasing temperature on the response of labile and resistant soil carbon pools has been shown by Fang et al. (2005). Experiments carried out on incubating organic matter fractions of different lability at different temperature showed that also the stable fraction is affected by increasing temperature. These results, together with model evidences, indicate according to the authors that by 2100, the SOM loss could be up to 46% in arable soil, 37% in grassland, and 32% in forest. Since organic matter is an important factor affecting the bio availability and degradation of contaminants (Puglisi et al. 2007a), these predicted changes in SOM levels will surely have an influence on organic contaminants fate worldwide.

4 Effects of Climate Change on Strategies for Risk Reduction

A number of techniques can be applied to reduce the risk related to the presence of organic contaminants in the environment. Here bioremediation, natural attenuation, phytoremediation, and mitigation by means of wetland, buffer strips, and biobed systems are presented and discussed in the context of climate changes.

4.1 Bioremediation and Natural Attenuation

Bioremediation is not a new concept since scientists have studied these processes since 1940s, but it became popular after the Exxon Valdez oil spill in Alaska in the late 1989. Bioremediation can be defined as a biological technique to reduce hazardous pollutants contamination in the environment to undetectable, nontoxic, or acceptable levels. Bioremediation is based on the biodegradation process, which can be defined as a natural process whereby bacteria or other microorganisms alter and breakdown organic molecules into other substances (Hoff 1993). Microbial metabolism is probably the most important process of pollutant degradation in soils (Kearney and Wauchope 1998). Indeed, microorganisms have the ability to interact both chemically and physically with a huge range of organic compounds, often leading to a structural change or the complete degradation of the molecule (Semple et al. 2001).

For bioremediation to be successful, it is necessary to have the right microbes in the right place with the right environment factors for degradation to occur (Boopathy 2000).

A particular example of bioremediation is represented by natural attenuation, a remediation strategy that is becoming much popular in the last years. It is possible to define natural attenuation processes as a number of chemical, physical, or biological process that, under favorable condition, act without human intervention to reduce the toxicity, mobility, or concentration of contaminants. The most common among these *in situ* processes are biodegradation, dilution, sorption, volatilization, and chemical or biological stabilization. In order to identify and understand the possible impacts of climate change on bioremediation strategies, it is however useful to discuss which are the most important limiting factors for biodegradation.

Table 22.2 Examples of the effect of temperature on the degradation rate of organic contaminants

Contaminants	Soil temperature (°C)	Degradation rate	References
Hydrocarbon (gasoline and diesel)	From 1 to 40	Degradation rate significantly increase from 1 to 20°C	(Walworth et al. 2001)
n-hexadecane	18 60	60°C enhanced the degradation rate	(Perfumo et al. 2007)
Rimsulfuron	10 25	Lower half life at higher temperature	(Vischetti et al. 1997)
DDT	15 45	45°C enhanced the degradation rate	(Samuel and Pillai 1989)
Acephate	15 25 35	Degradation was significantly faster at 35°C compared with 15°C	(Chai et al. 2010)
Metribuzin	20 +5/−5	T1/2 = 20 days; no significant degradation	(Stenrød et al. 2008)

The biodegradation of an organic contaminant does not depend only on the physicochemical property of the chemical, but it is the resultant of a number of factors related to both chemical and environmental conditions. Indeed, the fate and behavior of organic pollutants is usually limited by different factors such as the chemical (pH, temperature, moisture, oxygen, nutrients) and biological site conditions (biomass concentration, population diversity, enzyme activity). These parameters affect the acclimation period of the microbes to the substrate, while the molecular structure and contaminant concentration have been shown to strongly affect the feasibility of bioremediation strategy and the type of microbial transformation occurring (Boopathy 2000).

The pH of soil environments can vary widely. In general, biotic and abiotic factors can be influenced by soil pH and both of them can have thus an influence on contaminants dissipation. Low or high pH levels can inhibit growth and metabolism of the microbial community and microbial degradation process. Many researchers have demonstrated a positive correlation between pH and soil microbial biomass and activity (Singh et al. 2003). In general, most heterotrophic bacteria favor neutral pH, while fungi are less adversely affected by low pH value (Baath and Anderson 2003). Moreover, soil pH can also affect the solubility of pesticides and influence the adsorption and bioavailability of pesticides on organic matter and clay colloids. Commonly, acidic soil tends to increase pollutant solubility and reduce sorption on soil particles (Gavrilescu 2005).

Temperature is one of the important factors in bioremediation strategies since it controls the rate of enzymatic reaction of microorganisms (Worthington 1988), the rate of microbial growth, and it can also modify the physical nature of the contaminants. Many researchers studied the relationships between degradation and soil temperature of different organic contaminants. In general, it has been found that temperature is positively correlated with the degradation of organic contaminants. In Table 22.2, some examples of degradation of some organic contaminants in different temperature conditions are reported and discussed. Walworth et al. (2001) carried out a study to assess the effect of temperature on microbial activity and petroleum hydrocarbon degradation in contaminated cryic soils. They observed maximum microbial activity between 21 and 31°C, while average biodegradation rates increased from 1.7 mg kg^{-1} day^{-1} at 1°C to 8.2 mg kg^{-1} day^{-1} at 11°C, and to 15.1 mg kg^{-1} day^{-1} at 21°C. Average biodegradation rates were similar at 21 and 31°C, and decreased slightly when the temperature was increased to 41°C. The greatest increases of biodegradation rate occurred between 1 and 21°C. The decrease in degradation at 41°C

can be explained considering that the study was conducted on a sub-Arctic soil, which most probably harbored a microbial community adapted to low temperatures.

Perfumo et al. (2007) using instead an unpolluted soil spiked with hexadecane found that an increase in temperature up to 60°C resulted in significant increases in degradation, with or without the addition of biosurfactants or degrading strains. Vischetti et al. (1997) assessed the persistence of Rimsulfuron, a sulfonylurea, in a silty clay loam soil, and found that both DT50 and DT90 disappearance rates were increased when the temperature of the soil microcosms was raised from 10 to 25°C. Samuel and Pillai (1989) assessed the effect of raising the temperature from 15 to 45°C on the volatilization, soil binding, and degradation of DDT: at the higher temperature they found a significant increase in volatilization and degradation, but they also found a significant increase in the formation of bound residues. They also compared soils at 75% water holding capacity (WHC) with flooded soils, and found that in the latter both volatilization and degradation were higher.

Chai et al. (2010) studied three different soils and found that the degradation of acephate, an organophosphorus insecticide with high water solubility and low Kow, was significantly faster at 35°C compared with 15°C. Acephate degraded completely at 70 days for soils incubated at 25 and 35°C while periods of up to 160 days were required for complete acephate degradation at 15°C.

Stenrød et al. (2008) assessed degradation and leaching of Metribuzin, an asymmetrical triazine prone to leaching because of its weak adsorption in soils. They compared degradation at 20, 5, and −5°C, and they found a significant degradation only in the first case. They also found that freeze-thaw cycle increased the mobility of the pesticide but had no effect on degradation, which at temperatures below 5°C was found to be always negligible.

In order to describe the effects of temperature on degradation, Walker and Eagle (1983) developed a simplified degradation model, where the equations describing the temperature dependence of degradation are set so that a change in temperature of 10°C changes the half-life by a factor of two.

Another important parameter affecting the organic contaminant disappearance is soil moisture, since water availability has also an effect on microbial growth and enzymes production (Marín et al. 1998). Besides affecting the microbial activity, soil moisture can also influence the contaminant binding and distribution. Indeed, water plays a significant role in contaminant availability for microbial utilization (Atagana et al. 2003) and, by decreasing the sorption, it can also increase the rate of microbial degradation (Sims et al. 1991). Low levels of moisture may limit the microbial activity and the amounts of contaminants in solution, on the other hand wetter soil can affect oxygen diffusion because the free pore space may be blocked by water. Schoefs et al. (2004) have observed that hexadecane biodegradation rate decreases with increasing water content, concluding that the effect of a limitation of oxygen through the aqueous phase appears dominant. While Garcia-Valcarcel and Tadeo (1999) reported that simazine and hexazinone degradation rate increase with soil moisture content.

This different behavior is most probably due to differences in the physicochemical properties of the compounds, mainly water solubility and hydrophobicity (as expressed by the coefficient of partition between octanol and water – Kow): for hydrophobic compounds such as hexadecane the increase in water content may reduce or not affect biodegradation because of low water solubility, while for more polar compounds an increase in soil moisture can result in a higher fraction of contaminants in the water phase more susceptible to microbial attack.

Moreover, Chai et al. (2010) observed that there were no significant differences in the degradation rates of acephate among different soils for the same level of moisture content indicating that the soil type did not markedly affect degradation. However, several researchers reported that moisture levels of 60% of WHC are the

optimum for the dissipation of most contaminants, because it represents a sufficient amount of water for microbial processes, enough oxygen to support aerobic processes and solubilization of contaminant.

Microbial degradation rates in soil are often limited by ineffective supply of nutrients to the microorganisms (Morgan and Watkinson 1989). A number of studies on soils clearly demonstrated that nutrient supplementation (C, N, and P) can augment microbial growth rate-limiting components (Graham et al. 1999) and can further enhance biodegradation of organic pollutants (Atlas and Bartha 1972; Zhang et al. 2005; Qui et al. 2009). Adequate nutrients must be available to meet the metabolic and growth needs of the microbial populations performing the degradation reaction (Graham et al. 1999). In other case, nutrient stimulation does not improve the biodegradation rate, and this could be due to inadequate nutritional supplements that advance the activity of nondegrading microorganisms (Steffensen and Alexander 1995). Moreover, Alexander (1994) reported that the time necessary to biodegrade organic compounds containing P and N can be long because the organisms use the inorganic P and N from the environment in preference to the forms which would be released as results of degradation of the contaminant.

However, Davis and Madsen (1996) reported that microbial activity usually show greater results at a carbon:nitrogen:phosphorus ratio of 100:5:1, indicating that in soil containing high level of organic material, the toluene degradation appeared to be limited by the availability of N source in this soil.

Microorganisms such as bacteria and fungi play a key role in bioremediation process and generally they can use two different mechanisms in biodegrading pollutants (metabolic and cometabolic). Depending on the concentration of the compound and the environmental condition, the microorganisms can exhibit different acclimation period. This could be due to different factors such as enrichment of the capable microbial population, enzyme induction, and production of toxic metabolites.

4.2 Phytoremediation

Phytoremediation has been first defined in 1993 as the use of green plants to remove, contain, or render harmless an environmental contaminant (Cunningham and Berti 1993). Phytoremediation has emerged as a promising, cost effective, and solar-driven *in situ* technology for the remediation of contaminated sites (Vamerali et al. 2010). Most phytoremediation studies are aimed at inorganic pollutants through different approaches defined as phytoextraction (the use of metal-accumulating plants to transport and concentrate metals from the soil into roots and above-ground biomass), rhizofiltration (the use of plant roots to absorb, precipitate, and concentrate toxic metals from polluted effluents), and phytostabilization (the use of plants to reduce the mobility of metals) (Salt et al. 1995). These approaches can however be applied also for organic contaminants: phytoextraction has been used to remediate BTEX (benzene, toluene, ethylbenzene, and xylenes) mixtures, pentachlorophenol, and short-chained organic contaminants; phytostabilization has been successfully applied in the case of phenols and chlorinated solvents such as tetrachloromethane and tricholoromethane; and finally rhizofiltration to degrade different organic pollutants (Susarla et al. 2002).

Plants of course are not the only drivers of the remediation of pollution: rhizosphere-associated microorganisms also play a major role, and the term rhizoremediation thus often used. As for other bioremediation practices, the remediation is mainly due to the expression and activity of enzymes able to degrade, detoxify, or block the organic pollutants and these enzymes can indeed have a microbial or a plant origin. Among plants, many species are able to produce enzymes with a role in transforming organic pollutants. Some hybrid poplars (*Populus* sp.) produce aliphatic dehalogenases that are capable of degrading chlorinated pollutants such as trichloroethylene (TCE), one of the most polluting industrial solvents. Plants grown in TCE polluted soil are indeed able to extract TCE, transpire it, as well as improve the rhizodegradation by feeding

degrading microorganisms in the rhizosphere with root exudates (Meagher 2000). Interestingly, plant dehalogenases are thought to act with an oxidative pathway, while in microbes dehalogenation is only a reductive process. Other pollutants for which plants show good degrading abilities are nitroaromatic compounds such as 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and nitroglycerin (GTN): little is still known about the genetic basis of the degradation of these compounds, although a role of glutathione-S-transferases has been recently suggested by a study conducted on poplar plants exposed to TNT (Brentner et al. 2008). Another example is represented by the phytoremediation of PCB contaminated sites. PCB degradation is mainly a microbial process, as encoded by the biphenyl (*bph*) pathway: since the bioavailability of these pollutants is low (Puglisi et al. 2007b). Plants can improve the degradation not only by feeding microbes, but also by enhancing the bioavailability of PCB through rhizodeposition processes. A recent study also demonstrated for the first time a role of a plant nitrate reductase from *Zea mays* in the dechlorination of one representative PCB congener, PCB 153 (Magee et al. 2008).

As for other techniques discussed in this chapter, there are no published works about the possible effects of climate changes on the phytoremediation of organic pollutants. We can thus only speculate using evidence about phytoremediation in arid or wet areas and about general effects of climate change on plant physiology. As discussed in details in other chapters of this book, climate change is indeed expected to have a range of effects on the physiology of plant species and on their geographical distribution (Thuiller et al. 2005). The climatic variables which are considered to have the strongest effects on plant growth and physiology are mean annual precipitation, mean annual temperature, minimum temperature of the coldest month, and moisture availability were identified as the climatic variables influencing more plant growth and physiology. Although changes in these climatic variables will most probably alter the geographical distribution of plants, previsions are difficult to make about phytoremediation abilities. Some speculations of interest can, however, be made about effects of increasing atmospheric carbon dioxide concentrations on the soil–plant interface. There is a substantial agreement in the scientific community in associating increases in CO_2 air concentrations with a higher rhizodeposition and in turn with higher microbial densities and activities (Rogers et al. 1994; Smart et al. 1997; Marilley et al. 1999; Lu et al. 2004). Since organic contaminants degradation is a rhizobacterial activity thought to be increased by CO_2 (Shaw and Burns 2005), it can be even possible to predict a positive effect of some climate change scenarios on phytoremediation. Moreover, it should be taken into account how phytoremediation has proven to be a successful approach on different climatic and pedological conditions, ranging from arid zones (Mendez and Maier 2008; Cook et al. 2009; Zhu et al. 2010) to wetlands and buffer strips, as discussed in the following paragraphs. It is thus expected that climate change will not represent a limitation to phytoremediation: particular attention should be however devoted to the choice of species cultivars most adapted to specific pedoclimatic conditions.

4.3 Mitigation: Wetlands

Wetlands are transitional habitats between terrestrial and aquatic systems, where the water table is usually at or near the surface or the land is covered by shallow water (El-Refaie 2010). The ability of natural wetland to mitigate nonpoint source pollution and to limit surface water contamination has been extensively demonstrated (Salmon et al. 1998; Weaver et al. 2004; Moreno et al. 2007). In order to exploit these mitigation abilities, several artificial wetlands were constructed and studied. Indeed, constructed wetlands offer a cost-effective alternative to the traditional treatment systems to clean up water by organic and inorganic contaminants.

Compared to natural wetlands, artificial wetlands offer different advantages related to the possibility of controlling parameters such as substrate types and concentrations, plant species,

and hydraulic connectivity. Within wetlands, organic contaminants are removed as a resultant of chemical, physical, and biological processes such as sedimentation, precipitation, adsorption, plant uptake, and microbial degradation (Brix 1994). Indeed, the main processes in which wetland contribute to pollutant degradation are phytoremediation and bioremediation, which have been discussed earlier.

The active reaction zone for the degradation of contaminants in wetland is always the root zone (or rhizosphere), in which the interactions between plant, microorganisms, soil, and pollutants take place (Stottmeister et al. 2003).

Macrophytes play an important role in the water treatments of wetlands because they have a significant and positive effect on biological removal of organic contaminant. They take up inorganic pollutants, decrease the flow velocity favoring the absorption of solutes, transport gases and solutes between above-ground root zones, release oxygen and carbon compounds into the rhizosphere, and influence microbial diversity and activity (Brix 1994; Tanner 2001; Taylor et al. 2010).

On the other hand, microorganisms play the main role in the transformation and mineralization of organic contaminants. In subsurface flow systems, near roots and on the rhizoplane, aerobic processes are predominant in the zones that are largely free of oxygen, anaerobic processes such as denitrification, sulfate reduction, and/or methanogenesis take place (Stottmeister et al. 2003).

Gregoire et al. (2009) have extensively studied the artificial wetland in European countries and reported that the purification capability of wetlands can be optimized by developing both physical (hydraulic design, soil management, and water pathway) and biological processes (plant and bacteria development).

It is important to assess the possible impacts of climate changes on artificial wetlands in order to adapt and optimize future mitigation strategies. Indeed, the indirect effect of climate change can have an important implication on the efficiency of the systems. Since the change in demand of water, the variability of temperature, and the change in the land use may have an important effect on the fate of contaminants in the environment (Bloomfield et al. 2006; Gregoire et al. 2009).

Because the increasing variability in seasonal rainfall pattern leads to an unstable distribution of water resources, it is important to optimize the hydraulic design of wetlands to the specific site.

Hydraulic retention times are important parameters and commonly too low to permit an optimized adsorption and degradation of pollutants. Thus, the control of the hydraulic design and the use of adsorbing materials can be useful to increase the pollutant residence time and the contact between pollutants and biocatalyzers (Gregoire et al. 2009). Therefore, hydraulic dimensioning of artificial wetland should take into account the groundwater and surface water interaction and the evapotranspiration from wetlands.

Once the contaminants are sequestered in the wetlands, biological treatments can be activated. To improve biological treatments in the climate change scenario it is important to consider two aspects, the adsorption by selected macrophyte and the degradation by microorganisms induced. Thus, the introduction and stimulation of degrading microorganisms and plants adapted to the specific environmental conditions which can degrade and adsorb specific contaminant can be a solution to improve the efficiency of the system.

4.4 Mitigation: Buffer Strips

Buffer strips are filter zones inserted near the edge of the field, between the agricultural fields and the receiving water, permanently vegetated with trees, shrubs, or grasses (planted or indigenous). Buffers can reduce the amount of pollutants carried by runoff, erosion, spray drift, and drainage to the nearby surface water.

Buffer strips can have several different configurations such as grassed waterways, filter strips, vegetative barriers, riparian buffers, contour buffer strips, or field borders (Fig. 22.2). Grassed waterways are very simple and useful buffers to reduce soil erosion and capture most organic contaminants that would wash out of

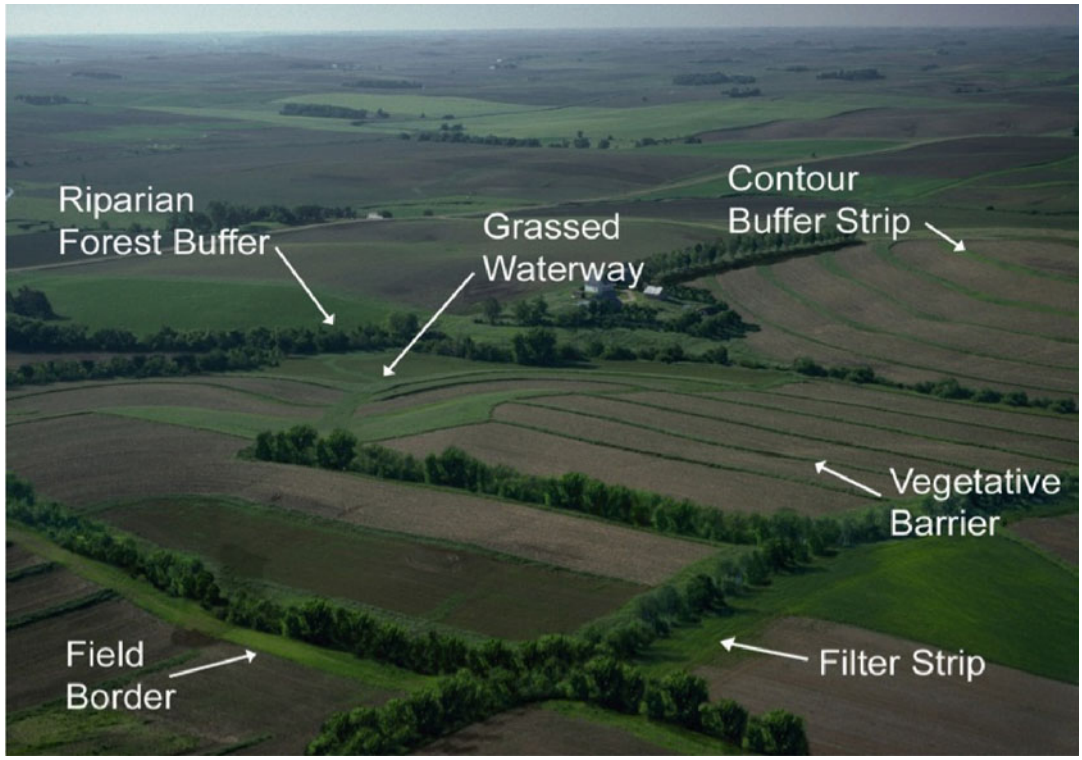

Fig. 22.2 Examples of different types of buffer strips and vegetated areas for mitigation of organic contaminants

fields into waters. Filter strips are important in protecting water quality, and they are composed of grass or permanent vegetation that protects riparian areas from pesticides, organics, nutrients, and sediment runoff. Vegetative barriers are narrower compared to the other types of buffer, and they are performed by perennial grasses or shrubs planted and are very effective in reducing water erosion which results in sediment or contaminant trapping and water infiltration. Riparian buffers, designed by diverse community of trees, shrubs, and perennial grasses, effective to intercept sediments, nutrients, pesticides, and other materials in surface runoff and reduce nutrients and other pollutants in shallow subsurface water flow. Contour buffer strips are simply strips of perennial vegetation that is interchanged with cultivated strips of crop. These types of strips are most effective when runoff water enters uniformly. Field borders are characterized by perennial vegetation on the edge of a field and they are effective in reducing wind and water erosion.

Due to their environmental benefit in treating non-point-source pollution, buffers are considered as natural solutions for pollutant removal. Several studies have demonstrated that buffers have the potential to improve water quality by reducing soluble nutrient concentrations and removing pollutants from field runoff (Patty et al. 1997; Hefting and de Klein 1998; Schmitt et al. 1999; Borin et al. 2005).

Different mechanisms are involved in contaminant removal such as reducing surface runoff, filtering surface and groundwater runoff, reducing erosion, and filtering pollutants from field (Borin et al. 2010).

Sediment deposition process largely controls the effectiveness of the filter area, whereas infiltration is the controlling factor for contaminant transport. Buffer strip alters flow hydraulics, reducing runoff speed and increasing water

infiltration and contaminant deposition before to their export to the water bodies (Misra et al. 1996; Vianello et al. 2005). Wilson (1967) observed that increasing the flow volume and velocity markedly reduced filtration efficiencies. Indeed, buffer strips are most effective when the flow is shallow, slow, and enters the buffer strip uniformly along its length (Barling and Moore 1994).

The presence of vegetation on buffer strips is strongly recommended mainly to limit the amount of surface and subsurface flow and erosion. Misra et al. (1996) determined that the major factor in reduction of herbicide transport was infiltration of inflow into the vegetative buffer strips. Abu-Zreig (2001) demonstrated that the conservation of grass cover in buffer strips prevents the formation of preferential flow channels, while Patty et al. (1997) showed that more than 99% of isoproturon and 97% of diflufenican residues in runoff were removed by grass buffer strips.

Plants also confer higher organic matter content in the filter zone, which increases pollutant adsorption capacity and microbial activity for degradation. An interesting work was presented by Benoit et al. (1999), which observed a rapid degradation of isoproturon in soil from grass buffer strips compared to the cultivated soils. Isoproturon half-live was significantly reduced from 72 days in the cultivated field to 8 days in the grass buffers. In the same study, authors observed that isoproturon sorption was enhanced in the grassed soil compared to the cultivated soil and especially in the surface layer (0–2 cm) containing a high proportion of nondecomposed plant residues.

It is generally recommended that the species to be used in buffer strips should be appropriate to the local soil, climate, and specific requirement, for example, plants with high nitrogen requirement or herbaceous plants with a long growing period, high moisture tolerance, and extensive root system (Delgado et al. 1995).

Traditionally buffer strips are usually relatively narrow, between 5 and 30 m, but different climatic conditions and cropping systems may require different types of buffer strips to assure the best performance (Vianello et al. 2005). However, it was noted that the width of the buffer zone controls the trapping of contaminants up to a maximum after which no improvements were observed. Establishing a buffer width is dependent on different factors including vegetation and hydraulic conditions, topography, geology, and local climatic conditions (Vischetti 2008).

The slope can affect the performance of the buffer strip since higher inclination can increase runoff flow velocity, and thus reduces the contaminant trapping efficiency.

Climate variability also affects the decontamination effectiveness of buffer strips and should be carefully studied to adapt the system to the future scenario. Buffer performance and efficiency could be affected by climate change.

In prevision of high rainfall events that can exceed the infiltration capacity of the soil and this can affect surface runoff and contaminants remobilization, hydrology parameter should thus be carefully implemented. For example, concentrated surface flow through buffer zones or flow through subsurface drains may affect buffer performance. Other consideration can be made on buffer strip width that should be proportioned on the basis of flow length to decrease the pesticides load in receiving water.

High temperature promotes infiltration, since evapotranspiration and soil water requirement are both higher during this period. Moreover, Ledwith (1996) observed that buffer width has an effect on air temperature and relative humidity. It should be taken into account indeed that buffer strips of insufficient width may allow an increase in direct and reflected solar radiation, thus increasing the air temperature and lowering the relative humidity on warm days.

On the contrary, it has been observed that during the winter when temperatures are lower the biological activity and the plant uptake declines, thus nutrient circulation in the environment is encouraged (Delgado et al. 1995). The introduction of plants adapted to the specific environmental conditions, adequate buffer configuration with appropriate buffer width and slope can be a solution to improve the efficiency of the system in different temperature conditions.

4.5 Mitigation: Biobeds

A system for reduction of point sources of organic contaminants was presented by Torstensson and Castillo (1997) with the introduction of biobed.

Biobed is a simple and cheap on-farm construction aimed at minimizing point source environmental contamination by means of pollutants adsorption and degradation.

Point source contamination usually derives from inappropriate managements of organic contaminants especially by the utilization of pesticides in agriculture. Indeed, unsatisfactory pesticides handling such as tank filling, spilling, and residues disposal procedure can be a major cause of pesticides contamination in water and groundwater.

The original biobed design proposed by Torstensson and Castillo (1997) consists of a lined hole in the ground (at least 0.6 m deep and a minimum surface area of 1 m^2 for every 1,000 L of liquid requiring treatment) filled with topsoil (25%), straw (50%), and peat (25%) which have the ability to retain and degrade contaminants from the water. This biomixture was used in order to ensure maximum binding capacity, moisture control, and to create optimal conditions for microbial degradation (Fogg et al. 2004). As extensively reported by Castillo et al. (2008), straw is the main substrate for pesticide degradation and microbial activity, especially from lignin-degrading fungi; the soil provides sorption capacity and should be rich in humus and sufficient clay content to promote microbial activity; the peat contributes to sorption capacity, moisture control, and abiotic degradation.

Biobeds are also made up of an impermeable clay layer (10 cm) in the bottom part, which decreases the water flow downward and increases the contaminants retention time in the system. Moreover, a grass layer covering the biobed regulates the moisture, enhances evapotranspiration, and produces root exudates to support cometabolic processes. The survival of this grass cover can also be used as an indicator of herbicide spills (Castillo et al. 2008).

Torstensson (2000) demonstrated that biobed can effectively retain and degrade pesticides in short time, thus it generates interest in other countries. Since environmental conditions and agricultural practices vary widely between Countries, the original biobed model has been modified to satisfy site-specific conditions and to utilize locally available organic materials (Fogg et al. 2003; Vischetti et al. 2004; Pigeon et al. 2005; Spliid et al. 2006; Coppola et al. 2007). A series of studies have shown that biomixtures consisting of composted materials instead of peat have high degradation and adsorption capacity (Fogg et al. 2003; Coppola et al. 2007; Fait et al. 2007; Vischetti et al. 2007; Monaci et al. 2009; Kravvariti et al. 2010; Coppola et al. 2011).

Since there are no studies correlating the biobed system with climate change scenarios, it is only possible to predict the activity of the system on the basis of previous work in different environmental conditions.

The future variations in temperature and moisture levels can affect the efficiency of the system. Temperature, as in all biological processes, affects the pollutant degradation, while moisture is a critical factor, which affects contaminants leaching, oxygen availability, and microbial activity.

Castillo and Torstensson (2007) carried out a laboratory study in order to determine the optimal conditions of temperature and moisture for pesticides degradation in a biomixture composed by straw–peat–soil in different proportion. They observed that increasing temperatures increase the microbial and enzymatic activity, which have a positive effect on the degradation of most of the pesticides tested. Moreover, it should also be considered that increase in temperature can also increase the solubility of the organic contaminants, which resulted in higher fraction bioavailable for microbial attacks.

However, increases in temperature have also effects on the degradation of organic matter and this may have drawbacks in terms of necessities for more frequent substitution of the substrate to ensure the system activity. Moisture content in the biomixture should be enough to leave oxygen for aerobic processes and to promote microbial activity and pesticides solubilization.

In the same work, Castillo and Torstensson (2007) observed that moisture at 60% of the

WHC gave the highest dissipation of most pesticides tested while moisture at 30 or 90% limited the microbial activity. As discussed for the wetlands and buffer strips, the most critical effect of climate changes on biobeds would probably be related to water management, while possible increase in temperature (as reported for bioremediation) would probably just result in an increased bioremediation. Given the negative effects of oversaturating WHC on the biobed performances, it will be at least necessary to work with covered biobed systems, to avoid negative effects of increased precipitations, and to promote the relative presence in the biomixture of substrates with higher water sorption capacities such as peat or compost. The development and introduction of closed-cycle drainage systems may also represent an improvement of biobed in order on one hand to reduce the moisture levels, on the other to increase the residence time of the contaminated water on the biomixture.

5 Conclusion and Future Perspectives

In the present chapter, we have addressed a topic still quite neglected in the scientific literature, which is the possible impact of climate changes on the fate, degradation, and mitigation of organic contaminants in the environment. Particular interest was devoted to techniques based on plants (phytoremediation, wetlands, buffer strips), organic biomass residues (biobeds), as well as on bioremediation processes controlled by microorganisms. Since in most cases no specific works on the effects of climate change on bioremediation and mitigation of organic contaminants could be found in the scientific literature, climate change scenarios were identified, and the obtained info critically correlated on available info about the effects of climatic parameters (temperature, precipitations, soil humidity, pH, organic matter, nutrients) on the fate, degradation, and mitigation of contaminants.

As widely known, it was evinced how the future scenarios will be dominated by increasing temperatures, increased precipitation (both in intensity and frequency) in some areas, and decreased precipitations in others. These changes will most probably affect other relevant ecological parameters, such as pH, soil moisture, organic matter levels, microbial activities, and chemicals bioavailability. According to the information gathered in this chapter, these changes will have a quite strong effect on the fate and behavior of contaminants, affecting their distribution in environmental compartments. Major processes controlling the distribution of contaminants will be indeed affected, with probable increases in photodegradation, volatilization, hydrolysis, deposition, runoff, and leaching. Persistence of contaminants may probably be reduced because of increased microbial activity, but on the other hand the reduction of SOM levels predicted by some authors will result in lower degradation and higher bioavailability of pollutants, with possible negative effects in terms of risk assessment.

While effects on fate and behavior can be considered quite relevant, it was found that biodegradation and mitigation will have limited and in some cases positive effects from climate changes. Temperature and moisture will probably increase biodegradation rates, high moisture levels may negatively affect some systems (e.g., wetlands, buffer strips, and biobeds), but this can be easily addressed by promoting more efficient water management systems. In plant-based bioremediation and mitigation systems (phytoremediation, wetlands, and buffer strips), it should be however taken into account how climate change will affect plant physiology and distribution. The solution here is local adaptation, that is the choice of species (in wetlands and buffer strips) or organic materials (in biobed mixtures) that will be more efficient in future climatic conditions. The history of biobed development described in the last chapter is already a successful example of this adaptation possibility, as it shows how a system developed in Northern Europe climatic conditions has been adapted with success to function properly in very different climates.

We can conclude that possible climate change will indeed have a significant effect on the fate and behavior of contaminants, and a more limited effect on bioremediation and mitigation

strategies. Since fate and behavior determine the exposure of biological receptor to contaminants toxicity, it will be very important to carry out risk assessment evaluations in the context of climate change. Bioremediation and mitigation will remain very powerful tools to contrast worldwide pollution: attention must be devoted to adapt these tools to climatic changes, in order to maintain and, if possible, improve their efficiencies.

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23 Exploitation of Weeds and Ornamentals for Bioremediation of Metalliferous Substrates in the Era of Climate Change

M.N.V. Prasad

Abstract

Weeds not only adversely affect the plant productivity but many of them also cause health hazards in human beings and animals. They are also known to seriously affect the biodiversity. Apart from this negative side, many weeds are known to have beneficial properties in one way or the other and have immense potential as food and fodder, medicinal, aromatic, phytoremediation, industrial, soil and water conservation resources, etc. A very little information is available on the use of weeds for such beneficial purposes. Therefore, this subject of research needs to be explored and expanded. Several of the weeds are utilized for (a) soil and water conservation, (b) alternative livelihood opportunities, and (c) industrial uses. Survey of published literature indicates that there is great scope for application of weeds in bioremediation. More research efforts are required for utilizing weeds for bioremediation of different type of pollutants from air, water, and soil. Ornamental plants have an added advantage of enhancing the environmental esthetics besides cleaning the environment. This approach has several advantages for environmental moderation, cleanup, and generation of revenue. Therefore, this approach will add a new dimension to the field of bioremediation of contaminated aquatic and terrestrial environments.

Keywords

Weeds • Ornamentals • Phytostabilization • Phytoremediation • Metalliferous substrates • Biocontrol

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1 Introduction

Bioremediation is an emerging and an effective technology for treatment of a wide variety of contaminants. This technology includes plant- and microbe-mediated processes (phytoremediation andrhizoremediation,respectively).Bioremediation approach is currently applied to contain contaminants in soil, groundwater, surface water, or sediments including air. These technologies have become attractive alternatives to conventional cleanup technologies due to relatively low capital costs and the inherently esthetic nature (Fig. 23.1). Quite a variety of plants, natural, transgenic, and/or associated with rhizosphere micro-organisms are extraordinarily active in these biological interventions for cleaning up pollutants by removing or immobilizing (Ma et al. 2011). Climate change will affect the ability of ecological systems that provide a range of essential ecological goods and services, such as food and fiber production; provision of clean and sufficient water maintenance of biodiversity; maintenance of human health; and storage and cycling of carbon, nitrogen, and phosphorus.

Technogenic and anthropogenic sources of metals is subject of importance not only to human health but also in general to the field of biogeochemistry, environment, and medicine (Figs. 23.2–23.4). Smelting and mining processes are the point source of a contamination of metals causing environmental contamination and pollution. Consequently, these pollutants get dispersed in natural resources (soil, water, and air) and ultimately enter the food chain. Physical stabilization (covering the metalliferous waste with geotextiles/geomembranes, etc.) and chemical stabilization (use of chemical binding agents) to reduce wind and water erosion are not a feasible proposition for large areas. However, phytostabilization – use of a specific type of vegetation is far more desirable than physical and chemical stabilization (Prasad 2006; Tordoff et al. 2000). Phytostabilization is an effective process of phytoremediation technology.

In order to cleanup large areas contaminated with toxic metals, plants producing very high biomass with limited inputs and simplistic management are desirable. It is a general belief that climate change promotes explosion of weeds in addition to other phenomena (Fig. 23.5).

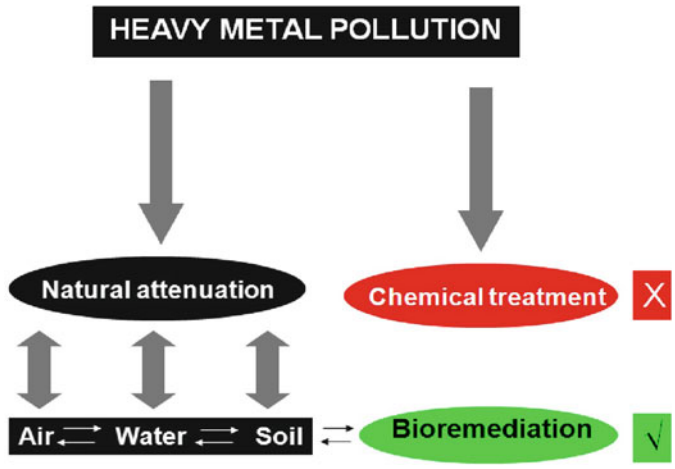

Fig. 23.1 Bioremediation, a schematic presentation. Bioremediaiton is based on use of the ecosystem services provided by its biotic compartment. Some examples of its application include the reduction and control of pollution through wetland systems, restoration of degraded natural systems or establishment of Eco-industrial parks, carbon sinks and ameliorating the effects and impacts of climate change

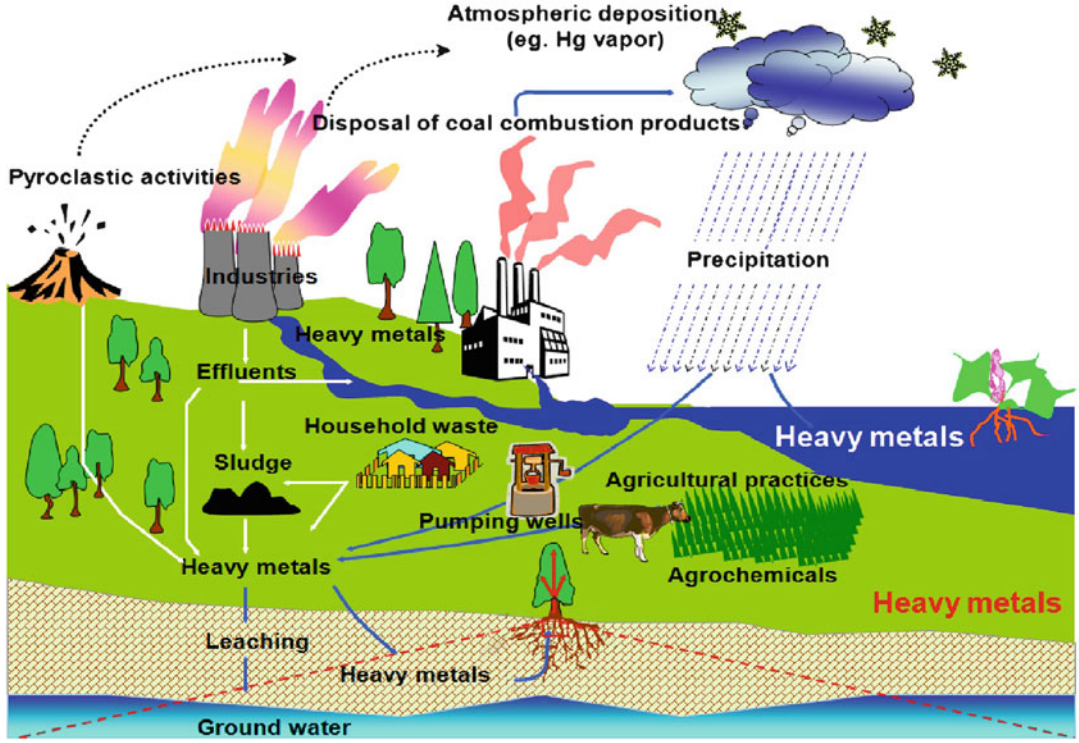

Fig. 23.2 Biogeochemical cycling of heavy metals in a generalized ecosystem

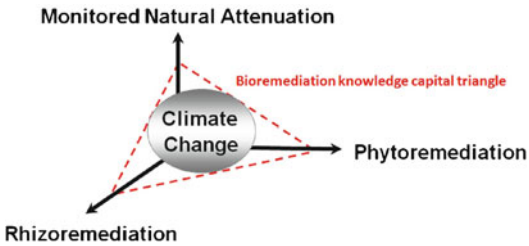

Fig. 23.3 Bioremediation knowledge capital triangle

Non-alien plants when they grow outside their niche become invasive. Invasives are widely distributed in a variety of ecosystems throughout the world. Many invasive alien species support farming and forestry systems positively in a big way. However, some of the alien species become invasive when they are introduced deliberately or unintentionally outside their natural habitats into new areas where they express the capability to establish, invade, and outcompete native species. According to International Union for Conservation of Nature and Natural Resources (IUCN), alien invasive species means, an exotic species which becomes established in natural or seminatural ecosystems or habitat, an agent of undesirable change which threatens the native biological diversity. Invasive species are therefore considered to be a serious hindrance to conservation and profitable use of biodiversity, with significant undesirable impacts on the services provided by ecosystems. Alien invasive species are supposed to have huge requirement and destructive modes of resource acquisition and consumption that would ultimately bring change in soil structure and nutrient composition, its profile, decomposition, moisture availability, etc.

Trace metal contamination and pollution in the environment is increasing due to technogenic and geogenic sources. The flux of trace metals deteriorates the quality of the environment since these are considered to be cytotoxic, mutagenic, and carcinogenic. In order to be healthy, physically and mentally, clean soil, water, and air are

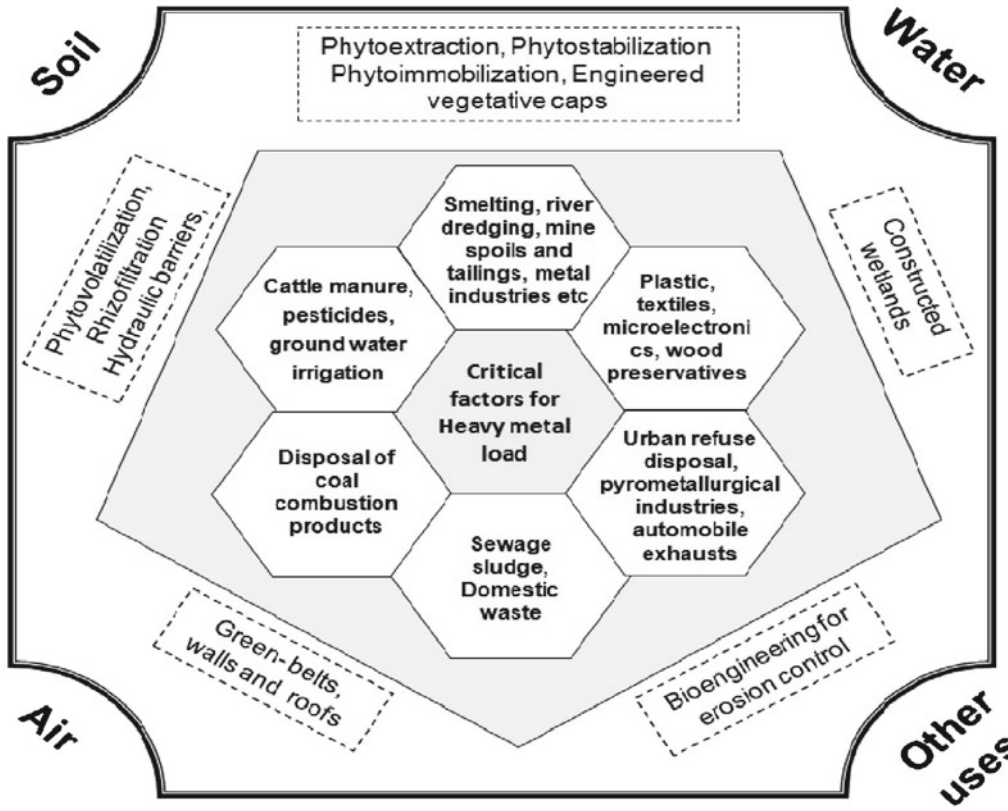

Fig. 23.4 Sources of heavy metals in the environment and various applications of bioremediation for treatment of natural resources and for miscellaneous applications

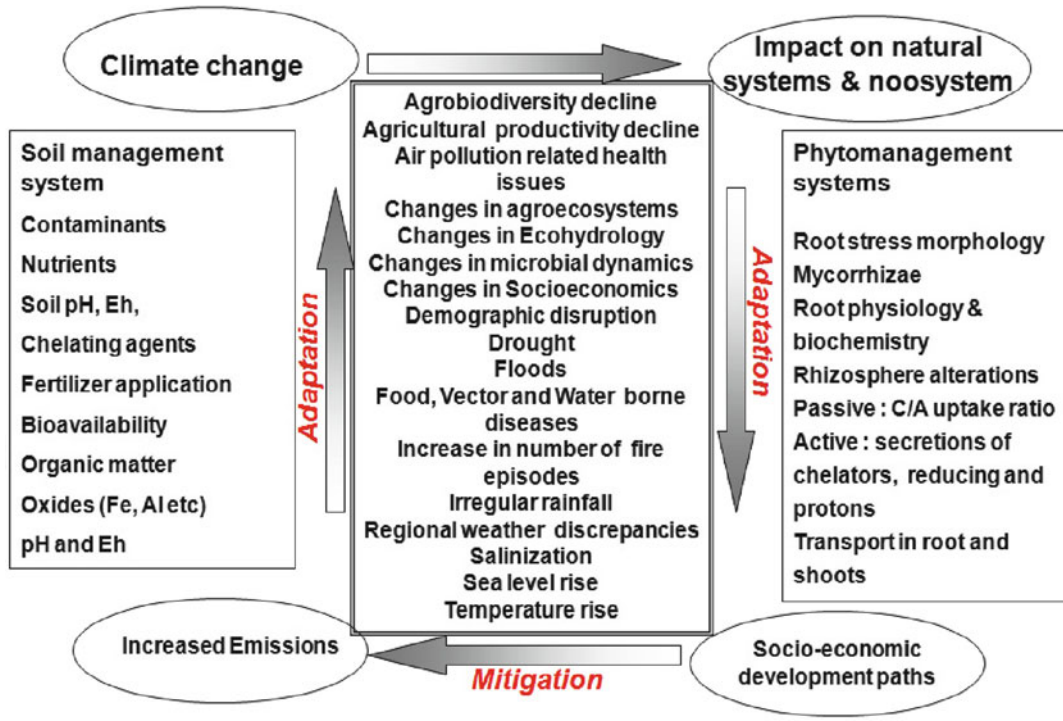

Fig. 23.5 Climate change induced adaptations and mitigation processes

prerequisites. Trace metal contamination and pollution would exert direct and indirect harmful effects that would eventually deteriorate biodiversity and economic wealth. In developed nations, trace metal contamination or pollution is often highly localized and the pressure to use such contaminated land and water for agricultural food production is minimal. In contrast, the technogenic/geogenic pollution and contamination is widespread in many Asian and eastern European countries and is dramatically increasing in Southeast Asia, India, and China (Cheng 2003; Meharg 2004). In order to contain trace metal pollution in soil, water, and air, phytoremediation is being considered as a low cost solution and a globally recognized technology (Garbisu and Alkorta 2001; Garbisu et al. 2002; McCutcheon and Schnoor 2003; Prasad and Freitas 2003; Macek et al. 2004; Gratão et al. 2005). Ornamental foliage plants have been suggested for the removal of arsenic (Alkorta et al. 2004). Therefore, this approach of involving weeds for bioremediation of metalliferous substrates in the era of climate change would yield desirable results with limited efforts and investments (Lorenzini et al. 2006; Prasad and Freitas 2003).

2 Grasses: Ideal for Phytostabilization

Grasses are tolerant to toxic metals and have played a convincing role in phytostabilization (Prasad 2006; Lai and Chen 2006; Li et al. 2009; Néel et al. 2007; Redondo-Gómez et al. 2011; Shu et al. 2002; Vernay et al. 2007; Wang et al. 2005; Zhang et al. 2010; Atabayeva et al. 2010). Abandoned mine soils and estuarine sediments are phytostabilized against erosion by grass species (Cambrollé et al. 2008; Comino et al. 2009; Mateos-Naranjo et al. 2008). Soil amendments and biosolids accelerate phytostabilization process (Santibáñez et al. 2008; Zhou et al. 2007). Grasses possess thickets of adventitious roots, unique root morphology (Li et al. 2009), high bioproductivity (Liu et al. 2009), therefore have an added advantage for application in phytostabilization. Further, grasses are often associated with mycorrhizal and endophytic fungi (Chen et al. 2008; Deram et al. 2007, 2008; Kuldau and Bacon 2008; Ortega-Larrocea et al. 2010; Punamiya et al. 2010). Grasses together with legume association have helped in situ stabilization of chemical waste (Hartley et al. 2009; Hartley and Lepp 2008). In climate constrained and carbon dioxide enriched era, grasses have physiological advantage (majority being C_4) of producing/increasing their biomass. Hence, grasses perform well in phytostabilization process (Wu et al. 2009). In view of their advantageous metabolic processes, hydroponic grass system based on plate or fabric is considered for the treatment of aquacultural wastewater (Pan et al. 2007).

2.1 *Lolium perenne* (Ryegrass)

It is a perennial exhibits luxuriant growth and produces large amounts of aboveground biomass. It has been used for phytostabilization of abandoned uranium mine (Abrutiga, Portugal) (Fig. 23.6).

2.2 *Panicum virgatum* (Switchgrass)

It is one of the perennial rhizomatous grasses being developed for the purpose of biomass production. It is a perennial C_4 grass propagated by seed that can be established at low cost and requires very low inputs while giving high biomass yields even on marginal soils. Attributes of switchgrass desirable for bioenergy cropping include its demonstrated high productivity across many environments, suitability for marginal and erosive land, relatively low water and nutrient requirements, and positive environmental benefits. There is need to examine its (a) adaptability across a range of contaminated sites, (b) fresh and dry matter yields.

2.3 *Prosopis juliflora* (Velvet Mesquite)

It is an evergreen phreatophyte, fast growing, drought resistant, widely distributed not only in

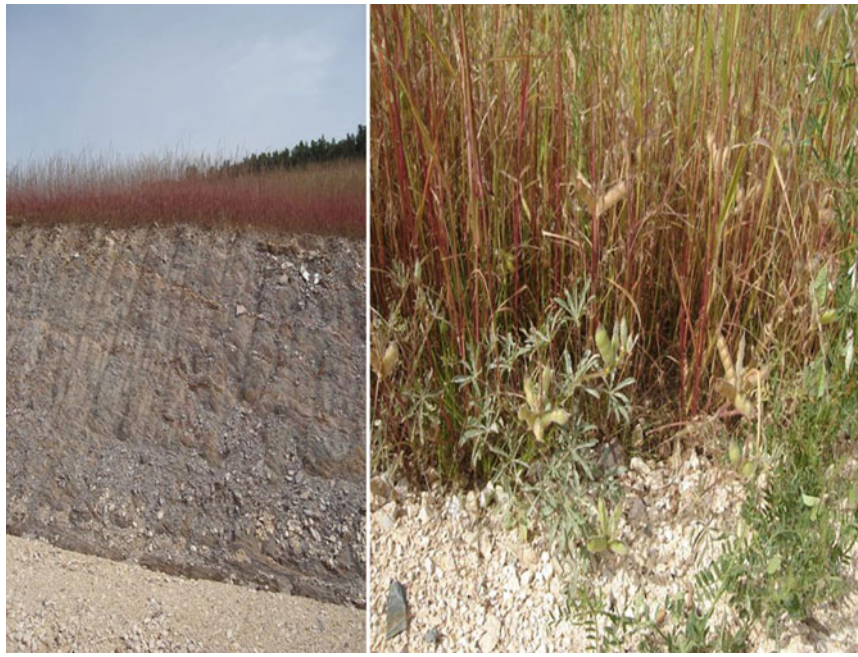

Fig. 23.6 *Lolium perenne* – phytostabilization of abandoned mine and soil profile

India but also in other arid and semiarid tropical countries. It is the only exotic species capable of growing on a wide variety of soils and climatic conditions. It is a valued tree for shade, timber, and forage. It is a thorny, deciduous, large crowned and deep rooted bush or tree which grows up to 10-m height or more, depending on the variety and climatic conditions. It is also widely distributed in the dry tropical and subtropical regions of Central America and Northern South America.

P. juliflora is an ideal species for stabilizing the pegmatitic tailings of mica mines in Nellore district of Andhra Pradesh, India (Nagaraju and Prasad 1998). It is also helpful for reclamation of copper, tungsten, marble, dolomite mine tailings and is a green solution to heavy metal contaminated soils (Varun et al. 2011). It is an appropriate species for rehabilitation of gypsum mine spoil in arid zone and restoration of sodic soils. It outperformed all other tree species in sand dune stabilization (Kailappan et al. 2000; Rai et al. 2003; Senthilkumar et al. 2005). Mycorrhizae are reported to greatly improve the growth of *P. juliflora* on high pH soils. *P. juliflora* was able to grow satisfactorily without amendments up to pH 9. Arbuscular mycorrhizal inocula isolated from its rhizosphere (low cost agrotechnology) were found to accelerate the growth of other agroforestry and social forestry legumes in perturbed ecosystems (Gardea-Torresdey et al. 2005; Siddhu and Behl 1997; Singh 1995).

3 Ornamentals for Environmental Moderation and Toxic Trace Metal Cleanup

Several ornamental plants have successfully been applied in environmental toxic cleanup. (Belmonth and Metcalfe 2003; Chintakovid et al. 2007; Davies et al. 2001; Madejon et al. 2003; Mazen 2004; McIntyre 2003; Mungur et al. 1995; Murillo et al. 1999; Negri and Hinchman 2000; Niu et al. 2007; Wei et al. 2009, 2010a, b)

Lemon-scented geraniums (*Pelargonium* sp. "Frensham," or scented geranium) accumulated large amounts of Cd, Pb, Ni, and Cu from soil in greenhouse experiments (Dan et al. 2000). Biotechnological interventions through hairy root regenerants are useful in floriculture (Giri and Narasu 2000; Giovanni et al. 1997). Pellegrineschi

et al. (1994) improved the ornamental quality of scented *Pelargonium* spp. This plant has pleasant odor that adds scent to the toxic metal contaminated soil.

Vetiveria zizanioides (Vetiver grass): It is known to have multiple uses. This plant had several popular names such as "the miracle grass," "a wonder grass," "a magic grass," "an unique plant," "an essential grass," "an amazing plant," "an amazing grass," "a versatile plant," "a living barrier," "a living dam," "a living nail," "a living wall," "an eco-friendly grass." This extraordinary grass is adaptable to multiple environmental conditions and it is globally recognized as an easy and economical alternative to control soil erosion and to solve a variety of environmental problems. It has been used for restoration, conservation, and protection of land disrupted by man activities like agriculture, mining, construction sites, oil exploration and extraction, infrastructure corridors, as well as used for water conservation in watershed management, disaster mitigation, and treatment of contaminated water and soil. Research at the global level has proved the relevance of vetiver in multiple applications. In Australia, *V. zizanioides* has been successfully used to stabilize mining overburden and highly saline, sodic, magnesic, and alkaline (pH 9.5) tailings of coalmines, as well as highly acidic (pH 2.7) arsenic tailings of gold mines. In China, it has been demonstrated that *V. zizanioides* is one of the best choices for revegetation of Pb/Zn mine tailings due to its high metal tolerance (Chen et al. 2004a, b; Chiu et al. 2005, 2006; Chong and Chu 2007; Rotkittikhun et al. 2007; Makris et al. 2007; Pang et al. 2003; Singh et al. 2008; Truong 2000; Wilde et al. 2005).

(a) *Hydrocotyle umbellata* (Pennywort): It is a wetland/marshy plant commonly found in many tropical countries. The plant grows very rapidly and serves as an ornamental and decorative purpose. It is reported to remove trace metals from aquatic systems.

(b) *Alternanthera philoxeroides* (Alligator weed): It is one of the most common aquatic weed in contaminated/polluted ecosystem. This is native to South America and naturalized in India (Naqvi and Rizvi 2000). Several Amaranthaceae produce large biomass and are suitable for environmental remediation and toxic metal cleanup (for e.g., *Amaranthaceae retrflexus*, Prasad 2001) (Table 23.1).

(c) *Talinum cuneifolium* (Portulacaceae): It is a succulent shrub of about 60-cm height with cuneate to obovate leaves, flowers in terminal panicles and purple colored corolla. It flowers and fruits throughout the year. It is widely distributed in India, Arabia, and Africa. Cuttings are a ready means of propagation of these plants. *T. cuneifolium* is and has been reported to accumulate high levels of copper in its leaves. These plants showed absorption barriers at high soil copper concentrations, indicating limits to uptake of the metal (Tiagi and Aery 1986; Adeniyi 1996).

4 Ornamental Hydrophytes for Phytoremediation

Several ecotechnological opportunities are available for aquatic plants (Lakshman 1987; Outridge and Noller 1991). The use of aquatic plants in water quality assessment has been in practice for centuries. The occurrence of aquatic macrophytes is unambiguously related to water chemistry and using these plant species or communities as indicators or biomonitors has been well recognized and established for in situ bioremediation (Deng et al. 2004). The noable examples are: *Azolla filiculoides*, *A. philoxeroides*, *Bacopa monnieri*, *Canna flaccida*, *Carex juncell*, *Carex pedula*, *Carex rostrata*, *Carex Sp*., *Ceratophyllum demersum*, *Chara*, *Nitella*, *Cladium jamaicense*, *Cyperus eragrostis*, *Distichlis spicata*, *Eichhornia crassipes*, *Elodea canadensis*, *Elodea densa*, *E. crassipes*, *Eriocaulon septangulare*, *Euryale ferox*, *Elodea nuttallia*, *E. canadensis*, *Eloea sptangulare*, *Eriophorum angustifolium*, *Eriophorum scheuchzeri*, *Glyceria fluitans*, *Hydrilla verticillata*, *Hygrophila onogaria*, *Isoetes lacustris*, *Lemna minor*, *L. trisulca*, *L. gibba*, *L. palustris*, *H. umbellata*, *Ipomea aquatica*, *Juncus articulatus*, *L. minor*, *Littorella uniflora*, *Ludwigia natans*, *Lysimachia nummularia*, *Myriophyllum spicatum*,

Table 23.1 *Alternanthera philoxeroides* (Mart.) Griseb: Potential for environmental remediation and cleanup

Tolerant to cadmium stress	Ding et al. (2007)
Responds rapidly to shoot removal	Wilson et al. (2007)
Accumulate Cd, Pb, and Zn from constructed wetlands	Liu et al. (2007a, b)
Herbivory, mowing, and herbicides differently affect production and nutrient allocation	Schooler et al. (2007)
Distribution and bioaccumulation of microcystins in water columns: a systematic investigation into the environmental fate and the associated risks with microcystins	Song et al. (2007)
Lead and zinc accumulation and tolerance in populations	Deng et al. (2006)
Exhibit phenotypic plasticity in relation to different water availability	Geng et al. (2006)
Differently respond to biological control	Li and Ye (2006)
Abiotic stress and phenotypic plasticity influenced riparian zone population	Pan et al. (2006)
Growth and reproduction simulated herbivory	Schooler et al. (2006)
Removes Ni(II), Zn(II), and Cr(VI) from aqueous solution	Wang and Qin (2006)
Genetic diversity has been established in *Alternanthera philoxeroides* in China	Wang et al. (2005)
Suitable for phytoremediation of small-scale oil spills in fresh marsh environments: a mesocosm simulation	Dowty et al. (2001)
Biologically controlled with fungi	Barreto et al. (2000)
Its extract had antiviral effect on epidemic hemorrhagic fever virus in vivo	Peng et al. (1997)
Contain phytochemicals, viz., alternanthin, A C-glycosylated flavonoid	Zhou et al. (1988)
Accumulate monosodium methanearsonate (MSMA)	Anderson et al. (1980)
The economics of its biological control	Andres (1977)
Insects as agents for biological control	Bennett (1977)
The biological control in the USA	Spencer and Coulson (1976)
Water hyacinths and alligator weeds for removal of lead, mercury, silver, cobalt, and strontium from polluted waters	Wolverton and McDonlad (1975a, b)

This is not an exhaustive

M. alterniflorum, *Melilotus indica*, *Mentha aquatica*, *Miscanthus floridulus*, *Miscanthus sacchariflorus*, *Mougeotia*, *Najas marina*, *Nasturtium officinale*, *Nuphar lutea*, *Nymphaea alba*, *Nymphaea violacea*, *Nymphoides germinate*, *Potamogeton natans*, *P. attenuatum*, *P. communis*, *Potamogeton crispus*, *P. filiformis*, *P. lapathifoilum*, *P. orientalis*, *P. pectinatus*, *P. perfoliatus*, *P. richardsonii*, *P. subsessiles*, *Phragmites karka*, *Pistia stratiotes*, *Ranunculus aquatilis*, *Ruppia maritima*, *Sagittaria latifolia*, *Salvinia acutes*, *Salvinia molesta*, *Scapania uliginosa*, *Schoenoplectus lacustris*, *Scirpus validus*, *Spartina alterniflora*, *Spirodela oligorrhiza*, *Sporobolus virginicus*, *Typha domingensis*, *Typha latifolia*, *Vallisneria americana*, *Vallisneria spiralis*, *Wolffia globosa, and Zizania aquatica* (Prasad 2007; McCutcheon and Schnoor 2003; Keskinkan et al. 2004; Peles et al. 2002; Hattink et al. 2000; Sheppard and Motycka 1997).

Aquatic plants have been frequently used to remove suspended solids, nutrients, trace metals, toxic organics and bacteria from acid mine drainage (AMD), agricultural landfill, and urban storm-water runoff. In addition, considerable research has been focused on determining the usefulness of macrophytes, as biomonitors of polluted environments and as bioremediative agents in waste water treatments. The response of an organism to deficient or excess levels of metal (i.e., bioassays) can be used to estimate metal impact. Such studies done under defined experimental conditions can provide results that can be extrapolated to natural environment. There are multifold advantages in using an aquatic macrophyte as a study material. Macrophytes are cost-effective, universally available with their ability to survive adverse conditions and high colonization rates and are excellent tools for studies of phytoremediation. Rooted macrophytes especially

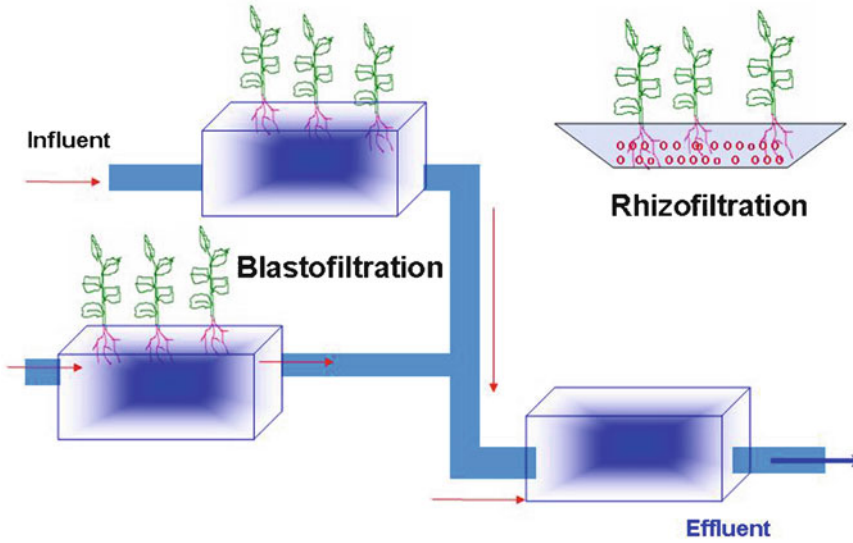

Fig. 23.7 Cascade model for removal of xenobiotics and treatment of waste streams. Most commonly employed species are *Spartina alterniflora*, Cord grass; *Sporolobus virginicus*, Coastal dropseed; *Salicornia virginica*, Perennial glasswort; *Cladium jamaicense*, Sawgrass; *Salicornia alterniflora*, Vermillon cordgrass; *Scirpus validus*, Great bulrush. A cascade model of treatment system was suggested for removal of radionuclides by rhizofiltration Dushenkov et al. 1995; 1997a, b; 2002 and blastofiltration (seedlings), e.g., sunflower

play an important role in metal bioavailability through rhizospheric processes. Macrophytes (floating, emergent, and submersed) readily take up metals in their reduced form from sediments, which exist in anaerobic situations due to lack of oxygen and oxidize them in the plant tissues making them immobile and bioconcentrate them in their tissues, thus reduced the toxic trace metal bioavailability in the interstitial waters. Rooted and emergent macrophytes make them particularly effective as bioindicator of metal pollution, as they represent real levels present at that site.

In the past research with macrophytes has centered mainly on determining effective eradication techniques for nuisance growth of several species such as *Elodea canadensis*, *Eichhornia crassipes*, *Ceratophyllum demersum*, etc. Scientific literature exists for the use of a wide diversity of macrophytes in toxicity tests designed to evaluate the hazard of potential pollutants. Estuarine and marine plant species are being used considerably less than freshwater species in toxicity tests conducted for regulatory reasons. *Lemna*, *Myriophyllum*, *Potamogeton*, *Ceratophyllu*, *Elodea*, *E. crassipes* have been exhaustively used in phytotoxicity investigations. Duckweeds have received the greatest attention for toxicity tests as they are relevant to many aquatic environments, including lakes, streams, and effluents.

The most important role of plants in wetlands is that they increase the residence time of water, which means that they reduce the velocity and thereby increase the sedimentation of particles and associated pollutants. Thus, they are indirectly involved in water cleaning. Plants also add oxygen providing a physical site of microbial attachment to the roots generating positive conditions for microbes and bioremediation.

Constructed and engineered wetlands (including naturalvwetlands) are in use for centuries for waste water treatment containing organic matter, nitrogen, phosphorus, (Kadlec and Knight 1996) (Figs. 23.7 and 23.8).

Aquatic macrophytes have paramount significance in the monitoring of metals in aquatic ecosystems (e.g., *L. minor*, *E. crassipes*, *Azolla pinnata*). Aquatic plants are important in nutrient cycling, control of water quality, sediment stabilization, and provision of habitat for aquatic organisms. The use of aquatic macrophytes in water quality assessment has been a common practice employing in situ biomonitors (Sobolewski 1999). The submerged aquatic

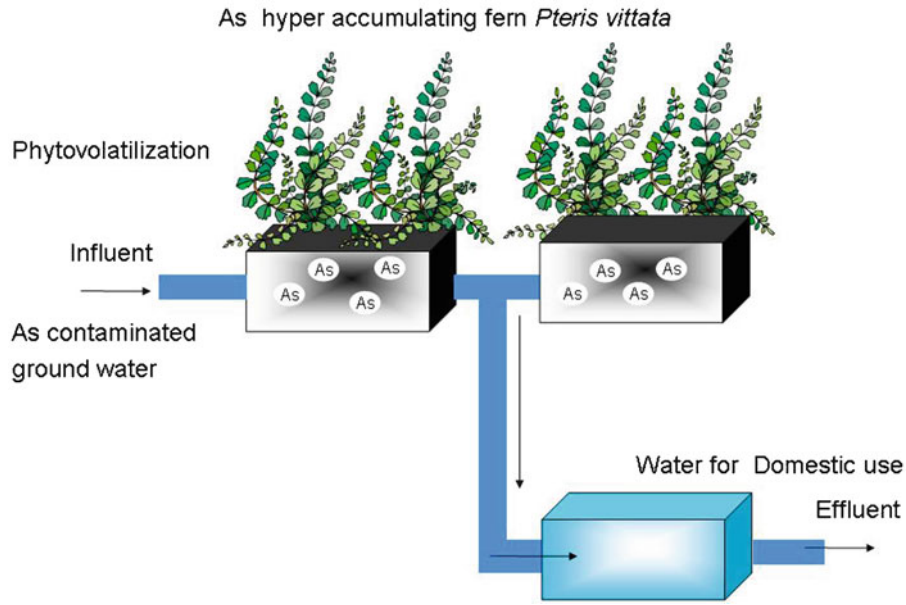

Fig. 23.8 Use of arsenic hyperaccumulator ferns and wetland vegetation (Carbonell-Barrachina et al. 1998; Rahman and Hasegawa 2011; Ma et al. 2001) for arsenic removal from water (Elless et al. 2005)

macrophytes have very thin cuticle and therefore readily take up metals from water through the entire surface. Macrophytes with their ability to survive adverse conditions and high colonization rate are excellent tools for phytoremediation. Further they redistribute metals from sediments to water and finally take up in the plant tissues and hence maintain circulation. Benthic rooted macrophytes (both submerged and emergent) play an important role in metal bioavailability from sediments through rhizosphere exchanges and other carrier chelates. This naturally facilitates metal uptake by other floating and emergent forms of macrophytes. Macrophytes readily take up metals in their reduced form from sediments, which exist in anaerobic situations due to lack of oxygen and oxidize them in the plant tissues making them immobile and thus get bioconcentrated in their tissues (Okurut et al. 1999).

A special group of plants may reduce element leakage from mine tailings by phytostabilization. Plants that are tolerant to elements of high concentrations have been found useful for reclamation of dry mine tailings containing elevated levels of metals and other elements. Mine tailings rich in sulfides, e.g., pyrite, can form AMD if it reacts with atmospheric oxygen and water, which may also promote the release of metals and As. To prevent AMD formation, mine tailings rich in sulfides may be saturated with water to reduce the penetration of atmospheric oxygen. An organic layer with plants on top of the mine tailings would consume oxygen, as would plant roots through respiration. Thus, phytostabilization on water-covered mine tailings may further reduce the oxygen penetration into the mine tailings and prevent the release of elevated levels of elements into the surroundings. Metal tolerance can be evolutionarily developed while some plant species seem to have an inherent tolerance to trace metals. Since, some wetland plant species have been found with the latter property, for example, *T. latifolia*, *G. fluitans,* and *Phragmites australis*, wetland communities may easily establish on submerged mine tailings, without prior development of metal tolerance. Some plant species have mechanisms that make it possible to cope with high external levels of elements. Low accumulators are plants that can reduce the uptake when the substrate has high

element concentrations or have a high net efflux of the element in question, thus the plant tissue concentration of the element is low even though the concentration in the substrate is high (Williams 2002; Wood and Mcatamney 1994; Woulds and Ngwenya 2004; Ye et al. 2001).

5 Utilization of Water Weeds for Bioremediation of Metalliferous Substrates

Weeds cannot be eradicated, hence there is a need to find appropriate and sustainable solutions. (Ji et al. 2011; Lin and Liu 2003); Liphadzi et al. 2003) Several of the wetland plants not only effectively purify metal contaminated water effectively (Horne 2000), Zhang et al. 2007 but also establish a dense vegetative cover (Ye et al. 2003).

For successful phytoremediation, plants chosen should have the following attributes:

(a) Adaptive and tolerance mechanisms.
(b) Fast growing with high bioproductivity such as duck weeds (Fig. 23.9) *Typha latifolia* (Cattail) and *Phragmites australis* (Ye et al. 1997a, b), *Eichhornia crassipes* (water hyacinth), *Alternanthera philoxeroides* (alligator weed), *Pistia stratiotes* (water lettuce), and *Potamogeton crispus*. The biomass production of these plants often exceeds the yield of most productive agricultural crops.

Further, the dried biomass of many of these aquatic macrophytes is an excellent biosorbent for removal of Cr(III), Ni(II), Cu(II), Zn(II), Cd(II), and Pb(II) (Andre et al. 1999). Wetland plants (water weeds) accelerate the sedimentation in constructed wetland and this being principal process for the removal of heavy metals from wastewater. Also wetland plants act as sites for metal precipitation (Mays and Edwards 2001). Water weeds for treatment of waste water are enumerated in Table 23.2

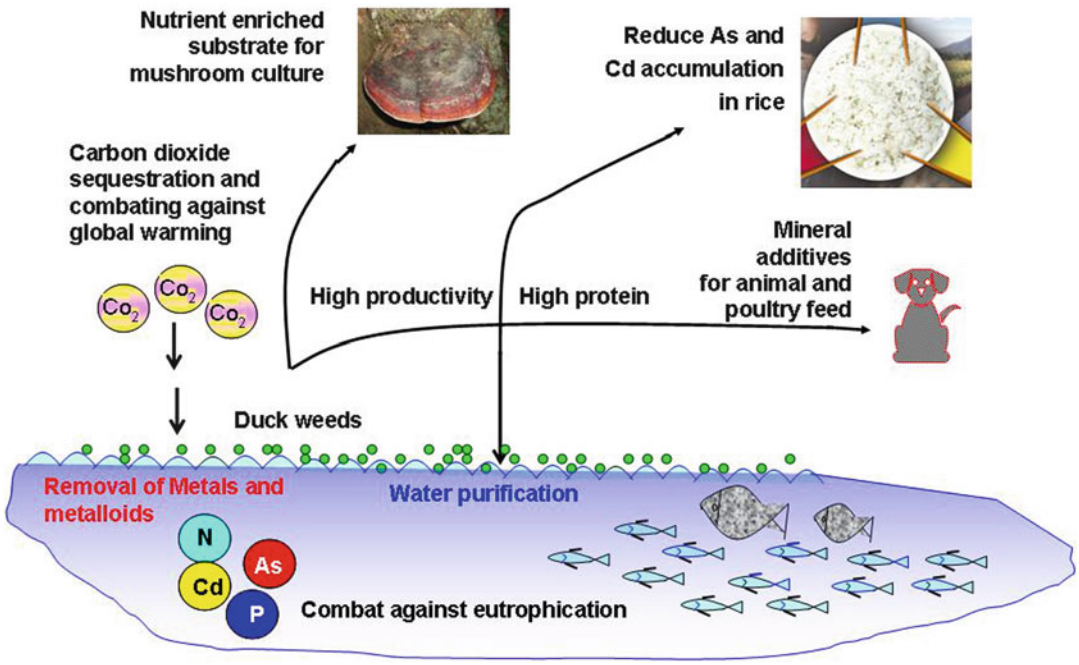

Fig. 23.9 Duck weeds for waste water treatment and for phytoproducts

Table 23.2 Water weeds for treatment of waste water: Lab, field, and pilot-scale experiments (not exhaustive)

Uptake of Zn, Cu, and Cd in metal loaded *Elodea canadensis*	Nyquist and Greger (2007)
Tolerance and phytoaccumulation of chromium by three *Azolla* species	Arora et al. (2006)
Tolerance and accumulation of copper and chromium in two duckweed species: *Lemna minor* L and *Lemna gibba* L	Ater et al. (2006)
Phytoremediation of chromium by model constructed wetland	Mant et al. (2006)
Wetland grasses for phytoremediation	Czako et al. (2005)
Accumulation of As in *Lemna gibba* (duckweed) in tailing waters of two abandoned uranium mining sites in Saxony, Germany	Mkandawire and Dudel (2005)
Potential of *Azolla caroliniana* for the removal of Pb and Cd from wastewaters	Stepniewska et al. (2005)
Lead accumulation in the aquatic fern *Azolla filiculoides*	Benaroya et al. (2004)
The ability of *Azolla caroliniana* to remove heavy metals such as Hg^{2+}, Cr^{3+}, Cr^{6+} from municipal wastewater	Bennicelli et al. (2004)
Responses induced by high concentration of cadmium in *Phragmites australis* roots	Ederli et al. (2004)
Capacity of *Salvinia minima* to tolerate and accumulate As and Pb	Hoffmann et al. (2004)
Phytoaccumulation of heavy metals by aquatic plants	Kamal et al. (2004)
Bioaccumulation of copper from contaminated wastewaters by using *Lemna minor* (aquatic green plants)	Kara (2004)
Heavy metal adsorption properties of a submerged aquatic plant (*Ceratophyllum demersum*)	Keskinkan et al. (2004)
Capacity of *Lemna gibba* (duckweed) for uranium and arsenic phytoremediation in mine tailing waters	Mkandwire et al. (2004)
Accumulation of trace elements by *Pistia stratiotes*: implications for phytoremediation	Odjegba and Fasidi (2004)
Metal uptake transport and release by wetland plants: implications for phytoremediation and restoration	Weis and Weis (2004)
Lead and nickel removal using *Microspora* and *Lemna*	Axtell et al. (2003)
Removal of heavy metals from aqueous solution by water hyacinth (*Eichhornia crassipes*)	Ingole and Bhole (2003)
Removal by marsh macrophytes *Spartina alterniflora* (cordgrass) and *Phragmites australis* (common reed)	Windham et al. (2003)
Phytoaccumulation and phytotoxicity of Cd and Cr in *Wolffia globosa*	Boonyapookana et al. (2002)
Chromium removal from tannery effluents by aquatic plants	Sinha et al. (2002)
Biosorption of cadmium and chromium in duckweed *Wolffia globosa*	Upatham et al. (2002)
Chromium phytoaccumulation from solution by selected hydrophytes	Zurayk et al. (2001)

5.1 *Ipomoea aquatica*

Ipomoea aquatica is a fast growing aquatic plant and has been applied widely to purify eutrophic water. It is a metal accumulator and metal removal potential depends upon levels of metal contamination in the water body in which they were growing. Water is regarded as a limited and susceptible resource, essential for life. It is widely distributed throughout tropical and warm climate regions in the world, especially in China and India. It is a fast-growing herbaceous vine commonly found in creeping on muddy stream banks or floating in freshwater marshes and ponds. Moreover, its leaves have high nutritive value and eaten as vegetables by human beings as well as fish and other grazing animals, and possess medicinal importance. In addition, in recent years, it is also used widely to purify wastewater (Gothberg et al. 2002, 2004; Cao et al. 2006; Hu et al. 2007). Rai et al. (1995) reported the toxic metals Pb, Cd, and Cr in *I. aquatica* accumulated highly from water resources of Eastern Ghats of India.

6 Biocontrol of Invasives Applied in Phytoremediation

Classical biocontrol agents or mycoherbicides are known for biocontrol of the following water weeds (Barreto et al. 2000): *Azolla xliculoides*, *Echinochloa polystachya*, *Eichhornia azurea*, *E. crassipes*, *Egeria densa*, *Myriophyllum aquaticum*, *Paspalum repens*, *Pistia stratiotes*, *Polygonum spectabile*, *Salvinia auriculata*, *S. molesta,* and *Typha domingensis*.

A triad approach of lab, pilot, and field studies are necessary for understanding the limitations and scope of bioremediation potential of weeds (Figs. 23.10 and 23.11).

7 Conclusions and Future Perspective

Screening of weeds and ornamentals capable of accumulating and hyperaccumulating metals for bioremediation of metalliferous substrates in the era of climate change has sufficient scope. Weeds, ornamentals, and grasses possess such properties. Some of these are extensively adaptive in capacity. Compared with crop, they possess adaptive and antistress properties which make them exceptional to grow in metalliferous substrates. With these characteristics, it is possible that weeds exhibit strong tolerance and exceptional functions to heavy metals. Identification of appropriate soil amendments that can enhance biomass production need to be investigated. Selected examples of weeds that might be useful for polishing soils contaminated with metals are *Solanum nigrum*, *Rorippa globosa*, *Bidens pilosa*, *Taraxacum mongolicum*, *Conyza canadensis,* and *Kalimeris integrifolia* (Wei and Zhou 2004a, b, 2008a, b; Wei et al. 2005, 2006, 2008a, b, 2009, 2010a, b). *Chromolaena odorata* (L) King & Robinson, (Asterceae), an invasive weed has the capability to phytoremediate soil contaminated with crude oil in the presence of metals (Atagana 2011; Tanhan et al. 2007). Critical functions of weeds for potential applications in cleanup of metalliferous substrates are:

(a) Translocation property: the contents of heavy metals in shoots should be higher than those in its roots, i.e., TF (transport factor) > 1.
(b) Enrichment factor: the concentration ratio in plant shoots to soils should be higher than 1 (EF > 1).
(c) Use of soil amendments for enhancing the biomass production for removal of metals.

Several potential and promising options for this emerging technology are forging ahead for environmental management., yet certain bottlenecks are to be investigated for wider applications such as (a) many tested plants have bioconcentration factor (BCF) less than 1, (b) usage

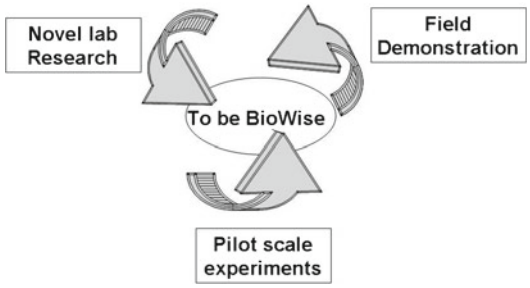

Fig. 23.10 The triad approach for successful bioremediation

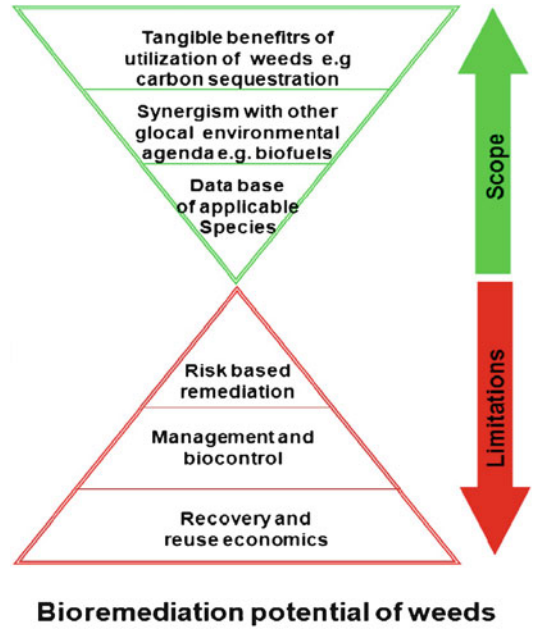

Fig. 23.11 Scope and limitations of weed application for bioremediation of heavy metals

of soil amendments and chelators may be necessary for achieving the hyperaccumulation and BCF > 10.

The regulatory bodies and environmental safety agencies are concerned about chelate and amendment assisted cleanup, as this approach would rapidly mobilize contaminant and increase the area of contamination including leaching of the toxic trace metals into ground water. Costs of the chelate applications need to be assessed. Arsenic hyperaccumulating fern, *Pteris vittata* (an important foliage ornamental), was used in the pilot-scale demonstration phytofiltration project to produce drinking quality water from arsenic contaminated ground water in New Mexico, a classic example of service to mankind (Elless et al. 2005).

The compost generated from the plants used in remediation serves as a compost and can be reused as growing media for production of ornamentals (Abad et al. 2001; Benito et al. 2006; Hernandez-Apaolaza et al. 2005; Hicklenton et al. 2001; Ingelmo et al. 1998) (Table 23.3). These questions have been satisfactorily answered for fostering phytoremediation using ornamentals. Phytoremediation technologies today have reached the site from lab to pilot-scale trials and field applications using aquatic, terrestrial (Prasad 2003, 2004a, b, 2007; Prasad and Freitas 2003) including space ecology (Kozyrovska et al. 2004, 2006). The debris generated from the ornamentals containing the toxic metal residues need to be treated as the biomass would be relatively less in view of high water contents and an appropriate

Table 23.3 Ornamentals for environmental cleanup and ecosystem service

Ornamental for environmental moderation and remediation	References
Calendula officinalis and *Althaea rosea* exhibited higher tolerance to Cd and Pb contamination and could effectively accumulate these metals	Liu et al. (2007a, b)
Ni(II) biosorption by *Cassia fistula* (its common names are Amaltas, Canafistula, Golden Shower, and Indian Laburnum)	Hanif et al. (2007)
Flindersia schottiana is a tree species used in the ornamental horticulture industry. Urea formaldehyde resin foam (UFRF) product used as a soil amendment. It is proposed to improve the physicochemical properties (viz., water relations and aeration) of the plant root zone. UFRFs are a relatively new class of soil amendment compared with hydro gels. Under plant nursery conditions, incorporation of 30% (v/v) Hydrocell™ into composted pine bark media and also into sand and loam soils led to limited, but significant ($P \leq 0.05$) growth benefits (e.g., increased leaflet number) for potted	Chan and Joyce (2007)
Biodegradable chelating agents, [*S*,*S*]-ethylenediaminedisuccinic acid (EDDS) and methylglycinediacetic acid (MGDA) assisted. Trace metals phytoextraction was demonstrated in *Mirabilis jalapa* including the growth of the associated bacterial population	Cao et al. (2007)
Phytoextraction trace metals with *Mirabilis jalapa*, combinatorial effect of biodegradable chelating agents and on its associated bacteria	Cao et al. (2007)
Crassula portulacea (Crassulaceae), *Hydrangea macrophylla* (*Hydrangeaceae*), *Cymbidium* Golden Elf (Orchidaceae), *Ficus microcarpa var. fuyuensis* (Moraceae), *Dendranthema morifolium* (Asteraceae), *Citrus medica var. sarcodactylis* (Rutaceae), *Dieffenbachia amoena cv. Tropic Snow* (Araceae), *Spathiphyllum Supreme* (Araceae), *Nephrolepis exaltata cv. Bostoniensis* (Davalliaceae), and *Dracaena deremensis cv. Variegata* (Dracaenaceae) had greatest capacity to remove benzene from indoor air	Liu et al. (2007a, b)
African marigold (*Tagetes erecta*), scarlet sage (*Salvia splendens*), and sweet hibiscus (*Abelmoschus manihot*) were investigated. According to the tolerant indexes, sweet hibiscus (*A. manihot*) was the most tolerance while scarlet sage (*S. splendens*) was the least and African marigold (*Tagetes erecta*) is in between	Wang and Zhou (2005)
Different compensatory mechanisms in two metal-accumulating aquatic macrophytes exposed to acute cadmium stress in outdoor artificial lakes	di Toppi et al. (2007)

(continued)

Table 23.3 (continued)

Ornamental for environmental moderation and remediation	References
Ficus microcarpa can provide useful information about the spatial variations of Ba, Cu, Fe, and Mg contents assuming the spatial differences are high enough. Temporal variations are evident for Al, Cu, Fe, Mg, Pb, V, and Zn. *F. microcarpa* foliage is not considered as a reliable biomonitor for Pb, Zn, and V in the urban areas	Oliva and Rautio (2005)
Tagetes patula and ornamental arum (*Syngonia* sp.) as phytoremediators of arsenic	Huq et al. (2005)
Araucaria angustifolia (Brazilian pine). The species is valuable for its wood, edible seeds, and ornamental use, and is today listed as a threatened species. This tree species establishes associations with arbuscular mycorrhizal fungus *Glomus clarum*, *Araucaria angustifolia* seedlings inoculated with *G. clarum* had a high degree of mycorrhizal colonization of their roots (81%). The inoculated seedlings grew significantly more (312% mass increase) than the controls	Zandavalli et al. (2004)
Cyperus papyrus and *Miscanthidium violaceum*-based constructed wetlands for wastewater treatment in a tropical country – a comparative study	Kyambadde et al. (2004)
Anorthosite is a poor support of the marigold growth, hence bacterial residents of alumino-silicate rocks to leach the plant essential ions from a substrate and therefore improved plant development and identified pioneer plants for a lunar base	Kozyrovska et al. (2004, 2006)
Ipomoea aquatica (water spinach, Morning glory, Convolvulace), is common in Southeast Asia. *I. aquatica* significantly accumulated toxic metals (such as cadmium, copper, and lead) in the roots, stems, and leaves	Costa-Pierce (1998), Kashem and Singh (2002), Gothberg et al. (2004)
Ornamental hydrophytes, viz., *Acorus gramineus* Soland and *Iris japonica* L., *Acorus calamus* L., *Lythrum salicaria* L., are suitable for sewage treatment. Performance of *Canna indica* on domestic sewage was better than *Phragmites communis*	Liu et al. (2003), Zhao et al. (2003)
Phosphate enhanced arsenic uptake by *Lolium perenne*, *Urtica dioica,* and *P. vittata*. Thus to alleviate arsenic toxicity, phosphate condition has to be managed	Otte et al. (1990), Cao et al. (2003)
Uptake and translocation of plutonium in two plant species using hydroponics. Comparative uptake of plutonium from soils by *Helianthus annuus*	Lee et al. (2002a, b)
Transgenic hairy roots in ornamentals: recent trends and applications	Giri and Narasu (2000)
Reed beds for water treatment	Lienard et al. (1995)
Nutrient removal from aquaculture wastewater using a constructed wetlands system	Lin et al. (2002)

techno-economic feasible options based on integrated model systems was recently suggested for the appropriate use of *E. crassipes* (water hyacinth) (Malik 2007). Similar solutions need to be worked out for the ornamental plants proposed for toxic metal cleanup, since each ornamental plant is a nonpolluting chemical factory producing a wide range of bioresource for the welfare of the mankind in addition to their ecosystem service, viz., environmental remediation and enhancing the beauty with esthetics and fragrance (Table 23.3).

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