Maryam Sarwat · Altaf Ahmad
M.Z. Abdin · Mohamed M. Ibrahim
Editors

Stress Signaling in Plants: Genomics and Proteomics Perspective, Volume 2

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Maryam Sarwat obtained her Ph.D. 2007 from Jamia Hamdard (Hamdard University). She then carried out postdoctoral research at the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi Chapter. Presently, she is working as a Senior Assistant Professor at Amity Institute of Pharmacy, Amity University, Noida, India. She has guided 15 master's research projects for her students, who are also awarded at various conferences and workshops. Dr. Sarwat has successfully handled three research projects funded by the Government of India and received extramural grants of US $15,980, US $31,961 and US $36,755, respectively, from D.S.T., D.B.T. and C.C.R.U.M., Government of India. She has presented her research work in various conferences nationally as well as internationally (France, Prague, and Germany). Based on her research findings, she has also filed two patents. Dr. Sarwat has 25 international publications with a cumulative impact factor of more than 65. Additional publications are in various stages of publication. Considering her achievements and outstanding contributions, the International Board of Awards of the National Academy of Environmental Sciences has bestowed her with the Scientist of the Year Award for 2015.

Chapter 1
Physiological, Metabolic, and Molecular Responses of Plants to Abiotic Stress

Vicent Arbona, Matías Manzi, Sara I. Zandalinas, Vicente Vives-Peris, Rosa M. Pérez-Clemente, and Aurelio Gómez-Cadenas

Abstract Plants respond to environmental challenges inducing several physiological, metabolic, and molecular responses. These responses are oriented to avoid or endure the adverse environmental condition in non-adapted plant genotypes. Under abiotic stress conditions, plants trigger mechanisms to minimize water loss through stomata; this affects photosynthetic ability of plants by reducing CO_2 intake and fixation, therefore favoring the production of ROS and the incidence of oxidative damage. Therefore, the main metabolic responses of plants to abiotic stress will be oriented to cope with water loss (inducing compatible osmolyte biosynthesis) and oxidative stress (inducing biosynthesis of antioxidant compounds). Integration of environmental stimuli and adequate modulation of the physiological response is achieved by synthesizing plant hormones (ABA, JA, SA, ET, PAs, CKs, or GAs), metabolites that act as endogenous regulators of different plant processes. Plant hormones usually act in cross talk so that different signaling pathways contribute to fine-tune specific stress and developmental responses. At the molecular level, this cross talk implies interaction with different transcription factors that bind to common and specific *cis*-acting elements in promoter regions of stress and hormone-inducible genes. Fundamental physiological and molecular information is essential to build up models and design strategies to improve or confer abiotic stress tolerance to elite crops. Based on this knowledge, different strategies are used to introgress these tolerance traits into cultivated species: marker-assisted selection of genotypes (QTLs, MABC, MARS, or GWAS), induction of polyploidy and mutagenesis followed by variant selection, and, finally, plant genetic transformation. Strategies used for crop improvement are discussed in detail, the physiological and molecular basis explained, and the potential advantages and drawbacks highlighted.

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Abbreviations

3-PGA	3-Phosphoglycerate
ABA	Abscisic acid
AB-QTL	Advanced backcross QTL
ABRE	ABA-responsive element
ADC	Arginine decarboxylase
APX	Ascorbate peroxidase
BABA	β-amino butyric acid
CAT	Catalase
CE	Coupling element
CK	Cytokinin
DHAR	Dehydroascorbate reductase
DRE	Dehydration-responsive element
EMS	Ethylmethanesulfate
ET	Ethylene
GA	Gibberellin
GR	Glutathione reductase
GWAS	Genome-wide association studies
JA	Jasmonic acid
MABC	Marker-assisted backcrossing
MARS	Marker-assisted recurrent selection
MDHAR	Monodehydroascorbate reductase
MeJA	Methyl jasmonate
NO	Nitric oxide
Pro	Proline
PSII	Photosystem II
ROS	Reactive oxygen species
SA	Salicylic acid
SAMDC	*S*-adenosyl methionine decarboxylase
SOD	Superoxide dismutase
SPDS	Spermidine decarboxylase synthase
SPMS	Spermine synthase

Plant Responses to Abiotic Stress

Introduction

Climate change represents one of the major challenges to cope with feeding an increasing world population. In this context, plants will be affected by adverse environmental conditions, as increasing temperatures might affect the crop cycle, metabolic processes such as photosynthesis and respiration, and finally affecting yield.

In addition, alterations in temperature distribution could be especially harmful during crops reproductive periods inducing gamete sterility, lower yields, and even complete crop failure (Teixeira et al. 2013). Increasing temperature would raise evapotranspiration and intensify the hydrological cycle (frequent flooding and run-off increasing drought conditions) resulting in changes in soil moisture. Increments in air pollutants such as CO_2 or ozone (O_3) are also expected and might affect plant performance. Most of the plant responses to individual abiotic stresses are well known. However, the interactions between various simultaneous stress factors are scarcely studied due to the complexity of the approach (Lobell and Gourdji 2012). In this chapter, some of these interactions and their implications to plant physiology will be discussed.

So far, most of the physiological information on the stress response has been generated using artificial approaches and model plants. Therefore, these approaches do not completely correlate with real field conditions: plant acclimation, age of plant material, and biased plant phenotyping could lead to misinterpretation of results, and only the plant response that takes place under severe stress conditions is clearly identified (Claeys et al. 2014a).

Tolerance and Adaptation

Abiotic stress is defined as the effect of an adverse environmental condition that limits plant growth and productivity (Boyer 1982). Therefore, this concept implies economic aspects and differs from those usually employed in controlled experimental conditions, where parameters such as plant survival or seed germination are usually used (Dolferus 2014). From the productive point of view, the gap between yields under suboptimal conditions and those achieved by unstressed plants is used as a magnitude of the stress indicator. However, irrespective of yield, plants are able to acclimate to diverse environmental conditions triggering different mechanisms to cope with the stressful situation (Gepstein and Glick 2013). All plant species have evolved mechanisms to cope with stressful situations, referred to as adaptation (involving genetic changes followed by selection over many generations, e.g., desiccation-tolerant seeds, pollen grains) (Minocha et al. 2014). Genetic and physiological traits conferring resistance to abiotic stresses are difficult to target at the organ or tissue level; however, physiological and molecular mechanisms leading to stress tolerance are usually restricted to particular tissues and organs (Minocha et al. 2014). Firstly, it is necessary to clarify several concepts regarding the strategy developed by plants to cope with abiotic stress factors (Verslues et al. 2006): *stress resistant* is an ambiguous term to refer to stress-tolerant plants for which the specific tolerance mechanism is not known, *stress avoidance* includes a number of strategies aimed to minimize the damaging effects of stress to tissues (e.g., stomatal closure to reduce transpiration and, hence, water requirements preventing a severe reduction in tissue ψ_w), and when avoidance mechanisms are not enough to keep plant tissues from experiencing stress *stress tolerance* mechanisms are then induced

(e.g., synthesis of compatible osmolytes and protective effector proteins, changes in metabolite composition, and induction of ROS detoxification mechanism). When tolerance mechanisms are induced and adverse conditions persist, plants reach a physiological "steady state" known as *acclimation* (also known as *hardening*) in which plants become less sensitive to the adverse conditions. It is important to note that whereas adaptation implies changes in the plant genome aimed to colonize a particular ecological niche, acclimation only involves physiological and biochemical changes orchestrated at the molecular level and modulated by plant hormones. As a general trait, crops do not possess any specific adaptation to adverse environmental conditions. Therefore, physiological, metabolic, and molecular mechanisms involved in stress resistance in crops are referred to as acclimation or tolerance.

The effects of the stress on plant growth and gene expression are dose-responsive, evidencing a fine-tuning machinery that allows sensing the stress level and adjustment of specific responses (Claeys et al. 2014a). A common response to different abiotic stress conditions is the downregulation of photosynthesis that is associated to several impairments to metabolism and growth. Under stress conditions, several metabolites are induced to cope with stress as osmoprotectants, antioxidants, or others that respond to specific stresses such as phytochelatins (see "Biochemical Responses of Plants to Stress: Basal Tolerance and Induced Tolerance" section). In addition, integration of environmental stimuli and physiological responses is mediated by an intricate network of plant hormones: ABA, jasmonates, SA, or ET that modulate stress responses (Peleg and Blumwald 2011).

Photosynthesis as a Central Process in the Response to Abiotic Stress

Abiotic stress conditions (drought, salinity, soil flooding, extreme temperatures, UV light, or O_3) reduce stomatal conductance restricting CO_2 diffusion to the substomatal chamber and limiting carbon assimilation required to maintain plant growth and development (Roy et al. 2014). Photosystem II (PSII) is particularly sensitive to CO_2 limitations, and the induction of photoinhibition reduces its efficiency enhancing ROS production (Noctor et al. 2014). Additionally, the combined effect of continuous light flux and abiotic stress accelerates the production of ROS having a synergistic impact on photoinhibition. The CO_2 limitation is linked to a reduction in the utilization of ribulose-1,5-bisphosphate by the Rubisco negatively affecting the synthesis of 3-PGA. This process uses NADPH generated in the photosynthetic electron transport chain. Increases in the $NADP^+$/NADPH ratio accelerate the reduction of O_2 to O_2^- and, subsequently, H_2O_2, derived from dismutation catalyzed by SOD. At the reaction centers, $^1O_2$ is generated after dissipation of excitation energy from chlorophylls (Asada 1999). ROS production, especially H_2O_2 and $^1O_2$, inactivates the translation of a pre-D1 protein, essential for D1 protein repair at the core of PSII reaction center (Nishiyama et al. 2004).

Plants have evolved diverse mechanisms to keep the photosynthetic apparatus functional, and photosynthetic rate is usually used as an indicator of plant/cultivar tolerance to abiotic stress. Nevertheless, there are certain plant species that show the opposite response to stress such as the NaCl-tolerant citrus rootstock Cleopatra mandarin that shows an early and strong repression of the photosynthetic machinery. Salt stress-induced stomatal closure impacts photosynthetic activity but also decreases transpiration hence reducing uptake of toxic saline ions (López-Climent et al. 2008; Brumós et al. 2009). In unstressed plants, ROS levels are tightly controlled by antioxidant systems. However, as stress conditions usually increase ROS production exceeding the cell antioxidant capacity, oxidative stress is likely to occur (Barcia et al. 2014). The cell antioxidant enzymatic system includes the enzymes SOD and CAT, operating in chloroplasts and peroxisomes; the enzymes of the ascorbate–glutathione cycle are found in chloroplasts and the cytosol: APX, MDHAR, DHAR, and GR (Asada 2006). An increase in antioxidant activity has been traditionally linked to stress tolerance (Arbona et al. 2009). Similarly, treatments that stimulate antioxidant activity have been correlated with enhanced photosystem protection and increased stress tolerance (Bandurska and Cieślak 2013).

Cross-tolerance, Memory, and Combined Stress Effects

Plants are frequently exposed to multiple stress conditions leading to synergistic, antagonistic, and sometimes neutral effects on plants. Evidently, the agronomical and physiological responses of plants exposed to several stressful factors can differ depending on the stress intensity or duration (Syvertsen and Garcia-Sanchez 2014). Literature describing the effects of combined stress conditions in plants is scarce. However, evidences point towards a negative effect of the individual stress conditions when applied together. For instance, in *Lotus japonicus*, combination of heat and drought induces the degradation of chloroplastic Cu/Zn SOD leading to increased O_2^- production, D1 protein degradation, and a decrease in PSII activity, therefore promoting photoinhibition (Sainz et al. 2010), although positive synergistic effects have also been observed (Colmenero-Flores and Rosales 2014). Environmental factors that reduce the transpiration rate such as high CO_2 concentration, low temperature, and high relative humidity could help to improve salt tolerance. In this sense, adequate stress management could result in important benefits (Syvertsen and Garcia-Sanchez 2014). Similarly, the application of heat stress could potentiate citrus and tomato fruit tolerance to other abiotic stresses such as cold damage during postharvest storage (Lu et al. 2010; Bassal and El-Hamahmy 2011). However, to be able to develop these strategies of stress management, more knowledge needs to be generated, since the specific responses to certain concurring stress conditions are not yet known.

Cross-stress tolerance is defined as physiological changes in response to previous stress conditions that protect plants from future stress events (Suzuki et al. 2012). This phenomenon is frequently linked to enhanced production of ROS such

as H_2O_2 together with the associated regulation of the expression of antioxidant and defense genes, also integrating the action of several plant hormones (reviewed in Bartoli et al. 2013 and references therein). In several research works, it was demonstrated that an increase in SA levels in response to UV-B radiation protects from drought-induced oxidative damage (Bandurska and Cieślak 2013). Similarly, S- and Cd-induced oxidative damage also promotes metabolic changes and accumulation of Pro contributing to stress damage mitigation (Bashir et al. 2013).

The "memory" concept (classically known as hardening) implies that a previous stress exposure makes the plant less susceptible to possible future stress onset. Abiotic stress conditions occur repeatedly throughout a plant life span. Therefore, plants "remember" past events and use the previous experience to build a robust response (Kinoshita and Seki 2014). This mechanism of acclimation could involve several hormone signaling pathways and the antioxidant system (Asensi-Fabado et al. 2012) triggering adaptive changes not present in relatives not previously exposed to such stress (Ding et al. 2012). Moreover, epigenetic DNA methylation appears as a plausible mechanisms underlying this "memory" effect that could be inherited (Bruce et al. 2007), representing an important mechanism driving adaptation (Munné-Bosch and Alegre 2013).

Biochemical Responses of Plants to Stress: Basal Tolerance and Induced Tolerance

Plant Tolerance to Abiotic Stress

Plants responses to abiotic stress conditions are oriented to the activation of several biochemical pathways leading to the production of defensive compounds and enhanced tolerance. These responses are referred to as "induced tolerance." However, when innate physiological status of the plant allows stress tolerance without significant induction of further defenses, it is known as "basal tolerance." Generalist species are able to induce stress tolerance to a more or less wide range, whereas basal tolerance is a result of an adaptation to harsh environmental conditions often restricted geographically and temporally.

Basal Tolerance

In basal tolerance, stressed plants have a genetically innate defense against the stress that helps them to tolerate it without any previous stimulus. Plants have developed this defense mechanism as a result of a constant and repeated exposure to the stress factors. Therefore, this has driven the conservation of certain genome changes resulting in improved physiological and metabolic defenses and adaptation to the stress conditions (Clarke et al. 2004). This is of especial relevance regarding temperature stress (Hong and Vierling 2000) or metal toxicity (Clemens 2006).

Induced Tolerance

In Induced tolerance, plants are acclimated to the adverse conditions by a gradual exposure to stress or by chemical treatments. Plant hormones such as ABA, MeJA, or SA and many other different chemical compounds can act promoting tolerance to abiotic stress. Exposure of whole plants or plant tissues in vitro to sublethal stress doses (salinity, temperature, drought, etc…) triggers progressive acclimation (or hardening) of plants to the adverse conditions (Janská et al. 2010; Mittler and Blumwald 2015). This has been traditionally used to generate stress-tolerant lines in vitro employing polyethylene glycol, mannitol, or hydroxyproline to simulate different abiotic stress conditions (Rai et al. 2011). Moreover, exogenous application of compatible osmolytes Pro and GB also induced tolerance to salinity and drought in tobacco BY-2 cell cultures and *Phaseolus vulgaris*, respectively (Hoque et al. 2007; Xing and Rajashekar 1999). In *Vicia faba*, the plant hormone SA induced tolerance to high salinity by inducing antioxidant defenses therefore reducing the impact of stress-induced oxidative damage (Orabi et al. 2013). In *Cucumis sativus*, the exogenous application of 24-epibrassinolide increased systemic photooxidative stress tolerance associated to overproduction of H_2O_2 and enhancing its signaling role (Xia et al. 2011). This response appeared also to be mediated by NO downstream the brassinosteroid signal (Cui et al. 2011). Treatment with plant hormones and other chemical compounds (such as BABA) act as *priming* agents inducing different defense mechanisms and pathways whereas progressive exposure to the stress agents increases the deleterious threshold, making plants less susceptible to stress.

Biochemical Responses to Abiotic Stress

Plants have evolved a compendium of biochemical resources to survive to stressful environmental conditions. Abiotic stress induces changes in primary and secondary metabolite composition. Regarding the primary metabolism, variations in the accumulation of carbohydrates, amino acids, and polyamines are the main responses observed in plants subjected to different abiotic stresses. The myriad of compounds produced in minute amounts and that are not essential for plant survival are referred to as secondary metabolites. These compounds have several functions depending on their chemical structure and properties: antioxidant, antimicrobial, signaling, etc. (Fig. 1.1).

Primary Metabolism

Carbohydrates

Under stress conditions, carbohydrates metabolism and accumulation is directly linked to photosynthetic performance. Plants mobilize starch and fructans from storage organs (roots, stems, amyloplasts in leaves) to obtain glucose and fructose

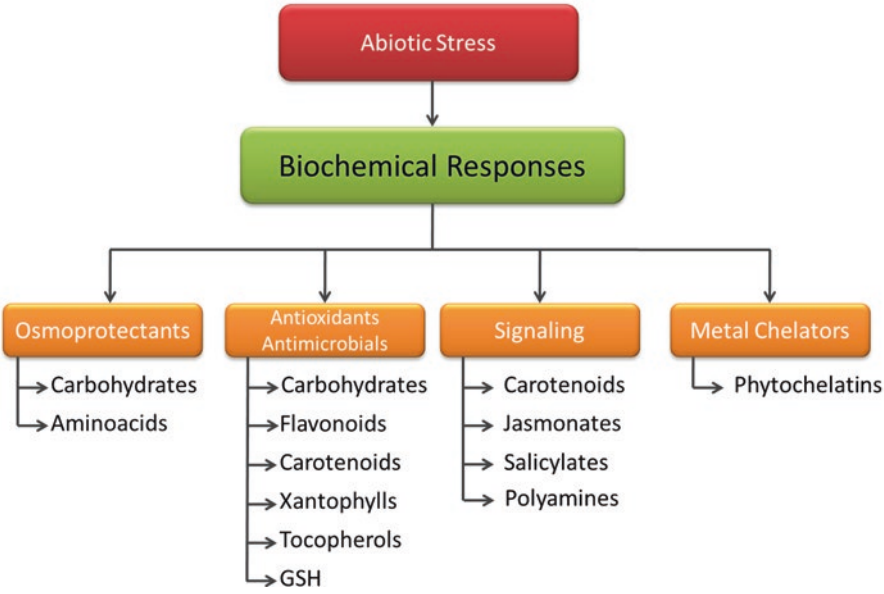

Fig. 1.1 Compound classes and roles exerted in response to abiotic stress

equivalents by increasing β-amylase activity. Besides being an important energy source, carbohydrates could act as osmoprotectants to maintain cell turgor, stabilize cell membranes, and avoid protein degradation (Sicher et al. 2012). Moreover, under stress conditions, several carbohydrates could interact with ROS, having an antioxidant activity and preventing oxidative damage (Keunen et al. 2013).

Several carbohydrate families have been shown to improve tolerance against different kinds of abiotic stress in several plant species through their action as osmoprotectants or antioxidants. Disaccharides such as trehalose are thought to have a protective role in plants subjected to drought, salinity, high or low temperatures, and oxidative stress. In transgenic tomato, overexpression the trehalose 6-phosphate 1 gene (TPS1) altered carbohydrate metabolism and improved stress tolerance (Cortina and Culiáñez-Macià 2005). Results obtained from transgenic *Arabidopsis thaliana* overexpressing a yeast TPS1-TPS2 gene fusion, suggested that trehalose was involved in altering sugar sensing and carbohydrate metabolism resulting in improved tolerance to multiple abiotic stresses (Miranda et al. 2007). Recently, trehalose has arisen as an important carbohydrate involved in the responses to heat and salt stress combination (Rivero et al. 2014). Raffinose family of oligosaccharides (RFOs) including galactinol and raffinose act as protecting macromolecules from drought, salinity, chilling, freezing, and oxidative stress (Nishizawa et al. 2008; Pennycooke et al. 2003). Overexpression of sucrose 1-fructosyltransferase gene under the control of a constitutive promoter increased oligo- and polysaccharide contents and improved tolerance to freezing in tobacco (Li et al. 2007) and chilling in rice (Kawakami et al. 2008). Moreover, transgenic plants with an enhanced

ability to accumulate sugar alcohols such as sorbitol and mannitol showed improved cell membrane stability and increased tolerance to drought, salinity, chilling, and oxidative stress (Pujni et al. 2007; Chiang et al. 2005).

Amino Acids

Amino acids are primarily present as constituents of proteins and peptides. However, this is not their only function in the plant, since some amino acids are accumulated under abiotic stress conditions in a great variety of plant species, potentially acting as osmoprotectants, antioxidants, and cell membrane stabilizers. Among these, Pro and the betaine conjugate GB are thought to play a role as compatible osmolytes, and the tripeptide glutathione (GSH, γ-L-Glutamyl-L-cysteinylglycine) is known for acting as the redox exchange molecule and also for being the precursor of PCs (Noctor et al. 2011).

Pro is synthesized from L-glutamic acid by the action of the enzymes P5CS (^{1}-pyrroline-5-carboxylate synthase) and P5CR (^{1}-pyrroline-5-carboxylate reductase) that has several roles in abiotic stress conditions, including osmotic adjustment, stabilizing subcellular structures, scavenging free radicals, and buffering cellular redox potential (Ashraf and Foolad 2007). Under drought and salt stress conditions, there is an accumulation of this amino acid in the cytosol, contributing to the osmotic adjustment. In addition, there is also an increase in Pro concentration in other abiotic stresses, as flooding or extreme temperatures (Kaplan and Guy 2004). Under stress conditions, Pro concentration is usually higher in stress-tolerant plants than in sensitive ones, contributing to plant resistance to stress. For example, in salt-tolerant alfalfa plants (*Medicago sativa*), Pro concentration under stressed conditions doubles that found in sensitive plants (Petrusa and Winicov 1997). Despite all these evidences, Pro accumulation is not a universal response associated to tolerance. In response to salt stress, Pro accumulation in the *Arabidopsis* mutant *eskimo-1* was nearly ten times higher than in wild type and 3.1-fold higher than in the halophyte *Thellungiella halophila*. Nevertheless, this mutant exhibited higher salt stress sensitivity than Thellungiella or even wild-type plants (Ghars et al. 2008).

Another molecule with a protective role to abiotic stress is the amino acid derivative GB, a quaternary ammonium compound synthesized in chloroplasts from ethanolamine, choline, and betaine aldehyde, and it is accumulated in plants in response to stress. Although its distribution among plant species is not universal, *Arabidopsis* and many crop plants do not accumulate GB (Krasensky and Jonak 2012). Like Pro, GB accumulation is usually higher in tolerant plants than in sensitive ones (Ashraf and Foolad 2007). In plants that do not produce GB naturally, introduction of biosynthetic genes improved their ability to tolerate abiotic stress, pointing to the beneficial effect of this metabolite in stress tolerance (Krasensky and Jonak 2012; Chen and Murata 2008). It has been shown that, under stressful conditions, GB protects vegetative as well as reproductive organs. Exogenously applied GB is translocated via phloem to actively growing and expanding tissues

and organs where it can act as osmoprotectant and as ROS-scavenging enzymes stabilizer (Chen and Murata 2008). It has been indicated that in salt-tolerant plant genotypes, GB accumulates to osmotically significant levels protecting PSII, stabilizing membranes and reducing the incidence of oxidative damage (Krasensky and Jonak 2012). Contrastingly, in tomato subjected to a combination of abiotic stress conditions, GB and the choline precursor did not show any significant accumulation in response to salt stress. Upon imposition of heat stress, GB and choline increased their tissue concentration about fourfold, but the combined imposition of salinity and heat reduced levels of both metabolites. In these experiments, Pro showed a significant accumulation only in response to salt stress and not to heat or combined abiotic stress conditions (Rivero et al. 2014). Taken together, these results indicate that the accumulation of osmoprotectants could be a species- and stress-specific response, probably related to the particular basal tolerance of the plant species.

As mentioned above, PCs are γGlu-Cys oligomers derived from glutathione act as intracellular chelators of toxic heavy metal ions, including As, Cd, Cu, Zn, and Mn in different plant species, such as *A. thaliana*, *O. sativa*, and *V. vinifera* (Huang et al. 2012; Dave et al. 2013; Yao et al. 2012). Nevertheless, PCs also might overaccumulate in response to other abiotic stresses.

Polyamines

PAs are low molecular weight nitrogenous aliphatic molecules derived from arginine that are involved in abiotic stress responses (Alet et al. 2012). The most widespread PAs are putrescine (Put), spermidine (Spd), and spermine (Spm) and can be present in both free and conjugated forms. The key biosynthetic genes are ADC, SPDS, SPMS, and SAMDC (Alcázar et al. 2010). Accumulation of PAs has been associated to the reduction of H_2O_2 by increasing peroxidase and CAT activities as well as Pro concentration. Abiotic stress conditions generally increase PAs concentration, and drought induces *ADC2*, *SPDS1*, and *SPMS* gene expression leading to Put accumulation but not Spd and Spm (Alcázar et al. 2006) probably as a result of efficient conjugation or removal. Salt stress also induces the expression of *ADC2* and *SPMS* subsequently inducing higher levels of Put and Spm (Urano et al. 2003). Cold stress also induces *ADC1*, *ADC2*, and *SAMDC2* gene expression therefore increasing Put concentration but, again, no effect on Spd and Spm levels is observed (Cuevas et al. 2008). In the constitutive halophyte *Prosopis strombulifera* (a native habitant of saline arid lands in Argentina), NaCl stress induces the accumulation of free Put in leaves, but not in roots, whereas the rest of polyamines reduce their concentration with respect to control value, probably as a result of NaCl-induced SAMDC inhibition (Reginato et al. 2012).

Secondary Metabolism

Phenylpropanoids

Phenolic compounds are secondary metabolites that have one or more phenolic rings, for example, flavonoids or coumarins. These compounds are derived from phenylalanine through reaction catalyzed by phenylalanine ammonia lyase (PAL) rendering cinnamic acid, the first precursor of phenylpropanoids. Phenylpropanoids constitute a highly diverse chemical class and several metabolites play important roles in abiotic stress tolerance: as important structural constituents of the secondary cell wall, ROS scavengers, protectants against UV radiation, signaling molecules and also modulators of auxin transport, etc. (Cheynier et al. 2013).

Flavonoids

Flavonoids are polyphenolic compounds derived from chalcone. According to their chemical structure, they can be classified into flavones, flavanones, flavonols, flavans, isoflavones, and anthocyanins (Djoukeng et al. 2008). In several plant species, abiotic stress induces the expression of flavonoid biosynthetic genes and the subsequent accumulation of flavonoids. However, it seems that this accumulation is not progressive and as the stress pressure increases the ability to synthesize flavonoids decreases (Bettaieb et al. 2011; Ithal and Reddy 2004). In response to soil flooding, tolerant citrus rootstocks accumulated more flavonoids than sensitive ones (Djoukeng et al. 2008), apparently constituting an adaptive response. As mentioned above, flavonoids are excellent protectants against UV radiation (Agati et al. 2011; Schenke et al. 2011).

Phenolic Acids and Other Non-flavonoid Phenylpropanoids

Phenolic compounds have several roles in plant cells: constituents of secondary cell walls (Moura et al. 2010), antioxidants (e.g., gallic and tannic acids, hydroxycinnamates and derivatives, etc.), signaling (e.g., salicylic acid), and also as phytoalexins (Arbona and Gómez-Cadenas 2015). In poplar, cadmium induced lignification of root secondary cell walls and reduced root expansion, in order to protect cells from heavy metal toxicity (Elobeid et al. 2012). On the contrary, lignification was reduced in maize roots subjected to water stress. Continuous accumulation of lignin in the absence of growth would lead to the lignification of the root elongation zone; therefore, this could be interpreted as an adaptive response to water stress allowing growth recovery after rehydration (Vincent et al. 2005). Phenolic compounds have shown an activity against high light intensities and UV-B radiation. Excess light acts as an important inducer of lignin biosynthesis in plants aiming to provide a barrier against high irradiation, and UV-B radiation induces the production of flavonoids and tannins (Moura et al. 2010). Moreover, phenolics were also induced in *Brassica*

rapa grown under elevated CO_2 (Karowe and Grubb 2011). In response to ozone stress, hydroxycinnamates can be conjugated to apoplastic polyamines acting as powerful ROS scavengers (Iriti and Faoro 2009). The role of SA in the regulation of responses to abiotic stress is not clear, and low or high levels of this compound can increase the susceptibility to abiotic stresses, being the optimal levels from 0.1 to 0.5 mM for most plant species (Yuan and Lin 2008). Furthermore, it has been demonstrated that exogenous applications of SA at low concentration induce tolerance to multiple abiotic stress conditions such as low temperatures or heavy metal toxicity. SA-induced acclimation to these adverse environmental conditions is thought to occur through the induction of ROS production as well as upregulation of the antioxidant machinery (Horváth et al. 2007).

Carotenoids and Other Terpenoids

These metabolites are generally overproduced in several abiotic stress conditions (Espinoza et al. 2013; Kim et al. 2012) and might have a protective role stabilizing the lipid phase of thylakoid membranes of chloroplasts (Volkova et al. 2009). Moreover, carotenoids act as absorbing excess light or UV radiation and also as powerful antioxidants (α-tocopherol) (Binder et al. 2009; Pateraki and Kanellis 2010). Besides, carotenoids are also sources of volatile (Beck et al. 2014) and non-volatile (Hauser et al. 2011) signaling compounds. This is the case of ABA, a sesquiterpene derived from lycopene with several functions in abiotic stress responses.

Molecular Responses to Abiotic Stress: Hormonal Regulation and Cross talk, Target Genes, and Gene Products

Regulators of Abiotic Stress Signaling: Signal Perception

Environmental signals are perceived by receptor proteins and sequentially transmitted to target signaling elements in order to respond to various external and developmental cues in a suitable and integrated manner. Receptor-like kinases (RLKs) constitute a large gene family in plants characterized by the presence of a cytosolic Ser/Thr kinase domain involved in the signal transduction to their target proteins by direct phosphorylation (Osakabe et al. 2013). RLKs control several plant mechanisms of plant growth and development as well as homeostatic processes underlying abiotic stress responses. Additionally, RLKs have been reported to have a key role in integrating environmental and plant hormone signaling (Shiu and Bleecker 2001; Diévart and Clark 2004) and can be considered as important regulators in growth and developmental processes in several environmental stress responses in the resulting adaptive mechanisms (Marshall et al. 2012). RLKs, such as RPK1, CYSTEINE-RICH RLK (CRK36), PROLINE-RICH-EXTENSIN-LIKE RLK4 (PERK4), and

GHR1 (GUARD CELL HYDROGEN PEROXIDE-RESISTANT1), have been reported to control water stress signaling directly in *Arabidopsis thaliana* (Tanaka et al. 2012; Hua et al. 2012; Osakabe et al. 2010).

Apart from RLKs, histidine kinases (HKs) are histidine-to-aspartate (His–Asp) phosphorelays similar to bacterial two-component signal transduction mechanisms involving a phosphotransferase activity that transfers a phosphate group from the sensor to the target protein. These receptors are plasma membrane- or endoplasmic reticulum membrane-bound and are involved in the control of different biological processes, including responses to different abiotic stresses such as drought, high salinity, and cold (Tran et al. 2010; Wohlbach et al. 2008; Jeon et al. 2010; Pham et al. 2012) and the perception of plant hormones. Specifically, five HK members (ETR1, ERS1, AHK2, AHK3, and AHK4) are implicated in the perception of the plant hormones ET and CK (Schaller et al. 2008). In addition, AHK2, AHK3, and AHK4 have been shown to negatively regulate ABA and stress signaling (Tran et al. 2007, 2010; Jeon et al. 2010). Moreover, AHK1 has been identified as a unique osmosensor with positive regulatory function in the expression of genes in both ABA-dependent and ABA-independent manner (Tran et al. 2007; Wohlbach et al. 2008).

Regulators of Abiotic Stress Signaling: Signal Transduction

Mitogen-activated protein kinase (MAPK) cascades constitute one of the most studied signaling mechanisms in plants. These comprise a group of highly conserved proteins with essential roles mediating perception of external stimuli and the induction of adaptive responses in all eukaryotic organisms (Hamel et al. 2012). MAPK cascades are composed of MAP kinase kinase kinases (MAP3Ks/MAPKKKs/MEKKs), MAP kinase kinases (MAP2Ks/MAPKKs/MEKs/MKKs), and MAP kinases (MAPKs/MPKs) sequentially activated by phosphorylation and have been involved in abiotic stresses responses (Rodriguez et al. 2010; Mittler 2002). The *A. thaliana* genome contains approximately 80 MAPKKKs, 10 MAPKKs, and 20 MAPKs that are activated by diverse stress signals, offering the possibility of cross talk between diverse stress signals. For instance, AtMPK6 is involved in O_3, H_2O_2, ET, ABA, and JA signaling pathways, and also in several developmental processes (such as epidermal cell patterning and anther and embryo development), probably acting as points of convergence of different stress and hormonal signaling pathways (Sinha et al. 2011). In *Arabidopsis*, the best characterized MAPK cascade in abiotic stresses is the MEKK1-MKK2-MPK4/MPK6 module. Mutants impaired in MKK2 activity also exhibited a deficient MPK4 and MPK6 activation and showed salt and cold hypersensitivity. Moreover, abolition of MKK2 activity altered expression of 152 genes involved in transcriptional activation in the *Arabidopsis* genome (Teige et al. 2004).

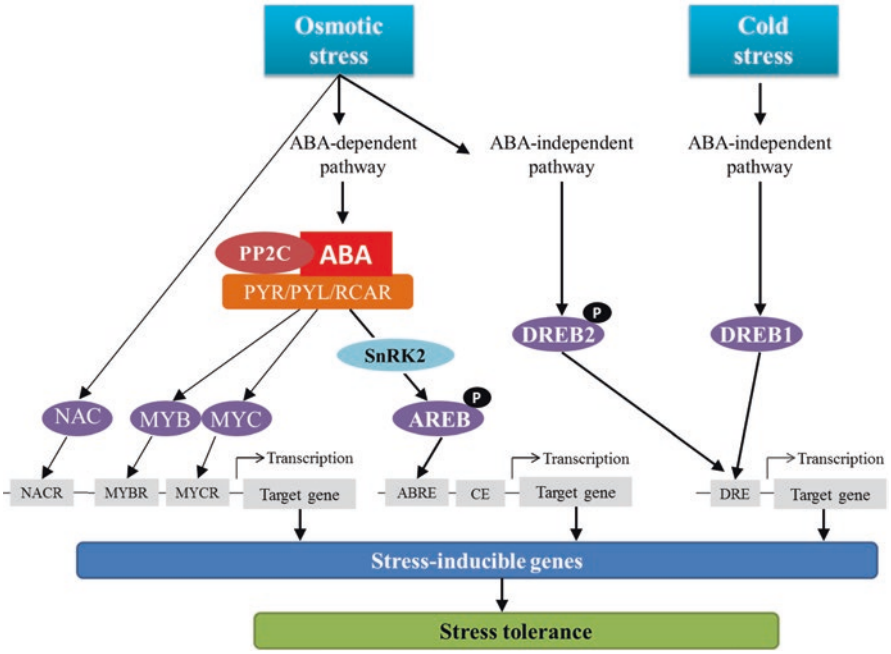

Fig. 1.2 Hormone and stress-dependent signaling pathways leading to stress-inducible gene expression

Hormonal Regulation of Abiotic Stress Responses

Plant hormones play central roles in the ability of plants to adapt to changing environments by mediating growth, development, and/or nutrient allocation (Peleg and Blumwald 2011). To this respect, ABA is a key regulator of many plant responses to environmental stresses, particularly osmotic stress (Hubbard et al. 2010). Recent studies have identified the components of the ABA signaling pathway (Fig. 1.2). Briefly, the core pathway consists of ABA receptors (PYR/PYL/RCAR), clade A protein phosphatases 2C (PP2C) such as ABI1 which act as negative regulators, and SNF-related protein kinases (SnRK2) which are positive regulators that phosphorylate several TFs and effectors, like the guard-cell SLAC-family anion channels, which mediate fast stomatal closure. Besides, other kinase families participate in ABA signal transduction leading to stomatal closure, such as Ca^{2+}-dependent CPK/CDPKs and the CBL-interacting CIPKs (Geiger et al. 2011). In the presence of ABA, PYR/PYL/RCAR inhibit PP2C activity resulting in release of SnRK2 proteins enabling ABA-mediated responses and the transcription of ABA-responsive genes (Santiago et al. 2012).

Experimental results have clearly shown that cross talk among different plant hormones is essential in integrating different environmental signals and readjusting

growth as well as acquiring stress tolerance. Other plant hormones have important roles in abiotic stress responses: GAs, ET, SA, JA, and auxin have recently been found to interplay with ABA at different levels. Vegetative and reproductive growth and development are regulated by GAs throughout the plant life cycle (Claeys et al. 2014b; Schwechheimer and Willige 2009; Wang and Irving 2011). DELLA (Asp-Glu-Leu-Leu-Ala) proteins act as repressors of GA-dependent gene expression, and their degradation results in GA response in plants (Hauvermale et al. 2012). Cross talk between GAs and ABA occurs at the biosynthetic level via the putative early DELLA target gene XERICO whose expression is induced by salt and osmotic stress and causes hypersensitivity to ABA. Moreover, overexpression of this gene results in elevated ABA content (Ko et al. 2006). ET/ABA cross talk regulates stomatal opening (Wilkinson and Davies 2010). Auxins have an epistatic role over ET/ABA cross talk in controlling root hair elongation and root branching under abiotic stress (Wang et al. 2013). Besides, ET induces the expression of ERF TFs that are also responsive to JA (Wu et al. 2009) and regulate a diverse range of processes associated to environmental cues (Wang et al. 2013).

The vast majority of studies on SA mode of action have predominantly been oriented toward its role in plant defense responses. However, recent reports have demonstrated that SA also plays an important role in modulating the plant response to many abiotic stresses (Kang et al. 2014; Miura and Tada 2014). SA interacts with GAs at different levels: SA application partially rescues seed germination in *ga1-3* mutant, and GA_3 application improved performance of SA-deficient *sid2* under salt stress (Alonso-Ramírez et al. 2009). Cross talk between ABA and SA signaling pathways was evidenced by the effect of SA on the synthesis of ABA-regulated proteins in *Arabidopsis* (Rajjou et al. 2006). Under stress, cross talk between jasmonates and ABA at the biosynthetic levels has been also observed in Arabidopsis and Citrus (Oa et al. 2009; De Ollas et al. 2013). In *Arabidopsis thaliana*, the TF MYC2 has been proposed to regulate the interaction between ABA and JA signaling pathways acting as a master switch between the two signaling pathways (Dombrecht et al. 2007; Kazan and Manners 2013). Auxins have also been shown to participate in the positive regulation of drought stress tolerance through regulation of root architecture, expression of ABA-responsive genes, ROS metabolism, and metabolic homeostasis (Shi et al. 2014). Under moderate drought, ABA accumulation modulates auxin transport in the root tip, which enhances proton secretion necessary to maintain root growth (Xu et al. 2013).

Transcriptional Factors Involved in Hormonal and Abiotic Stress-Associated Gene Expression

TFs interact with *cis*-elements in the promoter regions of several stress-inducible genes to regulate the expression of many stress-inducible genes involved in stress acclimation and tolerance (Agarwal et al. 2006). Studies of the transcriptional

regulation under drought and salinity have revealed the existence of ABA-dependent and -independent pathways (Yoshida et al. 2014). The ABA-dependent signaling system induces a family of TFs ABF/AREB/ABI5 (ABA-binding factor/ABA-responsive element-binding protein/ABA Insensitive 5) constituted by bZIP TFs that control ABA-mediated gene expression under abiotic stress conditions and developmental processes. These TFs are activated through multiple-site phosphorylation of their conserved domains by SnRK2s (Fujita et al. 2011, 2013; Furihata et al. 2006). Some of them also respond to specific environmental challenges; for instance, ABF1 is significantly induced by cold (Kim 2006) but not by osmotic stress (Fujita et al. 2004), whereas AREB1/ABF2, AREB2/ABF4, and ABF3 are induced both by ABA and high osmoticum (Yoshida et al. 2014). Besides ABF/AREB/ABI5, MYB2 TF belonging to the R2R3-type MYB family has been shown to be an important mediator of ABA-mediated gene expression under adverse conditions in *Arabidopsis* (Stracke et al. 2001). Other MYB TFs participate in ABA-mediated gene expression, including MYB102 that has been reported to respond to ABA, JA, salt stress, and wounding (Denekamp and Smeekens 2003); MYB41 that responds to drought, ABA, and salt treatments (Cominelli et al. 2008); MYB108 that functions as a positive transcriptional regulator of JA- and ABA-inducible genes, therefore playing an important role in abiotic and biotic stress tolerance (Mengiste et al. 2003); MYB44 which is activated by several hormone treatments (ABA, auxins, ET, JA, and GA) as well as by environmental conditions such as drought, high salinity, and low temperature (Jung et al. 2008; Persak and Pitzschke 2014).

On the other hand, AP2/ERF (reviewed in Lata and Prasad 2011) are a large family of plant-specific TFs that share a well-conserved DNA-binding domain. This family includes DRE-Binding proteins that activate the expression of stress-responsive genes, independent of ABA. This group includes CBF/DREB1 (cold-binding factor/dehydration responsive element binding 1) TF whose expression is strongly and transiently induced by low temperature stresses (Fowler et al. 2005) and DREB2 (e.g., DREB2A and DREB2B) that are induced by drought, high salinity, and heat stress but not by cold stress or exogenous ABA (Sakuma et al. 2006a, b).

There are other TFs involved in abiotic stress tolerance responses such as NACs or WRKYs (Chen et al. 2012; Nakashima et al. 2012). The encoded proteins show a conserved N-terminus region possessing five DNA-binding motifs, whereas the more divergent C-terminus contains domains that function as transcription activators (as extracted from yeast and plant assays) (Kleinow et al. 2009; Chen et al. 2014; Puranik et al. 2012). NAC TFs have been identified in Arabidopsis (ANACs) (Wang and Dane 2013) and crops such as banana (Cenci et al. 2014), cotton (Huang et al. 2013), tomato (Yang et al. 2011a, b, c), rice (Chen et al. 2014), poplar (Hu et al. 2005), and citrus (Puranik et al. 2012). Promoters in NAC genes contain domains known to be responsive to ABA (ABREs), JA, SA, drought (DREs), and low temperature (LTREs) as well as sites of recognition for MYB and MYC TFs (Puranik et al. 2012). In addition, in Arabidopsis, NAC factors such as ATAF1 have been recently shown to regulate expression of NCED3 and subsequent ABA accumulation (Jensen et al. 2013) and, at the same time, this TF is able to physically interact with SnRK2, involved in ABA signaling (Kleinow et al. 2009).

Cis-*Acting Elements Involved in ABA-Dependent and ABA-Independent Gene Expression*

Promoter sequences of stress- and hormone-inducible genes contain conserved boxes (or *cis*-acting elements) that allow binding of specific TFs acting as downstream stress or hormone signaling pathways (Shinozaki et al. 2003). Hence, promoter regions of ABA-inducible genes contain multiple conserved *cis*-elements globally named ABREs (PyACGTGGC) or combinations of ABREs and CE (Shinozaki et al. 2003). These are similar to ABREs and contain A/GCGT motif (Hobo et al. 1999). Genes containing ABREs in their promoter regions include several LEA class proteins, clade A PP2Cs, and diverse types of TF (Nakashima and Yamaguchi-Shinozaki 2013). In *Arabidopsis*, plant dehydrins RD29A and B are upregulated by stress and ABA, respectively. RD29B has two ABRE domains in its promoter region (Uno et al. 2000), whereas RD29A contains ABREs and also DREs. The existence of different *cis*-acting elements allows cross talk between different signaling pathways. For instance, DRE and ABRE motifs in RD29A promoter have been reported to act independently but, under certain circumstances, DRE could function cooperatively with ABRE (Narusaka et al. 2003). In addition, DREBs can physically interact with AREBs (Lee et al. 2010) suggesting potential cross talk between stress- and hormone-signaling pathways.

Stress-Responsive Effector Proteins

Stress-regulated genes and their products have important roles in stress response and tolerance. There are several groups of proteins that are induced in response to abiotic stress and are involved in stress acclimation mechanisms. For instance, hydrophilins are a group of proteins (including LEA proteins) which possess a 6% Gly content and hydrophilicity index higher than 1 (Battaglia et al. 2008). Other proteins induced by stress are small heat shock proteins (HSP) that are primarily induced upon heat stress (Liu et al. 2012). Small HSPs are molecular chaperones that assist correct protein folding. Under heat stress, mitochondrial HSP22 and chloroplast HSP21 were highly expressed in grapevine (Liu et al. 2012). Heat stress can also alter plasma membrane fluidity activating certain HSP genes encoding for proteins that act in preventing membrane disintegration (e.g., hspA) (Allakhverdiev et al. 2008). As mentioned above, plants alter their cell wall composition and stiffness in response to abiotic stress by inducing the phenylpropanoid pathway. In this process, there are a group of proteins called hydroxyproline-rich glycoproteins or HRGPs that are an integral part of cell walls and have functions in several aspects of plant growth and development. These can be divided into hyperglycosylated arabinogalactan proteins, moderately glycosylated extensins, and lightly glycosylated proline-rich proteins (Showalter et al. 2010). Although it is not absolutely clear, available evidence suggests a role of HGRPs in heavy metal stress tolerance (Yang et al. 2011a, b, c).

In addition, environmental stress also increases ROS production; hence, a myriad of proteins aimed to alleviate oxidative stress are induced: SOD, APX, CAT, GR, etc… Most of these enzyme activities have been targets of breeding programs aimed to improve abiotic stress tolerance (Gill and Tuteja 2010).

ABA- and stress-responsive genes, such as LEA class genes (RD29B, RAB18), cell cycle regulator genes (ICK1), PP2Cs (ABI1 and ABI2), and RD22 and RD26, have received special attention as potential targets for improvement of stress tolerance. The genes encoding for these proteins have conserved ABREs and DREs motifs in their promoter regions. RD22 gene expression is cooperatively mediated by ABA, MYC2, and MYB2 TFs (Shinozaki and Yamaguchi-Shinozaki 2007). In *Arabidopsis*, RD26 encodes a NAC protein induced by dehydration and ABA (Fujita et al. 2004). On the other hand, promoter of ERD1 gene (encoding a Clp protease regulatory subunit, ClpD) contains a MYC-like sequence (CATGTG) and a 14-bp *rps1* site 1-like sequence involved in dehydration response (Simpson et al. 2003). This aspect is reviewed in detail in the next Section of this Chapter.

Strategies for Engineering Abiotic Stress Tolerance: From Genotype Selection to Genetic Transformation and Genome Editing

A current challenge in applied plant physiology is the production of abiotic stress-tolerant crops able to endure water shortage, raising ambient temperature and CO_2 and other environmental cues (Suzuki et al. 2014). This aspect is becoming critical as world population, and food demand is steadily increasing while the land available for agriculture is being progressively reduced. Currently, more than one billion people have insufficient food to sustain life, and it is expected that by 2050 food supply would have to double to satisfy demand, as calculated on the basis of staple grain crops (maize, wheat, and rice, Reynolds et al. 2012). The four major strategies to induce stress tolerance in crops are: (1) identification of variability into wild populations relatives to the crops of interest and use them as rootstocks or parentals for breeding and transfer stress tolerance traits to crops, (2) polyploidization of crops of interest, (3) in vitro random mutagenesis and selection of variants, and (4) identification of particular genes from related or other plant species that might confer stress tolerance and transfer them into crops by genetic transformation.

Identification of Variability in Stress Tolerance from Natural Populations

Stress tolerance, high yield, and quality features do not come often together and, as a result, it is necessary to introduce these traits into economically valuable crops. Most of the stress tolerance traits observed in crops are controlled by a complex

network of genes. Therefore, accurate phenotype selection and development of genetic markers to aid in precocious selection of stress-tolerant variants is an active field in plant breeding. These genetic markers are referred to as Quantitative Trait Loci or QTLs, and their development for several crops and abiotic stress conditions has boosted up breeding strategies (Des Marais and Juenger 2010).

As a first step, extensive trials in mild to severe stress conditions are required to identify accessions with a significant tolerant phenotype (Atwell et al. 2014). Platforms for automated phenotyping of accessions and accurate control of environmental variables have facilitated this process (Granier et al. 2006). However, the use of these automated platforms is only feasible for small-sized plants that can be grown in a confined space (such as *Arabidopsis*, barley, or cotton) but for bigger plants or trees it is expensive and difficult to implement. This is partially overcome with the use of all the physiological information built up over the years and the genetic information made available recently for different crops such as barley (Muñoz-Amatriaín et al. 2014), citrus (Wu et al. 2014), or grapevine (Liu et al. 2012; Deluc et al. 2007) that allows identification of gene candidates and markers via genome-wide association studies (GWAS). Phenotyping of tolerance traits implies that hundreds or thousands of genetic lines have to be properly assessed in order to determine the alleles responsible of the stress tolerance. Typical phenotype parameters include yield as the primary trait but also other stress tolerance markers associated to metabolic and regulatory functions underlying stress tolerance and yield maximization are included (Setter 2012). As an example, in many rice-producing areas worldwide, submergence tolerance constitutes a key trait. Sub1 is a major QTL in rice landraces (FR13A) controlling submergence tolerance. This could be introgressed into cultivated rice genome by MABC, similar to backcrossing but genes and QTLs are transferred from donors to elite cultivars with the aid of marker-based background and foreground selections, thus eliminating the chances of receiving any undesirable linkage drag (Neeraja et al. 2007; Bailey-Serres et al. 2010). To this respect, introgression of tolerance genes from wild relatives to the cultivated rice constitutes a laborious task due to the high genetic distance existing among these species. Despite this issue, most overcrosses of wild relatives with domesticated *O. sativa* cultivars have been achieved so far, providing an excellent background for trait selection and improvement. Nevertheless, rice improvement in the near future will involve targeted transfer of genes from *O. sativa* landraces and wild germplasm into cultivated rice, probably along with traditional breeding programs (Atwell et al. 2014). Another example of marker-assisted breeding is chickpea, the second most important legume crop after beans. Several germplasm accessions exist that could be used as sources of resistance traits to different abiotic constraints such as drought, severe heat stress, low temperatures, or salinity (Jha et al. 2014). Abiotic stress tolerance is a complex attribute controlled by a myriad of major-effect QTLs but also by small-effect QTLs that also exert an effect on the plant phenotype. Besides this, genotype–environment interaction and epistasis hamper trait improvement by traditional breeding techniques (Jha et al. 2014; Atwell et al. 2014). Other strategies, such as MARS, help overcoming the problem of small-effects QTLs. In this strategy, a marker index is created and used for

genotype selection and intercrossing of selected individuals in advanced generations. Other working schemes designed are AB-QTL that does not need a predefined gene/trait association and, more recently, GWAS in which DNA marker data and phenotyping scores are used instead of detecting significant QTLs. In general, all these genetic marker-assisted approaches share the fact that phenotyping is only needed once (Jha et al. 2014; Dumas 2012), constituting an advantage with respect to laborious stress tolerance assays needed on every round of individual selection.

Polyploidy as a Stress Tolerance Trait

Polyploidy refers to the duplication of an entire genome and is a common feature in the plant kingdom. More than 70 % of flowering plants are polyploids: 75 % allopolyploids (polyploids arisen from merging two divergent genomes into the same genome) and the rest autopolyploids (result of duplication of the same genome). Allopolyploids exhibit changes in gene expression due to the increase in heterozygosity constituting the basis of subfunctionalization (Moghe and Shiu 2014), whereas in autopolyploids no gene complementation is possible as genome duplicates have the same origin. In these individuals, variations in gene expression induce profound changes in phenotype with respect to the diploid parentals or relatives (Allario et al. 2011). Polyploidy could be a response to the strong environmental stress pressure constituting an adaptation mechanism (Madlung 2013; Moghe and Shiu 2014). In a study performed on wild populations of the annual grass *Brachypodium distachyon*, ploidy level positively correlated with improved WUE, constituting a possible adaptive strategy (Manzaneda et al. 2012). In citrus, autotetraploid Rangpur lime conferred stress tolerance to the grafted variety when used as a rootstock through decreased stomatal conductance and increased ABA content (Allario et al. 2012a, b). Autopolyploidization could be artificially achieved by using colchicine and allopolyploidization of incompatible species by in vitro protoplast fusion and embryo rescue. Nevertheless, in this process crop characteristics such as quality and yield may vary (bread wheat, a hexaploid or strawberry, an octaploid) with respect to their diploid parental lines.

In Vitro Mutagenesis and Selection of Stress-Tolerant Variants

This is probably the most classical approach by which chemically or physically induced variations in DNA sequence are at the basis of phenotype-driven selection. In *Arabidopsis*, homogeneous seed populations were massively treated with EMS or gamma radiations. Mutagenized plant material is subsequently subjected to environmental selection pressure (e.g., abiotic stress: high salinity, osmotic pressure, etc.); germinating seeds, which cotyledons do not turn senescent after exposure to stress, are maintained until the reproductive growth stage and homozygous lines

generated in subsequent reproductive stages (Mba et al. 2007; Ben-Hayyim and Moore 2007). This procedure can be carried out in vegetative tissues, cell cultures, calli, etc., and it is totally random. The success rate is a function of the population of different variants generated. In the ornamental plant species *Chrysanthemum morifolium*, plant explants were established in vitro and treated with EMS and the resulting mutant tissues subjected to organogenesis to regenerate shoots, which were subsequently subjected to NaCl stress and those showing better performance selected (Hossain et al. 2006). Potato tuber shoot explants were cultured in vitro and subjected to gamma radiation and the regenerated plants exposed to salinity recovering the most tolerant (Yaycili and Alİkamanoğlu 2012). Sugar-beet explants grown in vitro were mutagenized by gamma radiation and the resulting variants selected for their drought tolerance. Drought tolerance was correlated with increased antioxidant enzyme activity (Sen and Alikamanoglu 2014). In rapeseed seedlings, cold tolerance was induced by treating in vitro-cultured microspores with UV radiation, inducing point mutations, and variants were subjected to a chemical in vitro selection assay. Selected lines showed increased SA, JA, and Pro production (McClinchey and Kott 2007). In conclusion, random chemical or physical mutation is a feasible strategy without previous genetic information on the crop to improve stress tolerance or other traits of agronomic interest. However, to ensure acceptable success rates using this approach, it is necessary to finely adjust the selection media and to screen large populations of mutants, which often make this strategy unfeasible.

Genetic Transformation and Genome Editing

Engineering of plants for enhanced response to abiotic stress has classically consisted in strengthening the own plant response systems by intervening at different steps of the response: from sensors, signaling/regulatory components (e.g., receptors, kinases, etc.), to direct action genes or effectors (e.g., biosynthetic genes, antioxidants enzymes, heat shock proteins, etc.) (Cabello et al. 2014). To this respect, the quest for target genes that might confer stress tolerance to crops arises from the physiological and molecular characterization of wild relatives of plant genotypes of interest tolerant to abiotic stress: genes involved in osmolyte biosynthesis, regulation of oxidative stress (Jin and Daniell 2014; Balestrazzi et al. 2009), PC biosynthesis (Liu et al. 2011), or root-to-shoot transport of solutes (Wojas et al. 2009; Barabasz et al. 2013). The extremophile *Thellungiella salsuginea*, a close relative of *A. thaliana* (Amtmann 2009), tolerates cold, salinity, and drought as well as nutrient constraints. Some genes from *Thellungiella* have been associated to stress tolerance, cloned, and functionally characterized. Their transfer to Arabidopsis or other plant systems (such as tobacco) resulted in improved abiotic stress tolerance. Overexpression of ThCBL9, encoding for a Calcineurin B-like Calcium Sensor Protein 9 a protein that interacts with CBL-protein kinase 23 (CIPK23) targeting it to the plasma membrane, in *Arabidopsis* resulted in improved

K^+ uptake under saline conditions, thus reducing the damaging effects of NaCl stress (Ren et al. 2013; Alemán et al. 2009). Overexpression of a ^{1}-pyrroline-5-carboxylate synthase (P5CS) in citrus increased tolerance to drought by ameliorating osmotic adjustment through Pro biosynthesis (Molinari et al. 2004). Similarly, overexpression in poplar of *mtl*D gene, encoding a mannitol-1-phosphate dehydrogenase, improved salinity tolerance through the accumulation of mannitol and influencing osmotic adjustment under salt stress (Hu et al. 2005). Despite the evidences supporting the efficiency of several effector genes as stress tolerance inducers, their applicability to "real life" environmental conditions seems quite restricted. When plants are grown under controlled conditions, the stress factor is perfectly defined and its pressure adjusted. However, in the field several stress factors concur at the same time inducing different physiological and molecular responses than the individual stresses (Suzuki et al. 2014). Moreover, it seems that stress combination has a different effect on physiology and/or gene expression than isolated stress conditions (Rivero et al. 2014). This makes TF and other regulator genes ideal targets for engineering stress tolerance (see "Molecular Responses to Abiotic Stress: Hormonal Regulation and Cross talk, Target Genes, and Gene Products" section). *Arabidopsis* plants overexpressing AtMYB44 were more sensitive to ABA and more tolerant to salinity and water deprivation (Jung et al. 2008). A novel MYB TF from soybean, GmMYBJ, whose expression is induced by high osmoticum, salinity, cold, and ABA, induced drought tolerance in Arabidopsis by reducing oxidative damage and the rate of water loss (Su et al. 2014). As discussed before, AP2/ERF TF mediate a myriad of physiological responses to environmental stresses such as submergence and hypoxia (Yang et al. 2011a, b, c; Hess et al. 2011), drought, and osmotic stress (Golldack et al. 2014). In soybean, GmERF3 expresses in response to several abiotic elicitors as well as to hormonal treatments and its overexpression in tobacco enhanced tolerance to salinity and osmotic stress (Zhang et al. 2009). In *Arabidopsis*, overexpression of *Triticum aestivum* ERF1 increased survival rate under cold, drought, and high salinity conditions as well as reduced expansion of *Botrytis cinerea* and *Pseudomonas syringae* (Xu et al. 2007). bZIP TFs (ABF/AREB/ABI5 clade) have also been implicated in the induction of stress responses and in the switching off of ABA signal through direct interaction with PP2Cs (Lynch et al. 2012) suggesting the existence of additional regulatory mechanisms. Hence, overexpression of a maize ABI5 orthologues in tobacco decreased tolerance to mannitol, NaCl, and temperature stress (Yan et al. 2012). On the contrary, overexpression of SlAREB (orthologous of *Arabidopsis* ABF2/AREB1) in *Arabidopsis* and tomato led to increased abiotic stress tolerance and higher relative water content (Hsieh et al. 2010). These contrasting results suggest that not all TF regulate the same responses and also that temporal regulation is an important aspect in transcriptional responses. Recently, NACs (NAM, ATAF, CUC2 family of TFs) such as ATAF1/2 or CUC1 have drawn much attention as potential targets for conferring stress tolerance in transgenic plants. Expression of sequences identified in wheat (TaNAC2 or TaNAC67), cotton (GhNACs), or rice

(OsNAP) rendered plants with enhanced tolerance to abiotic and biotic stress conditions (Mao et al. 2012; Huang et al. 2013; Chen et al. 2014; Redillas et al. 2012; Xue et al. 2011). In rice, OsNAP overexpression induced clade A PP2Cs, genes like OsDREB1A or OsMYB2 resulting in improved stress tolerance (Chen et al. 2014). Targeting TF and other signaling components broadens the effect on stress tolerance in clear contrast to effector proteins that constitute a more restrictive "symptom-driven" approach.

In recent years, targeted genome editing using artificial nucleases has become a reality providing a tool to modify genomes rapidly, precisely, and in a predictable manner (Bortesi and Fischer 2014). Before 2013, the only genome editing tools available were zinc finger nucleases (ZNF) and transcription activator-like effector nucleases (TALENs). Both systems are based on artificial fusion proteins comprising an engineered DNA-binding domain fused to the nonspecific nuclease domain of the FokI restriction enzyme. Conversely, CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-associated 9) from *Streptococcus pyogenes* is based on RNA-guided engineered nucleases. The system is a part of the immune system of *S. pyogenes* that integrates invader DNA fragments (spacers) between two adjacent repeats at the proximal end of the CRISPR locus (Bortesi and Fischer 2014). Guiding RNAs are 20 nucleotide sequences that can be engineered to have homology with any part of the genome; these sequences guide the Cas9 nuclease to the action site. This endonuclease cleaves homologous double-stranded DNA at the target site. This technique has been successfully applied to model plants *Arabidopsis thaliana* (Schiml et al. 2014) and *Nicotiana benthamiana* (Nekrasov et al. 2013) but also to crops such as rice (Shan et al. 2014) or Citrus (Jia and Wang 2014). An important aspect of CRISPR/Cas9-engineered crops is that this technique leaves no trace of transgenes or selection genes, therefore it is possible that CRISPR/Cas9-engineered crops would not be considered genetically modified organisms anymore, according to the current regulatory framework. To date, several crops have been engineered by means of genome-editing tools such as CRISPR/Cas9: *Citrus sinensis*, targeting the CsPDS gene that encodes a phytoene desaturase (Jia and Wang 2014); rice, targeting several genes involved in carotenoid biosynthesis (OsPDS) or signal transduction such as genes encoding for MAP kinases or TFs such as MYB1 (Bortesi and Fischer 2014), to name a few. This technique has quickly developed over the last 2 or 3 years allowing an increasing number of research groups to implement CRISPR/Cas9 genome editing tools. This will make possible further advances in the understanding and control of the CRISPR/Cas9 system and the development of new genome-editing tools.

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Chapter 2
Molecular Network of Monoterpene Indole Alkaloids (MIAs) Signaling in Plants with Reference to *Catharanthus roseus* (L.) G. Don

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Abstract Terpenoids are the most functionally and structurally varied group of plant metabolites. These are synthesized in all organisms but are especially abundant and diverse in plants. Despite their diversity of functions and structures, all terpenoids are derived from the common five-carbon (C5) building unit, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) through MEP pathway in all living organisms. The MEP pathway synthesizes IPP and DMAPP in plastids in plants and more complex terpenoids are usually formed by "head-to-tail" or "head-to-head" addition of isoprene units. Monoterpene indole alkaloids (MIAs) are one of important class of terpenoids which are C10 terpenoids, consisting of two isoprene units and are the components of more than 2000 defined compounds. These are a large group of plant-derived natural products with a range of pharmacological properties. These MIAs are potent drugs, such as anticancer, antimalarial, and antiarrhythmic agents. These MIAs have been found in eight different plant families, being most common in Apocynaceae, Rubiaceae, and Loganiaceae. Madagascar periwinkle, *Catharanthus roseus*, the best-characterized MIA-producing plant species, is the source of the valuable MIAs. It has approximately 130 alkaloids of indole group, out of which 25 are dimeric in nature. Some of these compounds have important medicinal properties such as anticancerous property (vincristine and vinblastine) and antiarrhythmic property (ajmalicine and serpentine). In the present chapter, molecular network of monoterpene indole alkaloids (MIAs) signaling in plants with reference to *Catharanthus roseus* (L.) G. Don has been reviewed.

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Introduction

Medicinal plants are the resources of new drugs. They have been playing an essential role in the development of human culture and are used from the ancient times in the treatment of diseases. Most of the plants with medicinal properties are consumed by humans as spices and seasonal food (Tapsell et al. 2006). This formation of secondary metabolites is important due to the medicinal effects of the plant species. These secondary metabolites are produced to cope up the plants with the harsh environmental condition and to interact the plant with environmental conditions. These secondary metabolites also play a significant role in the defense mechanism of plants. Approximately, 80 % of active compounds among the isolated 120 compounds are currently used traditionally as well as in modern therapeutic use (Fabricant and Farnsworth 2001). In the modern pharmacopoeia, at least 7000 medicinal compounds are derived from plants. The World Health Organization (WHO) estimates that approximately 80 % of the Asian and African population presently use herbal medicines. In 2011, the annual global export value of medicinal plants alone being over was US$ 2.2 billion.

Catharanthus roseus (L.) G. Don is one of the most important medicinal plants which synthesize a large group of structurally diverse secondary metabolites collectively known as Monoterpenoid Indole Alkaloids (MIAs). More than 130 types of MIAs are derived in the whole plant with important pharmaceutical activities (Van der Heijden et al. 2004). At present, it is widely used as plant of interest due to the presence of chemotherapeutic agents, having activity against several kinds of diseases (Schmeller and Wink 1998). Vincristine and vinblastine are the most important alkaloids with anticancerous activities (Mukherjee et al. 2001). Both of these agents have an ability to form tubulin para crystals that help in preventing tubulin polymerization and interfere with the mitotic spindle during cell division which causes metaphase arrest (Dumontet and Sikic 1999). Their antineoplastic activity may actually arise from the combined disruption of multiple microtubule-dependent processes, inhibition of the cell cycle, and induction of programmed cell death (Dumontet and Sikic 1999). Some other MIAs like serpentine are used for the treatment of anxiety. For example, ajmalicine is used for treating arterial hypertension (Kruczynsky and Hill 2001) and antiarrhythmic disorders (Morant et al. 2003). Water extract of *C. roseus* leaves is beneficial in the treatment of fever, arrest of bleeding, and diabetes (Ross 2003). The cost of extracted vincristine and vinblastine is very high and the level of this compound in the leaves of *C. roseus* is very low (around 0.0005 % DW) (De Luca and Laflamme 2001).

All these MIAs of *C. roseus* are obtained from the central precursor strictosidine, which forms after the fusion of tryptamine moiety of shikimate pathway and secologanin moiety, derived from the plastidic nonmevalonate pathway. The biosynthesis of these monomeric and dimeric alkaloids in *C. roseus* involves at least 35 intermediates and 30 enzymes, 30 biosynthetic agents, 2 regulatory genes, and 7 inter and intracellular compartments (St-Pierre et al. 1999; Van der Heijden et al. 2004). However, information about the regulatory steps involved in these pathways is meager. Given this, information about the biochemical and molecular aspects of MIA pathway is reviewed here.

Monoterpene Indole Alkaloids

Alkaloids containing structural moiety of indole are called indole alkaloids. It is one of the largest classes of alkaloids containing more than 4100 known different compounds (Seigler 1998). On the basis of their biosynthesis, the indole alkaloids are distinguished as isoprenoids and nonisoprenoids (Dewick 2002). Monoterpene indole alkaloids (MIAs) are most extensively distributed in secretory tissues of higher plants, insects, fungi, and marine organisms. These are composed of ten carbons of two condensed isoprene units. Many of the MIAs exhibit biological activities and is used for fragrance, ingredients in cosmetics, and as medicinal agents. They are synthesized either through Mevalonic acid (MVA) pathway or Methylerythritol pathway (MEP) and are divided as acyclic, monocyclic, and bicyclic types. Because of their biological activities, progress has been made on the biotransformation, biogenesis, isolation, structure determination, and bioactivity studies of monoterpenoids in the recent years (Tholl and Lee 2011).

Biosynthesis of Monomeric and Dimeric Indole Alkaloids of *C. roseus*

Formation of indole alkaloids involved a long and complex biosynthetic pathway that led to the characterization of numerous biosynthetic intermediates and enzymes. To collect the information about the involvement of precursors and intermediate and regulation of pathway, characterization of enzymes has been found very useful.

Indole Pathway (Biosynthesis of Tryptamine Precursor)

Biosynthesis of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan occurs through a common shikimate pathway in plants, microorganism, and in some animals (Herrmann and Weaver 1999). Five enzymatically controlled steps are involved in the formation of L-Tryptophan (Bongaerts et al. 2001; Whitmer et al. 2002a, b). The first step is the formation of anthranilate from chorismate which is catalyzed by the enzyme anthranilate synthase (AS, E.C. 4.1.3.27). *N*-(5-phosphoribosyl) anthranilate formation takes place in the second step, catalyzed by the enzyme phosphoribosyl diphosphate (PR)-anthranilate transferase (E.C. 4.1.1.48). Enzyme PR-anthranilate isomerase (E.C. 5.3.1.24) catalyzed the third step which involved the formation of 1-(*O*-carboxyphenylamino)-1-deoxyribulose phosphate. This is followed by the formation of indole-3-glycerol phosphate catalyzed by the enzyme indole-3-glycerol phosphate synthase (E.C. 4.1.1.48). Next step is catalyzed by the enzyme tryptophan synthase (E.C. 4.2.1.20) which catalyzes the formation of tryptophan (Jacobs et al. 2005). Tryptophan, an indole

precursor is converted into tryptamine by pyridoxal-dependent decarboxylation of L-tryptophan by tryptophan decarboxylase (TDC) enzyme, encoded by *tdc* gene (Back and Chappell 1996; Facchini 2001).

Anthranilate Synthase (AS, E.C. 4.13.27)

Anthranilate synthase catalyzes the formation of anthranilate. It is a tetramer and was first isolated and purified from *C. roseus* (Poulsen et al. 1993). It consisted of two large and two small subunits. The large α-subunit converts chorismate into anthranilate and the smaller β-subunits are responsible for the generation of the substrate NH3 from glutamine (Verpoorte et al. 2002). Among tissue analysis the enzymatic activity of AS was found highest in roots as compared to the other organs in *C. roseus* (Jacobs et al. 2005). Some studies on *C. roseus* cell suspension culture revealed tryptophan synthase (E.C. 4.2.1.20) activity for the short period of time during the growth phase and in immobilized cells (Facchini and DiCosmo 1991). The molecular characterization of alpha subunit of AS is studied in *Arabidopsis thaliana* (Niyogi and Fink 1992), *Camptotheca acuminate* (Lu et al. 2005), and *Ruta graveolens* (Bohlmann et al. 1996). All these showed that α-subunit of AS is encoded by two genes *ASA1* and *ASA2*. Mutation in *Saccharomyces cerevisiae* and in *E. coli* confirmed that both the genes encode functional AS proteins. The level of mRNA of *ASA1* is observed comparatively much higher than *ASA2* in all the plants. Increased expression level of *ASA1* induces alkaloid biosynthesis. In *Ruta graveolens*, differential expression of AS genes regulates the biosynthesis of primary and secondary metabolites in the pathway.

Tryptophan Decarboxylase (TDC; EC 4.1.1.28)

The enzyme, tryptophan decarboxylase (TDC) links the primary metabolism with the secondary metabolic pathway for further MIAs biosynthesis (Goddijn et al. 1994). It is a substrate specific, soluble dimeric cytosolic enzyme, having molecular weight of 115 kDa and consists of two 54 kDa monomeric subunits with a pl of 5.9. A cDNA clone encoding TDC was first isolated from *C. roseus*. Identity of isolated cDNA was confirmed through expression in *Escherichia coli* (De Luca et al. 1989) and *Nicotiana tabacum* (Li et al. 2003). Under the response of external factors like fungal extract and hydrolytic enzymes treatment the expression of *tdc* gene is induced. It is also regulated by abiotic stresses and tissue-specific developmental control (Meijer et al. 1993). In *C. roseus* cell cultures, overexpression of *tdc* gene did not result in an increased production of MIAs. This indicated that the activation of *tdc* expression is not only responsible for MIA biosynthesis but also it is not a rate-limiting enzyme involved in metabolite synthesis (Meijer et al. 1993). Reduction and loss of TDC activity in hairy root cultures was observed to reduce the accumulation of MIAs (Moreno-Valenzuela et al. 2003).

Iridoid Pathway (Formation of Secologanin)

Biosynthesis of secologanin is initiated from isopentyl diphospate (IPP) through a number of steps, some of which are still unknown. In the beginning, IPP forms monoterpene 10-hydroxygeraniol from geraniol by the enzyme geraniol 10-hydroxylase (G10H) (Collu et al. 2001). Formation of IPP is itself occurred through the cytosolic mevalonate (MVA) pathway and plastidial methyl-erythritol phosphate (MEP) pathway. Recently, it was discovered that besides the MVA pathway, mevalonate-independent pathway was used by plants and bacteria for the formation of IPP and is widely known as 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. MEP pathway in plants leads to the formation of monoterpenes, diterpenes, chlorophylls, carotenoids, phytohormones, and trans-cytokinins. Some studies performed on cell cultures also proved that the biosynthesis of secologanin occurs through the MEP pathway in *C. roseus* (Contin et al. 1998) but now some evidences showed that the MVA pathway serves as a minor source of precursors for iridoid biosynthesis and contributes in the fine regulation of the MEP gene expression through protein prenylation (Oudin et al. 2007).

In *C. roseus*, biosynthesis of secologanin is divided into two phases. First includes the biosynthesis of isopentenyl diphosphate (IPP) and other involve the synthesis of secologanin which starts with the isomerization of IPP to dimethylallyl diphosphate (DMAPP). In *C. roseus*, MEP pathway involves the seven steps out of these four steps are identified and well characterized at molecular level (Chahed et al. 2000).

1-Deoxy-D-Xylulose-5-Phosphate Synthase (DXS, EC 2.2.1.7)

Enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS, EC 2.2.1.7) catalyzed the first step where the condensation of hydroxyl ethyl thiamine (formed by the decarboxylation of pyruvate in the presence of thiamine diphosphate, TPP) with glyceraldehydes 3-phosphate at C1 aldehyde group occurs to yield 1-deoxy-D-xylulose-5-phosphate (DXP) after the loss of TPP. The step requires divalent cations Mg^{2+} or Mn^{2+} for its activity (Chahed et al. 2000). The enzyme belongs to the family transketolases. The gene (*dxs*) encoding the enzyme was first cloned from *E. coli* (Lois et al. 2000). In plants, it has been cloned from *A. thaliana* (Araki et al. 2000), *W. somnifera* (Gupta et al. 2013) are some to name. The cDNA (*Crdxs)* encoding for the enzyme in *C. roseus* is also isolated and characterized which encodes an ORF of 716 aa (76.8 kDa) which is 70 % identical with the *A. thaliana* DXS (Chahed et al. 2000). The expression of *dxr* gene showed the positive correlation with the isoprenoid biosynthesis under light and dark conditions in case of *A. thaliana* seedlings (Carretero-Paulet et al. 2002), tomato (Enfissi et al. 2005), *C. roseus* cell cultures etc. (Veau et al. 2000). In plants, it was reported that the final products of the pathway vary between two and seven folds. This analysis supported the fact that DXS catalyses a rate-limiting step for the synthesis of IPP and DMAPP building blocks (Kuzuyama et al. 2000; Matthews and Wurtzel 2000).

1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase (DXR, EC 1.1.1.267)

Second step involved reduction and rearrangement of DXP to branched chain polyol2C-methyl-D-erythritol 4-phosphate (MEP) which is catalyzed by NADPH-dependent 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR, EC 1.1.1.267) (Proteau 2004). The enzyme encoded by the gene *dxr* has been characterized in *E. coli* (Querol et al. 2002) and its orthologs are isolated and characterized from plants like *A. thaliana* (Schwender et al. 1999), *C. roseus* (Veau et al. 2000), and many other plants. Enzyme DXR exists as homodimers with molecular weights between 42 and 45 kDa. In *C. roseus*, cDNA of Cr*dxr* encodes a peptide of 474 aa (51 kDa) with 89 and 76 % identity with the DXR from *A. thaliana* and *Mentha Piperata*, respectively. In *C. roseus*, upregulation of gene *dxr* contributed in increased production of MIAs (Veau et al. 2000). The antibiotic fosmidomycin acts as a specific inhibitor for DXR and widely used in MEP pathway regulation studies (Phillips et al. 2008).

4-Diphosphocytidyl-2C-Methyl-D-Erythritol Synthase (CMS, EC 2.7.7.60)

In the third step, MEP is converted into 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) by an enzyme 4-diphosphocytidyl-2C-methyl-D-erythritol synthase (CMS, EC 2.7.7.60). For this conversion, enzyme requires the participation of divalent cation preferably Mg^{2+} and can use several nucleotide triphosphates as a substrate besides a cytidine 5′-triphosphate (CTP). The enzyme has been isolated and characterized in *A. thaliana* and its catalytic domain is 30 % identical to *E. coli* enzyme (Rohdich et al. 2000). Whereas in *C. roseus* the enzyme is not yet characterized (Oudin et al. 2007).

CDP-ME Kinase (CMK, EC 2.7.1.148)

Phosphorylation of CDP-ME occurs by the enzyme CDP-ME kinase (CMK, EC 2.7.1.148) which requires the cofactor Mg^{2+} for this activity. The enzyme is a member of the GHMP protein family and has not been characterized in *C. roseus* till now.

2-C-Methyl-D-Erythritol 2,4-Cyclodiphosphate Synthase (MECS, EC 4.6.1.12)

Fifth step involved the conversion of CDP-ME into the unusual cyclodiphosphate containing intermediate 2-C-methyl-D-erythritol 2,4-cyclo diphosphate (MECDP) in a Mg^{2+}/Mn^{2+}-dependent reaction catalyzed by the enzyme 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECS, EC 4.6.1.12) (Richard et al. 2002). The gene *crmecs* is well characterized in *C. roseus* that encodes a protein of 236 aa having molecular weight of 25 kDa. The gene is 48 % identical to *E. coli* and 90 % identical to *A. thaliana* gene products. It is also reported that the expression level of MECS showed positive response to the accumulation of MIAs (Veau et al. 2000).

HMBPP Synthase (HDS, EC 1.17.4.3)

MECDP is then converted by the enzyme HMBPP synthase (HDS, EC 1.17.4.3) into 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBPP). The enzyme is encoded by the gene *hds* that has been characterized in *A. thaliana* and *L. esculentum* (Querol et al. 2002). A cDNA clone of *hds* gene (*crgcpe*) is available for *C. roseus* (A. Oudin, unpublished results). A polypeptide from *A. thaliana* contains two structural domains (N-terminal extension and the central domain) of 30 kDa, which are absent in *E. coli.* This indicates that N terminal region targets the protein to chloroplasts in *A. thaliana* under in vivo conditions and also has been concluded that the enzyme is not a rate-limiting enzyme but act as an important housekeeping gene in plants in MEP pathway (Rodriguez-Concepcion et al. 2004).

HMBPP Reductase (HDR, EC 1.17.1.2)

HMBPP in the final step is reduced to form the mixture of IPP and DMAPP in 5:1 ratio. The reduction is catalyzed by the enzyme HMBPP reductase (HDR, EC 1.17.1.2) (Cunningham et al. 2000). Like HDS, HDR is a [4Fe–4S] protein (Wolff et al. 2003). The *hdr* gene has been cloned from *A. thaliana*, but not yet from *C. roseus*.

Acetoacetyl-CoA Thiolase (AACT, E.C. 2.3.1.9)

The first step for the formation of IPP in mevalonate pathway initiated with coupling of two molecules of acetyl-CoA. AACT enzyme catalyzed the reaction which belongs to the family thiolase. It contains two conserved cysteine residues essential for thiolase activity. One cysteine molecule, which involved in the formation of an acetyl enzyme intermediate, is located at the N-terminal section of the enzymes while the other acts as active site for the deprotonation in the condensation reaction that is located at the C-terminal extremity (Montamat et al. 1995). The enzyme is unstable and sensitive to high salt concentrations. Its full length cDNA is isolated and characterized in *A. thaliana*, rice, and radish (Vollack and Bach 1996) and partially purified from a cell suspension culture of *C. roseus* (Van der Heijden et al. 1994).

HMG-CoA Synthase (HMGS, E.C. 4.1.3.5)

In second step, an enzyme HMGS catalyzed the condensation of acetoacetyl-CoA with another molecule of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Using HPLC, catabolic activity of three HMG-CoA was observed in *C. roseus* cell suspension culture (Van der Heijden et al. 1994). Like AACT, HMGS is also unstable and sensitive to high concentration. It is partially characterized in suspension cultured cells of *C. roseus* and in para rubber tree. The enzymatic activity of enzyme and expression level of mRNA showed the positive relationship with the accumulation of rubber. These researches indicate that the enzyme showed high regulation of gene.

HMG-CoA Reductase (HMGR, E.C. 1.1.1.34)

HMG-CoA reductase (HMGR) reduced HMG-CoA to form mevalonate. It is a rate-limiting enzyme, inhibited by lovastatin (Caelles et al. 1989). It consists of three domains: the N-terminal region, a linker region, and a C-terminal catalytic domain which is highly conserved (Istvan and Deisenhofer 2000). The enzyme is well studied in plants, fungi, bacteria, archeobacteria, and animals. The gene encoding HMGR has been purified and characterized from number of species including *C. roseus, Camptotheca acuminate* (Maldonado-Mendoza et al. 1997), and *A. thaliana* (Learned and Connolly 1997). HMGR is regulated by different environmental and developmental signals like infection, wound, light, sterols, herbicides, and hormones (Bach 1995). In *C. roseus* hairy roots, overexpression of *hmgr* gene increased MIAs levels.

Mevalonate Kinase (MVAK, E.C. 2.7.1.36) and 5-Diphosphomevalonate Kinase (MVAPK, E.C. 2.7.4.2)

Mevalonate is phosphorylated by enzymes mevalonate kinase (MVAK) and 5-diphospho mevalonate kinase (MVAPK) to form 5-diphospho mevalonate and is extensively studied in cell cultures and plants of *C. roseus* (Schulte et al. 2000). Both the enzymes are of GHMP kinase ATP-binding protein family having Gly/Ser-rich region in the N-terminal section (Lee et al. 2005). In cell cultures of *C. roseus*, enzyme MVAK was purified to homogeneity and characterized. The enzyme is stable and strongly inhibited its activity by farnesyl diphosphate. Its enzymatic activity depends on the presence of the divalent ions, Mg^{2+} and Mn^{2+} (Schulte et al. 2000). In *A. thaliana*, the expression pattern of genes suggested that the enzyme MVAK plays an important role in biosynthesis of different classes of isoprenoids and also involved in cell functioning (Lichtenthaler et al. 1997). The full length cDNA encoding MVAPK has been characterized in *C. roseus* (Schulte et al. 2000), *A. thaliana*, and *H. brasiliensis* (Lichtenthaler et al. 1997) but functional characterization is unrevealed.

5-Diphospho-Mevalonate Decarboxylase (MVAPP Decarboxylase, E.C. 4.1.1.33)

The MVAPP decarboxylase catalyzed the last step of MVA pathway which leads to the conversion of MVPP to IPP. In *A. thaliana*, there is a small gene family for the enzyme which may function as homodimer or heterodimer (Cordier et al. 1999). Little is known about this enzyme and characterization is done only in a few plant species (Contin et al. 1998; Verpoorte et al. 2000).

IPP Isomerase (IDI, E.C. 5.3.3.2)

The reversible conversion of IPP to DMAPP is one of the important steps required for the biosynthesis of different classes of isoprenoids and MIAs are catalyzed by the enzyme IPP isomerase (IDI; EC 5.3.3.2). This activity is found in all living organisms (Ramos-Valdivia et al. 1998). The enzyme is classified as two types on the basis of cofactors required for their activity. Type I IDIs are zinc metalloproteins that require Mg^{2+} for their catalytic activity (Lee and Poulter 2006), encoded by two distinct genes. The short and long isoforms of both the genes have been expressed in *A. thaliana.* The difference lies in the different lengths at their N-terminal ends (Phillips et al. 2008). Type II IDIs are tetrameric flavoproteins that require reduced form of flavin mononucleotide and Mg^{2+} for its activity (Sharma et al. 2010). The enzyme was also isolated and partially purified from *C. roseus* cultures (Ramos-Valdivia et al. 1998). All the organs of *C. roseus* show the high expression of both long and short transcript of *CrIDI 1,* on the other hand *CrIDI 2* is barely transcribed in mature leaves (Guirimand et al. 2012). In 5-day-old, *C. roseus* cell culture IDI activity was inhibited during the first 120 h after the treatment with *Pythium aphanidermatum* elicitor (Moreno et al. 1996). In *Nicotania benthamania,* silencing of plastidial IDI revealed the decrease in chlorophyll and carotenoid contents (Page et al. 2004).

Geranyl Diphosphate Synthase (E.C. 5.1.1.1)

Geranyldiphosphate (GPP)is formed by the condensation of DMAPP and IPP in a head-to-tail manner. This condensation is catalyzed by geranyl diphosphate synthase (GPPS) which belongs to the family of short-chain prenyl transferases (Ogura and Koyama 1998). It exists as homomeric and heteromeric structures and has been characterized in *Abies grandis, Arabidopsis thaliana*, *Lycopersicon esculentum* (as homomeric) (Schmidt and Gershenzon 2008; Schmidt et al. 2010) and as heteromeric GPPS in *Mentha piperita*, *A. majus* (Wang and Dixon 2009). The heteromeric GPPS in *A. thaliana* was found in plastids but in some species it also possess cytosolic isoforms (Bouvier et al. 2000) and is composed of a non catalytic small subunit (SSU) and a large subunit (LSU) (Wang and Dixon 2009). Despite the fact that the enzyme catalyzes the branch point reaction for the biosynthesis of monoterpene moiety of all MIAs, very little information about the type of GPPS involved in MIA biosynthesis is known so far. It has been reported that homomeric GPPS in gymnosperms and SSUs of heteromeric GPPS regulate monoterpene biosynthesis (Hsiao et al. 2008; Schmidt et al. 2010). In *C. roseus,* the gene *CrGPPS* that encode the enzyme is characterized and found that its large subunit form an active heteromeric GPPS to catalyze the efficient biosynthesis of GPP and play an important role in maintaining the relative amounts of precursors for both primary as well as secondary metabolism. On the other hand, homomeric *CrGPPS* might be involved in mitochondrial isoprenoid biosynthesis for ubiquinones. Expression of small subunit (SSU) under abiotic and biotic stress showed induced activity level of GPPS, resulting in increased GPP synthesis which enhances MIA biosynthesis (Rai et al. 2013).

Geraniol 10-Hydroxylase (CYP76B6; EC 1.14.14.1)

In the diverse range of plant species, hydroxylation of geraniol at its C-10 position is catalyzed by cytochrome P450 monooxygenase enzyme geraniol 10-hydroxylase (G10H) and initiates the first committed step in the formation of iridoid monoterpenoids. G10H was first characterized in *C. roseus* (Meehan and Coscia 1973) and also purified from *C. roseus* by means of solubilization with cholate in cell culture suspension in four steps (Meijer et al. 1993). After certain modifications, molecular cloning and the functional expression of full length cDNA encoding G10H enzyme was reported in *C. roseus* cell culture. The encoded CYP76B6 protein (493 aa) is able to hydroxylate geraniol and nerol into their 10-hydroxy derivates and hence catalyzes G10H activity when expressed in yeast cells and transgenic *C. roseus* (Collu et al. 2001). The enzyme CPR is required for the activity of G10H as it transfers electrons from NADPH to the cytochrome P450 monooxygenase (Collu et al. 2001). Overexpression of G10H in the hairy roots improved the production of several TIAs under stress and when combined with hormones and precursors (Peebles et al. 2009; Wang et al. 2010). This indicated a close relationship between G10H activity and alkaloid accumulation. In *C. roseus* cell culture, the activity of enzyme can be repressed by ketoconazole and induced by phenobarbital (Contin et al. 1999).

Cytochrome P450 Reductase (CPR; E.C. 1.6.2.4)

CPR is an essential enzyme required for the activity of enzyme G10H as it transfers electron from NADPH for all cytochrome P450 monooxygenases including G10H (Meijer et al. 1993). CPR for functioning requires the FMN, FAD, and NADPH cofactors. The CPR isoforms and corresponding cDNAs were isolated from plant species, such as *Vigna radiate* (Shet et al. 1993), *Catharanthus roseus* (Meijer et al. 1993), and *Papaver somniferum* (Rosco et al. 1997). The enzyme is also partially purified from etiolated seedlings of *C. roseus* plant (Madyastha and Coscia 1979) and it was also found that the *cpr* transcripts are detected in all the tissues of 3-month-old *C. roseus* plants. CPR mRNA level is enhanced by fungal elicitor treatments in *C. roseus* (Lopes Cardoso et al. 1997). The N-terminus of CPR enzyme possesses a hydrophobic domain which serves as a membrane anchor. Its promoter contains strong GT-1 binding sites at −632 to −366 regions, elicitor-inducible expression found to be absent when this region was deleted (Lopes Cardoso et al. 1997).

10-Hydroxygeraniol Oxidoreductase/Acyclic Monoterpene Primary Alcohol Dehydrogenase (10HGO/ADH)

Few early studies suggested that 10-oxogeranial/10-oxoneral and iridodial are intermediates between 10-hydroxygeraniol/10-hydroxynerol and secologanin in *C. roseus* (Uesato et al. 1984). The oxidization of 10-hydroxygeraniol and 10-hydroxynerol takes place at C1 and C10 positions to give 10-oxogeraniol and 10-hydroxyl geranial which further oxidized to form 10-oxogeranial. This reaction is catalyzed by 10-hydroxygeraniol

oxido reductase/acyclic monoterpene primary alcohol dehydrogenase (10HGO/ADH) which contains Zn ions and requires NADP+ or NADPH as the cofactor. The enzyme is not purified from *C. roseus* till now but cDNA clone encoding the enzyme from *C. roseus* is available (GenBank accession number AY352047). Purified ADH is available from the leaves of *Nepeta racemosa* (Hallahan et al. 1995) and cell culture of *Rauwolfia serpentine* (Ikeda et al. 1991). Another NADPH-dependent enzyme 10-oxogeranial cyclase converts 10-oxogeranial into iridodial (Uesato et al. 1984). The enzyme if partially purified from *C. roseus* hairy roots (Sanchez-Iturbe et al. 2005) and from cell free extracts of *R. serpentine* (Ikeda et al. 1991).

Loganic Acid Methyltransferase (LAMT, E.C. 2.1.1.50) and 7-Deoxyloganin 7-Hydroxylase (DL7H, EC 1.14.13.74)

Iridoidal form 7-deoxyloganic acid in the presence of some unknown enzymes. The enzyme is suggested as possibly P450-dependent enzyme (Contin et al. 1999) which has not been described so far. In the next step, loganic acid is synthesized from 7-deoxyloganic acid by means of two ways which in the first way include hydroxylation step precedes a methylation step. Here, an enzyme 7-deoxyloganic acid hydroxylase initiates hydroxylation of 7-deoxyloganic acid at the C-7 position to give loganic acid which further undergo methylation process to give loganin by an enzyme *S*-adenosyl-L-methionine: loganic acid methyl transferase (LAMT). It is localized in the leaf epidermis (Guirimand et al. 2011). This enzyme is isolated in partially purified form from *C. roseus* seedlings (Madyastha and Coscia 1979) and catalyses a methyl group transfer to loganic acid to form loganin. On three different media of *C. roseus* cell culture, LAMT activity was measured. It was observed that the enzyme showed maximum activity in the early period of growth (Contin et al. 1999). Similar results were also observed in the seedlings of *C. roseus*, where the enzyme maximum activity was noticed just after germination (Madyastha and Coscia 1979). A full length cDNA of LAMT enzyme was cloned and functionally characterized from *C. roseus* leaf epidermis with the length of the cDNA clone 1396 bp. It exhibited moderate similarities with 44% amino acid sequence identity to *Medicago truncatula* carboxyl methyl transferase (Murata et al. 2008). Second way involved methylation step precedes hydroxylation. In this step, 7-deoxyloganic acid is first methylated to form 7-deoxyloganin and then hydroxylated by an enzyme 7-deoxyloganin 7-hydroxylase (DL7H, EC1.14.13.74) into loganin. The enzymatic activity of DL7H was detected in microsomal preparations from *Lonicera japonica* cell cultures and *C. roseus* cell suspensions (Irmler et al. 2000).

Secologanin Synthase (SLS; E.C. 1.3.3.9)

A cytochrome P450 family enzyme secolognin synthase catalyzed the final of step of indole pathway in which loganin is converted into secologanin. In *C. roseus* leaves, the enzyme is epidermis specific and encoded by the gene *CYP72A1*. It accepts only loganin as substrate (Yamamoto et al. 2000) and is NADPH dependent.

The enzyme was observed first in the microsomal fraction of cell suspension cultures of *C. roseus*. It was suggested that the enzyme involved in early steps of the MIA biosynthesis (Irmler et al. 2000).

Biosynthesis of Strictosidine

In *C. roseus* all monomeric and dimeric indole alkaloids are derived from the central intermediate strictosidine, the building blocks for its biosynthesis are provided by the stereo-specific condensation of their iridoid glucoside secologanin with tryptamine. This condensation is catalyzed by the enzyme strictosidine synthase (STR) (Verpoorte et al. 1997).

Strictosidine Synthase (STR, E.C. 4.3.3.2)

Strictosidine synthase (STR) is a vacuolar-specific enzyme, which plays an important role in the formation of central intermediate (strictosidine) for the MIAs biosynthesis compartment (Stevens et al. 1993). The molecular weight was estimated between 34 and 38 kDa when it was first time purified from *C. roseus* (Treimer and Zenk 1979). Extracts purified from leaves and cell suspension cultures revealed six STR isoforms out which four were characterized (De Waal et al. 1995; Yamamoto et al. 2000). The biological roles of these isoforms are not exactly known but studies suggested that the posttranslation modifications resulting in the formation of STR isoforms (Meijer et al. 1993). Western blotting and two-dimensional electrophoresis of *C. roseus* cell suspension cultures detected seven STR isoforms (Jacobs et al. 2005) and it was also observed that the expression of these isoforms varied by the *P. aphanidermatum* elicitation in *C. roseus* cell suspension culture. STR is encoded by single copy gene *str* and the complete cDNA sequence has been purified and cloned from cell culture of *C. roseus* and *Rauvolfia serpentine* (Facchini 2001). High substrate specificity for secologanin and tryptamine was observed when recombinant STR was expressed in *E. coli*. Studies done on in vitro cultures showed that the influence of hormones, growth regulators, and elicitors show positive correlation with *str* gene expression and alkaloid accumulation (Meijer et al. 1993). Alkaloids biosynthesis increased when the *str* gene is overexpressed but after subculturing, decreased alkaloid biosynthesis even in the presence of high *str* activity indicated that the expression of *str* may not be rate limiting (Whitmer et al. 2002a).

Biosynthesis of Ajmalicine, Serpentine, and Tetrahydroalstonine

After the biosynthesis of central intermediate strictosidine, routes toward the different specific end products are thought to diverge in the biosynthetic pathway of MIAs in *C. roseus*. An enzyme strictosidine-β-D-glucosidase plays an important role to

give specific direction to MIAs biosynthesis. In the first step, ajmalicine and serpentine are synthesized which themselves have medicinal properties as well as they act as monomeric precursors for the synthesis of important dimeric vinblastine and vincristine.

Strictosidine-β-D-Glucosidase (SGD; E.C. 3.2.1.105)

Enzyme strictosidine-β-D-glucosidase (SGD) catalyzes the de-glucosylation of strictosidine glucose moiety to produce an unstable aglycon. The aglycon then rapidly converted into a dialdehyde intermediate like 4,21-dehydrocornynanteine aldehyde and 4,21-dehydrogeissoschizine is formed which further converted into cathenamine (Van der Heijden et al. 2004). Strictosidine-β-D-glucosidase is a glycoprotein encoded by a single copy gene. It is localized to the ER with high strictosidine specificity (Geerlings et al. 2000). The enzyme is observed in a form of complex with approximately 63 kDa of molecular weight for each subunit. It was first partially purified from *C. roseus* and *Tabernaemontana divaricata* cell cultures (Stevens 1994). Later, it was completely purified and the gene is characterized from cell suspension cultures of *C. roseus* (Luijendijk et al. 1998; Geerlings et al. 2000). Its expression is observed in a developmentally regulated manner in flowers, stems, leaves, and roots. The gene encoding SGD enzyme is cloned from *C. roseus* cell cultures and found that mRNA level of the enzyme is proportional to its activity (Geerlings et al. 2000).

Cathenamine Reductase (CR) and Tetrahydroalstonine Synthase (THAS)

Ajmalicine formation from cathenamine occurs after its reduction. The step is catalyzed by an enzyme cathenamine reductase (CR). For the activity of enzyme it utilizes cathenamine as substrate and NADPH as a cofactor. In *C. roseus* cell culture, two different CRs have been identified at low levels (El-Sayed and Verpoorte 2007). One reduced to form ajmalicine or 19-epiajmalicine while the other converts the iminium form of cathenamine into tetrahydroalstonine by using an enzyme tetrahydroalstonine synthase (THAS) (Hemscheidt and Zenk 1985). Subcellular fractionation assays of *C. roseus* cell cultures suggested that the enzyme THAS is localized in the vacuoles and used iminium form of cathenamine as a substrate while the localization of CR was not determined because of its very low activity below the detection limit (Luijendijk 1995). The enzyme THAS is partially purified (35 folds) from *C. roseus* cell culture suspension with 81 kDa molecular weight.

Vacuolar Peroxidase

Ajmalicine and its tetra hydroderivative serpentine are present in major amount in roots of the *C. roseus*. After the biosynthesis, ajmalicine moved across the plasma membrane until it gets trapped inside a vacuole at a low pH. Then, the enzyme

vacuolar peroxidase oxidizes ajmalicine into serpentine (Blom et al. 1991). Vacuolar peroxidase activity is found to be increased by 20-folds when the cell cultures of *C. roseus* were grown in light as compared to dark. This gives the conclusion that chloroplast might play an important role in serpentine biosynthesis (Misra et al. 2006). In *C. roseus* cell culture, it was observed that the activity of basic peroxidase is directly related to the serpentine formation. In an experiment, isolated vacuoles when incubated with ajmalicine and H_2O_2 resulted in increased production of serpentine (Blom et al. 1991). Serpentine is found to be trapped in vacuoles as it is an anhydronium compound and could not pass the tonoplast (Blom et al. 1991).

Catharanthine Pathway

Information for catharanthine (member of iboga family) biosynthesis is very limited. Intensive research efforts are needed in this regard. Geissoschizine was found to be incorporated into catharanthine when fed to the *C. roseus* plant. In *C. roseus* cell culture, stemmadenine feeding resulted in the formation of catharanthine and tabersonine in few hours (El-Sayed et al. 2004). In the support of these studies, still it is believed that the pathway through which catharanthine is formed from strictosidine involve the formation of intermediates 4,21-dehydrogeissoschizine, stemmadenine, and dehydrosecodine (Verpoorte et al. 1997).

Vindoline Pathway

Vindoline is a member of Aspidosperma classes of alkaloids and itself act as precursor for the accumulation of important terpenoid indole alkaloids in leaves of *C. roseus*. Biosynthesis of vindoline starts with transformation of tabersonine (derived from precursor geissoschizine and stemmadenine) into vindoline which requires the involvement of the sequence of six enzymatic steps (Schroder et al. 1999). Catharanthine and tabersonine can be synthesized in the cell suspension cultures of *C. roseus* but it lacks the expression of enzymes necessary for their transformation into vindoline. So the researchers are more focused on the studies based on the involvement and regulation of enzymes in whole plants rather than cell culture systems for vindoline biosynthesis. Vindoline biosynthesis requires a sequence of six steps: Aromatic hydroxylation, O-methylation, hydration of the 2,3 double bond, N(1)-methylation, hydroxylation at position 4 and 4-O-acetylation. These steps are catalyzed by six different enzymes (De Luca et al. 1986).

Tabersonine-16-Hydroxylase (T16H; EC 1.14.13.73)

T16H is a cytochrome P450-dependent enzyme which catalyzes the hydroxylation of tabersonine at C-16 position (Schroder et al. 1999; Van der Heijden et al. 2004). Recently, another cytochrome P450 (CYP71D351) is isolated showing T16H

activity and having high affinity for tabersonine. It is a first enzyme displaying two isoforms T16H1 (CYP71D12) and T16H2 (CYP71D351) encoded by distinct genes. The cDNA for both the genes is characterized in *C. roseus* (Besseau et al. 2013). The expression of CYP71D12 (T16H1) is restricted to flowers and undifferentiated cells. On the other hand, the expression of mRNA of T16H2 (CYP71D351) is specifically located in leaf epidermis. Vindoline accumulation is directly proportional to the level of CYP71D351 transcript. Studies revealed that the hydroxylation by T16H worked in an organ-dependent manner by two genes (Besseau et al. 2013).

16 *O*-Methyltransferase (OMT; EC 2.1.1.94)

In the second step, methylation of hydroxyl moiety of 16-hydroxytabersonine occur to give 16-methoxytabersonine. The step is catalyzed by the enzyme 16-hydroxytabersonine 16-*O*-methyltransferase (OMT) which requires Sadenosyl-L-methionine (SAM) as a methyl group donor (co-substrate) (Pierre and De Luca 1995). The localization of the enzyme is reported in leaf epidermal cells (Murata and De Luca 2005). It has been cloned and its cDNA is characterized from *C. roseus* (Levac et al. 2008).

Unidentified Enzyme (Hydratase)

The hydratation of the 2,3 double bond of 16-methoxytabersonine is the third step, catalyzed by an unidentified hydratase to form 16-methoxy-2,3-dihydro-3-hydroxytabersonine (Kutchan 1998).

N-Methyltransferase (NMT; EC 2.1.1.99)

The enzyme *N*-methyltransferase or *S*-adenosyl-L-methionine 16-methoxy-2,3-dihydro-3-hydroxytabersonne-*N*-methyltransferase catalyzes the fourth step by transferring a methyl group from *S*-adenosyl-L-methionine to form 16-methoxy-2,3-dihydro-3-hydroxy-*N*-methyltabersonine (desacetoxyvindoline). Here, O-methylation preceded N-methylation because the enzyme NMT is highly specific for the indole-ring nitrogen of 16-methoxy-2,3-dihydro-3-hydroxy-tabcrsoninc (Facchini 2001). NMT is localized in the chloroplast thylakoids and has been partially purified (Dethier and De Luca 1993).

Desacetoxyvindoline-4-Hydroxylase (D4H; EC1.14.11.11)

The hydroxylation at C-4 position of desacetoxyvindoline is the fifth step, catalyzed by highly substrate-specific desacetoxyvindoline 4-hydoxylase (D4H). It is a cytosolic enzyme, purified to apparent homogeneity and characterized from *C. roseus*. The molecular weight in native condition is 45 kDa and in denatured condition is 44.7 kDa; this suggested that the enzyme has monomeric structure. Under

denaturing conditions, isoelectric focusing purified D4H in three isoforms with pI 4.6, 4.7, and 4.8 (De Carolis and De Luca 1993). Studies on substrate interaction kinetics and product inhibition suggested an ordered terter mechanism for the enzyme where 2-oxoglutarate is the first substrate to bind followed by O2 and desacetooxyvindoline. On the other hand, the first product released after reaction is deacetylvindoline followed by CO_2 and succinate. Highest activity of D4H enzyme was found in leaves followed by stems (8 %) and fruits (5 %) and is absent in flowers and roots of *C. roseus* plants. *C. roseus* seedlings exposure to light and methyl jasmonate induce the activity of enzyme (Vazquez-Flota and De Luca 1998). Tryptic digestion of the purified protein and its micro sequencing isolated three oligo peptides from cloned *d4h* gene of *C. roseus*. Authenticity of all the three clones as *d4h* clones was confirmed by the recombinant protein heterologous expression showing D4H activity. Southern blotting confirms that the D4H encoding gene (*d4h)* is a single copy (Vazquez-Flota et al. 1997).

Deacetylvindoline-4-*O*-Acetyltransferase (DAT; EC 2.3.1.107)

The final step of vindoline biosynthesis is an acetylation of deacetylvindoline which occurs by the transfer of an acetyl group from acetyl-CoA to the four position of deacetylvindoline (Pierre et al. 1998). The step is catalyzed by the cytosolic enzyme deacetylvindolne 4-*O*-acetyltransferase (DAT), which is highly specific for deacetylvindoline and used acetyl-Co-A as a co substrate for its activity. It is present in maximum amount in leaves, stems contain less percentage of DAT enzyme, and is completely absent from roots (Fahn et al. 1985). DAT enzyme is purified to homogeneity from *C. roseus* and the gene *dat* that encodes the respective enzyme was isolated and characterized (Powers et al. 1990). In the previous studies, due to the proteolytic artifact of protein purification, the enzyme was thought to be a heterodimer with two subunits of molecular weight 33 and 21 kDa. Later, it was confirmed by the cloning of *dat* gene that the enzyme is a monomer of molecular weight 50 kDa (St-Pierre et al. 1999). The purified DAT enzyme is weekly inhibited by tryptamine, vindoline, on the other hand, tabersonine and coenzyme A are found to be the strong inhibitors for that (Powers et al. 1990). Recently, the DAT gene promoter is cloned, sequenced, and analyzed which revealed that DAT promoter contains several potential regulatory elements required for the regulation of *dat* gene expression. It is also reported that in methyl jasmonate signal transduction pathway, three TGACG motifs and one inverted motif (CGTCA) between −808 and −1086 bp of DAT promoter are involved (Wang et al. 2010).

Biosynthesis of Bisindole Alkaloids (Vinblastine and Vincristine)

Vinblastine and vincristine are the bisindole alkaloids of great interest as they are used as anticancerous agents. For their biosynthesis, an oxidative enzymatic coupling of previously formed precursors catharanthine and vindoline occurred. The product

α-3′,4′-anhydrovinblastine (AVLB) is formed by this coupling which is further converted into vinblastine and then into vincristine (Verpoorte et al. 1997). The enzyme anhydrovinblastine synthase (AVLBS) catalyzes the coupling process. Despite of abundance of precursors, vindoline and cataranthine, the dimeric alkaloids are produced in traces (0.003 % dry weight). This fact led to work on different approaches including a semisynthetic process for coupling the monomers either chemically (Kutney et al. 1976; Langlois et al. 1976) or enzymatically (Goodbody et al. 1988), tissue culture approach, using of transgenic cell lines and genetic engineering, etc.

Anhydrovinblastine Synthase (EC: 4.1.3.27)

Anhydrovinblastine synthase is a H_2O_2-dependent enzyme, catalyzing the formation of α-3′,4′-anhydrovinblastine (AVLB). From *C. roseus* leaves some protein fractions are purified containing basic peroxidase with AVLBS activity (Sottomayor et al. 1998). Highly unstable intermediate AVLB (true precursor) is formed after the oxidization process, for which electron is provided by the monomeric substrates of vindoline and catharanthine coupling and AVLB. The enzyme is localized in *C. roseus* mesophyll vacuoles associated to the inner surface of tonoplast. The enzyme is high spin ferric heme protein belonging to the class III peroxidase family with the molecular weight of 45.4 kDa and pI of the protein around 10.7. A full length cDNA clone of *CrPrx1* gene encode basic peroxidase is prepared by using a PCR strategy and screening of a cDNA library prepared from *C. roseus* leaf tissue (Hilliou et al. 2002).

Regulation of MIA Pathway in *C. roseus*

Developmental and exogenous signals have the ability to control the regulation of alkaloids (monomeric or dimeric). The idea for studying the regulation came after observing the reversible inhibition of G10H activity by catharanthine in *C. roseus* (McFarlane et al. 1975). G10H plays an important role in the initiation of the first committed step needed for the formation of strictosidine, a central precursor of all the biosynthesis of MIAs. Thus, suggested as a regulatory gene of MIA pathway. When a *C. roseus* cell line was allowed to grow on production media then significant increase in G10H activity was observed on the first day of culture. The activity decreased after the addition of phosphate in the medium (Schiel et al. 1987). It is also experimentally proved that there is a relationship between the accumulation of MIAs with G10H activity (Collu et al. 2002) and with the transcript levels of *g10h* gene (Papon et al. 2005). During the exponential phase, feeding of 10-hydroxygeraniol to hairy root cultures also result in increased amount of MIA accumulation (Hong et al. 2003). In case of MEP pathway, experimental evidences proved that under the developmental and environmental signals transcript level of genes of MEP pathway

expressed and vary which regulate the flux of intermediates for various isoprenoid biosynthesis. The enzymes DXS, DXR, and HDR also showed posttranscriptional regulation. In some cases inverse regulation corresponding to their respective transcript genes accumulation also noticed (Cordoba et al. 2009). The genes *dxs* and *hdr* encoding DXS and HDR enzymes, respectively, have rate-limiting roles for the pathway in several plants which seems to play a major role in the efficiency and control of isoprenoid biosynthesis (Botella-Pavia et al. 2004). Transcript accumulation of *crdxs, crdxr,* and *crmecs* in *C. roseus* cell cultures showed strongly positive correlation with the MIA biosynthesis (Chahed et al. 2000). The coordination between the MEP pathway and the downstream pathways (including indole pathway) through the control of key genes was also evidenced by the reference of some studies. So it can be said that all the pathways associated with MIA biosynthesis contribute to a fine regulation and coordination (Hughes et al. 2004). Evidences of coordination between MIA accumulation and expression of different genes including *crdxs, crdxr, crmecs,* and *g10h* in young and actively growing aerial parts of organs in several plants are also reported (Burlat et al. 2004). In suspension cells, treatment of C20 cells with inducers, cytokinin, and ethylene stimulates the expression of *crdxs, crdxr,* and *g10h* and enhances MIA production significantly (Papon et al. 2005). In *C. roseus* cell cultures, application of chemicals such as betaine, malic acid, tetramethyl ammonium bromide, and rare elements enhanced the production of ajmalicine and catharanthine up to five- to sixfolds more (Zhao et al. 2001a), cadmium treatment enhances TDC enzyme activity and ajmalicine content (Zheng and Wu 2004). In hairy root cultures, treatment with inhibitors like oxygenase restricted the formation of lochnericine and horhammericine suggesting that these chemicals affect the activity of cytochrome P450 enzymes, since the enzymes are essentially required for these alkaloids biosynthesis (Morgan and Shanks 1999). Addition of phenobarbital increased the enzymatic activity of G10H (a cytochrome P450 enzyme) (Contin et al. 1999). Rate-limiting steps in MIA pathway decreased the production of alkaloids; this can be overcome by feeding precursors. Feeding of secologanin or loganin (Moreno et al. 1993) and tryptamine and loganin to the transgenic cell cultures resulted in the high production of alkaloids (Whitmer et al. 2002a, b). Feeding with geraniol, 10-hydroxygeraniol, and loganin to a *C. roseus* hairy root culture also significantly increased tabersonine formation (Morgan and Shanks 2000).

Role of Plant Growth Regulators in Regulation of MIAs in C. roseus

Regulation of *C. roseus* indole alkaloids by using plant growth hormones affects the growth and metabolites biosynthesis (Verpoorte et al. 1997). Cytokinins regulate many aspects of plant growth and differentiation. In auxin-free *C. roseus* cell cultures, addition of zeatin (cytokinin) increased the alkaloids production (Decendit et al. 1992). It also enhanced the activity of enzyme G10H and rate of secologanin to ajmalicine bioconversion in *C. roseus* cell cultures (Decendit et al. 1993).

Additions of auxins decrease alkaloid biosynthesis at all the levels. Its addition to the cell cultures rapidly decreases the level of *tdc* transcripts. On the other hand, *tdc* and *str* mRNA levels increased when the cells are subcultured on an auxin-free medium. During the growth phase, alkaloid formation is strongly inhibited after the addition of 2,4-D in cultured cell suspensions (Arvy et al. 1994). Abscisic acid (ABA) is one of the important regulators widely used in plants tissue culture techniques since they regulate plant growth, development, and adaptation to abiotic environmental stress (Davies and Jones 1991; Beaudoin et al. 2000). ABA application to the precursors of *C. roseus* cells increased the catharanthine and vindoline formation. This was due to delayed catabolism of strictosidine caused upon addition of ABA (El-Sayed and Verpoorte 2002). Jasmonic acid (JA) is a lipid-based hormone signal that regulates plant growth, photosynthesis, reproductive development, as well as plant responses to poor environmental conditions and other kinds of abiotic and biotic challenges. Alkaloid yield increased after the addition of JA to the hairy root cultures (Rijhwani and Shanks 1998), seedlings (Aerts et al. 1994) of *C. roseus*. Precusors feeding and JA treatment to *C. roseus* cell cultures increased strictosidine and ajmalicine accumulation (El-Sayed and Verpoorte 2002). Activation of transcriptional factors such as APETALA2, ORCA2, and ORCA3 by JA induces the biosynthesis of *C. roseus* cell cultures based MIAs by subsequently increasing of *str* expression (Gantet and Memelink 2002). The activation of ORCA3 expression in turn activates the expression of *dxs, cpr, tdc,* and *str* genes of *C. roseus* (Vander Fits and Memelink 2000; Memelink et al. 2001). Like ORCA2, overexpression of ORCA3 (AP2-domain transcriptional factor) also induces the expression of genes of MIA biosynthesis in *C. roseus* cell cultures because ORCA3 protein binds to the promoter regions of MIA biosynthetic genes *str, tdc, cpr* and hence increased the accumulation of alkaloids (Vander Fits and Memelink 2001). Salicylic acid (SA) plays an important role in the defense mechanism of plants. Addition of SA to the cell culture (El-Sayed and Verpoorte 2002) or seedlings (Aerts et al. 1996) of *C. roseus* did not affect the MIAs content accumulation but induces transcript level of *str* and *tdc* genes in low amount (Pasquali 1994).

Role of Biotic Stress in the Regulation of MIAs in C. roseus

Many studies revealed that the fungal elicitors affect the regulation of MIAs biosynthesis (Verpoorte et al. 1997). Biotic stress (pathogenic attack) on the plants cell surface initiates producing cellular signaling processes that activate plant defense responses like accumulation of defense-related chemicals and oxidative burst gene activation (Scheel 1998; Radman et al. 2003). Fungal elicitation induces the enzymatic activities of MIA pathway where the enzyme TDC activity is increased in a good proportion while the activity of enzymes G10H and IPP isomerase is induced slightly in *C. roseus* cell cultures (Moreno et al. 1996). Screening of 12 different fungal elicitors was performed which revealed that the different fungal elicitors and dosages increased the accumulation of

different alkaloids and enzymatic activities (Zhao et al. 2001a, b). In *C. roseus* cell culture, addition of tetramethyl ammonium bromide and *Aspergillus niger* homogenate increased the production of ajmalicine and catharanthine (Zhao et al. 2001a, b). In *C. roseus* leaves, phytoplasma infection increased the level of loganic acid, secologanin, and vindoline. The amount of vindoline is well correlated with the increased level of secologanin biosynthesis in the same study (Choi et al. 2004). The leaves of *C. roseus* which are experimentally infected with spiroplasma show the relatively high expression of *sgd* gene as compared to expression of same gene in the naturally infected leaves (Nejat et al. 2012). HPLC analysis of *Agrobacterium*-mediated transformation in *C. roseus* enhanced the level of *dat* expression with an increase in the production of vindoline content in the transgenic plants (Wang et al. 2012).

Role of Abiotic Stress in the Regulation of MIAs in C. roseus

Light plays an important role in the regulation of biosynthesis of alkaloids in MIA pathway by induction and activation of several enzymes (Pierre and De Luca 1995). It was found that stimulation of serpentine and oxidation of ajmalicine in cells and tissue cultures depend on light (Loyola-Vargas et al. 1992). Vindoline biosynthesis is significantly influenced by light in *C. roseus* callus cultures as well as plastidial basic and acidic peroxidases stimulation by it induced the production of serpentine (Zhao et al. 2001a). Comparative study of light grown and dark grown *C. roseus* hairy root cultures shows that there is a significant increase in the levels of ajmalicine and serpentine with slight increase in the amount of tabersonine in the former one (Shanks and Bhadra 1997). In the multiple shoot cultures and cell suspension cultures of *C. roseus,* contents of vincristine and vinblastine increased under the influence of UV-B light treatment (Binder et al. 2009). UV exposure to *C. roseus* seedlings also increased the level of biosynthesis of vindoline, vincristine, and catharanthine. Under the influence of salt stress, the vincristine biosynthesis increased in leaves of *C. roseus* (Misra and Gupta 2006; Osman et al. 2008). Vincristine content showed significant increase in roots and shoots of *C. roseus* culture upon addition of a medium in 100 and 50 μM concentrations, respectively (Rai et al. 2014).

Compartmentalization of Monomeric and Dimeric Alkaloids of *C. roseus*

Improvement in the biosynthesis of alkaloids of *C. roseus* can be achieved by understanding the regulation of TIA pathway. In the past few years, studies on in situ hybridization and immunocytochemistry provided interesting details about

the compartmentalization, transportation, and formation of TIAs in *C. roseus* (Mahroug et al. 2007). An understanding of subcellular compartmentation provided information about the enzymatic characteristics under in vitro conditions. In *C. roseus,* MIAs formation is a highly dynamic, complex, and compartmentalization process which co-ordinate in a well-organized manner (Kutchan 2005). Different cellular compartments of root tips and leaves are the sites for different enzymes localization (Murata and De Luca 2005). Enzymes TDC and STR were localized in the stems, leaves, and flower buds epidermis in shoot system and also seen in cortical and protoderm cells around the apical meristem of the root tips (Collu et al. 2001). On the other hand, laticifer and idioblast cells of leaves, stems, and flower buds carried *D4H* and *DAT* mRNAs. Immunocytochemical localization studies determined that the sites for TDC, D4H, and DAT enzymes are different in the early and late stages of vindoline biosynthesis (Pierre et al. 1999). Northern blot and in situ hybridization studies revealed the identical cell-specific expression patterns of *DXS, DXR, MECS,* and *G10H* genes of MEP pathway in the internal phloem parenchyma of young aerial organs of *C. roseus* (Burlat et al. 2004). The expression of SLS enzyme encoding gene CYP72A1 is localized in *C. roseus.* The end products produced from tabersonine are localized in the different tissues of the plant (Rodriguez et al. 2003). In MIA pathway, translocation of the intermediates has been carried out from the internal phloem parenchyma to the epidermis and then to the lactifiers and idioblasts. During this translocation and MIA biosynthesis, photosynthetic primary metabolites and hormone were supplied by MEP pathway. MIA formation involved chloroplasts, cytosol, vacuoles, and endoplasmic reticulum of the plant which were absent in hairy root culture resulting in poor performance of alkaloid contents. In MEP pathway, enzymes found to be located in the chloroplast or plastid (Lange et al. 2000). Biosynthesis of tryptamine from tryptophan catalyzed by TDC in the cytosol (Stevens et al. 1993), whereas G10H activity occurred in endoplasmic reticulum (ER) (Guirimand et al. 2009). For the coupling with secologanin, tryptamine is transported across the tonoplast since the enzyme (STR) required for the coupling and is localized in the vacuoles (McKnight et al. 1991). The next enzyme SGD is found in ER when studied in vivo (Geerlings et al. 2000). During vindoline biosynthesis, T16H enzyme was found in ER (Pierre and De Luca 1995), NMT localized in thylakoid membranes and the rest of the enzymes D4H, DL7H, and DAT are localized in the cytosol (De Carolis et al. 1990). But northern-blot analysis determined that the expression of *DAT* gene is carried out in specialized idioblast and laticifer cells only in light exposed tissues like leaves and stems (Laflamme et al. 2001). Further, the coupling of vindoline and catharanthine to form the bisindole alkaloid anhydrovinblastine by peroxidase enzyme occurred in the vacuoles (Costa et al. 2008). Localization of the biosynthesis of vinblastine and vincristine in both, biosynthesis is not exactly known in both, whole plant and in vitro plant models (Table 2.1).

Table 2.1 Enzymes involved in biosynthesis of indole alkaloids of *C. roseus*

Enzymes	Abbreviation	Cofactors	Product	Localization
Anthranilate synthase	AS	Mg^{2+}	Anthranilate	Plastid
Tryptophan decarboxylase	TDC	PP, PQQ	Tryptamine	Cytosol
Isopentenyl diphosphate isomerase	IPP	Mg^{2+}, Mn^{2+}	3,3-Dimethyl allyl diphosphate	Plastid
Geraniol 10-hydroxylase	G10H	Haeme	10-Hydroxygeraniol	Provacuolar membrane
NAPDH:cytochrome P450 reductase	CPR	NADPH, FAD, FMN	–	Provacuolar membrane
SAM:loganic acid methyl transferease	LAMT	SAM	Loganin	–
Secologanin synthase	SLS	NADPH	Secologanin	–
Strictosidine synthase	STR	–	Strictosidine	Vacuole
Strictosidine β-glucosidase	SGD	–	Cathenamine	Endoplasmic reticulum
Cathenamine reductase	CR	NADPH	Ajmalicine	–
Geissoschizine dehydrogenase	–	NADP+	4,21-Dehydro geissoschizine	–
Tetrahydroalstonine synthase	THAS	NADPH	Tetrahydro alstonine	–
Tabersonine 16-hydroxylase	T16H	Haeme, NADPH	16-Hydroxy tabersonine	Endoplasmic reticulum
11-Hydroxytabersonine *O*-methyltransferase	OMT	–	16-Methoxy tabersonine	–
SAM:methoxy 2,16-dihydro-16-hydroxytabersonine *N*-methyltransferase	NMT	SAM	Desacetoxy vindoline	Thylakoid membranes
Desacetoxyvindoline 17-hydroxylase	D4H	2-	Oxoglutarate, Fe^{2+}, ascorbate	Desacetyl vindoline
Cytoplasm acetyl CoA: deacetylvindoline 17-*O*-acetyltransferase	DAT	Acetyl CoA	Vindoline	Cytoplasm
Acetyl CoA: minovincinine-*O*-acetyl transferase	MAT	Acetyl CoA	Echitovenine	Cytoplasm
Anhydro vinblastine synthase	AVLB synthase	–	Anhydro vinblastine	Vacuole

(El-Sayed and Verpoorte 2007)

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Chapter 3
Glutamate Receptor-Like Ion Channels in *Arabidopsis thaliana*

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Abstract Ionotropic glutamate receptors (iGluRs) are glutamate-gated nonselective cation channels (NSCC) that mediate rapid conduction of impulses through the synapses in central nervous system of animals. At one time, signaling through the iGluRs was thought to be limited to the animal system but with the discovery of 20 glutamate receptor-like genes (GLRs) in *Arabidopsis thaliana* has paved the way for the study of glutamate receptors in an organism lacking nervous system. These 20 genes expressed in diverse tissue throughout the plant and designated as putative glutamate receptor. They were named as putative glutamate receptor on the basis of high similarity of their deduced amino acid sequences with members of the iGluR superfamily. Furthermore, these nonselective ion channels share the properties similar to those of animal iGluRs. The information based on sequence similarity predicts that *A. thaliana* glutamate receptors (AtGLRs) also exist as the integral membrane proteins like iGluRs. Through the application of specific antagonists or agonists, designated as inhibitors or stimulators respectively, the putative function of AtGLRs has been associated to an array of processes. Comprehensive information about glutamate receptors of *A. thaliana* is given in this chapter.

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Introduction

Like animals, glutamate receptor-like genes (GLRs) have also been identified in a number of plant species including *Arabidopsis thaliana*. Twenty genes in *A. thaliana* genome have been uncovered after completion of whole genome sequencing project that encode subunits of glutamate-like receptors (AtGLRs) (Lam et al. 1998; Lacombe et al. 2001). These AtGlr subunits are so named due to their similarity in primary sequence and predicted secondary structure to animal ionotropic glutamate receptor (iGluR) subunits (Lam et al. 1998). On the basis of parsimony analysis, these 20 glutamate-like genes have been divided into 3 protein families, Clade I, Clade II, and Clade III, that contain all the signature domains of animal ionotropic GluRs (Chiu et al. 2002; Davenport 2002). In animals, iGluRs predominantly function as a glutamate-gated Na^+ and Ca^{2+} ion channels of influx pathways at neural junction (Dingledine et al. 1999). These iGluRs are nonselective cation channels (NSCC), selectively permeable for cations over anions but do not discriminate strongly between monovalent cations. In vivo, the iGluRs exist either as homo- or heteromultimers. All the subunits share the same structural pattern and are known to contain six conserved domains. Subunits located in the hydrophobic region of membrane comprise three transmembrane domains (M1, M3, and M4), loop between the first two domains comprises the pore forming domain (M2), facing cytoplasm and two domains located on the external face of the membrane form the channel ligand binding site (S1 and S2). On the basis of high similarity of their deduced amino acid sequences, *Arabidopsis* GLR genes encode all the conserved domains of animal iGluR, including the two glutamate binding domains (GlnH1 and GlnH2), the three transmembrane domains (M1, M3, and M4), and the putative pore region (P) between M1 and M3. While comparing AtGLRs and animal iGluRs, the highest level of sequence similarity was observed in pore region, in which the percentage of identity was above 60 % (Chiu et al. 1999). Because of the sequence similarity between animal iGluR and AtGlr gene, sequences of AtGlr gene span all the important functional domains defined in animal iGluRs.

Glutamate Receptors

Ionotropic Glutamate Receptors in Mammals

In the central nervous system of mammals, wide range of pharmacologically distinct receptors which include ionotropic glutamate receptors and G-protein linked receptors [also called metabotropic glutamate receptors] are activated by glutamate (Asher and Nowak 1988; Collingridge and Lester 1989; Monaghan et al. 1989). Ionotropic glutamate receptors are functionally known for their role in fast excitatory neurotransmission and involvement in higher brain functions like learning, synaptic plasticity, and memory. The ionotropic glutamate receptors (iGluRs)

encompass a large family of ligand-gated ion channels which are mainly situated on presynaptic or postsynaptic membranes of neurons and are implicated in fast excitatory signal transmission between neurons.

Gene Families

In vivo, ionotropic glutamate receptors exist as tetramers, formed by four subunits that surround a central pore which is permeable to various cations like Na^+, K^+, and Ca^{2+}. Mammalian iGluRs encompass a large family of genes and more than 20 subunits have been discovered so far. Pharmacologically, iGluRs are categorized into three distinct subfamilies in mammals and are named on the basis of agonist that activates them efficiently. These subfamilies include *N*-methyl-D-aspartic acid receptors (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPA), and kainate receptors. Each subfamily consists of multiple subunits that form functional ion channels only when they co-assemble among themselves (Wenthold et al. 1992). Besides, there is a fourth subfamily called delta or orphan receptors which do not form functional receptors. In AMPA family, homomeric or heteromeric combination of subunits forms functional ion channels while functional NMDA receptors are obligatory heteromers. For kainate receptors, some subunits form the possible homomeric and heteromeric combination while other subunits only form the functional channels as heteromers. Because of the availability of sequence for various iGluRs subunits, it becomes possible in deciphering the basic structure of iGluR subunits and their evolutionary connections with other proteins and ion channels.

Receptor Structure

The fascinating feature about the iGluRs is the fundamental structure of the receptor subunit itself. Ionotropic receptors are oligomers formed by the combination of four subunits. Study on the basic structure of a receptor subunit reveals that iGluRs are ancient integral membrane proteins that emerged before the divergence of plants and animals, and were found to be expressed in bacteria. However, over the time iGluR evolved and gained more complexity in mammals than others. Figure 3.1 shows organization of iGluR subunit. The NTD denotes the N-terminal domain which is homologous to the leucine-isoleucine-valine bacterial periplasmic amino acid binding protein (O'Hara et al. 1993). The N-terminal domain of NMDA receptors contains binding sites for cations and protons (Banke et al. 2005). Certain deletions in the NTD of AMPA receptors have been found to impair assembly and expression of the subunits (Xia et al. 2002). The S1 and S2 domains constitute the ligand binding site while the P domain consists of hairpin loop which dips into the membrane from the cytosolic site and constitutes the lining of ion channel. The

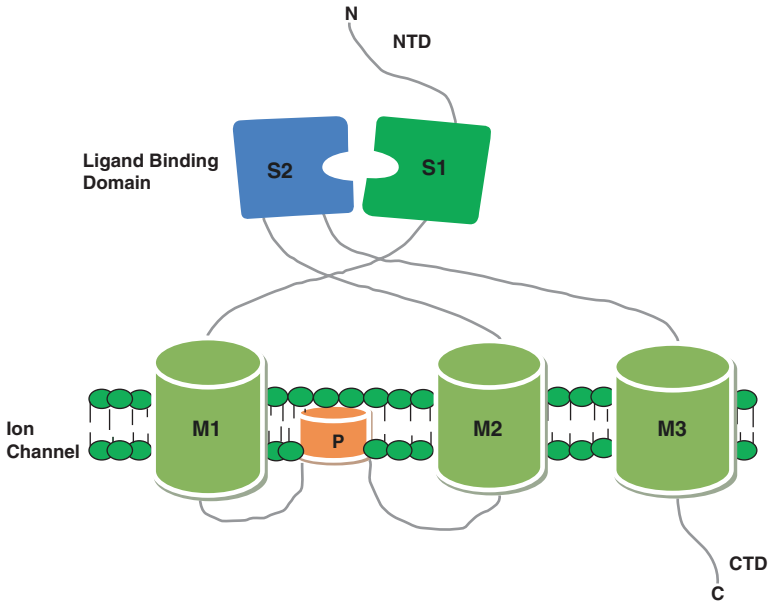

Fig. 3.1 Domain organization of iGluR

CTD denotes the C-terminal domain that differs in length ranging from 50 amino acids in most of the iGluRs to 400–600 amino acids in NR2 subunits of the NMDA receptors. Moreover, C-terminal domain is altered by alternate splicing. Like other ligand-gated ion channels, iGluRs subsist mainly in three conformational states. In the absence of ligand, receptors attain the resting state during which the ion channel remains closed. During ligand binding, receptor gets activated and causes the ion channels to open for the passage of cations. In the presence of prolonged binding of ligand to receptor, the receptor becomes desensitized and channel is closed even in the presence of ligand. The state of conformation and desensitization rate varies with the receptor subtypes. The desensitization is a reversible process where ion channel is resensitized after the ligand is detached.

NMDA Receptors

NMDA receptors are composed of different subunits. These subunits are acquired from three related groups: NR1, NR2, and NR3 (Fig. 3.2) which share 27–31 % identity among themselves. The key feature of NMDA receptor is that it can form functional ion channel only as heteromers in the presence of NR1 and NR2 subunits. NMDA receptors are exclusive among iGluRs because they are ligand-gated voltage-activated ion channels. The voltage dependency of NMDA receptor is due to a block of its ion channel by Mg^{2+} at −70 mV resting membrane potential (Mayer

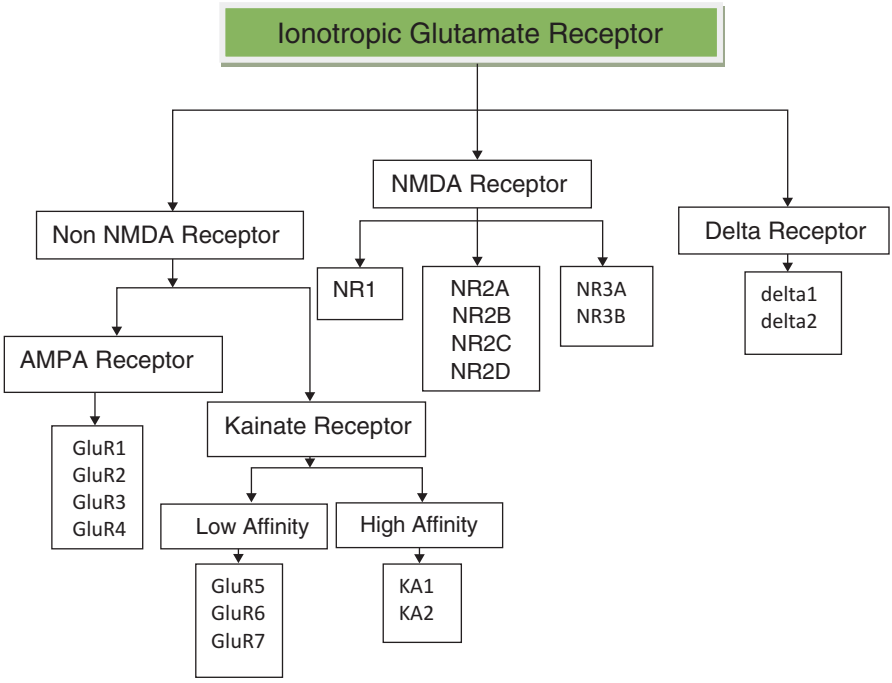

Fig. 3.2 Systemic representation of mammalian glutamate receptors and their subunits

et al. 1984; Nowak et al. 1984). The Mg^{2+} ion-induced block is reduced when the membrane is depolarized and this depolarization allows the influx of ions which further depolarize the membrane and reduce the block by Mg^{2+} ion. Moreover, NMDA receptors require glutamate as an agonist which binds to NR2 subunit and glycine as a co-agonist which binds to NR1 subunit. Besides, NMDA receptors also respond to a wide range of chemical signals, like zinc (Zn^{2+}), protons (H^{+}), polyamines, etc. at synapse.

NMDA receptors in mammals perform physiologically discrete roles like synaptic plasticity, memory, and learning. Malfunctioning of these receptors involves cell death under various neuropathological conditions (Collingridge and Singer 1990; McBain and Mayer 1994). All these distinct properties revealed by NMDA receptors are due to their higher Ca^{2+} permeability, ligand-gated, and voltage-dependent activation of channels. The incursion of Ca^{2+} leads to interaction of Ca^{2+} with various Ca^{2+} sensitive proteins such as calmodulin, calcium-calmodulin kinase II, nitric oxide synthase, etc. in Ca^{2+}-dependent signaling pathways (Kennedy 1998). These elicit series of events in neuron lead to increase (Long-term potentiation: LTP) or decrease (Long-term depression: LTD) in the strength of synaptic transmission.

AMPA Receptors

AMPA receptors are formed from the combination of four distinct subunits named GluR1, GluR2, GluR3, and GluR4 (Fig. 3.2). These four subunits assemble as homomers or heteromers to constitute a functional ion channel (Keinanen et al. 1990). Each subunit of AMPA receptor has distinct properties imparted on the ion channel: for example, GluR4 homomers show more rapid desensitization (Erreger et al. 2004). Two splice variants named flip and flop exist for all four subunits of AMPA receptors. The spliced region is located in transmembrane domain just before the C-terminal region in the mature receptor subunit (Monyer et al. 1991). Compared to flip variants of subunits, the flop variants desensitize more rapidly (Mosbacher et al. 1994).

Kainate Receptors

Kainate receptors are tetramers formed by combination of five distinct subunits named Glu5, Glu6, Glu7, KA1, and KA2. On the basis of affinity for kainate, the subunits are classified as low affinity kainate binding subunit and high affinity kainate binding subunit. Subunits Glu5–7 belonging to low affinity binding group constitute low affinity receptors with dissociation constant (K_d) of 50–100 nM, while subunits KA1 and KA2 form high affinity receptors with K_d values of 5–15 nM (Jaskolski et al. 2005). Kainate receptors can exist as homomers or heteromers. Co-assembly of Glu5–7 among themselves can form functional homomers or heteromers and with KA1 and KA2 to form heteromers (Lerma 2003). By contrast, the co-assembly of KA1 and KA2 subunits with Glu5–7 only forms functional heteromers.

Kainate receptors were expressed predominantly in presynaptic and postsynaptic membrane. In presynaptic membrane, kainate receptors modulate the release of neurotransmitters like glutamate or GABA.

Delta Receptors

Delta receptors, also known as orphan receptors, formed by the combination of two subunits named: delta1 and delta2 (Lomeli et al. 1993). Delta2 subunit shares 18.5 % sequence homology with NMDA receptors and 25 % with non-NMDA receptors. Both the subunits do not form functional channels.

Glutamate Receptors in *Arabidopsis Thaliana*

Discovery of glutamate receptors like genes in an organism lacking nervous system came into light with the completion of the *Arabidopsis thaliana* genome sequencing project (Lam et al. 1998; Kim et al. 2001; Lacombe et al. 2001). Plants appear to have multiple animal glutamate receptors like genes. Twenty glutamate receptors like genes have been uncovered in the complete genome of *A. thaliana* with the completion of genome sequencing project. These 20 genes expressed in diverse tissue throughout the plant and designated as putative glutamate receptor (AtGlr). Based on preliminary parsimony analysis, a nomenclature system has been established that separates these 20 members of AtGlr gene family into three clades (Lacombe et al. 2001) (Table 3.1).

Table 3.1 Nomenclature for the *Arabidopsis* Glr gene family with genbank accession numbers (Chiu et al. 2002)

		cDNA		Genomic	
Clade		Full length	Splice variants	BAC	Protein ID
I	AtGlr1.1	AF079998		AC016829	AAF26802.1
	AtGlr1.2	AY072064	AY072065	AB020745.1	BAA96960.1
	AtGlr1.3			AB020745.2	BAA96961.2
	AtGlr1.4	AY072066	AY072067	AC009853	AAF02156.1
II	AtGlr2.1			AF007271	AAB61068.1
	AtGlr2.2	AY072068		AC007266.1	AAD26895.1
	AtGlr2.3			AC007266.2	AAD26894.1
	AtGlr2.4			AL031004	CAA19752.1
	AtGlr2.5			AL360314.1	CAB96656.1
	AtGlr2.6			AL360314.2	CAB966653.1
	AtGlr2.7			AC005315.1	AAC33239.1
	AtGlr2.8	AY072069		AC005315.2	AAC33237.1
	AtGlr2.9	AJ311495		AC005315.3	AAC33236.1
III	AtGlr3.1	AF079999	AF038557	AC002329	AAF63223.1
	AtGlr3.2	AF159498		AL022604	CAA18740.1
	AtGlr3.3			AC025815	AAG51316.1
	AtGlr3.4	AF167355	AY072070	AC000098	AAB71458.1
	AtGlr3.5	AF170494		AC005700.1	AAC69939.1
	AtGlr3.6			AL133452	CAB63012.1
	AtGlr3.7	AF210701		AC005700.2	AAC69938.1

Phylogenetic Relationship Between Animal iGluRs and AtGLRs

Results based on parsimony analysis of all 20 *Arabidopsis* GLR genes, all rat iGluRs, and two prokaryotic iGluRs (*Synechocystis* GluRO and *Anabaena* iGluR) using bacterial periplasmic amino acid binding protein as an out-group suggested that the primitive signaling mechanism existed before the divergence of plants and animals and evolved from the common ancestor (Chiu et al. 2002). Compared to the amino acid sequences of GLR to various kinds of ion channels such as animal iGluRs, potassium channel, acetyl-choline receptors and GABA receptors, the plants GLRs showed most close relationship to animal iGluRs (Chiu et al. 1999). However, it is unclear whether AtGlr genes may encode functional ion channel subunits as in the case of animal but *in planta* electrophysiological measurements suggest the existence of glutamate-gated ion channels in *Arabidopsis* (Dennison and Spalding 2000). Moreover, *Synechocystis* GluRO, a gene that evolved before the divergence of *Arabidopsis* GLRs and animal iGluRs, has been shown to encode the functional ion channel subunits (Chen et al. 1999). This suggests that AtGlr genes are likely to encode the ion channel subunit and may have general importance in plant physiology.

Characteristics of Plant Glutamate Receptors

Ligands to the Glutamate Receptors

In mammalian nervous system, iGluR subunits respond to a number of different endogenous substrates, including glutamate, aspartate, glycine, L- and D-serine, and homocysteine (Wolosker 2006). On the basis of the study of more than 100 high resolution crystal structures for multiple iGluR subtypes, it came to know that the ligand binding domain (LBD) of receptors undergoes "venus flytrap" like movement when ligand binds to the cleft between the two lobes. Before the discovery of GLR, ligands for plant GLRs were not exactly known. In fact, the effectiveness of an agonist for an iGluR is very well interrelated with the degree of domain closure provoked by the compound (Pohlsgaard et al. 2011).

On the basis of initial sequence analysis, plant GluRs were found to contain a mutation in the pore-forming M3 region which makes the mammalian D2 receptor constitutively active (Chiu et al. 1999) suggesting the likelihood that plant iGluRs might not function as ligand-gated channels. On the other hand, several workers suggested the existence of amino acid-gated calcium channels in plants after reporting the fact that glutamate and other amino acids were able to induce membrane depolarization and Ca^{2+} ion conductance in plants (Dubos et al. 2003; Qi et al. 2006; Stephens et al. 2008). In the line of these findings, Michard et al. (2011a, b) showed that in the apical region of pollen tube, D-serine activates GLRs and allows the influx of Ca^{2+} ion into cytoplasm. They suggested that plant GLRs form calcium

channels in the pollen tube on the binding of pistil D-serine. In recent study, when Vincill et al. (2012) expressed the AtGlr3.4 in mammalian cells, it was shown to be gated by Asn, Ser, and Gly, revealing the fact that at least one plant GLRs is capable of forming an amino acid-gated channel. Though the ligand binding domain (LBD) structures of plants GLRs are not known, it is highly expected that the binding of these amino acids to the LBD of AtGlr3.4 causes opening of the channel.

Channel Selectivity

In the central nervous system, iGluRs are called the nonselective cation channels (NSCC) that mediate the conduction of Na^+, K^+, and Ca^{2+} in the presence of glutamate (Furukawa et al. 2005). The amino acid residues of the M2 and M3 regions that line the pore-forming domain play an important role in the selectivity of cations (Panchenko et al. 2001). So far, the studies conducted suggest that GLRs are a candidate family that may encode plant Ca^{2+} permeable NSCCs (Meyerhoff et al. 2005). Kim et al. (2001) observed that overexpression of AtGlr3.2 in *Arabidopsis* not only exhibited the sensitivity to Ca^{2+} but also shown increased sensitivity to K^+, Na^+, and Mg^{2+} cations and result obtained again proved their putative role as NSCCs. Moreover, Roy et al. (2008) further provided evidence that supports the fact that plant GLRs exist as NSCCs. In their study, they observed the enhanced plasma membrane conductance of Ba^{2+}, Ca^{2+}, and Na^+ ions after the expression of AtGlr3.7 in *Xenopus oocytes*. In addition to the Clade III genes, Tapken and Hollmann (2008) reported that AtGlr1.1 and AtGlr1.4 genes from Clade I family also function as NSCC. In their study, they observed that pore domain transplantation of AtGlr1.1 and AtGlr1.4 into rat GluR1 and GluR6 produced functional K^+, Na^{2+}, and Ca^{2+} channels.

Pharmacological Characteristics of Plants GLRs

Discovery of glutamate-like receptor homologs in plants presents a provocation for genetic approaches due to potential for functional redundancy. To outwit this, the use of pharmacology to act upon multiple members of the family is a common approach. Pharmacological and electrophysiological approaches are starting to reveal the functions of this protein family. At amino acid level, the ligand binding domain of plant GLRs shares homology with mammalian iGluRs. As a result, several groups have examined whether plants GLRs are influenced similarly as their mammalian counterparts in response to known iGluR agonist and antagonists (Brenner et al. 2000). In order to relate the AtGlr genes to light signaling and other biological functions, initial attempt was made by Lam et al. (1998) by examining the responses of plants to the known competitive antagonist 6,7-dinitroquinoxaline-2,3-(1*H,4H*)-dione. When *Arabidopsis* seedlings were grown in media containing DNQX, plants grown in light have showed a light-dependent increase in hypocotyls elongation with reduced chlorophyll synthesis than those grown in dark. In the line

of these findings, Brenner et al. (2000) observed that when light-grown *Arabidopsis* seedlings were treated with *S*(+)-β-Methyl-α,β-diaminopropionic acid (BMAA), iGluRs agonist, there was an increase in hypocotyls elongation with the impairment of cotyledon opening. BMAA-induced hypocotyls responses were lessened when exogenous glutamate applied, suggesting that there may exist a conserved mechanism for the BMAA activity between mammalian and plant glutamate receptors and it also suggested a role of AtGLRs in photomorphogenic development (Brenner et al. 2000). Besides DNQX, 6-cyno-7-nitroquinoxaline-2,3-dione(CNQX) and AP-5 were also found to restrain pollen tube growth in tobacco (Michard et al. 2011a, b). Thus, the result obtained revealed the direct evidences that plant GLRs do response to mammalian glutamate receptor agonist and antagonist.

Subcellular Localization of Plant GLRs

Through the application of various protein tagging approaches such as reporter or antigenic tag, only two plants AtGLRs have been characterized so far. Vincill et al. (2012) have shown the AtGlr3.4 localization on plasma membrane. Similarly, numerous AtGLRs (AtGlr3.4, 3.7, and a radish GLR) have been localized to plasma membrane of *Arabidopsis*, onion epidermis cells, and tobacco protoplasts by using GFP or GUS tags (Kang et al. 2006). Moreover, in addition to plasma membrane, Teardo et al. (2010) reported the presence of AtGlr3.4 in the chloroplast.

Physiological Roles of Plant GLRs

Presence of limited evidences reported that plants GLRs function as active nonselective cation channels. Although there is no experimental proof showing that the membrane topology of plant GLRs is identical to animal iGLRs, the perceptible conservation of ligand binding domains S1–S2 makes it possible that at least some or subunit combination show sensitivity to ligand. The attribution of GLR function in the literature is mostly indistinct but generally points that GLRs function in ligand-gated Ca^{2+} signaling of the extracellular amino acid levels at plasma membrane. It appears that there is little suspicion about at least one, possibly more, of plant GLRs being amino acid-activated channels since the ligand-gated Ca^{2+} signaling of AtGlr3.4 has been reported in heterologous expression system (Vincill et al. 2012). In fact, presence of limited type of NSCC gene candidate suggests that GLRs may have a variety of physiological roles and could differ in ligand sensitivity and membrane localization. Thus, some GLR subunits work as constitutively active NSCCs, and play roles in ligand-gated Ca^{2+} signaling and nutrient uptake at the plasma membrane whereas others could sense amino acids at the outside of the membrane in which the GLRs are localized.

A number of studies suggest that factors such as carbon and nitrogen supplies and stress have an effect on the amino acid content in the apoplast. Microarray study

using an inhibitor of glutamine biosynthesis performed by Gutierrez et al. (2008) suggested that a considerable fraction of N-responsive genes responds to the extracellular glutamate/glutamine, indicating a sensory mechanism of GLRs expressed at plasma membrane for apoplasmic amino acids. Transcript analysis of antisense plants for AtGlr1.1 showed altered transcript expression and the protein abundance related to C:N metabolism enzymes when compared to wild plants. These results suggested a role for AtGlr1.1 in C:N perception, metabolism, and signaling (Kang et al. 2004).

Recent studies report the presence of GLRs in plastid indicating an additional role of GLRs in this organ. In fact, plants carrying T-DNA insertions in *AtGlr*3.4 illustrated weak photosynthetic phenotypes (Teardo et al. 2011). Thus, the presence of GLRs in plastid exhibits the dual localization in plastid as well as plasma membrane. However, the exact functions of GLRs in plastid anticipate further investigation.

Interestingly pharmacological and genetic approaches to understand the function of GLR revealed their roles in biological process of extracellular amino acid that were not previously studied. Recently, it was reported that gene integration in AtGlr1.2 and 3.7 caused pollen tube phenotype and altered Ca^{2+} signature in AtGlr1.2 mutant. Moreover, it was also observed that D-serine, an agonist to animal iGluR, is competent of activating calcium changes in the growing pollen tube and pollen tube growth is hindered in knockout mutant of serine racemase. These results suggested a potential participation of D-Ser and GLRs in male gametophyte-pistil communication (Michard et al. 2011a, b). It is probable that more members of the GLR family are implicated in such cell-to-cell communication in plants. Similarly, it was revealed that AtGlr3.1 is expressed preferentially in guard cells and overexpression of AtGlr3.1 leads to the hindrance in stomatal closure that is stimulated by external Ca^{2+} ions (Cho et al. 2009). Since evidences regarding amino acid-gated channel activity for AtGlr3.1 are lacking, whether the channel conductivity is influenced by apoplasmic amino acid needs to be studied.

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Chapter 4
Phosphate Signaling in Plants: Biochemical and Molecular Approach

Gurjeet Kaur, V. Prabhavathi, Kiran Bamel, and Maryam Sarwat

Abstract Phosphate (P) being an essential macronutrient plays a central role in virtually all metabolic processes in plants. Despite the importance, it is one of the least available nutrients and thus, a frequent limiting factor for plant productivity. A substantial amount of P is fixed in soils and unavailable for plants. Due to critical nature of Pi nutrition, plants are compelled to evolve an efficient P-signaling system developing a series of interconnected responses to conserve and remobilize internal Pi and to increase Pi acquisition from the external environment. In this chapter, various adaptations in plants in response to P-starvation have been discussed along with various signaling molecules like Pi, sugars, hormones, and microRNA (miRNA), both at biochemical and molecular level. Some light is also thrown on several important transcription factors involved in Pi signaling pathways. P-Zn interaction and effect of intercropping and root interactions on P-uptake has also been examined.

Introduction

Phosphate (P) an essential macronutrient plays a central role in virtually all metabolic processes in plants, including photosynthesis and respiration. It is required for the constitution of cellular components like membrane, nucleic acids, and ATP, and is a key component for the regulation of many enzymatic reactions (Marschner 1995). It is also important for signal transduction and genetic information transmission and expression, as it is a critical element for phosphate esters (NTP and NDP) and phospholipids.

The uptake form of P by roots of plants is orthophosphate ($H_2PO_4^-$ and HPO_4^{2-}), designated as Pi (inorganic phosphate). Despite the importance of Pi, it is one of the

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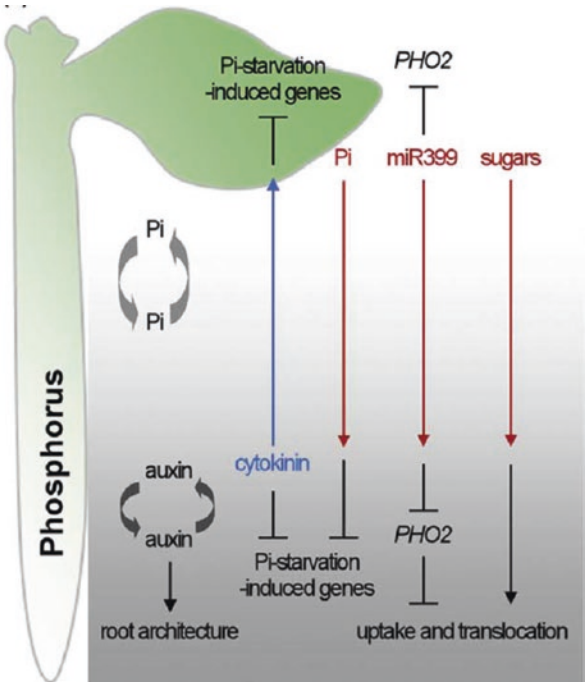

Fig. 4.1 Schematic representation of long-distance signaling pathway of phosphorus between shoots and roots to coordinate the nutrient demand of shoots and nutrient supply from roots. The recycling of Pi between shoots and roots and the redistribution of auxin within the roots are indicated as *circular arrows*. The *blue arrows* show the root-to-shoot movement of signals whereas the *red arrows* represent the shoot-to-root movement of signals. *Source*: Liu et al. (2009). Current Opinion in Plant Biology

least available nutrients and thus, a frequent limiting factor for plant productivity. This is because most of the Pi that remains in the soil is either converted to organic compounds by microorganisms or it becomes insoluble by interacting with cations forming an integral component in Ca, Fe, or Al salts (Marschner 1995; Raghothama 1999). As a result, a substantial amount of P is fixed in soils and unavailable for plants. For a typical plant, Pi concentrations are at the ranges of 1 μM in the soil, 10,000 μM in the cells, and 400 μM in the xylem (Fang et al. 2009).

Pi nutrition and its availability are very critical to plants, which compel them to evolve an efficient signaling system to regulate Pi acquisition, utilization, and homeostasis to adapt to diverse environmental Pi conditions. Thus, understanding how plants sense and respond to external variation of Pi concentrations at the biochemical and molecular level is very important for the sustainability of P resources. It has been observed that in spite of large fluctuations of Pi in soils, the intracellular concentrations of Pi in plants are highly regulated to maintain homeostasis. For this, plants have developed a series of interconnected responses to conserve and remobilize internal Pi and to increase Pi acquisition from the external environment (Theodorou and Plaxton 1993, Vance et al. 2003). The adaptive responses to Pi

starvation are at the developmental and biochemical levels (Raghothama 1999). The level of phosphate within the plant is also nonuniform as it depends on its physiological demands. In general, the accumulation of Pi is more in shoot tissues, metabolically active cells, vacuoles, and plastids than root tissues, less active cells, and cytoplasm, respectively (Mimura 1999)

Signaling Routes

Local Versus Systemic Signaling

Plants respond to variations in mineral nutrient availability by adapting their growth metabolically, physiologically, and developmentally. Plant growth depends on integration of both endogenous signals and external environmental factors. Usually, two types of action are undertaken. One involves local signaling which depends on external Pi concentrations and the other involves systemic or long-distance signaling and is determined by the Pi status of the whole plant. To elicit localized signaling cascades, the signaling molecules need to move intracellularly or intercellularly but the molecules acting as long-distance signals have the ability to travel through the vascular system, either via xylem or phloem, to distant target sites (Liu et al. 2009).

Many specific changes in root system architecture (RSA) are controlled by local Pi supply independent of the internal Pi content implicating a local sensing and signaling route (Bates and Lynch 1996; Lopez-Bucio et al. 2003; Linkohr et al. 2002; Svistoonoff et al. 2007; Thibaud et al. 2010). The regulation of local and systemic signaling operates in a specific manner, which requires delicate communication. It is possible that the dynamic Pi recycling between roots and shoots, rather than Pi concentrations may provide the signal for systemic regulation (Drew and Saker 1984).

Transcriptomic analysis based on a split-root system experiments have revealed that genes involved in Pi uptake and recovery are generally systemically regulated, whereas genes associated with stress- or hormone-related responses are locally regulated (Thibaud et al. 2010). An alternative strategy by which P-deprived vascular plants can acquire Pi from the soil is symbiotic association with mycorrhizal fungi. During the development of arbuscular mycorrhizal (AM) symbiosis, systemic signals are also involved in Pi-starvation responses, such as the downregulation of Mt4 expression in *Medicago truncatula* (Burleigh and Harrison 1999). However, the knowledge about such long-distance signaling remains quite limited and further research is needed.

Root Tip as a Local Sensing Site

Arrest of primary root growth by low Pi, which is a determinate developmental program, is regulated locally around the root tip area (Linkohr et al. 2002; Svistoonoff et al. 2007). Regardless of sufficient Pi status in shoots, physical contact of the

primary root tip with a low Pi medium was necessary and sufficient to attenuate primary root growth. Experiments on *Arabidopsis* showing unusual change in primary root growth under low Pi indicate that the root tip, including the meristem region and root cap, may represent a site to sense local Pi (Svistoonoff et al. 2007).

Molecular Studies

Disruption of *PDR2* (*Phosphate Deficiency Response 2*) encoding a P5-type ATPase exhibits an inflated short-root phenotype under Pi deficiency owing to meristem exhaustion (Ticconi et al. 2004, 2009). Irrespective of the whole-plant Pi status, this phenotype is a local response. Local application of phosphite (Phi), an analog of Pi, on the root tip rescues the phenotype, suggesting that Pi likely functions as a local signal in this response. In contrast to *pdr2* mutants, *lpi* (*low phosphorus insensitive*) (Sanchez-Calderon et al. 2006), *lpr* (*low phosphate root*) (Reymond et al. 2006; Svistoonoff et al. 2007), and *psi (phosphate starvation insensitive*) (Wang et al. 2010) mutants display long primary roots under Pi deficiency.

Whereas the molecular identity of *LPI* is unknown, *LPR1* and *LPR2* are paralogs encoding multicopper oxidases (Svistoonoff et al. 2007). The gene responsible for *psi* mutant phenotype is the same allele as *LPR1/LPR2* (Wang et al. 2010). *PDR2* interacts genetically with *LPR1/LPR2*, which is epistatic to *PDR2* (Ticconi et al. 2009). Moreover, *PDR2* and *LPR1/LPR2* are coincidently expressed in the root meristematic region and both reside in the endoplasmic reticulum (ER) (Svistoonoff et al. 2007; Ticconi et al. 2009). It was proposed that PDR2 may function together with LPR1/LPR2 to regulate the meristem activity in an ER-resident pathway, once changes in external Pi are sensed. PDR2 may act upstream to regulate negatively the output of LPR1/LPR2. These observations established an association between pattern of root development and root meristem activity in response to Pi availability (Chiou and Lin 2011).

Various Adaptations in Plants to P-Starvation

Adaptation 1: Regulation of the Root Systems

During Pi starvation, in *Arabidopsis*, primary root growth is inhibited (Lopez-Bucio et al. 2002; Williamson et al. 2001) whereas lateral root growth alterations are more clear and visible. Local Pi status at the root tip (Linkohr et al. 2002) controls primary root inhibition by Pi starvation while lateral root alterations are controlled by systemic Pi status (Franco-Zorrilla et al. 2004). Among the major hormones, auxin seems to play the most central role in alterations root development (Hardtke 2006) during Pi starvation. Significant increase in both the length and density of root hairs was observed in *Arabidopsis* during Pi starvation (Bates and Lynch 1996; Ma et al. 2001) which contributes significantly to Pi acquisition (Bates and Lynch 2000, 2001)

Adaptation 2: Phosphate Mobilization and Utilization

Induced Production of Acid Phosphatases and Ribonucleases

Acid Phosphatases

Pi is accessible into plant cells with the help of various high- and low-affinity Pi transporters in the cellular membrane systems. But on the other hand, organic P, such as phosphomonoesters and nucleic acids, is difficult for direct translocation. Hydrolases such as phosphatases and ribonucleases may play an important role as they are involved in releasing Pi from such organic sources for efficient transport and subsequent utility. Some key processes include mobilization of the organic P in soil for root absorption, remobilization of organic P in senescing organs, storage tissues, and intracellular compartments (Fang et al. 2009).

Pi starvation stress could be reduced by activating and enhancing these processes since numerous reports suggest members of phosphatases and ribonucleases are induced by Pi starvation. Among these enzymes, acid phosphatases are greatly induced by Pi deficiency. Their function is to release Pi from phosphomonoesters and have optimum activity in acidic conditions (Duff et al. 1984). Several isoforms of APases are Pi starvation inducible (Trull et al. 1997; Tomscha et al. 2004). One such isoform that has been purified and characterized from Pi-starved seedlings is a 34 kDa monomeric protein AtACP5/AtPAP17 (Li et al. 2002; del Pozo et al. 1999). AtACP5 (At3g17790) is usually expressed in flowers and senescent leaves during Pi sufficiency but it is transcriptionally upregulated in root and leaf during Pi starvation (Misson et al. 2005; Müller et al. 2007; Morcuende et al. 2007). Its promoter contains binding sites for AtPHR1, a transcriptional regulator involved in Pi-starvation response (Rubio et al. 2001). There is a possibility that AtACP5 is linked to the cell wall or plasma membrane (del Pozo et al. 1999) no APases were extracted from apoplastic fluid. Thus, AtACP5 may function in Pi starvation-inducible Pi mobilization, possibly from extracellular sources.

Another purified *Arabidopsis* APase from Pi-starved suspension cell cultures is a 100 kDa dimeric vacuolar protein AtPAP26 (encoded by At5g34850). It is upregulated at the protein level during Pi starvation (Veljanovski et al. 2006) suggesting its role in remobilizing intracellular phosphomonoesters in a Pi starvation-induced manner. AtPAP12, AtACP5/AtPAP17, and AtPAP26 are all purple APases (PAPs) which contain a conserved motif forming a binuclear metal ion complex, and they display a characteristic purple color (Schenk et al. 2000; Olczak et al. 2003). In the *Arabidopsis* genome, 29 PAP genes have been identified (Li et al. 2002), of which at least 11 of them are upregulated during P-starvation (Misson et al. 2005), indicating their potential role in Pi nutrition.

Of many Pi starvation-inducible APases studied in *Arabidopsis*, three are widely studied including AtPAP12, which is likely to be secreted into the soil to mobilize organic P. AtACP5/AtPAP17 which may function in internal P remobilization and AtPAP26 which may perhaps be involved in intracellular P remobilization.

Ribonucleases

Certain types of ribonucleases (RNases) are hypothesized to participate in mobilization of Pi from RNA during Pi stress. In *Arabidopsis*, three genes have been identified which code for S-like RNases that could be involved in Pi mobilization—AtRNS1 (At2g02990), AtRNS2 (At2g39780), and AtRNS3 (At1g26820) (Taylor and Green 1991; Bariola et al. 1994) AtRNS1 encodes a secreted protein (Bariola et al. 1999) expressed mainly in flowers (Bariola et al. 1994) and is upregulated during Pi starvation at both mRNA and protein (Bariola et al. 1994) levels. AtRNS2 encodes an intracellular protein (Bariola et al. 1994) expressed in various organs (Taylor et al. 1993) and is strongly induced at both the mRNA level in leaf (Müller et al. 2007; Taylor et al. 1993) and the protein level (Bariola et al. 1994) during Pi starvation. It is also induced at the mRNA level during senescence (Taylor et al. 1993). AtRNS3 is expressed in various tissues, but during Pi starvation and senescence, it is only modestly regulated at the mRNA level (Bariola et al. 1994). It is likely that AtRNS1 and AtRNS2 are involved in Pi mobilization from extracellular and intracellular RNA sources during Pi starvation as well as senescence or wounding (Fang et al. 2009).

Induced Synthesis and Secretion of Organic Acids

Organic acids exuded from plant roots, such as citrate and malate, may possibly have a role in liberation of Pi from Al–, Fe–, and Ca–P complexes and reducing toxic cations like Al^{3+}, and are thus stimulated during Pi starvation or Al^{3+} accumulation (Ryan et al. 2001). Pi starvation-induced organic acid exudation has been reported in *Arabidopsis* (Narang et al. 2000) and rice (Begum et al. 2005). Such exudation can be largely accredited to increased synthesis and secretion in the root systems. It includes increased activity of enzymes like phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), and citrate synthase (CS), as well as reduced activity in aconitase and reduced respiration rates, based on evidences mainly from cluster roots of white lupin (Vance et al. 2003). In rice, overexpression of a PEPC gene increased organic acid synthesis and exudation (Begum et al. 2005). Similarly, overexpression of a citrate synthase gene in *Arabidopsis* increases citrate synthesis and secretion (Koyama et al. 2000).

Usually, organic acids exist in their anion forms in cytoplasm pH conditions. So the mechanisms of secretion may perhaps involve anion transporters (Vance et al. 2003). In white lupin cluster roots, a correlation was observed between proton secretion and organic acid anion exudation during Pi starvation (Sas et al. 2001). Regulation of some plasma membrane H^+-ATPase may explain proton extrusion enhancement by Pi starvation (Yan et al. 2002).

Adaptation 3: Phosphate Transport

Three important processes involve Pi transport: Pi acquisition, Pi homeostasis, and Pi redistribution. Regulation of these three processes is an important adaptive strategy. For Pi-acquisition, the mycorrhizae between some plants and their symbiosed fungi markedly extend the root rhizosphere, thus improving the acquisition (Bucher 2007; Javot et al. 2007; Paszkowski et al. 2002). During Pi starvation, it had been found that Pi uptake ability increases in various root systems (Lee 1982; Mimura et al. 1998). Induction of high-affinity Pi transporters could be the underlying mechanisms of this phenomenon.

Pi homeostasis usually occurs at two different scales. In the whole-plant level, a flexible regulation of Pi acquisition is required in response to in vivo Pi status and complicated tissue Pi supply coordination; whereas in the scale of one cell, it primarily depends of the homeostatic function of vacuole. The vacuole stores up to 85–95 % of the total Pi under sufficient supply but during Pi deficiency, this Pi storage is remobilized and exported for use in the cell, constituting an intracellular homeostasis mechanism (Mimura et al. 1996; Tu et al. 1990; Sakano et al. 1992; Ohnishi et al. 2007).

Pi redistribution is the remobilization of Pi from old tissues and its retranslocation to young and/or actively growing tissues. Although it occurs during the normal developmental process also, it is noticeably enhanced in response to Pi starvation. This adaptation accelerates the remobilization and protects the newly growing tissues from an immediate damage caused by Pi deficiency as young tissues are more sensitive to Pi declining than the older ones. Regulators of this adaptation probably include miR399s, PHO2, and the AtIPS1/At4 family (Shin et al. 2006; Martin et al. 2000), which might function by inducing APases (e.g., AtACP5 and AtPAP26) and RNases (e.g., RNS1 and RNS2) to hasten Pi remobilization.

Adaptation 4: Metabolism

Adjustment of Cellular Respiratory Pathways

Long-term Pi starvation has a marked effect on cytoplasmic Pi and nucleoside phosphates such as ATP and ADP. They begin to decline whereas the level of pyrophosphate (PPi) is more constant (Duff et al. 1989). This situation would severely affect normal glycolysis and oxidative phosphorylation pathways as they involve several ADP-/ATP-/Pi-dependent reactions. This could have a rather unfavorable impact on the systemic metabolism. However, plants seem to have evolved an adaptive strategy where they change the respiratory pathways so as to bypass the ADP-/ATP-/Pi-dependent reactions (Plaxton 1996; Theodorou and Plaxton 1993).

Several experiments on Pi-starved Brassica nigra suspension cells have shown some alterations of the glycolytic pathways. During reduced ATP concentration, the metabolic by-product PPi is used as an alternative energy donor. These bypasses serve to maintain essential carbon flow and to recycle phosphate esters to Pi. The rotenone-insensitive NADH dehydrogenase pathway and the cyanide-resistant alternative oxidase pathway have been suggested as new pathways which may be activated to bypass the normal oxidative phosphorylation pathways in the mitochondria: These alternative pathways can work under the limited ATP production upon severe Pi starvation.

Alteration of Membrane Lipid Composition

Pi starvation induces severe changes in lipid composition of plant membranes, which includes a decline in phospholipids and an enhancement in non-phosphorous lipids in many species, including photosynthetic bacteria (Benning et al. 1993) and *Arabidopsis* (Essigmann et al. 1998) In Pi-starved *Arabidopsis*, sulfolipids were found increased in thylokoid membranes (Essigmann et al. 1998), whereas galactolipids were found to be increased in both the thylokoid membranes (Essigmann et al. 1998) and extraplastidic membranes (Hartel et al. 2000; Jouhet et al. 2004). Microarray results also show similar observations that several genes encoding phospholipase C [e.g., At3g03530 (Nakamura et al. 2005), At3g03540 (Misson et al. 2005)], and D [e.g., At3g05630 (Misson et al. 2005)] are upregulated in the leaf and root, which is consistent with the results on phospholipid degradation enhancement during Pi starvation.

The Signaling Molecules

Pi, sugars, hormones, and microRNA (miRNA) have been considered as signals. These signals either act locally and/or serve as systemic signals to bring responses at different sites. The signaling pathways are usually interconnected and elicited responses are often the output of many signaling routes.

Phosphate as a Signal

Increased intracellular Pi concentrations could repress Pi-starvation responses (PSR). Convincing evidence obtained from the results of Phi application supports the opinion that Pi serves as a signal (Chiou and Lin 2011). Though Phi is taken up by plants through Pi transporters; it cannot be oxidized to Pi or further metabolized once it enters the cell (Carswell et al. 1996, 1997). Under low Pi conditions, exogenous application of Phi eases wide range of PSR, including a reduction in the

root-to-shoot biomass ratio, root hair elongation, lipid remodelling, anthocyanin accumulation, and expression of many Pi starvation-induced (PSI) genes (Carswell et al. 1996, 1997; Kobayashi et al. 2006; Ticconi et al. 2001; Varadarajan et al. 2002).

The interference of gene expression by Phi is specific to PSR and is an early event occurring at the level of transcription. As there is large structural similarity between Phi and Pi, the Pi signaling machinery is not capable of discriminating Phi from Pi. In addition, Phi inhibits the influx of Pi in a competitive manner and it usually accumulates in the cytoplasm (Danova-Alt et al. 2008; Pratt et al. 2004). It is probable that elevated intracellular Phi concentrations imitate adequacy of Pi, thus interfering with Pi signal transduction pathways, even though plants are starving for Pi. Jointly, these findings reveal that Pi is capable of acting as an initial signal.

Molecular Studies

Interference of Phi with the Pi signaling pathway has also been observed in Pi starved yeast (*Saccharomyces cerevisiae*). Pho84, high-affinity Pi transporter is one of the potential targets for Phi action (McDonald et al. 2001). It is strange that methylphosphonate, which is a non-metabolized analog of Pi usually triggers degradation of Pho84 protein instead of reducing Pho84 transcript levels (Pratt et al. 2004). This finding suggests that Phi- or Pi-mediated signaling can operate posttranslationally also.

Many split-root experiments have demonstrated that several PSR and the expression of PSI genes are dependent on the whole-plant Pi status, which in turn is determined by the transport and mobilization of Pi within different plant parts (Burleigh and Harrison 1999; Franco-Zorrilla et al. 2005; Liu et al. 1998). In one portion of roots which was grown in Pi-depleted medium, upregulation of PSI genes was repressed, most probably by a systemic suppressor which has been transported from the other portion of the roots grown in Pi-replete medium. On the contrary, the observation that reduced induction of the *Medicago Mt4*, a PSI gene, in the Pi-depleted compartment initiated before a rise in Pi concentration and reduction in Pi flow had no effect on the systemic suppression argues against Pi being a signal (Burleigh and Harrison 1999; Thibaud et al. 2010).

MicroRNA-Mediated Signaling: miR399 as a Systemic Signal

To maintain Pi homeostasis proper regulatory mechanisms are required. Regulation of gene expression at transcriptional, posttranscriptional, and posttranslational levels (Franco-Zorrilla et al. 2004; Chiou and Lin 2011) is one of the adaptive responses to P deficiency. Transcription factors are responsible for the transcriptional control of P levels. Besides the transcription factors, microRNAs (miRNAs) have also

come up as new regulators in regulating phosphate starvation response genes at the posttranscriptional level. Several studies have shown that miRNAs play a significant role in P deficiency signaling and regulation of Pi homeostasis. A microRNA (miRNAs) is a small endogenous noncoding RNAs containing about 20–24 nucleotides generated from an ssRNA precursor with a hairpin secondary structure. They negatively regulate gene expression at the posttranscriptional level by base pairing to their target mRNAs, which often directs mRNA cleavage in plants (Reinhart et al. 2002). miRNAs have complementary sequences within mRNA (Bartel 2009). The mRNA strand is cleaved and silenced by the miRNAs, destabilize the mRNA by shortening its poly A tail and less efficient translation (Bartel 2009).

The first miRNA that is upregulated due to P deficiency and rapidly decreased upon P re-addition is miR399 (Fujii et al. 2005). The other two miRNAs—miR827 and miR2111—are demonstrated to be highly and specifically induced by P deficiency just like miR399 in *Arabidopsis* (Hsieh et al. 2009). All these three miRNAs (i.e., miR399, miR827, and miR2111) targets the genes that code for proteins involved in the ubiquitin-mediated protein degradation pathway. It shows the importance of posttranslational regulation of protein levels in the adaptive responses of P deficiency.

Studies on *Arabidopsis thaliana* shows that six *MIR399* genes (*MIR399A* to *F*) are coded by its genome that are upregulated to different levels in response to the P-starvation. The overexpression of miR399 stimulates increased Pi uptake and its translocation to the shoot in transgenic *Arabidopsis* when grown under P-sufficient conditions (Fujii et al. 2005; Aung et al. 2006; Bari et al. 2006; Chiou et al. 2006). Doerner (2008) also showed in *Arabidopsis* that a protein-*PHO2*, microRNA-399 and a ribo-regulator are the molecular components of a module that regulate the systemic Pi homeostasis, its uptake and transport. *PHO2* controls the protein stability and regulates the systemic Pi homeostasis. *pho2* plants do not mobilize Pi from mature leaves to young leaves (Chiou et al. 2006). Molecular studies have shown that *pho2* plants are due to mutation in the UCB24 gene (Aung et al. 2006; Bari et al. 2006). Pi homeostasis is dependent on a complex cascade of molecular signals (Chiou and Lin 2011; Zhang et al. 2014). This protein in turn is regulated by miR399 (a Pi deficiency-responsive gene). When the levels of Pi are low the expression of miR399 is elevated in shoot tissues. miR399 is translocated to the root via the phloem where it degrades the *PHO2* mRNA.

Low *PHO2* activity in turn stimulates the expression of phosphate transporter genes pht1;8 and pht 1;9. In IPS/At4 gene family, the noncoding RNAs are also highly expressed and they in turn inhibit the miRNA399 charged silencing complexes on *PHO2* mRNA and thereby lets the UBC24 protein induction resulting in an increase in Pi uptake and transport (Franco-Zorrilla et al. 2004; Aung et al. 2006; Bari et al. 2006). When the P levels are sufficient, UBC24 is expressed in the roots and under low P levels it is downregulated. The levels are kept in check by the miR399 (Fujii et al. 2005; Bari et al. 2006; Chiou et al. 2006).

The results were strengthened by *ubc24* mutant and miR399-overexpressing transgenic *Arabidopsis* plants (Chiou et al. 2006). Similarly, when *Arabidopsis*

miR399 was overexpressed in tomato (*Solanum lycopersicum*) it increased accumulation of Pi. miR399 and *UBC24* homologs have also been observed in rice and common bean in response to Pi starvation (Valdés-López et al. 2008; Liu et al. 2010b). PHO2 homologs (Bari et al. 2006) and miR399 (Barakat et al. 2007) have been identified in eudicots and monocots. PHO2/UBC24 is important for Pi homeostasis. The miR399-PHO2 regulatory mechanism is evolutionarily conserved in angiosperms and is a systemic signal regulating Pi homeostasis.

In addition of a Pi deficiency-responsive microRNAs mR399 other miRNA families such as miR156, miR159, miR166, miR319, miR395, miR398, miR399, miR447, and miR827 are commonly responsive to P deficiency among species. These miRNAs also regulate P deficiency signaling pathways in plants, as exemplified by miR399. The majority of miRNA target genes are involved in transcriptional regulation.

Many studies are there which uncover the function of miR399, the molecular identity of *PHO2* (*PHOSPHATE2*), and the biological function of the *AT4/IPS1* family in Pi signaling. miR399 is highly upregulated in Pi-depleted tissues (Aung et al. 2006; Bari et al. 2006; Chiou et al. 2006). It directs the cleavage of *PHO2* mRNA encoding a ubiquitin-conjugating E2 enzyme (UBC24). *Arabidopsis* and rice-overexpressing miR399 display excessive accumulation of shoot Pi, which results in Pi toxicity even under Pi-sufficient conditions (Ciereszko et al. 2005; Fujii et al. 2005), a phenotype similar to *ubc24* T-DNA knockout lines and a previously identified *pho2* mutant (Aung et al. 2006; Bari et al. 2006; Ciereszko et al. 2005; Delhaize and Randall 1995). The phenotype is caused due to increased transcript levels of *Arabidopsis PHT1;8* and *PHT1;9* as well as rice *OsPT2* coding for Pi transporters (Liu et al. 2010a).

It is likely that Pi transport is regulated by PHO2 through ubiquitin-mediated protein degradation. Thus, suitable expression of *PHO2* is critical to maintain Pi homeostasis under Pi-sufficient conditions by preventing overloading of Pi into plants. During Pi deficiency, *PHO2* is downregulated and miR399 is upregulated, leading to active Pi uptake. Analysis suggests that miR399s and *PHO2* are expressed predominantly in vascular tissues (Aung et al. 2006). Steady with these observations, miR399 was detected in the phloem sap of rapeseed and pumpkin (Buhtz et al. 2008; Pant et al. 2008). Reciprocal grafting experiments between wild-type and miR399-overexpressing plants led to the conclusion that shoot-to-root movement of mature miR399 could suppress *PHO2* in roots (Pant et al. 2008). Thus, movement of miR399 serves as a systemic signal for activation of Pi uptake in roots and for communication of Pi status in shoots to Pi uptake activity in roots. It has also been observed that induction of miR399 under Pi starvation is positively regulated by the transcription factor PHR1 (PHOSPHATE STARVATION RESPONSE1) and by the availability of photoassimilates (Rubio et al. 2001; Valdés-López et al. 2008; Zhou et al. 2008). In many plant species, regulation of miR399 and *PHO2* is found to be conserved and homologous genes of miR399, *PHO2*, and *At4/IPS1* have also been identified (Liu et al. 2010a; Valdés-López et al. 2008).

Other Phosphate-Responsive miRNAs

Additional Pi-responsive miRNAs (apart from miR399) have been identified from several plant species (Hsieh et al. 2009; Lundmark et al. 2010). Similar to miR399, some of them (e.g., miR156, miR169, miR395, and miR398) are evolutionally conserved, whereas others are species specific. Several miRNAs are differentially regulated under various nutrient deficiencies which suggest the existence of a coordinated cross talk mediated by these miRNAs (Hsieh et al. 2009; Pant et al. 2009).

Hormone-Mediated Systemic Signaling

Hormones affect many mechanisms by influencing the signaling pathways in response to the nutrient availability or starvation in the soil (Rubio et al. 2009). Many hormones have been found to have a role in Pi signaling. Changes in Pi availability can modify hormone production, sensitivity, and transport. Variation in transcript levels of genes involved in hormone biosynthesis under Pi deficiency was revealed by microarray analyses (Misson et al. 2005; Morcuende et al. 2007).

Cytokinin

The involvement of cytokinin signaling in regulating P-starvation responses (PSR) is well documented. Cytokinin usually effects only the Pi-starvation responses that are dependent on whole-plant Pi status but not the local Pi-dependent responses, such as increased root hair number and length, which indicates that cytokinin signaling may be involved in the systemic repression responses of Pi signaling (Martin et al. 2000).

The cytokinin level decreases when the plants sense phosphate starvation (Salama and Wareing 1979; Horgan and Wareing 1980). Cytokinins affect only Pi-starvation responses that are controlled by systemic signals, and not local Pi-dependent responses, which again suggest it as a probable candidate for the systemic repression signaling system (Martin et al. 2000). Pi starvation represses the action of cytokinin by reducing its concentrations (Kuiper et al 1988) and by down-regulating the expression of CRE1, a cytokinin receptor (Franco-Zorrilla et al. 2002). On the other hand, cytokinin negatively regulates many PSI genes (Franco-Zorrilla et al. 2002, 2005; Martin et al. 2000). A decline in the level of endogenous cytokinin or in its action could ensure a full PSR upon Pi deficiency. As cytokinin affects a broad range of PSI genes, it is assumed to be involved in the systemic repression of Pi signaling (Martin et al. 2000). Cytokinin-induced repression of the PSR was observed to be partially attributed to a rise of Pi concentration in shoots (Wang et al. 2006); however, cytokinin treatment does not mimic the repression

effect of Pi resupply, arguing against the role of cytokinin as a Pi long-range signal (Franco-Zorrilla et al. 2005).

Auxin

Auxin signaling correlates strongly with modification of RSA caused by Pi deprivation. Exogenous auxin application triggered localized alterations in RSA including arrest of primary root growth and induced formation of lateral roots in Pi-deprived plants (Gilbert et al. 2000; Lopez-Bucio et al. 2002). Moreover, Pi-deprived plants are more sensitive to exogenous auxin than Pi-replete plants. Auxin and cytokinin signaling are implicated in membrane lipid remodeling during Pi starvation by regulation of gene expression (Kobayashi et al. 2006; Nacry et al. 2005).

Arabidopsis lpr1 (low phosphate-resistant root) mutants displaying reduced lateral roots formation under Pi limitation conditions were isolated as allelic to BIG, which is required for polar auxin transport (Lopez-Bucio et al. 2005). After detailed analysis of root architecture parameters with the application of auxin or auxin transport inhibitor in wild-type plants and auxin-responsive mutants, it was found that auxin redistribution within the roots triggered the Pi-starvation-induced changes of root development (Nacry et al. 2005).

Ethylene

Similar to auxin, ethylene has also been implicated in changing the RSA in response to Pi deficiency, as increased levels of ethylene have been detected under these conditions and imitating phenotypes of Pi-starved roots were obtained after exogenous application of ethylene (Gilbert et al. 2000; Ma et al. 2003). It has been found that during Pi starvation, ethylene is important for inhibition of primary root growth and for promotion of lateral root elongation but was not found necessary for lateral root initiation.

Abscisic Acid

It has been speculated that abscisic acid (ABA) signaling is involved in PSR because some similarities in phenotypes, such as increased root-to-shoot ratio and root hair density were found between plants subjected to Pi starvation and those treated with ABA (Ciereszko and Kleczkowski 2002; Trull et al. 1997). However, a direct relation between ABA signaling and PSR has not been established. Though, ABA could inhibit the expression of several PSI genes possibly via the control of ABI1 type 2C protein phosphatase (Ribot et al. 2008; Shin et al. 2006).

Gibberellin

Adaptations to low Pi are controlled by GA via a DELLA-dependent mechanism (Jiang et al. 2007). Pi starvation reduces the level of bioactive GA, which in turn may lead to accumulation of DELLA proteins. Pi starvation-induced changes in RSA and anthocyanin accumulation are found to be repressed by exogenous GA or in DELLA-deficient mutants. MYB62, a PSI transcriptional factor, further revealed cross talk between GA biosynthesis and PSR (Devaiah et al. 2009). The expression of early GA biosynthetic genes were found to be repressed by overexpression of *MYB62* and resulted in GA-deficient symptoms. This finding suggests that the previously observed reduction in GA biosynthesis under Pi deficiency is caused by upregulation of MYB62 to a certain extent. In addition, overexpression of *MYB62* suppresses the expression of many PSI genes and affects Pi uptake, RSA, and acid phosphatase activity (Devaiah et al. 2009). The regulatory role of MYB62 on these PSR may function through the modulation of GA metabolism or signaling.

Sugar-Mediated Signaling

Besides hormones carbohydrates also affect the nutrient homeostasis. Many studies have shown the importance of sugar signaling in regulating PSR, including increased expression of PSI genes and changes in RSA (Hammond and White 2008; Jain et al. 2007; Karthikeyan et al. 2007). Moreover, Pi starvation has been found to have a role in expression of various sugar-responsive genes (Ciereszko and Kleczkowski 2002). The observation that increased translocation of phloem mobile carbohydrates to the root of P-deprived plants raised the possibility that shoot-derived sucrose serves as a long-distance signal to regulate the nutrient uptake in roots (Hammond and White 2008; Hermans et al. 2006). Limitation of Pi results in decreased level of photosynthesis and an increased level of sugars and starch in Pi-deprived leaves (Morcuende et al. 2007; Nilsson et al. 2007). The built-up sugars are translocated to roots via enhanced loading of sucrose into the phloem. The root-to-shoot biomass ratio increases because of this resource allocation.

A plethora of evidences support the positive bidirectional interaction between sugar and Pi sensing (Sadka et al. 1994; Nielsen et al. 1998; Ciereszko et al. 2001; Ciereszko and Kleczkowski 2002; Hammond et al. 2003; Wu et al. 2003; Rubio et al. 2009). A strong association was found between increased gene expression and enzymatic activities relating to carbohydrate biosynthesis and PSR in response to Pi deficiency (Müller et al. 2007; Morcuende et al. 2007; Wasaki et al. 2006). Both hexokinase-dependent and -independent signaling pathways have been suggested to be involved in interactions between sugar sensing and PSR (Karthikeyan et al. 2007; Muller et al. 2005). Sugars demonstrate clear temporal and spatial control of PSR. Increased sucrose concentrations in roots precede the induction of PSR

(Hammond and White 2008). Moreover, it was observed that exogenous application of sugars magnifies PSR (Karthikeyan et al. 2007; Müller et al. 2007). It was proposed that carbon assimilation and its partitioning are checkpoints for the onset of Pi deficiency and sugars, mainly sucrose, are candidates for the shoot-derived systemic signal, which in turn contributes to the regulation of PSR in roots. It seems that signaling pathways mediated by sugar and different hormones are interconnected (Gibson 2004). Under Pi starvation, sucrose may promote auxin transport and increase the sensitivity of the root system to auxin (Jain et al. 2007; Karthikeyan et al. 2007). Moreover, sugars and cytokinins act antagonistically to regulate the expression of PSI genes (Franco-Zorrilla et al. 2005).

The *Arabidopsis pho3* mutant, which consists of a defective copy of the SUC2 gene encoding a phloem-expressed sucrose transporter, was isolated from a screen for reduced induction of root acid phosphatase (ACP) activity upon Pi starvation (Zakhleniuk et al. 2001). Characterization of this mutant pointed towards declined phloem loading of sucrose which accounted for the hyposensitivity of pho3 to internal Pi concentrations (Lloyd and Zakhleniuk 2004). Moreover, several Pi-starvation-responsive genes were shown to be upregulated in the cluster roots of Pi-deprived white lupin plants in a photosynthesis-dependent manner (Liu et al. 2005).

In wild type, *cre1* and *ahk3* mutants of *Arabidopsis*, the study of Pi-starvation-responsive genes indicated that a functional link exists between cytokinin, sugar, and Pi-starvation signaling that involves the CRE1 and AHK3 receptors (Rubio et al. 2009). Studies on transgenic plants overexpressing genes that activate cytokinin signaling indicated a bidirectional antagonistic interaction between sugars and cytokinins (Moore et al. 2003).

Transcriptional Regulation

Transcription Factors

There are several important transcription factors in Pi signaling pathways, including members in the families of MYB, WRKY, and bHLH. Even during Pi sufficiency, negative regulation of several PSI genes appears to be common. Activation of these genes at low Pi turns could be a result of derepression. *Arabidopsis* PHR1, an R2R3MYB protein, and a homolog of *PSR1* (*PHOSPHORUS STARVATION RESPONSE 1*) in *Chlamydomonas reinhardtii* (Wykoff et al. 1999) is the most well-characterized transcription factor implicated in PSR (Rubio et al. 2001). PHR1 and PHL1 (PHR1-like1) are central integrators in transcriptional regulation of PSR because most of transcriptional activation and repression responses to Pi starvation are affected in *phr1* and *phr1 phl1* mutants (Bustos et al. 2010). PHR1 plays a crucial role in regulating genes involved in Pi transport and remobilization, RSA, anthocyanin biosynthesis, and carbohydrate metabolism (Bustos et al. 2010; Nilsson et al. 2007; Rubio et al. 2001).

Another MYB transcription factor involved in PSR is MYB62 (Devaiah et al. 2009). Although MYB62 is upregulated during Pi deficiency, it functions as a negative regulator suppressing several PSI genes. Two basic helix-loop-helix domain-containing transcription factors *Arabidopsis* bHLH32 and OsPTF1, which are involved in PSR, have been characterized. Both are upregulated under Pi deprivation. *Arabidopsis* bHLH32 plays a negative role, whereas OsPTF1 serves as a positive regulator for PSR (Yi et al. 2005). ZAT6 (zinc finger of *Arabidopsis*), a cysteine-2/histidine-2 zinc finger transcription factor, is involved in regulating RSA and Pi homeostasis (Devaiah et al. 2007). ZAT6 is upregulated at low Pi concentrations and functions as a repressor because overexpression of ZAT6 inhibits primary root growth and expression of many PSI genes.

Potential Regulators in Phosphate Signaling

Calcium and Inositol Polyphosphates

Calcium ion (Ca^{2+}) and inositol polyphosphates (IPs) are secondary messengers which are involved in eliciting multiple cellular responses (Kudla et al. 2010; Monserrate and York 2010). Moreover, inositol 1,4,5-trisphosphate (IP3) can very well regulate intracellular Ca^{2+} release, suggesting the presence of cross talk between these two signaling pathways. Two mutants, *cax1cax3* and *ipk1*, which are either defective in two vacuolar Ca^{2+}/H^{+} exchangers or impaired in inositol polyphosphate kinase, result in an excessive accumulation of Pi in shoots (Stevenson-Paulik et al. 2005). IPK1 is the last enzyme participating in the biosynthesis of phytate (inositol hexakisphosphate, IP6) (Verbsky et al. 2002). In the *ipk1* mutant, the excessive accumulation of Pi is associated with a reduced level of IP6 and an increased amount of inositol tetrakisphosphate (IP4) and inositol pentakisphosphate (IP5) intermediates (Stevenson-Paulik et al. 2005). This finding provides the earliest link between IP metabolism and regulation of Pi homeostasis in plants. Inositol heptakisphosphate (IP7) has been found to play a major role in transmitting low Pi signals in yeast (Lee et al. 2008). Upon Pi starvation, intracellular concentrations of IP7 increase, primarily by the activity of VIP IP6 kinase.

Reactive Oxygen Species

It has been observed that the concentration of reactive oxygen species (ROS) in roots increases rapidly (within a few hours) after deprivation of nitrogen, potassium, sulfur, or P (Schachtman and Shin 2007; Shin et al. 2005) and triggers the expression of several nutrient starvation-responsive genes. Variation in ROS concentration and distribution in specific root cells occurs in response to Pi deficiency although

various patterns were reported (Shin et al. 2005). Alterations in ROS distribution in Pi-starved roots may be associated with changes of RSA.

P-Zn Interaction and Homeostasis in Nodule

Phosphorus and zinc are two essential nutrients, which are required for normal plant growth and development. These nutrients are mutually antagonistic in certain circumstances that can cause yield reductions in many crops due to either P or Zn deficiencies. These deficiencies are caused when nutrients are available in small quantities. The Zn induces P deficiency because farmers commonly use large amounts that cause the deficiency of the other. The Zn-induced P deficiency is a very rare phenomenon because users commonly apply large amounts of P fertilizer as compared to Zn fertilizer. The P-induced Zn deficiency is related to the application of phosphatic fertilizers at high dose to the soils that are low or marginal in available Zn.

There are four hypotheses to explain this phenomenon: (a) P may interfere with the translocation of Zn from roots to top. Cakmak and Marschner (1987) observed that the total Zn concentration in plant tissues was not changed with P supply but water-soluble Zn was decreased, and hence visual Zn deficiency symptoms were observed in cotton, grown in nutrient solution. (b) High availability of P can accentuate Zn deficiency in plant tissues. (c) Zn concentration may reduce because of the dilution caused by growth response of P. Another observation that P application increased the growth of upper plant parts enough to dilute the Zn concentration in bean plants (Loneragan et al. 1979; Singh et al. 1988). (d) Wijebandara (2007) explained the phenomenon; an imbalance between Zn and P can cause metabolic disorder with plant cells. In sweet corn plants grown in nutrient solution, Soltangheisi et al. (2013) stated that P/Zn ratio could be a better indicator of Zn nutritional status than Zn concentration alone. Soltanpour (1969) explained that P-Zn interaction was related more to plant physiology than to soil reactions, since in his work the Zn and P bands did not contact one another in the soil.

In plants, Pi and Zn homeostasis are known to have strong interaction (Loneragan et al. 1982; Cakmak and Marschner 1987; Huang et al. 2000; Zhu et al. 2001; Bouain et al. 2014; Khan et al. 2014). The interaction between Pi and Zn on molecular level so far is poorly understood. Misson et al. (2005) reported that the long-term Pi deprivation leads to Zn over-accumulation and inversely Zn starvation appears to cause an over-accumulation of Pi. Transcriptome data from roots of Pi- or Zn-deficient *Arabidopsis* plants (Hammond et al. 2003; Wintz et al. 2003; Wu et al. 2003; Misson et al. 2005; van de Mortel et al. 2006; Müller et al. 2007; Bustos et al. 2010; Rouached et al. 2011; Woo et al. 2012).

Legume plants are used in crop rotations and their root nodules fix the atmospheric N_2 or alternative source of nitrogen is chemical fertilizers (Oldroyd and Dixon 2014). These plants that are affected by abiotic and biotic stresses, such as drought, salinity, low pH, extreme temperatures, heavy metals, and low nutrient availability, are major limitations for legume production in most of the areas where

these plants are cultivated (Lopez-Arredondo et al. 2014; Araujo et al. 2015; Valentine et al. 2011). Suboptimal soil phosphorus availability is the environmental limitation that has received considerable attention. Phosphorus is a major component of essential structural molecules and is an important element for energy transformation and regulation of various enzymatic activities (Schulze et al. 2006).

In nucleic acids, proteins, lipids, sugars, and adenylate etc., P is central to majority of the molecular constituents required for the functioning of plant cell. In plants, P plays an important role in various metabolic processes like respiration, photosynthesis, carbohydrate metabolism, glycolysis, redox reaction, and signaling (Vance et al. 2003). Low availability of P in the soil plants reduces plant growth, development, and photosynthetic ability per unit leaf area (Chaudhary et al. 2008; Sulieman et al. 2013). The availability of P also has an effect on symbiotic tissues and has serious implication on the growth and functioning of the nodule because of the specific requirement for symbiotic N2 fixation as an energy requiring process. Many reports support that N fixation of legumes needs more P for functioning than non-nodulating plants (Sulieman and Schulze 2010; Rotaru and Sinclair 2009). When there is P deficiency, the growth of N_2 fixing legumes is severely retarded, and the ammonium assimilation into amino acids in the plant cell fraction of nodules is not sufficient to support the development for plant growth (Hernandez et al. 2009; Cabeza et al. 2014).

The P and Zn play an important role in various metabolic processes. Other than this, in plants Pi and Zn play an important role in molecular mechanism. *Arabidopsis* genome contains nine PHT1 family members and most of them are controlled by endogenous Pi of the plant (Poirier and Bucher 2002; Nussaume et al. 2011). Some PHT1 genes are expressed in roots and works as high-affinity Pi uptake transporter (Bayle et al. 2011; Nussaume et al. 2011). In plants, Pi and Zn loading into root xylem are highly regulated and complex processes. Deeper investigations into the Pi-Zn nutrition interaction at the molecular level will be necessary to untangle their interconnected signaling networks, but also to improve Pi or Zn nutrition in higher plants. For example, understanding that how Zn deficiency induces Pi translocation to the shoot as well as its accumulation can be exploited to improve the Pi nutritional stress response in major crop plants (Kisko et al. 2015).

Molecular Studies

At molecular level, Khan et al. (2014) reported that direct molecular evidence for the cross talk between Pi and Zn nutrition in *A. thaliana*. There are genes involved in the cross talk. They showed genetic programmes that regulate Pi-Zn nutrition interaction in plants and molecular evidence for the Pi-Zn homeostasis and interaction in *A. thaliana* with an emerging role of PHO:H3. Zn deficiency is associated with over-accumulation of Pi in the shoots of dicotyledons and monocotyledons although genes can underline this problem (Huang et al. 2000; Misson et al. 2005). Khan et al. (2014) identified genes that are necessary for the increase in Pi

over-accumulation in response to Zn deficiency in *Arabidopsis*. In *Arabidopsis*, loading of Pi involves PHO1 and PHO1;H1 and HMA2 and HMA4 genes are for Zn. Though the co-expression analysis prove that expression of these genes correlate directly with PHO1 and HMA4 and indirectly with PHO1;H1 and HMA2. These results show that the presence of cross talk between Pi and Zn regulatory network in plants with the help of these genes. Poirier et al. (1991) identified *Arabidopsis* mutant AtPHO1. This mutant was affected in Pi transport.

Effect of Root Interactions on P-Uptake and PUE

Root interactions among plants play an important role in determining the performance of individuals in natural communities as well as crop productivity in agroecological systems. It is dependent on morphological (root architecture) and physiological plasticity of roots (e.g., exudation of organic and inorganic compounds, nutrient uptake) in response to the soil environment. To compete for soil resources (water and nutrients), plants largely invest in root growth (Cahill et al. 2010).

P-use efficiency could be enhanced by intercropping, i.e., growing two or more crops together in the field. For instance, growing mixtures of maize and faba bean (*Vicia faba*) are being used widely to improve P-use efficiency and grain yield in cropping ecosystems (Li et al. 2007; Zhang et al. 2010; Shen et al. 2011, 2013). The reason for which could be root interactions, which have a profound impact on P-uptake and yield in the maize/faba bean system (Li et al. 2003, 2014).

Usually, any neighbors with maize potentially represent competition, but findings of Zhang et al. (2015) indicated that neighboring faba bean was less competitive than neighboring maize. Presence of faba bean near maize resulted in improved maize shoot growth and nutrient uptake. They found that the effect of soil P availability on shoot P content and root length of target maize was significantly greater in the maize/faba bean than maize/maize mixtures. P availability induced by faba bean stimulated root morphological plasticity in maize (Zhang et al. 2015).

Faba bean causes rhizosphere acidification which in turn results in increased mobilization of organic/inorganic P sources, thus facilitating P-uptake by target maize (Li et al. 2007). Furthermore, larger amount of carboxylates, citrate, and acid phosphatase in the faba bean rhizosphere soil improves mobilization of sparingly soluble soil P, thus effectively increasing the amount of accessible P for target maize (Hinsinger 2001; Jones et al. 2003). Enhanced acquirement of limiting resources was possible because of decrease in intensity of interspecies competition through niche complementarity which means maize and faba bean accessing different P fractions where faba bean accessed sparingly soluble P (unavailable to maize) through root exudation, but maize mainly used soluble soil P (Hinsinger et al. 2011; Shen et al. 2011, 2013; Brooker et al. 2014; Li et al. 2014).

Hence, increased P uptake may be attributed to root interactions between neighboring plants.

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Chapter 5
Glycinebetaine-Mediated Abiotic Oxidative-Stress Tolerance in Plants: Physiological and Biochemical Mechanisms

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Abstract Plants face many stressful conditions during their lifetimes and because of their sessile nature they have to adapt to these conditions in order to survive. One unfortunate and unavoidable consequence of all major biotic and abiotic stresses is the overproduction of reactive oxygen species (ROS). ROS are highly reactive and toxic chemical entities and can cause serious damage to cellular proteins, lipids, carbohydrates and DNA, leading to irreparable metabolic dysfunction and cell death. Plant cells and their organelles, particularly the chloroplasts, mitochondria and peroxisomes have antioxidant defence systems, composed of enzymatic and non-enzymatic components, to counter the deleterious effects of ROS and/or to perform signalling functions. It is an established fact that the timely induction of antioxidant defences is a key to protection of plant cells from oxidative damage due to stress. Enzymatic antioxidants include superoxide dismutase, catalase, peroxidases and glutathione reductase, while the major non-enzymatic antioxidants are compatible osmolytes (glycinebetaine, GB; and proline), ascorbic acid, reduced glutathione, α-tocopherol, amino acids and polyphenols. Stimulated biosynthesis and accumulation of low molecular weight

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compatible osmolytes is one of the most effective mechanisms evolved by plants to maintain their cellular integrity and ensure survival when exposed to multiple abiotic stresses. Glycinebetaine, an *N*-trimethyl derivative of glycine and a quaternary ammonium compound, is one of the most studied and efficient compatible solutes. Due to its unique structural features, it interacts both with the hydrophobic and hydrophilic domains of macromolecules, including enzymes and proteins. GB has been reported to protect plants from the antagonistic effects of a range of abiotic stresses, by maintaining the water balance between plant cells and environment, osmotic adjustment, protecting the thylakoid membrane system, protein stabilization, photosystem and photosynthetic electron transport chain protection and by modulating ROS detoxification. In recent years, GB has attained unprecedented attention due to its multifunctional roles in plants under stressful conditions. In this chapter, we summarize our understanding of ROS formation under abiotic stress and GB biosynthesis and accumulation, as an adaptive mechanism, with particular emphasis on the new insights into the biochemical and molecular mechanisms involved in GB-mediated abiotic oxidative stress tolerance in plants.

Introduction

Plants face many stressful conditions during their lifetimes and because of their sessile nature they have to adapt to these conditions in order to survive. In their natural environment plants are exposed to various biotic and abiotic stressors. Abiotic stressors include drought, flooding, salinity, extreme temperatures, heavy metals, nutrient deficiency, high light intensities and UV radiation, all of which can have negative impacts on plant growth, development and crop production and can reduce product quality. Fuelled by the ever-increasing human population, increased crop productivity, from available lands, coupled with minimization of crop losses due to abiotic stress has become the highest agricultural priority, in recent times (Tuteja et al. 2011). However, production of abiotic stress tolerant plants requires a comprehensive understanding of the complex mechanisms associated with how plants respond to stress. One of the most active fields of plant science research focuses on understanding the molecular, physiological and genetic responses of plants to environmental stress, coupled with the development of approaches to improve abiotic stress acclimation and tolerance (Cabello et al. 2014).

One unfortunate and inevitable consequence of abiotic stress is the induction of oxidative stress. Therefore, oxidative stress is considered as a component of all major abiotic stresses. A common feature of plants' responses to these stressors is the overproduction of reactive oxygen species (ROS) (Petrov et al. 2015). They are formed by the incomplete reduction or excitation of molecular oxygen and are major causative factors of oxidative damage to lipid membranes and other essential macromolecules found in plants, including pigments, proteins, DNA and RNA. Overproduction of ROS can lead to irreparable metabolic dysfunction and ultimately causes cell death (Qureshi et al. 2013; Petrov et al. 2015). However, ROS also have the ability to work as signalling molecules, at lower cellular concentrations, and regulate plant development, as well as

various aspects of stress tolerance (Ismail et al. 2014). Interestingly, plants have evolved cellular repair mechanisms to maintain cellular redox balance and to convert oxidized macromolecules back to their reduced states (Krishnamurthy and Rathinasabapathi 2013). These mechanisms involve the induction of plant antioxidant systems, with enzymatic and non-enzymatic components, to detoxify or scavenge ROS (Khare et al. 2015). Upon exposure of plants to abiotic stressors, metabolic shifts occur, which result simultaneously to changes in the levels and range of cellular metabolites (Chen and Murata 2011). As a result, plants accumulate common cell solutes such as carbohydrates, organic acids and inorganic ions, which contributes to enhanced stress tolerance. However, high concentrations of these common solutes can if not localized inhibit enzyme activities and so plants often accumulate these solutes in vacuoles, where their increasing concentrations do not harm cellular metabolism (Kurepin et al. 2015).

In addition to these common solutes, plants often produce compatible solutes, or compatible osmolytes, which are membrane-impermeable solutes that accumulate in the cytoplasm to very high concentrations ($C \geq 0.2$ M) in response to stress (Kurepin et al. 2015). One of the best-documented and important abiotic stress-responsive mechanisms adopted by plants is the biosynthesis and accumulation of compatible osmolytes. Compatible osmolytes are found in many living organisms, ranging from bacteria to plants and animals, and show considerable chemical diversity among living organisms. As they accumulate in the cytoplasm and remain non-toxic, even at molar concentrations, in response to water deficit they are also often called osmoprotectors. The most common compatible osmolytes include amino acids (proline, glutamate, glutamine and alanine) and their derivatives (ectoine and hydroxyectoine), quaternary amines (glycinebetaine, polyamines and dimethyl sulfoniopropionate), sugars (trehalose) and polyols including sugar alcohols (mannitol, sorbitol, pinitol, glycerol and galactinol) (Khan et al. 2009; Jewell et al. 2010; Kumar and Khare 2015). These osmoprotectors have wide-spectrum functions including scavenging of ROS, balancing cell redox, acting as osmoprotectants or osmoticums, and the stabilization of cytosolic pH, proteins, enzymes and membranes, in addition to acting as a potential source of carbon and nitrogen for plants both during stress events and the subsequent recovery phases (Kumar and Khare 2015). Amongst these, one of the most efficient compatible solutes is glycinebetaine (abbreviated as GB; *N,N,N*-trimethylglycine), which helps to protect plants against the stress-induced oxidative damage (Wani et al. 2013). The numerous properties of GB include a antichaotropic function related to its zwitterionic nature (Papageorgiou et al. 1985), a low molecular weight, a high solubility and a low viscosity, all of which make GB one of the most efficient osmoregulators (Yancey 2005; Kurepin et al. 2015). This chapter aims to describe and discuss various aspects of GB-mediated oxidative stress tolerance in plants.

Biosynthesis of Glycinebetaine

GB is a quaternary ammonium compound that occurs naturally in most biological systems ranging from prokaryotes, eukaryotic microorganisms, most animals, plants and microorganisms like cyanobacteria, algae and fungi. GB is synthesized

via two pathways, using choline and glycine as respective substrates. In plants, the key enzyme for choline synthesis is phosphoethanolamine *N*-methyltransferase (PEAMT; EC 2.1.1.103), a cytosolic enzyme that catalyses all three of the methylation steps required to convert phospohoethanolamine to phosphocholine, the precursor to choline biosynthesis (McNeil et al. 2001). In plants choline is then transported into the chloroplast where it undergoes a two-step oxidation reaction: first choline is oxidized to betaine aldehyde, a toxic intermediate, which then is oxidized to GB. The first oxidation is catalysed by choline monooxygenase (CMO, EC 1.14.15.7), an unusual ferredoxin-dependent soluble protein with a motif characteristic of Rieske-type iron-sulphur proteins. In animals and bacteria this oxidation reaction is catalysed by choline dehydrogenase (CDH; encoded by the *betA* gene), but some bacteria may also use choline oxidase for the first step GB synthesis. The second oxidation step is catalysed by NAD^+-dependent betaine aldehyde dehydrogenase—BADH, EC 1.2.1.8, in most organisms (Rathinasbapathi et al. 1997), although in some bacteria CDH and choline oxidase can also catalyse the second step. In higher plants, GB can also be synthesized in the chloroplast from serine via ethanolamine and betaine aldehyde (Rhodes and Hanson 1993). Although both CMO and BADH are localized in the stroma of chloroplasts, they are encoded by nuclear genes and contain transit sequences targeting them to chloroplasts. An alternate biosynthetic pathway of betaine from glycine, catalysed by two *N*-methyltransferase enzymes, has been reported for cyanobacterium and *Arabidopsis*, and it was found that the co-expression of *N*-methyltransferase genes caused accumulation of betaine that confers stress tolerance. Interestingly, in some naturally GB-accumulating plants, including mangrove (Hibino et al. 2001) and barley (Fujiwara et al. 2008), no CMO activity was detected in chloroplasts.

Though, GB is found in many plant species, its distribution is sporadic amongst them. For instance, many higher plants, including *Arabidopsis* and tomato, have been reported not to accumulate GB. While GB is a small organic metabolite and is highly soluble in water, it also contains a non-polar moiety consisting of 3-methyl groups. Owing to its unique structural features, it has the ability to interact with the hydrophobic and hydrophilic domains of macromolecules including proteins (Gupta and Huang 2014).

Transportation and Translocation of Glycinebetaine in Plants

Though, little is known about the transport of GB in plant cells, it is likely that transporters of GB are located in the plasma and chloroplast membranes, but no GB-specific transporters have been reported to date (Chen and Murata 2011). The transport of GB from the cytosol to various subcellular compartments is also poorly understood. In spinach, GB levels in the chloroplasts of unstressed spinach plants were ~0.7 μmol mg^{-1} against 6.6 μmol mg^{-1} chlorophyll in stressed plants' chloroplasts indicating most GB accumulation in response to salt stress was found in the chloroplasts. This concentration gradient across the chloroplast envelope suggests the existence of a specific transport mechanism. Schwacke et al. (1999) demonstrated

that the product of the tomato gene *LeProT1*, a homologue of a proline transporter in *Arabidopsis*, transported GB with high affinity and both proline and γ-amino butyric acid (GABA) with low affinity, when expressed in yeast. Similar results were also reported for the GABA and proline transporter, ProT2, whose gene was cloned from *Arabidopsis* (Breitkreuz et al. 1999). Here, the ProT2-mediated transport of GABA/proline was strongly inhibited by GB, indicating that GB had strong affinity for the transporter. These results suggest that the transporters of both proline and GABA might also transport GB. However, Ueda et al. (2001) cloned the gene for a proline transporter (*HvProT*) from the roots of salt-stressed barley and demonstrated that the uptake of proline by the yeast cells expressing *HvProT* was not inhibited by GB, suggesting that *HvProT* does not act as a transporter of GB.

GB translocation was studied with (^{14}C)-labelled GB in barley (Ladyman et al. 1980), tomato, pea, soybean and turnip (Makela et al. 1996) and the results revealed the translocation of GB within 2 h from the roots to the leaves via the phloem and labelled GB was found throughout the plant within 24 h. Heat girdling of the leaf sheaths of barley plants prevented the export of [^{14}C]-GB from the leaf blades. It appears that GB, synthesized by mature leaves during exposure of plants to abiotic stress, behaves as an inert end product, which upon re-watering of plants is translocated to the expanding leaves. Makela et al. (1996) found similar results for tomato plants.

The GB biosynthesis pathway is present in many higher plant species, including alfalfa (*Medicago sativa* L.; Wood et al. 1991), algarrobo (*Prosopis alba* Griseb.; Meloni et al. 2004), barley (*Hordeum vulgare* L.; Ladyman et al. 1983; Kishitani et al. 1994; Hattori et al. 2009), bean (*Phaseolus vulgaris* L.; Gadallah 1999), cotton (*Gossypium hirsutum* L.; Desingh and Kanagaraj 2007), corn (*Zea mays* L.; Quan et al. 2004), pea (*Pisum sativum* L.; Takhtajan 1980), sorghum [*Sorghum bicolor* (L.) Moench; Mickelbart et al. 2003], spinach (*Spinacia oleracea* L.; McCue and Hanson 1990), strawberry (*Fragaria* × *ananassa* Duchesne; Rajashekar et al. 1999), townsend's cordgrass (*Spartina* × *townsendii* H. Groves and J. Groves; Storey et al. 1977) and wheat (*Triticum aestivum* L.; McDonnell and Jones 1988; Wang et al. 2010). However, some plant species exhibit undetectable levels of GB when exposed to abiotic stress, e.g. *Arabidopsis thaliana* (Hibino et al. 2002), eggplant (*Solanum melongena* L.; de Zwart et al. 2003), potato (*Solanum tuberosum* L.; de Zwart et al. 2003), tobacco (*Nicotiana tabacum* L.; Nuccio et al. 1998), tomato (*Solanum lycopersicum* L.; Park et al. 2004) and rice (*Oryza sativa* L.; Sakamoto and Murata 1998) are reported to have no detectable accumulation of GB in response to abiotic stress. Furthermore, in sugar beet (*B. vulgaris*), betaine applied exogenously to old leaves was translocated preferentially to young leaves and roots (Yamada et al. 2009). When GB was applied to individual mature leaves of tomato plants, a large fraction of the incorporated GB were translocated to meristem-containing tissues, which included flower buds and shoot apices (Park et al. 2006). Variations in the levels of GB in different plant organs indicate active and, possibly, regulated translocation from the original site of application accumulation/storage. Translocation of GB with photosynthetic assimilates to actively growing and expanding parts of plants has also been reported, indicating that the

long-distance transport of GB is phloem related (Makela et al. 1996). In GB-accumulating transgenic *Arabidopsis* (Sulpice et al. 2003) and tomato plants (Park et al. 2004, 2007), the highest levels of GB are found in actively growing tissues, such as flowers and shoot apices, indicating that GB is efficiently translocated from source to sink tissues via the phloem.

Cellular Glycinebetaine Accumulation and Abiotic Stress Tolerance

As detailed earlier, although GB biosynthesis takes place in many plant species, it does not appear to be ubiquitous in all species. Plant species such as eggplant, potato, *Arabidopsis*, tomato and many cultivars of rice are reported not to accumulate the detectable amounts of GB (Kurepin et al. 2015). Therefore, genes associated with GB biosynthesis, of plant or bacterial origin, have been introduced/overexpressed in these non-accumulators of GB (Chen and Murata 2011). Such studies have resulted in a better understanding of the roles of GB in plants stress responses. Levels of accumulated GB are generally directly correlated with the plants' tolerance to abiotic stresses (Giri 2011; Chen and Murata 2011; Wani et al. 2013). GB has been reported to accumulate intra-cellularly to high concentrations as a result of biosynthesis, uptake, or both, abiotic stresses in a variety of plants (Bhuiyan et al. 2007; Hassine et al. 2008; Hattori et al. 2009; Wang et al. 2010). However, Sarwas et al. (2006) reported that endogenous GB levels varied greatly between various cotton (*Gossypium hirsutum* L.) genotypes, as did the tolerance to drought stress, and the authors observed that genotypes with increased drought tolerance had higher endogenous GB levels.

Many halotolerant plants accumulate GB in their chloroplasts and plastids to increase tolerance against range of abiotic stresses (Chen and Murata 2008). GB-mediated enhancement of tolerance to abiotic stresses may be attributed to the ability of GB to protect the functioning of the photosynthetic apparatus, by protecting the enzymes and lipids required to maintain optimal linear electron flow through the electron transports chains embedded in the thylakoid membranes and to maintain CO_2 assimilation (Sakamoto and Murata 2002; Chen and Murata 2011). Besides the earlier protective roles, GB also helps to limit stress-induced inactivation of the PSII complex, which is considered the most vulnerable component of the photosynthetic apparatus and plays a pivotal role in the photosynthetic responses of plants to abiotic stress (Murata et al. 1992; Allakhverdiev et al. 2003, 2007; Adams et al. 2013).

Due to its properties as a compatible osmolyte, GB protects cells against osmotic inactivation via increasing water retention (Sakamoto and Murata 2002; Ashraf and Foolad 2007; Kurepin et al. 2015). Since compatible osmolytes like GB remain uncharged at neutral pHs and are highly soluble in water, they are excluded from the hydration sphere of proteins and help to stabilize the proteins (Low 1985). GB stabilizes the quaternary structures of enzymes and other complex proteins, as well as maintaining the ordered state of membranes, at non-physiological temperatures and

high salt concentrations (Papageorgiou and Murata 1995). The effects of GB in mitigating the detrimental effects of oxidative bursts induced by various abiotic stressors are well established. GB acts as an activator or stabilizer of some ROS-scavenging enzymes or as a repressor of ROS production, by the mechanisms largely unknown. GB is known for its strong protective roles in the reproductive organs of plants growing under abiotic stress conditions, which is considered critical for maintaining high crop yields (Chen and Murata 2008).

ROS Production Under Abiotic Stress

As described earlier, the electrons that have a high-energy state are transferred to molecular oxygen (O_2) to form ROS (Mittler 2002), which comprise of singlet oxygen ($^1O_2$), super oxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). In plants, ROS are primarily formed at low levels as by-products of several aerobic metabolic processes like photosynthesis and respiration in the organelles such as chloroplasts, mitochondria, peroxisomes, plasma membranes, endoplasmic reticulum, cell walls and the apoplastic space (Rhoads et al. 2006; Møller et al. 2007; Ahmad et al. 2010a, b; Sharma et al. 2012). During abiotic and biotic stress conditions, the generation rates of ROS are highly elevated (Ahmad et al. 2009, 2010a, b; Sharma et al. 2012; Mostofa et al. 2015a, b) leading to the onset of oxidative stress (Mittler 2002; Mittler et al. 2011; Kotchoni et al. 2006; Hossain et al. 2015).

The light-dependent electron transport chains (ETCs) in photosystems I (PS I) and II (PS II) are considered to be the main source of ROS in plant cells (Doyle et al. 2010; Khanna-Chopra 2011; O'Brien et al. 2012). ROS production by these sources is enhanced by conditions limiting CO_2 fixation, such as drought, salt, extreme temperatures and high light (Sharma et al. 2012). In response to salinity and drought stress, plants decrease their stomatal conductance, to reduce excess water loss, which ultimately leads to a decrease in internal CO_2 concentrations and slows down the reduction of CO_2 by the Calvin cycle and induces photorespiration (Abogadallah 2010; Sanda et al. 2011). High temperatures suppress the carboxylation reaction catalysed by ribulose 1,5-bisphosphate carboxylase (RuBisCO) by reducing the specificity of the enzyme for CO_2 (Kim and Portis 2004; Kaushal et al. 2011), whereas low temperatures slow the activities of the Calvin cycle enzymes, causing $NADP^+$ depletion (Wise 1995). With respect to cadmium (Cd) stress metal ions, such as Ca^{2+} and Mn^{2+}, present in the PS II centre can be replaced by Cd ions, thereby limiting photosystem reactions and leading to uncoupling of electron transport in the chloroplast (Mohanty and Mohanty 1988; Atal et al. 1991).

Mitochondria can also produce ROS in plants. Under normal aerobic conditions, electron transport and ATP syntheses are tightly coupled, but stress can lead to changes in the mitochondrial electron transport chains (ETC) that can lead to overreduction of electron transporters and the excess production of ROS (Noctor et al. 2007; Blokhina and Fagerstedt 2006). Increased ROS production as a result of ETC perturbations has been reported in plants exposed to chilling (Prasad et al. 1994a, b; Purvis et al. 1995), salinity (Hernández et al. 1993; Mittova et al. 2003), high temperatures (Schwarzlander

et al. 2009), exposure to Cd (Schwarzlander et al. 2009) and phosphate deficiency (Juszczuk et al. 2001; Malusa et al. 2002). Metal ions such as Fe, Cu and Zn are essential for the proper functioning of the mitochondrial enzymes involved in the TCA cycle, ATP synthesis, electron transport and antioxidant defences (Tan et al. 2010; Nouet et al. 2011). ROS production in the endoplasmic reticulum (ER) could facilitate the transmission of toxic Cd^{2+} ions at the ER–mitochondria interface. Again, H_2O_2 could diffuse out of the ER and attack the membranes of neighbouring mitochondria, bypassing the protection conferred by mitochondrial SOD that is located in the mitochondrial matrix (Karuppanapandian et al. 2011).

Peroxisomes compartmentalize the enzymes involved in the β-oxidation of fatty acids, the C_2 photorespiratory cycle and they are major sites of intracellular H_2O_2 production due to their essentially oxidative metabolism (del Río et al. 2006). Peroxisomes produce $O_2^{\bullet-}$ as a consequence of normal metabolism (Corpas et al. 2001), with three integral peroxisomal membrane polypeptides (PMPs), with molecular masses of 18, 29 and 32 kDa embedded in the membrane, having been shown to form $O_2^{\bullet-}$ (del Río et al. 2002).

In addition to the earlier sites of ROS production, plant cells have several other potential sites of ROS production. Electron transporting oxidoreductases are ubiquitous in plasma membranes and can generate ROS. Plasma membrane-bound NADPH oxidases have been proposed to play key roles in the production and accumulation of ROS in plants and are involved in responses to various abiotic stressors (Orozco-Cardenas et al. 2001; Kwak et al. 2003), including nutrient deficiency or excess of Cd, copper (Cu) and nickel (Ni) (Quartacci et al. 2001; Hao et al. 2006). Cell walls are also regarded as active sites for ROS production as cell wall-associated peroxidases and oxidases are involved in H_2O_2 generation. ROS generation by cell-wall-located peroxidases has been shown to occur during the hypersensitive response (HR), triggered in cotton by the bacterium *Xanthomonas campestris* pv. malvacearum (Martinez et al. 1998), and potassium (K) deficiency in Arabidopsis (Kim et al. 2010; Higuchi 2006). Production of $O_2^{\bullet-}$ and H_2O_2, was noted in the cell walls of maize roots (Liszkay et al. 2004) and $OH^{\bullet}$ generation was demonstrated in vivo and in vitro in the cell walls of several other plant species (Schopfer 2001; Spiteller 2003). The apoplast is also an important site for ROS accumulation in response to abiotic stressors, such as drought, salinity, high and low temperatures, ozone and high light (Hernández et al. 2001; Zhu 2001; Miller et al. 2009; Vahisalu et al. 2010) and the cell-wall-located enzymes have been shown to be responsible for apoplastic ROS production (Apel and Hirt 2004; Heyno et al. 2011). H_2O_2 accumulation in the apoplast is involved in the acclimation responses of plants, such as modulation of growth rate and cell wall strengthening, to drought and salt stress (Hernández et al. 2001; Zhu 2001; Rodríguez et al. 2004).

ROS Scavenging and Detoxification by Antioxidants

Plants possess complex antioxidant defence systems to protect cellular components from oxidative damage. These systems include enzymatic and non-enzymatic components to scavenge and detoxify ROS. The enzymatic antioxidants include multiple

superoxide dismutases (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), glutathione reductase (GR; EC 1.6.4.2), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.9), glutathione *S*-transferase (GST; EC 2.5.1.18), peroxidase (POX, EC 1.11.1.7), guaiacol peroxidase (GPOX; EC 1.11.1.7), and the non-enzymatic antioxidants include ascorbate (AsA), glutathione (GSH), tocopherol, carotenoids, flavonoids, proline and GB (Ahmad et al. 2010a, b; Gill and Tuteja 2010; Hossain et al. 2011a, b; Sharma et al. 2012; Mostofa et al. 2015a, b). Increased levels of ROS scavenger enzymes, in response to various abiotic stress, are found in different cellular locations, e.g. chloroplasts, mitochondria, the plasma membrane and cell wall (Das et al. 2015; Hossain et al. 2015). ROS scavenging and detoxifying in the different cellular compartments represents a coordinated response (Pang and Wang 2008). The key ROS detoxification systems found in plants are shown in Fig. 5.1. Various components of the antioxidant defence systems found in plants have been

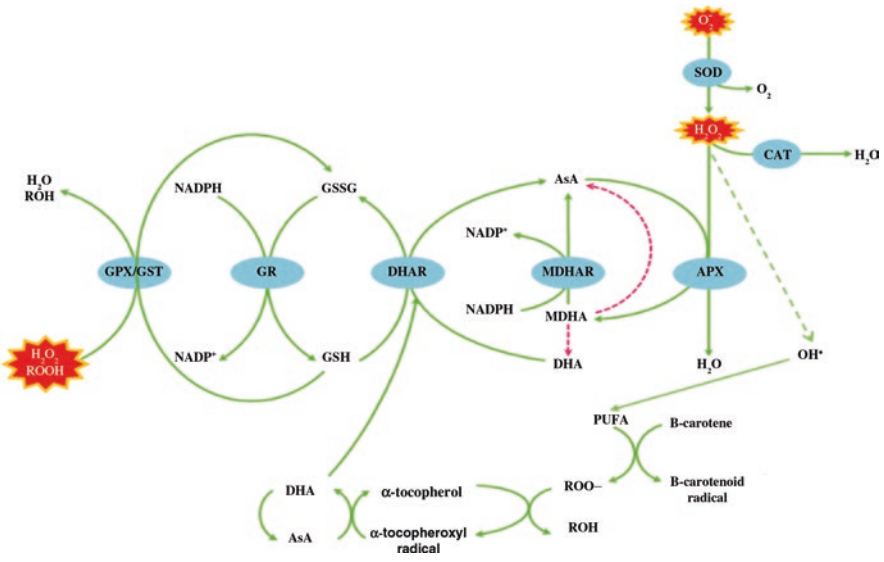

Fig. 5.1 Reactive oxygen species detoxification systems in plants (modified from Hossain et al. 2014). SOD, CAT, APX, GPX and GST are the proteins responsible for eliminating ROS. Enzymes that promote the elimination of ROS via the ascorbate-glutathione cycle are APX, MDHAR, DHAR and GR. The elimination of ROS by non-enzymatic processes is carried out by carotenoid (vitamin A), α-tocopherol (vitamin E), AsA (vitamin C) and glutathione. Superoxide produced in different cell organelles is rapidly converted to H_2O_2 by SOD, which, in turn, is converted to H_2O by APX and CAT. The oxidation of AsA caused by ROS or by APX leads to the formation of monodehydroascorbate (MDHA) and dehydroascorbate (DHA). MDHA is reduced to AsA by MDHAR with the utilization of NADPH and DHA is converted to AsA by DHAR with the utilization of GSH. GR is responsible for recycling of GSSG to GSH by the expense of NADPH. GST and GPX catalyse the GSH-dependent reduction of H_2O_2 and organic peroxides, including lipid peroxides to H_2O or alcohols. During lipid peroxidation, carotenoid, α-tocopherol and AsA help regenerate GSSG back into GSH through vitA, vit E and vit C cycle. Abbreviations are defined in the text

manipulated, overexpressed or down-regulated to aid in our understanding of the roles antioxidant systems play in the responses of plants to stress (Das et al. 2015).

The Molecular Mechanisms of ROS Quenching by Glycinebetaine in Plants Under Stress

Though, GB does not appear to scavenge the ROS directly, various studies have attributed the ability of GB to protect photosystems to ROS scavenging (Murata et al. 2007; Chen and Murata 2011; Giri 2011). Excessive light causes inactivation of PSII, a phenomenon known as photoinhibition, which is an unavoidable process in photosynthetic organisms, due to light being the driving force of photosynthesis (Chen and Murata 2011). Under normal conditions plants can efficiently repair of PSII (Aro et al. 1993), but excessive generation of ROS in stressed plants can interfere with PSII repair, mainly via suppression of de novo protein synthesis (Takahashi and Murata 2006).

Various transgenic studies support the role of GB in ROS scavenging and detoxification. For example, Kathuria et al. (2009) concluded that GB plays a significant role in reducing the accumulation of and in the detoxification of ROS in transgenic plants overexpressing genes encoding enzymes involved in GB biosynthesis, compared to their wild-type plants. In addition, GB has been reported to lower the rate of membrane lipid peroxidation, a consequence of oxidative stress, via inducing the expression of fatty acid desaturase and lipoxygenase genes, and therefore helping to maintain membrane integrity in tomato plants subjected to low temperature stress (Karabudak et al. 2014). GB has also been reported to help maintain ROS homeostasis in wheat plants under salinity stress by up-regulating the transcription of alternative oxidase (AOX), H^+/Na^+ antiporter exchanger (NHX1) and salt overly sensitive 1 (SOS1) genes (Badran et al. 2015). Cruz et al. (2013) critically examined the effectiveness of application of GB to *Carapa guianensis* plants growing under water deficit, and they observed that GB caused a significant increase in APX activity and attenuated lipid peroxidation in stressed plants.

Enhanced Abiotic Oxidative Stress Tolerance via the Exogenous Application of Glycinebetaine

All forms of abiotic stress, such as salinity, drought, chilling, freezing, heat and heavy metals, can cause an excessive accumulation of ROS leading to irreparable dysfunction and death in plants. In this section, we will discuss the involvement of exogenous GB in modulating ROS and MG detoxification systems as a means of inducing oxidative stress tolerance.

A series of recent experiments have shown that exogenous application of GB to plants increases abiotic oxidative stress tolerance (Hossain et al. 2010, 2011a, b,

2014; Hu et al. 2012; Anjum et al. 2012; ffSorwong and Sakhonwasee 2015). Park et al. (2006) reported that exogenous application of GB induces chilling tolerance in a GB non-accumulating variety of tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker). After 2 days of chilling treatment GB-treated plants had lower H_2O_2 levels and higher CAT activities than control plants. In was concluded that GB-induced chilling tolerance involves the induction of H_2O_2 detoxifying antioxidant defence systems. Even pre-treatment of seeds with GB can enhance chilling tolerance in hybrid maize (*Zea mays* L.), through the maintenance of higher water contents; reduced electrolyte leakage (EL) and higher SOD, CAT and APX activities (Farooq et al. 2008a).

The role of GB in modulating salinity-induced oxidative stress tolerance has been well documented in plants. Hoque et al. (2007) showed that exogenous GB enhances salinity-induced oxidative stress tolerance in cultured tobacco (BY-2) cells, by modulating the activities of AsA-GSH cycle enzymes. In addition, it has also been shown that exogenous GB application enhances salt tolerance by reducing protein oxidation and by modulating GST, GPX and glyoxalase system enzymes activities (Hoque et al. 2008). Nawaz and Ashraf (2010) studied the role of exogenous GB application as a modulator of salt tolerance in two maize (*Zea mays* L.) genotypes. Salt stress led to a decrease in photosynthetic activity, chlorophyll content and SOD activity in both of maize cultivars. Exogenous application of GB significantly enhanced the photosynthetic capacity and the activities of SOD, CAT and POD in treated plants compared to controls. These results suggested that GB-induced enhancement in antioxidant enzyme activities might help to protect chloroplasts from salt-induced oxidative damage. In a study of mung bean (*Vigna radiata*) seedlings under salinity stress, salt led to a robust increase in ROS and MDA levels. Exogenous application of GB significantly enhanced the activities of ROS and MG detoxification systems and reduced salt-induced oxidative damage, with lower ROS and MDA levels compared to the seedlings not treated with GB (Hossain et al. 2011a, b). Hu et al. (2012) showed that exogenous application of GB increased salinity tolerance in perennial ryegrass (*Lolium perenne*). Plants subjected to salt stress showed higher EL, MDA and proline contents than control plants, but exogenous application of GB reduced the EL, MDA and proline contents under salt stress. Salt stress significantly reduced the activities of the antioxidant enzymes SOD, CAT and APX. Importantly, addition of GB increased activities of ROS detoxifying enzymes. In addition, GB treatment reduced the Na^+ accumulation whereas increased the K^+ content of shoots, which led to a higher K^+/Na^+ ratio under saline conditions. These results indicated that GB-induced salt tolerance is at least in part due to higher SOD, CAT and APX activities, and improved ion homeostasis, resulting in less ROS induced damage. Recently, Hasanuzzaman et al. (2014) showed that exogenous application of GB (5 mM) to rice seedlings enhanced salinity-induced oxidative stress tolerance through the up-regulation of the ROS and MG detoxification pathways. Yildirim et al. (2015) showed that exogenous application of GB to lettuce plants could ameliorate the harmful effects of salt stress by reducing lipid peroxidation, H_2O_2 levels and membrane permeability. Importantly, exogenous application of GB also found to increase the gib-

berellic acid (GA) and salicylic acid (SA) and indole acetic acid (IAA) content under salt stress condition.

The protective roles of GB have also reported in plants subjected to drought stress. Farooq et al. (2008b) showed that exogenous application of GB enhanced drought tolerance in fine grain aromatic rice (*Oryza sativa* L.). Drought stress greatly reduced rice growth while GB application improved plant growth both under well watered and drought conditions. Importantly, foliar application of GB under drought stress significantly altered the level of ROS and MDA and increased the activities of SOD, APX and CAT. Farooq et al. (2008b) concluded that GB-induced increased antioxidant production reduced the oxidative damage in plants under drought stress. Anjum et al. (2012) showed that exogenous application of GB modulates drought stress tolerance in two maize cultivars contrasting of their drought stress tolerance. Prolonged drought stress increased lipid peroxidation whereas GB treatment significantly reduced oxidative damage, as indicated by lower MDA levels. The activities of POD, SOD and CAT increased initially but subsequently declined with continued drought stress. Importantly, GB-treated plants maintained higher levels of ROS detoxifying enzymes that would have contributed to greater stress tolerance and improved growth and yields. Recently, Molla et al. (2014) showed that exogenous application of GB reduces drought-induced oxidative stress in lentil (*Lens culinaris*) seedlings. Drought stress led to a significant increase in oxidative stress, as indicated by higher H_2O_2 and increased glutathione disulphide levels (GSSG). Exogenous application of GB (15 mM) to drought stressed plants resulted in a significant increase in the GSH content and the activities of the enzymes GST and Gly I, with a simultaneous reduction in GSSG and H_2O_2 levels. Molla et al. (2014) also suggested that exogenous GB enhances drought stress tolerance by limiting H_2O_2 accumulation and by increasing the activities of the antioxidant and glyoxalase systems. Additionally, Hossain et al. (2014) showed that exogenous application of GB to mustard (*Brassica juncea*) modulated drought-induced oxidative stress tolerance as indicated by higher ROS and glyoxalase pathway enzymes along with the lower level of H_2O_2 and lipid peroxidation (Hossain et al. 2014).

The roles of exogenous GB in Cd stress tolerance were studied by Islam et al. (2009a) in cultured tobacco BY-2 cells under Cd stress (100 μM Cd). Cd stress caused growth inhibition and oxidative damage, as indicated by higher MDA levels. Addition of GB caused an increase in endogenous GB, CAT activity, decreased MDA levels and lower Cd accumulation. In a second study, Islam et al. (2009b) showed that exogenous application of GB application also increased the activities of APX, DHAR, MDHAR and GR, which helped to protect key cellular components from Cd-induced oxidative damage (Islam et al. 2009b). Consequently, we (Hossain et al. 2010) showed that exogenous application of GB increased Cd tolerance in mung bean (*Vigna radiata* L.) seedlings. Imposition of short-term (24 h) Cd stress (1 mM) led to a significant increase in H_2O_2 and MDA levels in mung bean leaf tissues in comparison to control plants. Inactivation or insufficient up-regulation of MG and ROS detoxifying enzymes such as APX, MDHAR, DHAR, GPX, GST, CAT, Gly I and Gly II and AsA and GSH contents was found in seedlings subjected to Cd stress. Surprisingly, 5 mM GB application favourably modulated the ROS and MG detoxifying enzyme activities and the glutathione redox state, making the plants more tolerant to Cd stress-induced oxidative damage.

Duman et al. (2011) studied the effects of exogenous GB application on the responses of duckweed (*Lemna gibba* L.) to Cd exposure. Duckweed samples were subjected to various concentrations of Cd for 6 days in the absence or presence of GB. Treatment with GB had no significant influence on Cd accumulation, but GB had a significant influence on endogenous proline accumulation, ROS detoxifying enzyme activities, the level of lipid peroxidation and photosynthetic activity. They concluded that GB has a defensive role in plants exposed to Cd, reducing both ROS and MDA levels. Cao et al. (2013) showed that exogenous application of GB induces oxidative stress tolerance in rice seedlings exposed to Cd. Rice seedlings, pre-treated with 100 μM GB and then exposed to Cd for 5 days, had greater root lengths, fresh and dry weights, higher chlorophyll contents and less ROS-induced damage, as shown by lower MDA levels, and higher SOD activities, in stem tissues, compared to control plants not pre-treated with GB. Recently, Ali et al. (2015) showed the exogenous application of GB enhanced chromium tolerance in wheat (*Triticum aestivum* L.). Cr stress significantly inhibited growth, chlorophyll and protein contents, and increased antioxidant enzyme activities. Foliar application of GB (0.1 mM) under Cr stress reduces Cr accumulation in grains and modulated the activities of APX and CAT in root and shoot tissues. Increased antioxidant enzyme activities with GB application under Cr stress might be one of the possible mechanisms of GB-induced metal tolerance in plants. Additionally, Lou et al. (2015) showed that GB application induces Cd stress on perennial ryegrass (*Lolium perenne*). Cd stress resulted in a decrease in turf quality, shoot growth, transpiration rates and Chl contents, with significant increases in EL, MDA content, SOD, CAT, POD activities, and oxalic and tartaric acid levels. Exogenous applications of GB (20 mM) reversed the adverse impacts of Cd stress. Their findings suggested that GB could alleviate the detrimental effects of Cd on perennial ryegrass and that amelioration was mainly related to elevation of SOD, CAT and POD activities and higher stress responsive gene expression.

Sorwong and Sakhonwasee (2015) showed that GB can enhance heat stress tolerance in marigold cultivars. Heat stress caused photoinhibition and lower CO_2 assimilation, stomatal conductances and transpiration rates in heat-treated marigold plants compared to control plants grown at a constant 25 °C. Significant increase in H_2O_2, lipid peroxidation and cell death in all cultivars were observed under heat stress. Foliar application of GB significantly reduced the levels of H_2O_2, superoxide and MDA. Sorwong and Sakhonwasee (2015) conduced that the mechanisms of GB-induced heat stress tolerance involved protection of the photosynthetic machinery, increased gas exchange and ROS detoxification.

From the above reports it has become clear that GB plays a pivotal role in keeping ROS levels, induced by various abiotic stressors, under control by regulating the activities of enzymes involved in ROS scavenging and detoxification, and also by regulating the glyoxalase system. However, more in-depth studies might also reveal subtler regulatory roles for GB in modulating abiotic stress tolerance.

Glycinebetaine-Accumulating Transgenic Plants and Abiotic Oxidative Stress Tolerance

The cloning of various genes (*codA and BADH*) encoding enzymes that catalyse the biosynthesis of GB has been reported, and many lines of transgenic plants have been produced expressing GB biosynthetic genes with enhanced abiotic and abiotic oxidative stress tolerance in plants (Yang et al. 2007; Ahmad et al. 2010a, b; Zhang et al. 2011; Fan et al. 2012; Li et al. 2014a, b; Di et al. 2015).

Yang et al. (2007) reported that transgenic tobacco plants overexpressing a *BADH* gene showed enhanced heat stress tolerance. The activities of antioxidant enzymes (APX, MDHAR, DHAR, GR and CAT) all decreased in response to heat stress in wild-type (WT) plants, but in the transgenic plants the activity of many of these enzymes increased significantly or remained unchanged, and the levels of AsA and GSH were higher in the transgenic plants. These findings suggest that over-accumulation of GB in transgenic plants could lower ROS levels, which contributes to heat stress tolerance. Ahmad et al. (2010a, b) found that overexpression of multiple genes (*codA, SOD* and *APX*) in potato plants enhanced stress tolerance, as compared to plants overexpressing only a *SOD* or *APX* gene. Transgenic plants expressing multiple genes showed higher methyl viologen (MV)-induced oxidative stress tolerance, as compared to the single gene transgenic plants. Additionally, plants overexpressing three genes showed higher SOD, APX and CAT activities as compared to wild type or APX or SOD-expressing plants, under salt or drought stress. The synergistic effects of GB, SOD and APX appeared to help the transgenic plants grow and develop better under conditions of abiotic stress.

Zhang et al. (2011) showed that transgenic cotton (*Gossypium hirsutum* L.) plants overexpressing a *betA* gene (a gene for GB synthesis) showed enhanced salinity tolerance, as indicated by higher rates of photosynthesis, better osmotic adjustment, higher relative water contents, and lower levels of lipid peroxidation and ion leakage. Fan et al. (2012) showed that transgenic sweet potato (*Ipomoea batatas*) plants overexpressing a GB biosynthetic gene (*BADH*) had enhanced abiotic stress tolerance. Transgenic plants maintained higher photosynthetic activities, and lower H_2O_2 and MDA levels under salt, chilling and MV-induced oxidative stress. Transgenic plants also showed higher *SOD* gene expression and enzyme activities. Higher expression levels of *CAT, APX, MDHAR, DHAR, GR, GPX* and *POD* genes were also observed in plants under salt, drought and MV stress. Fan et al. (2012) concluded that better abiotic stress tolerance in transgenic plants was in part due to improved ROS scavenging. Similarly Li et al. (2014a) showed that transgenic tomato (*Lycopersicon esculentum* cv. 'Moneymaker') plants overexpressing a *BADH* gene exhibited higher heat stress (42 °C) tolerance as compared to WT plants. Transgenic plants showed higher photosynthetic activities, and lower levels of H_2O_2 and superoxide and lipid peroxidation as compared to WT plants. In addition, transgenic plants showed higher antioxidant enzyme activities under stressful conditions. In a second study, Li et al.

(2014b) showed that transgenic alfalfa (*Medicago sativa* L. cv. Xinjiang Daye) plants overexpressing a *codA* gene showed enhanced tolerance to abiotic stress. Transgenic plants showed better tolerance to MV-induced oxidative stress and better salinity-induced oxidative stress tolerance, as indicated by higher Chl contents and lower MDA levels as compared to the WT plants. From the above reports it is evident that transgenic plants overexpressing GB biosynthetic genes have enhanced abiotic stress tolerance and oxidative stress tolerance.

Conclusion and Future Perspectives

Extensive work in recent past has confirmed that GB is an important compatible osmolyte with multiple functions in plant growth and survival, both under normal as well as stressful conditions. Plants accumulate GB in their tissues in response to and to counteract the deleterious effects of abiotic stresses and usually higher GB levels are not only correlated with, but also attributable to better plant stress tolerance. Besides protecting vital enzymes and membranes, GB can also mitigate ROS-mediated oxidative damage to plant cells and help to maintain the cellular redox balance, as well as controlling potential oxidative bursts.

Due to its wide spectrum of functions, GB biosynthetic pathway genes have been used to generate transgenic plants that accumulate GB and exhibit enhanced tolerance to various abiotic stresses, including secondary oxidative bursts. Recent scientific advancements have supported the role of GB in the prevention of excess ROS generation and oxidative stress in plants cells, when coupled with increased levels of ROS-scavenging enzymes. However, further research focused more on identifying genes associates with GB biosynthesis and explore the advantages of chloroplast engineering over its genome counterpart is required. Recent findings also suggested GB-mediated up-regulation of gene cascades, some of them with ROS scavenging roles, demonstrates a possible interaction between oxidative stress, gene expression and the accumulation of GB under condition of abiotic stress. The possibility of gene stacking and co-expressing various genes, with known antioxidant potential, with genes associated with GB biosynthesis needs to be explored to produce plants with enhanced oxidative stress tolerance. Further work is also needed to establish whether the transcript changes are direct targets of GB or is just produced by transgenic plants via metabolic adjustment.Acknowledgements The financial support from the Science and Engineering Research Board, Government of India [grant number SR/FT/LS-93/2011] to V.K. as a Young Scientist Project is gratefully acknowledged.

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Chapter 6
Role of Plant Calreticulins in Calcium Signaling

Maryam Sarwat and Narendra Tuteja

Abstract Plant calreticulins (CRTs) are ubiquitously expressed and are similar to animal CRTs in structural organization and basic functioning. Its expression is affected by developmental and environmental stimuli showing its role in the physiology of plants. Though it is an important member of the Ca^{2+} homeostasis of the endoplasmic reticulum, the underlying mechanism of growth modulation by CRT is still unclear and is an area of recent research.

Introduction

CRT is a ubiquitous protein, present from algae to higher plants (Michalak et al. 1999). It is highly conserved from humans to plants (Chen et al. 1994; Michalak et al. 1999; Smith 1992a, b). It is constitutively expressed in both meristematic and mature cells and is the most abundant protein of the ER. The animal CRTs are very well studied. It plays complex role in the cellular physiology (Bem 2011; Liu and Li 2013a). In case of plant CRTs, the research is still in infancy. The domain organization of plant CRTs is highly conserved with mammalian CRTs. By visualizing its high level of sequence similarity with animal CRTs, we can assume similar functions for them, but we cannot predict any plant-specific functions for them.

In animals, the CRTs are of two types: CRT1 and CRT2, where CRT1 is the main CRT isoform, and CRT2 may function in specialized tissue and cell types (Persson et al. 2002; Thelin et al. 2011). In plants, there are at least two distinct groups of calreticulin isoforms found in higher plants (Jia et al. 2009). Arabidopsis contains three CRT proteins, where AtCRT1a and CRT1b are members of one subgroup, the other CRT, AtCRT3, is a member of a different subgroup (Christensen et al. 2010).

But as CRTs are important calcium-binding proteins, their role in calcium signaling is inevitable (Sarwat and Tuteja 2007; Sarwat et al. 2013). As, calcium signaling

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is involved in various physiological events, CRTs role in these events can be predicted. Some of the Ca^{2+}-dependent processes where CRT's role has been very well studied are apoptosis and chaperonic activity (Gelebart et al. 2005; Jia et al. 2009; Williams 2006; Waterhouse and Pinkoski 2007).

Structural Organization of Calreticulins

The CRTs as their transmembrane homologs, calnexin (CNX) possesses three distinct domains: the N-, P-, and C-domains (Figs. 6.1 and 6.2) (Michalak et al. 1999). The N- and C-domains are present on the N-terminus and C-terminus, respectively (Michalak et al. 2002). The N-domain of calreticulin possesses both the polypeptide- and carbohydrate-binding sites (Leach et al. 2002; Kapoor et al. 2004). This domain is responsible for the formation of a core which is resistant to proteolysis in the presence of Ca^{2+} (Corbett et al. 2000). N-domain has a signal sequence which helps in targeting the proteins to the ER, and this domain along with the P-domain is responsible for protein folding (Michalak et al. 1999). As soon as the oligosaccharide binds to the N domain, it changes its conformation to facilitate polypeptide binding (Saito et al. 1999). Studies have shown that both the N- and P-domains are needed for the full chaperone function of calreticulin (Leach et al. 2002). Tyr109 and Asp135 are responsible for abolishing interaction of the protein with oligosaccharides (Kapoor et al. 2004). Other residues which might be involved in sugar binding to the protein are Lys111, Tyr128, and Asp317 (Thomson and Williams 2005; Gopalakrishnapai et al. 2006). Two disulfide bonds (Cys88-Cys120 and Trp244-Trp302), in the globular domain, play an important role in the chaperone function of calreticulin (Martin et al. 2006). The middle portion of calreticulin, the P-domain is proline-rich and thus provides flexibility (Figs. 6.1 and 6.2). P-domain possesses two highly conserved proline-rich segments (repeat A, IXDPXA/DXKPEDWDX, and repeat B, GXWXPPXIXNPXYX) which are repeated three times each (Figs. 6.1 and 6.2) (Persson et al. 2003; Michalak et al. 1999). These repeats may be involved in the lectin function of calreticulin (Vassilakos et al. 1998). The P domain helps in the

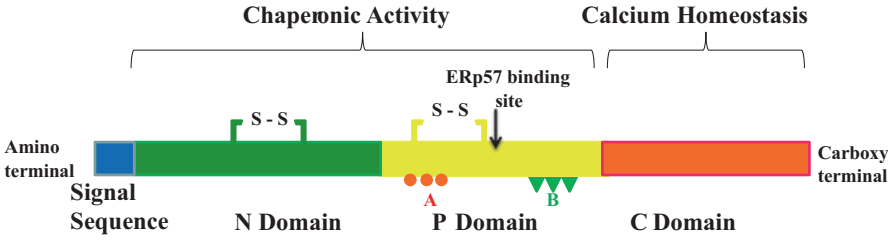

Fig. 6.1 Linear representation of calreticulin domains. The protein contains an N-terminal amino acid signal sequence (*blue*), N-domain (*green*), P-domain (*yellow*), C-domain (*orange*) and a C-terminal ER retrieval signal. Repeats A (amino acid sequence PXXIXDPDAXKPEDWDE) and B (amino acid sequence GXWXPPXIXNPXYX) are indicated by *red circles* and *green triangles*, respectively. The amino acids involved in S–S bonds are shown

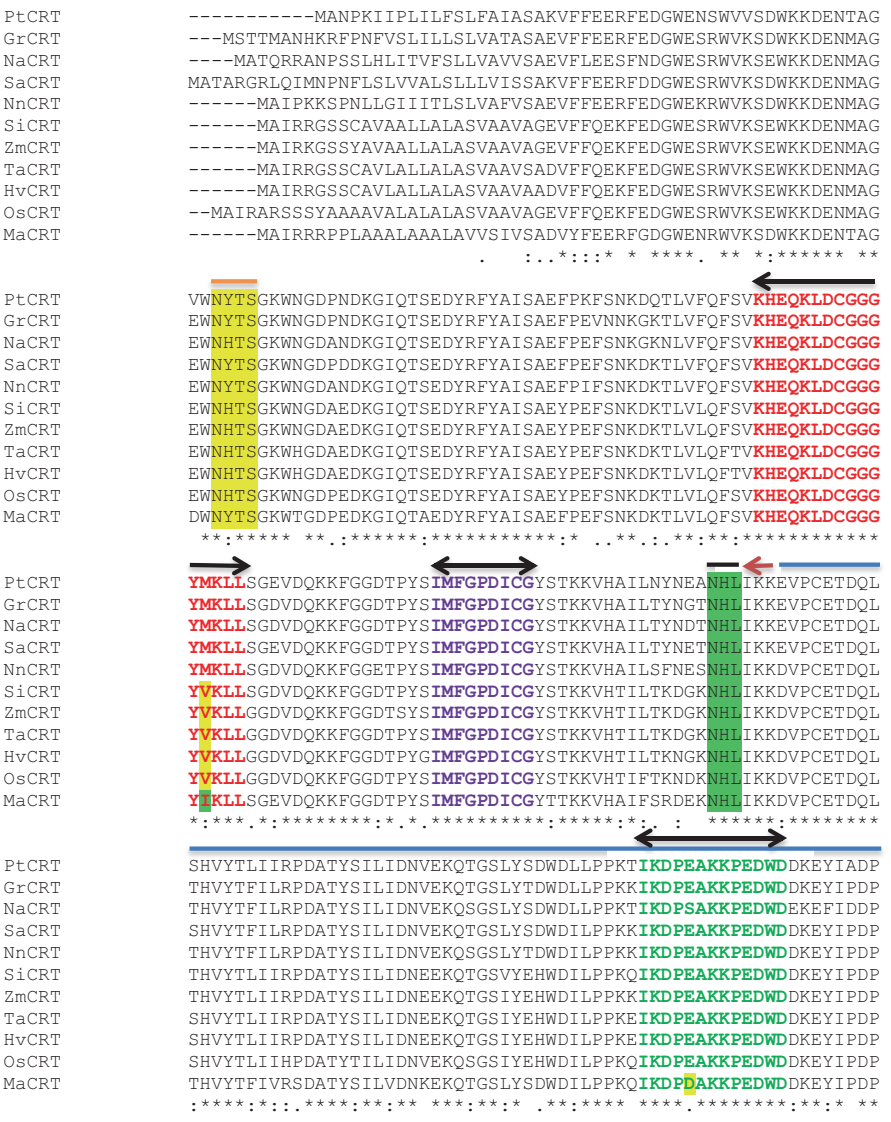

Fig. 6.2 Comparison of CRT protein sequences from 11 different plant species as described in Table 6.1. Identical and similar amino acids are marked with an *asterisk*. The approximate positions of the histidine-rich N-terminus (N domain, marked with *brown arrow*), the proline-rich internal region (P domain, with *solid blue line*), and the negatively charged C-terminus (C domain, marked with *brown arrow*). CRT-family signature motifs 1 (KhEQkldCGGGYXKLL) are in *red* and CRT-family signature motifs 2 (IMFGPDiCG) are in *purple* and are indicated by *black two-sided arrows*. Two Calreticulin family repeated motif signatures (IkDpXakKPEDWD) are shown in *green*, indicated by *black two-sided arrows*. The N-glycosylation site is highlighted in *yellow* and marked by *orange line*. The microbodies targeting site is highlighted in *green* and marked by a *solid black line*. The ER retention motif is highlighted in *aquablue*

Fig. 6.2 (continued)

interactions between CRTs and protein disulfide isomerases (Frickel et al. 2002). The C-domain has an ER-retention signal (typically K/HDEL) and is the main domain responsible for the Ca^{2+}-binding ability of CRT (Christensen et al. 2008, 2010; Michalak et al. 1999). It possesses a large number of negatively charged residues which gives the protein its Ca^{2+}-buffering property (Sarwat and Tuteja 2007). CRT is known to bind 50 % Ca^{2+} present in the ER lumen (Nakamura et al. 2001) with high capacity and low affinity.

Characteristics of Plant Calreticulin

The rule of "one protein, one gene" does not fit for plant CRTs. There is nearly about 80 % similarity in the amino acid sequence of plant CRTs (Fig. 6.2) whereas they share only 50 % similarity with animal CRTs. The molecular structure of plant CRTs is also conserved. They share with their animal counterpart similar three domain organizations and similar biochemical properties. Multiple Sequence Analysis of CRTs from 11 different plants (Table 6.1) showed many conserved sequences across these genus (Fig. 6.2).

N-Glycosylation

Most of the plant CRTs have conserved sites for N-glycosylation. Among them, the highly conserved one is located at position 32 in the N-domain. *Arabidopsis* CRT has an N-glycosylation site in the C-domain (Navazio et al. 1996; Pagny et al. 2000). *Euglena* and *Chlamydomonas* lack the N-glycosylation site altogether. The functional role of N-glycosylation is still unclear. It may prove stability to nascent proteins and help in their proper folding (Helenius and Aebi 2001).

Phosphorylation

CRT can be phosphorylated both in vitro and in vivo by CK2 enzyme. These phosphorylation sites are located at the C-terminus (Baldan et al. 1996). In case of *Euglena* (Navazio et al. 1996) and *Chlamydomonas* (Zuppini et al. 1999), these sites are not free for phosphorylation. Other results also suggest that only high plant CRTs can get phosphorylated by CK2. Protein phosphorylation is important for

Table 6.1 Specifications of CRT protein used for multiple sequence analysis

S. no.	NCBI no.	Codes	Plant name
1.	NP_001059283.1	OsCRT	*Oryza sativa Japonica*
2.	XP_004987426.2	SiCRT	*Setaria italica*
3.	CAA86728.1	ZmCRT	*Zea mays*
4.	AAW02798.1	TaCRT	*Triticum aestivum*
5.	BAJ89536.1	HvCRT	*Hordeum vulgare* subsp. *vulgare*
6.	XP_009393180.1	MaCRT	*Musa acuminata* subsp. *malaccensis*
7.	ACH72686.1	NtCRT	*Nicotiana tabacum*
8.	XP_011092140.1	SiCRT	*Sesamum indicum*
9.	XP_002318957.1	PtCRT	*Populus trichocarpa*
10.	XP_012462922.1	GrCRT	*Gossypium raimondii*
11.	XP_010268013.1	NnCRT	*Nelumbo nucifera*

modulation of biological activity of that particular protein, and it is important for signaling events.

When CRT is fully saturated with Ca^{2+}, its phosphorylation with CK2 is very much reduced, but CK2 performs its other functions normally. The conformational changes of C terminal tail of CRT negatively regulates the Ca^{2+}-binding activity of CRT as the phosphorylation and low affinity high capacity Ca^{2+}-binding sites are located at the C-terminal tail.

Intracellular Localization of Calreticulin

On the C-terminal site of CRT, it contains a consensus HDEL sequence which helps in its ER retention. In *Euglena* CRT, it is KDEL sequence (Navazio et al. 1996). These sequences are a part of K/HDEL retrieval mechanism which helps in the exit and reentry to ER of plants, yeast, and mammals. This mechanism is mediated by ERD-2 receptors present within the membrane (Sanderfoot and Raikhel 1999).

CRT has been reported to be present in the plasmodesmata of maize (Baluska et al. 1999, 2001). The ER associated with the plasmodesmata are rich in CRT. CRT is also co-localized with Myosin VIII (Baluska et al. 2001). These observations suggest CRT to be a component of plasmodesmal regulation by modulating Ca^{2+} levels. Ca^{2+} also regulates the permeability of plasmodesmata. Elevation of cytosolic Ca^{2+} causes rapid closure of plasmodesmata. In pollen tubes and root hairs, cytosolic Ca^{2+} concentrations are maintained at high levels during cell growth (Baluska et al. 2000; Lenartowska et al. 2002). CRT has also been localized in the Golgi apparatus (Torres et al. 2001). It is found to be absent from the vacuoles, the major Ca^{2+} stores of plant (Opas et al. 1996).

Inducible Expression of Calreticulin

Through expression analyses of calreticulin protein and gene, it has been evident that calreticulin is involved in a number of plant functions.

Reproduction

It has been observed for the first time in barley that the expression of calreticulin gene in its ovaries is found to be increased 1 day after pollination and remained so during the early stages of embryogenesis (Chen et al. 1994). The same has been observed in other plants as well. For example, high CRT level was observed in maize cells after fertilization; in immature embryos; and in floral tissues of tobacco, *Arabidopsis* (Nelson et al. 1999), and *Ricinus*. These findings suggest important

role of CRT in plant reproduction. High CRT expression was also observed in the early developmental stages of the somatic embryos and ovules of *N. plumbaginifolia* (Borisjuk et al. 1998) post-fertilization. CRT has also found to be elevated in gametes of *Chlamydomonas reinhardtii* when compared to its vegetative cells (Zuppini et al. 1999). Strong expression of CRT has also been observed in mature anthers, dry and germinated pollen, growing pollen tubes, and unpollinated/pollinated pistils of *Haemanthus albiflos* (Lenartowska et al. 2009).

The mechanism of involvement of CRT in plant reproduction is not clear, but the possible explanation can be given by the buffering capacity of CRT which regulates cytosolic Ca^{2+}. Studies suggest Ca^{2+} to be an important ion for gamete activation. Accumulation of Ca^{2+} has been observed during gamete differentiation of *Chlamydomonas* (Harris 1989), and rapid internalization of Ca^{2+} ion has been reported in maize sperm cells when exposed to Ca^{2+} (Williams et al. 1997).

A transient elevation of Ca^{2+} can induce post-fertilization phase of plants, and this is coupled with an upregulation of the calreticulin gene (Faure 2001). These findings suggest CRT plays important role(s) not only in pre-fertilization stage but also in the post-fertilization events of plants.

Cell Division

Calreticulin gene expression has been found to be increased in the proliferating and secreting tissues (Coughlan et al. 1997; Nelson et al. 1997), such as root tips, nodes, and leaf base (Coughlan et al. 1997); this suggests that CRT might play a role in plant cell division. CRT has also found to be high in those cells which are active in secretion; this suggests that calreticulin possibly assists the assembly of nascent proteins and enzymes (Coughlan et al. 1997; Nelson et al. 1997; Borisjuk et al. 1998).

Stress Tolerance

CRT expression can be modulated by different stress treatments, i.e., OsCRO1, a rice gene from CRT family exhibited strong induction in cold stress (Li et al. 2003). The *Arabidopsis* CRT (AtCRT3) was induced by high salt (Persson et al. 2003), drought (Jia et al. 2008) and cold treatment (Komatsu et al. 2007). Wheat gene TaCRT showed drought tolerance when it was overexpressed in tobacco (Jia et al. 2008).

Other scientists have shown all the three CRT types to be involved in stress-related signaling in plants. A triple knockout Arabidopsis mutant, lacking CRT1, CRT2, and CRT3 and named as t123, exhibits an increased sensitivity to water stress. This reflects involvement of all three calreticulins in water stress (Kim et al. 2013). These three CRTs may play distinct role too (Christensen et al. 2010; Thelin et al. 2011). In brassinosteroid (BR) signaling, absence of CRT3 causes the production of

defective BR receptors, although the other too CRTs (CRT1 and CRT2) are present and calnexin was also present. This shows CRT1, CRT2, and CNX were not able to supplement the function of CRT3 (Hong et al. 2008; Jin et al. 2009).

Hormonal Treatments

A number of phytohormones when applied exogenously to plants showed modulation of CRT expression (Borisjuk et al. 1998). Application of gibberellic acid on the barley aleurone cells showed enhanced CRT transcript.

Different hormones modulate CRT level in a different manner, as α-naphthaleneacetic acid showed increased expression of CRT protein, whereas 2,4-dichlorophenoxyacetic acid showed decreased levels of CRT, when studied in *N. plumbaginifolia* (Borisjuk et al. 1998).

Treatment with naphthaleneacetic acid and 6-Benzyladenine also showed elevation of CRT expression in rice suspension culture.

Growth

Several reports indicate involvement of CRT in growth-related signaling in plants. The gravity stimulus caused an induction of CRT transcripts to several folds in the stem pulvinus cells of maize. In the early stages of this response, the protein synthesis of CRT has found to increase suggesting its important role during the process. Overexpression of the *Brassica rapa* CRT (BrCRT1) has reported to promote robust shoot production and root formation even when the concentrations of BA/NAA were low (Jin et al. 2005).

Immunity

Increased synthesis of CRT protein has been reported during plant pathogen attack also suggesting its role in the early stage of plant pathogen attack where it may activate certain pathogenesis-related protein (Verchot 2014). Wheat CRT (TaCRT1) has shown to participate in the regulatory processes of defense and stress tolerance in wheat (An et al. 2011). The Arabidopsis CRT2 has also shown to be a regulator of plant innate immunity. However, during *Pseudomonas syringae* infection, the CRT2 overexpressing tomato plants exhibit reduced resistance despite of increased salicylic acid levels and constitutive expression of resistance-associated marker genes PR1, PR2, and PR5 (Qiu et al. 2012). This can be understood by the phenomenon that the overexpression of His173 mutant of CRT2 reduces the susceptibility

Fig. 6.3 *cis*-regulatory elements in 1.0 kb upstream promoter region of the calreticulin (CRT) gene. Figure shows several *cis*-motifs, *viz.* ABRE, MeJA-responsive motifs, and also several gene-specific *cis*-motifs, *viz.* those for gibberellin responsiveness and salicylic acid responsiveness, thus depicting multifarious role of CRT

of transgenic plants to the Pst DC3000 infection; CRT2 causes its self-inhibition during SA-dependent immune responses, where His173 plays a critical role.

Both the processes of gravitropism and plant pathogen interaction involves Ca^{2+} signaling; the role of CRT in these processes can be attributed to Ca^{2+}-buffering property of CRT.

The Arabidopsis CRT1, CRT2, and CRT3 has reported to act as defense-related activities for certain viral and microbial pathogen (Chen et al. 2005; Qiu et al. 2012; Saijo et al. 2009). Besides this, upregulation of CRT has also been observed after mannitol stress and aluminum treatment (Baluska et al. 1999). Both of them cause callose deposition at plasmodesmata, suggesting the possible role of CRT in the structure or function of plasmodesmata. Thus, the above studies have shown CRT to play an important role in various activities related to Ca^{2+} signaling based on its Ca^{2+}-buffering property. These activities are in addition to its chaperonic activity.

The distribution of *cis*-regulatory elements in 1.0 kb upstream promoter region of CRT revealed various important *cis*-motifs (Fig. 6.3, Table 6.2). These motifs might provide an indication for the possible function CRT (Table 6.2). The *cis*-regulatory elements present in the promoter sequences are light-responsive elements (G-box), heat-responsive element (HSE), hormone-responsive elements for ABA (ABRE), gibberellin (TATC), and elements for pathogen defense (TC-1). The presence of these predicted *cis*-elements exhibit strong indications of the involvement of these genes in abiotic stress responses.

Table 6.2 Type of *cis* elements in CRT promoters

S. no.	Name of *cis* element	Sequence of motif	Assigned function
1.	ACE	ACGTGGA	*cis*-Acting element involved in light responsiveness
2.	ARE	TGGTTT	*cis*-Acting regulatory element essential for the anaerobic induction
3.	Box-W1	TTGACC	Fungal elicitor responsive element
4.	CAAT/CCAAT box	CAAAT	Common *cis*-acting element in promoter and enhancer regions
5.	CCGTCC-box/A-Box	CCGTCC	*cis*-Acting regulatory element related to meristem-specific activation
6.	CE3 element	GACGCGTGTC	*cis*-Acting element involved in ABA and VP1 responsiveness
7.	CGTCA motif	CGTCA	*cis*-Acting regulatory element involved in the MeJA responsiveness
8.	G-box	CACGTA	*cis*-Acting regulatory element involved in light responsiveness
9.	GC-motif	CCCCCG	Enhancer-like element involved in anoxic-specific inducibility
10.	HSE	AAAAAATTTC	*cis*-Acting element involved in heat stress responsiveness
11.	MBS	TAACTG	MYB binding site involved in drought inducibility
12.	TATA	TATA	Core promoter element around −30 of transcription start
13.	TATC box	TATCCCA	*cis*-Acting element involved in gibberellin responsiveness
14.	TC-1	ATTTTCTTCA	*cis*-Acting element involved in defense and stress responsiveness

Earlier research (Oh et al. 2003) have shown the similarities between plants and other organisms. Recently, plant-specific characteristics have also been observed by Hayashi et al. (2013). The characteristic features of ER elements in plants are reported as pERSE (CCAAT-N10-CACG), pUPRE (ATTGGTCCACGTCATC), and pUPRE-II (GATGACGCGTAC) in tobacco (Katagiri et al. 1989), Arabidopsis (Liu et al. 2007; Oh et al. 2003), rice (Hayashi et al. 2013), etc.

Role of ER in Quality Control

Similar to the eukaryotic ER, an effective quality control mechanism is present in the plant ERs where a number of enzymes and factors work synergistically and ensure proper folding of newly synthesized glycoproteins. The proteins are not able to fold properly due to various reasons. Some research reports are as follows:

Phaseolin: The vacuolar storage protein of common bean (*Phaseolus vulgaris*) is targeted to the vacuole when it is correctly folded, but its transport to the vacuole is hampered when it is improperly folded.

Brassinosteroid Receptor: The Arabidopsis CRT3 caused the retention of a mutant brassinosteroid receptor, brassinosteroid-insensitive 1–9 (bri1–9) in the ER. The C-terminal domain of CRT3, which is rich in basic residues, plays an important role in retaining the bri1–9 within the ER. CRT3 has also been observed to retain an incompletely folded plant immunity receptor EF-Tu Receptor (EFR).

In order to further investigate the CRT sequences responsible for this type of quality control, experiments were done with insertional mutant of CRT3, ebs2–8 (EMS mutagenized bri1 suppressor 2–8). The Complementation experiments of ebs2–8 bri1–9 with mutant CRT3 (M) transgenes revealed two motifs in the C-terminal domain of CRT3. One is a highly conserved basic tetrapeptide Arg (392) Arg (393) Arg (394) Lys (395) which is essential for the quality control function of CRT3. The other motif is a less conserved basic tetrapeptide Arg (401) Arg (402) Arg (403) Arg (404), which is found to be dispensable (Liu and Li 2013b).

When these misfolded proteins get accumulated in the ER, they cause a trigger in the ER stress termed as Unfolded Protein Response (UPR). This causes an upregulation of various ER chaperones. The overexpression of these chaperones then mitigate the ER stress. As reported from *Nicotiana*, UPR causes increased transcription of BiP genes. This increase then alleviates the UPR in *Nicotiana*.

Among all the ER chaperones, BiP is very well studied both in normal as well as stress conditions. Other chaperones like CRT and its closely related ER protein Calnexin (CNX) are known only for their chaperonic activities in plants. Their other roles in stress responses, growth modulation, reproduction, etc. are beginning to unfold.

Plant ER as Ca^{2+} Store

Ca^{2+} can be stored in various sites within a cell, but all of these sites cannot be considered as rapidly exchangeable sites. Ca^{2+} binding occurs on the pectin polymer of cell wall. While, in chloroplast and nucleus, the Ca^{2+} is controlled independent to the cytosolic Ca^{2+}, so both of these organelles have their own independent Ca^{2+} signals and they regulated their own Ca^{2+} dependent processes. The role of plant mitochondria in Ca^{2+} signaling is still unclear. The plant vacuole is considered as the major Ca^{2+} reservoir. Several Ca^{2+} transporters and Ca^{2+} release channels are present within the plant vacuolar membrane supporting its major role as stimulus releasable reservoir. Vacuolar sap has an acidic pH of 3.6, so the Ca^{2+}-buffering role is carried out by organic or inorganic ions or Ca^{2+}-binding protein reticuloplasmins. These proteins do not show any sequence similarity with the ER proteins CRT and calnexin (CNX).

The ER is considered as the second largest storehouse of intracellular Ca^{2+} in plants. It has important role in calcium signaling in plants. Calmodulin-regulated Ca^{2+} ATPases are present in the ER membranes.

Various types of Ca^{2+} channels are present in plant endoplasmic reticulum. These are voltage-gated and ligand-gated channels and are activated by the pyridine nucleotide derivatives such as nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (cADPR) and, possibly, by inositol 1,4,5-trisphosphate ($InsP_3$). These Ca^{2+} ATPases and Ca^{2+} channels suggest the important role of ER in Ca^{2+} signaling within the cell. CRT is one of the important ER resident protein which modulates Ca^{2+} ions within the ER as well as the mobilization of ER Ca^{2+} store when it is required.

The ER membranes are present as a dynamic, three-dimensional network within the cell. This well-developed network is important for Ca^{2+} homeostasis at the local areas of the cell. The ER which is present just below the plasma membrane in plant is known as cortical endoplasmic reticulum and is highly developed polygonal structure. It is physically attached to the plasma membrane through cytoskeletal proteins and transmembrane integrin-like proteins on well-defined adhesion sites. This type of arrangement facilitates the rapid dissemination of signals emerging from the plasma membrane, and the cortical ER is a very good respondent of these signals.

The Ca^{2+} signals are stimulated depending upon the type of trigger. Cold stress triggers Ca^{2+} fluxes starting from the plasma membrane progressing downwards, as seen in the aequorin-transformed tobacco seedlings. The mechanical stimulation causes Ca^{2+} release from in house Ca^{2+} stores.

Generation of Ca^{2+} oscillations has been observed in some specialized cell types, such as stomata guard cells and pollen tubes, due to a wide variety of stimuli. In the unicellular green alga, cycles of Ca^{2+} release and Ca^{2+} reuptake by the endoplasmic reticulum have been observed; they are reportedly repetitive Ca^{2+} spikes. Studies on plants are rare concerning these types of Ca^{2+} oscillations, whereas in animals they are numerous.

Calreticulin and Ca^{2+} Signaling

The relationships between calreticulin and Ca^{2+} signaling have been very well studied in animals. Calreticulin has emerged as a player of chaperonic activity having interactions with other endoplasmic reticulum chaperones, regulating $[Ca^{2+}]_{ER}$ and participating in the signaling network of the endoplasmic reticulum (Corbett et al. 2000).

The knowledge about plant CRT is very sparse.

Evidences about the modulation of Ca^{2+} status of the ER by plant CRT has recently came into picture. CRT overexpression in tobacco changes the Ca^{2+} pool of the ER. An ATP-dependent Ca^{2+} accumulation has been observed when CRT level was increased in the microsomes enriched with endoplasmic reticulum membranes.

When CRT is overexpressed *in planta*, it enhances the survival of transgenic plants growing in a limiting, low Ca^{2+} medium (Persson et al. 2001). In *Arabidopsis*, only the expression of the C-domain of calreticulin (targeted to the endoplasmic reticulum) enhances survival of these plants growing on Ca^{2+}-depleted medium (Wyatt et al. 2002). The explanation for the above phenomenon is that an overproduction of calreticulin causes increased Ca^{2+}-buffering ability which maintains their Ca^{2+} homeostasis under adverse growth conditions.

Further, it has been observed that expression of Arabidopsis H^+/Ca^{2+} antiporter (sCAX1) gene in agricultural crops causes an increase in the total calcium (Ca^{2+}), but the yield may be adversely affected and Ca^{2+} deficiency-like symptoms were seen. Wu et al. (2012) have demonstrated that when maize CRT was co-expressed with sCAX1, it mitigates these adverse effects. The explanation of this phenomenon is that the co-expression of these proteins could alleviate the hypersensitivity to ion imbalance in the experimental plants (Wu et al. 2012).

The role of plant CRTs is not very well studied in spite of their high sequence conservation, constitutive expression, and ubiquitous distribution. Recent studies have shown plant CRTs as a multifunctional player.

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Chapter 7
DNA Damage, Response, and Repair in Plants Under Genotoxic Stress

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Abstract Several environmental and endogenous factors create a variety of lesions in the genome of an organism. These lesions could potentially be genotoxic and might lead to mutations, which could be lethal. All organisms exhibit a prompt response against DNA damage, which is referred to as the DNA damage response. The existence of elaborate, evolutionarily conserved systems to repair the damage, mostly at the expense of huge amount of energy, points to the importance of safeguarding the integrity of DNA. Most of the current understanding about DNA damage response and repair pathways has been distilled through decades of research on prokaryotes, yeast, and mammalian systems. The response to genotoxic stresses and the repair mechanisms involved in plants has only recently begun to be investigated. Herein, we present a comprehensive account of the types of DNA damage, the DNA damage response, and the repair pathways with reference to the recent insights gained from the plants. Although, the underlying common theme runs through to the plants, the mechanisms in plants demonstrate some unique features. Also, there are several missing links that need to be unravelled for inferring a complete picture. A thorough understanding of the mechanisms involved would aid in devising strategies to help plants avoid irreparable damages to their genome upon exposure to genotoxic agents.

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Introduction

The genome contains the blueprint of life. Protecting the sanctity of the information in DNA is a cellular imperative for maintaining and perpetuating life. Though intrinsically more stable than other macromolecules, DNA is nonetheless prone to damages that can compromise its function and the eventual fate of an organism. A variety of processes, some endogenous and other catalyzed by environmental agents, can undermine the integrity of DNA. The endogenous sources of damage include metabolic by-products and stalled replication forks while environmental agents like ultraviolet (UV) and ionizing (IR) radiation and chemical mutagens are the exogenous ones. To combat the DNA damage, organisms contain elaborate cellular networks termed the DNA damage response (DDR) that are critical to maintain the integrity of the genome (Ciccia and Elledge 2010). The components of this response include processes to detect the damage, to relay the signal to specific regulators in the cell, and to elicit the production of effector molecules (Vespa et al. 2005; Culligan et al. 2006). The orchestration of these processes ultimately leads to the repair of DNA lesions or the induction of cellular death, if the damages are beyond repair. The DDR pathways are fundamental to life and most of them have been conserved through the evolution. Much of the current understanding of these mechanisms derives from the research done in prokaryotic, yeast, and mammalian systems. The delineation of DDR in plants has only been attempted lately.

Plants are continuously exposed to environmental, edaphic, and other anthropogenic stresses. These include extremes of temperature, drought, UV-B, IR, as well as air and soil pollutants. In addition to severely impacting the structural, enzymatic, and nonenzymatic components of plants, these stresses potentially threaten the plant genomes (Wasi et al. 2013). Unrepaired DNA damages can lead to mutations, which can impact the stability of a plant's genomes, its growth and productivity, and might threaten the survival of the plant (Singh et al. 2010; Biedermann et al. 2011). Being sessile, plants cannot evade these stresses and combat is the only alternative for their survival and growth. It is, thus, imperative that an efficient and specific DDR system be in place in plants to cope with DNA damage (Yoshiyama et al. 2013). The sequencing of several plant genomes has given huge impetus to the study of DDR in plants, with many components of the system having been discovered in plant genomes (Mannuss et al. 2012). Although the basic mechanism remains conserved in eukaryotes, several plant-specific regulators have been reported, alluding to the existence of some unique DDR systems in plants.

In this chapter, we have endeavored to summarize the current understanding of DDR in plants. The following sections in the chapter provide details on the DNA damaging agents, types of DNA damage, mechanism of genotoxic stress perception, transduction of the signal, and repair of the damage.

DNA Damaging Agents

Different intrinsic and extrinsic factors, classified either as physical or chemical, are capable of damaging the genetic material.

Physical Agents

Ultraviolet and ionizing radiation are the most common physical factors contributing to DNA damage. The innocuous sunlight, essential for photosynthesis in plants, also contains radiations that are potentially genotoxic. These energy-rich radiations include UV-C (100–280 nm), UV-B (290–320 nm), and UV-A (320–400 nm). UV-C is filtered out by absorption in the upper stratospheric and ozone layers but UV-B and UV-A enter the atmosphere, their amount reaching the earth's surface dependent on latitude and elevation, as well as the cloud cover and canopy density. UV-B, on account of its higher energy, is potentially more deleterious to living organisms. Besides damaging proteins and biomembranes, UV-B is strongly mutagenic (Pang and Hays 1991; Britt 1999). It is absorbed by the DNA and may lead to the generation of cyclobutane pyrimidine dimers (CPDs) and to a lesser extent pyrimidine (6-4) pyrimidone dimers (6-4 PPs; Friedberg et al. 2006). These lesions affect the transcription and also result in error-prone replication. Besides, UV light is strongly absorbed by many environmental organic contaminants, thereby, increasing their toxicity (Huang et al. 1993). The UV-A and visible spectrum of light cannot be absorbed by the native DNA. However, they can still damage DNA through reactive oxygen species (ROS) generation via photosensitizing reactions (Alscher et al. 1997; Iovine et al. 2009). ROS can induce a variety of DNA damages like base and nucleotide modifications, especially in guanine-rich sequences and may even cause strand breaks (Wiseman and Halliwell 1996; Tuteja et al. 2001; Tuteja and Tuteja 2001).

IR is another major mutagen that damages DNA, either directly or indirectly through the production of free radicals (Ward 1975). Chromosomal breaks, inversions, duplications, and translocations can result from double-strand (DSBs) and single-strand breaks (SSBs) caused by the absorption of IR by the sugar–phosphate backbone in the DNA. The indirect effect of IR is mediated through the radiolysis of water which generates OH radicals, the most damaging of all the ROS that attack the DNA along with proteins, lipids, and other cellular constituents.

Chemical Agents

Chemically, genotoxic agents could either be inorganic or organic. Heavy metals like Cd^{2+}, Cu^{2+}, Ni^{2+}, and Pb^{2+} are the main inorganic genotoxic compounds which cause the generation of free radicals that damage the DNA (Imlay and Linn 1988).

Metal ions can also influence the efficiency and fidelity of DNA replication. Ions like Ni^{2+} and As^{3+} change the stability of DNA by altering their methylation pattern (Davis et al. 2000).

Genotoxic organic compounds can affect the DNA in different ways. Alkylating agents like ethylmethane sulfonate (EMS) and methylmethane sulfonate (MMS) cause DSBs while aromatic compounds such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are mutagenic because they intercalate between the DNA strands.

Types of DNA Damage

DNA damage caused by physical or chemical agents can be broadly classified into three types of lesions: mismatched bases, double-strand breaks, and chemically modified bases. The damages induced by the different agents are discussed below.

UV-Induced DNA Damage

A major type of DNA damage induced by UV-B is the formation of dimers between adjacent pyrimidines—the CPDs and 6-4 PPs (Mitchell and Nairn 1989; McGregor 1999; Fig. 7.1). CPDs can occur in any of the diastereoisomeric forms, i.e., cis/trans

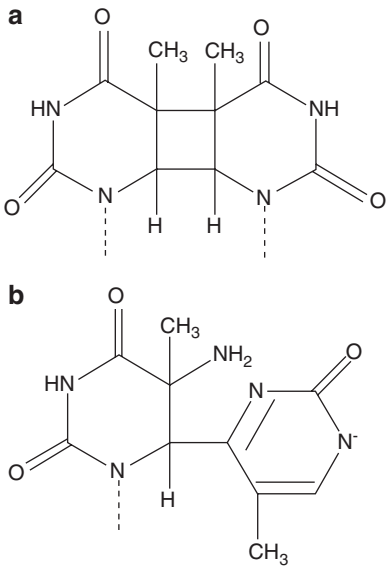

Fig. 7.1 Pyrimidine dimers formed upon UV-B irradiation. Structure of a cyclobutane pyrimidine dimer (**a**) and a pyrimidine 6-4 pyrimidone dimer (**b**)

(with respect to the relative position of pyrimidine rings) and syn/anti (with reference to relative orientation of C5–C6 bonds). Whereas cis-syn is the predominant CPD form in DNA, the trans-syn occurs exclusively in single-stranded DNA. The 6-4 PPs are formed between adjacent TT, TC, and CC nucleotides depending upon the UV wavelength and adjacent sequence (Mouret et al. 2006). CPDs and 6-4 PPs make up approximately 70–80 % and 20–30 %, respectively, of the total UV photoproducts (Mitchell and Nairn 1989). The pyrimidine dimers inhibit DNA replication and transcription by inducing structural distortions within the DNA, produced as a consequence of bending and unwinding of the DNA helix (Demple and Harrison 1994).

UV-induced ROS can damage DNA mostly by the formation of 8-hydroxydeoxyguanosine (8-OHdG) at the 5′-site of –GG– sequence in the double-stranded DNA. This modified guanine pairs with adenine at 50 % probability causing G:C to T:A transversions (Ito et al. 1993).

IR-Induced DNA Damage

Like UV, IR, too, can induce DNA damage both by being directly absorbed and by production of ROS as a consequence of radiolysis of water. The most common DNA lesions generated by IR include sugar and base damage, single- and double-strand breaks, and DNA–DNA and DNA–protein cross-links (Belli et al. 2002). Direct absorption of IR by the sugar–phosphate backbone of DNA generates nicks and DSBs. IR-induced SSBs are characterized by the presence of a one nucleotide gap containing a 5′-phosphate and either a 3′-phosphoglycolate or a 3′-phosphate (Henner et al. 1983).

Hydrolysis-Induced DNA Damage

Hydrolysis of glycosidic bonds between bases and sugar–phosphate backbone in DNA results in the formation of apurinic/apyrimidinic (AP) sites. These sites prevent normal DNA replication and transcription and are the most common locations for the creation of DSBs because of the collision of replication forks. These lesions are generally recognized and repaired under normal conditions, but their occasional bypass during replication can be mutagenic (Gentil et al. 1984).

Cytosine and methyl-cytosine can undergo hydrolytic deamination to uracil and thymine, respectively. During replication, this can lead to C:G to T:A transition because of mispairing with adenine. Though, the unusual presence of uracil in the DNA is promptly recognized and the lesion is repaired, the transition to thymine is difficult to detect and frequently leads to point mutation.

Alkylation-Induced DNA Damage

7-Methylguanine, 3-methyladenine, and O^6-methylguanine are the most common lesions produced by alkylating agents like methyl methane sulfonate (MMS) and ethyl methane sulfonate. While 7-methylguanine is a neutral mutation, 3-methyladenine blocks DNA synthesis creating DSBs and O^6-methylguanine pairs with thymine to produce G:C to A:T transition.

Oxidation-Induced DNA Damage

Increased ROS production is a common manifestation of many abiotic stresses in plants with chloroplasts and mitochondria being the major sites of production. ROS-induced oxidative damage to DNA includes modified bases and sugar, abasic sites, strand breaks, DNA–protein cross-links. The most common oxidation product of purines is 7,8-dihydro-8-oxoguanine, also known as 8-oxoguanine, which can base pair equally well with adenine and cytosine (Maki and Sekiguchi 1992). Thymine glycol is the main oxidatively modified form of pyrimidines and can block DNA replication. Furthermore, ROS can react with sugar of the sugar–phosphate backbone leading to SSBs and DSBs.

DDR Pathway: Perspectives from Animals and Plants

The DDR pathway has mostly been investigated in animals and yeast. At the molecular level, it consists of a number of components including DNA damage sensors, signal transducers, mediators, and effectors. In mammals, ataxia telangiectasia mutated (ATM), ATM, and Rad3-related (ATR), and DNA-dependent protein kinase subunit (DNA-PKcs) are the major regulators of DDR and are rapidly activated in response to DNA damage (Sirbu and Cortez 2013). DSBs are mostly sensed by ATM which initiates a cascade of steps that comprise the ATM pathway. ATR, on the other hand, can respond to a variety of DNA lesions, especially those associated with DNA replication to commence the ATR pathway (Cimprich and Cortez 2008).

Sensing the Damage

The different types of DNA damages are sensed by either the ATM or ATR pathway and culminate into a suitable response depending upon the severity of the damage. In mammals, the DSBs are recognized in the ATM pathway by a ternary MRN complex that comprises the proteins MRE11, RAD50, and NBS1 (Rupnik et al. 2010). This

complex assembles at the site of DSBs and recruits ATM whereby the interaction with NBS1 activates the kinase activity of ATM resulting in the phosphorylation of target proteins, especially the histone variant H2AX. γH2AX, the phosphorylated form of H2AX, further recruits other DDR proteins (Dickey et al. 2009). Homologs of MRE11, RAD50, and NBS1 have been reported in *Arabidopsis thaliana* (Hartung 1999; Gallego and White 2001; Akutsu et al. 2007). Phosphorylation of H2AX by AtATM and AtATR, the *A. thaliana* homologs of ATM and ATR, has also been reported in response to DSBs (Friesner et al. 2005). That the induction of γH2AX was observed to be impaired in the *rad50* and *mre11* mutant plants suggested the involvement of the MRN complex in the phosphorylation of H2AX via the ATM and ATR homologs in plants (Amiard et al. 2010).

In mammals, replication protein A (RPA), a single-strand DNA (ssDNA)-binding protein acts as a sensor in the ATR pathway. The RPA-ssDNA complex formed at the site of stalled replication fork (near a DNA lesion) is bound by an ATR-interacting protein (ATRIP) which recruits ATR, eventually activating the check point kinase 1 (CHK1; Zou and Elledge 2003). Alternatively, the ATR pathway can be initiated by recognition of the damage by the RAD9/RAD1/HUS1 (9-1-1) complex. This complex is a ring-like clamp that is loaded onto ssDNA at the damage site with the help of a clamp loader, RAD17 (Bermudez et al. 2003). The homologs of RPA, 9-1-1 complex, and RAD17 have been identified in the *A. thaliana* genome (Takashi et al. 2009; Heitzeberg et al. 2004). Functional characterization of mutants of these homologs suggests that the DNA sensing mechanisms via the ATM and ATR pathway are conserved between animals and plants.

Transducing the Signal

For proper response, information about the damage detected by the sensors is amplified and transduced to the effector proteins by the mediation of transducers. These transducers are mostly serine/threonine kinases which initiate a sequence of phosphorylation steps. ATM/ATR and CHK1/CHK2 are the main transducers in animals. Activation of ATM involves its autophosphorylation and subsequent monomerization of the ATM dimer (Bakkenist and Kastan 2003), whereas ATR is brought into action by its recruitment to RPA-ssDNA complex by ATRIP (Ball and Cortez 2005; Warmerdam et al. 2010). The downstream substrates for phosphorylation by ATM and ATR include CHK1, CHK2, p53, NBS1, and BRCA1 (Matsuoka et al. 2000; Gatei et al. 2003; Zhao and Piwnica-Worms 2001). Of these, CHK1 and CHK2 are the main transducers which have common phosphorylation substrates like p53, BRCA1, E2F1, and CDC25A (Kim et al. 2007).

AtATM and AtATR, the *Arabidopsis* homologs of mammalian ATM and ATR have been identified. Analysis of the *atm* and *atr* mutants revealed their sensitivity to DNA damage inducing agents signifying the conservation of the role of these proteins as signal transducers in both animals and plants (Garcia et al. 2003; Culligan et al. 2004). Orthologs of ATRIP have also been reported in *A. thaliana*

(Sweeney et al. 2009; Sakamoto et al. 2009). Although the orthologs of CHK1 and CHK2 have not been identified in plants the presence of other kinases having functions similar to these two proteins has been alluded to, in view of the presence of mediator proteins like BRCA1 and E2F in plants (Lafarge 2003; Inze and de Veylder 2006).

Between the Signal and Response: The Mediators

Different mediator proteins that regulate DDR have been identified and their roles have been described. The two most common mediators involved in the ATM pathway are BRCA1, mediator of DNA-damage checkpoint protein 1 (MDC1), and p53-binding protein (53BP1; Stewart et al. 2003; Stucki and Jackson 2004). DNA topoisomerase-2-binding protein 1(TOPBP1) and CLASPIN have been demonstrated to regulate the ATR pathway (Garcia et al. 2005; Kumagai et al. 2004). Many of these proteins interact with other phosphorylated proteins through a conserved BRCA1 associated C-terminal (BRCT) domain (Manke et al. 2003). These mediator proteins function as scaffolds for many DNA damage repair proteins. AtBRCA1, a homolog of BRCA1 with two BRCT domains, which is strongly induced by IR, has been reported in *A. thaliana* (Culligan et al. 2006; Lafarge 2003). Also, an *Arabidopsis* MEIOSIS DEFECTIVE 1 (ME1) protein with five BRCT domains having 40 % similarity to the human TOPBP1 has been reported although its role as a mediator in DRR remains to be confirmed (Mathilde et al. 2003). Other mediators are yet to be identified in plants.

Responding to the Damage: The Effectors

Cellular response to DNA damage depends on the severity of the damage. A cell tries to repair the damage by halting its progress in the cell cycle and stopping DNA replication followed by the activation of DNA repair pathways. If the damage is extensive and beyond repair, the cell prefers to initiate apoptosis. The ultimate result of DNA damage is decided by the action of effector proteins. In animals, the tumor suppressor protein p53 is the most important effector (Helton and Chen 2007). The amount and activity of this transcription factor is regulated very precisely by post-translational modifications, of which phosphorylation is the most crucial (Taira and Yoshida 2012). Under normal conditions, p53 levels are maintained at low levels by MDM2-mediated ubiquitination and proteasomal degradation (Xirodimas et al. 2004). DNA damage leads to phosphorylation of p53 by ATM, ATR, CHK1, and CHK2; phosphorylation inhibits the interaction of p53 with MDM2, thereby, resulting in its stabilization and activation (Shieh et al. 1997; Appella and Anderson 2001). Despite the conservation of many DRR proteins between animals and plants,

a plant p53 homolog has long eluded plant scientists. Presence of factors with functions similar to that of p53 has been envisaged; SOG1 has been described as one such factor.

An essential prerequisite for initiating DNA repair is the halt or arrest of cell cycle to prevent the replication of damaged DNA or allow the division of cells carrying such DNA. Cyclin-dependent kinase (CDK) inhibitors, p21 and WEE1 kinase, are the major effectors that halt the cell cycle in response to DNA damage in animals (Abbas and Dutta 2009; Sorensen and Syljuasen 2012). While p21 arrests the cells at G1 in a p53-mediated manner (Reinhardt and Schumacher 2012), WEE1 prevents mitotic entry (McGowan and Russell 1995). In addition, several CDC25 phosphatases are also cell-cycle effectors. These phosphatases remove the inhibitory phosphate groups on CDKs to control the progression of cell cycle. Upon DNA damage, CDC25 is itself inactivated by phosphorylation mediated by CHK1 and CHK2 leading to the cell-cycle arrest (Karlsson-Rosenthal and Millar 2006). Although, homologs of p21, like its immediate regulator p53, have not been identified in plants several other CDK inhibitors (CDKIs) have been reported from *A. thaliana*. These CDKIs belong to two families, namely Kip-related protein (KRP) family and SIAMESE/SIAMESE-RELATED (SIM/SMR) family. DSBs strongly induce the expression of SMR4 and SMR5, indicating their involvement in cell-cycle arrest (Yoshiyama et al. 2009). AtWEE1 is the WEE1 homolog in *A. thaliana* that is activated by DNA damage or DNA replication arrest in an AtATM- or AtATR-dependent manner, respectively (de Schutter et al. 2007). It has been demonstrated to control cell-cycle arrest in the DDR pathway. Although a CDC25-like protein has been identified in *A. thaliana*, its role in cell-cycle regulation is not confirmed (Spadafora et al. 2011).

By applying brakes on the cell cycle, cell buys time to make amends to the damaged DNA. A number of conserved DNA repair pathways viz., nucleotide excision repair (NER), base excision repair (BER), homologous recombination (HR), nonhomologous end-joining (NHEJ), and direct repair (DR) have been identified in yeast, animals, and plants. If the damage is beyond repair, animal cells activate apoptosis, the exquisitely regulated pathway to kill cells with compromised genomes. The diversion of cells from DNA repair pathways to apoptosis is mediated by the transcriptional activation of genes such as *BAX*, *PUMA*, and NOXA by p53 (Vousden and Lu 2002). Plants, too, have programmed cell death (PCD), but it is distinct from the apoptotic cell death in animals because plants lack the core apoptotic machinery. PCD involving AtATM and AtATR has been observed in shoot and root apical meristem in plants in response to DNA damage (Fulcher and Sablowski 2009). Metacaspases (MCs) that are structurally similar to animal caspases, the initiator proteins for apoptosis have also been reported in plants (Uren et al. 2000; Lam and Zhang 2012). Overexpression of some of these MCs increased the cell death upon treatment with ROS-inducing agents while their loss resulted in delay or decrease of cell death (Lam and Zhang 2012). It has been suggested that some of these pathways might be regulated by SOG1 in plants.

DNA Repair Pathways in Plants

Maintenance of genomic integrity is crucial to life. This comes at a huge premium in terms of energy expenditure and elaborate repair framework that organisms need to maintain. Although damages that do not interfere with the replication or transcription can sometimes be innocuous, those that hinder the activity of DNA and RNA polymerases are very harmful for the organism and need to be compulsorily repaired. Most of the repair mechanisms have been conserved through evolution and homologs of different elements have been identified across kingdoms. The whole genome sequence of *A. thaliana* and rice has revealed the presence of many repair proteins that are similar to those in humans. Characterization of mutants and overexpressing lines has provided functional validation for these proteins. A comprehensive list of DNA repair genes in the rice genome was provided by Kimura and Sakaguchi (2006). Like animals, plants are also equipped with a variety of repair pathways catering to the different types of DNA damages. These damages can be rectified through a number of pathways. Herein, we have described the main pathways, namely photoreactivation/photorepair (PR), base excision repair (BER), and nucleotide excision repair (NER).

Photoreactivation/Photorepair (PR) Pathway

As inherent in the name, photoreactivation is a light-dependent pathway majorly responsible for the repair of UV-B-induced DNA damage, especially CPDs. This pathway utilizes light-mediated reversion of the damage by photolyases (Yasui and Eker 1998). Photolyases generally contain two chromophoric co-factors, one of which is always the two electron reduced form of FAD ($FADH^-$; Sancar 2003). $FADH^-$ acts as a transient electron donor to reverse the cross-link between the bases. The second chromophore acts as an antenna pigment to excite the electron donor. The reaction mechanism proceeds via a free radical mechanism. Photolyases bind specifically to DNA lesions absorbing blue/UV-A (320–400 nm) light and reducing pyrimidine dimers to monomers (Kimura et al. 2004; Fig. 7.2). A number of factors including quality, timing, and quantity of photoreactivating light as well as the severity of the damage affect this repair (Sutherland et al. 1996; Takeuchi et al. 1996; Stapleton et al. 1997). Although, PR is present in prokaryotes and eukaryotes including some species of plants and animals, it is not universal, with many species, including humans, lacking it (Todo 1999). The genes for photolyases have been cloned from higher plants such as *A. thaliana*, *Cucumis sativus*, *Oryza sativa*, and *Spinacia oleracea*. Six genes with photolyase activity have been identified in the *Arabidopsis* genome. The common UV-induced lesions, CPD and 6-4 PP, are specifically recognized by photolyases PHR1/UVR2 and UVR3, respectively. *Arabidopsis* mutants for these photolyases were defective in PR (Jiang et al. 1997). Similarly, rice cultivars with deficient PR of CPDs were reported to carry a mutation

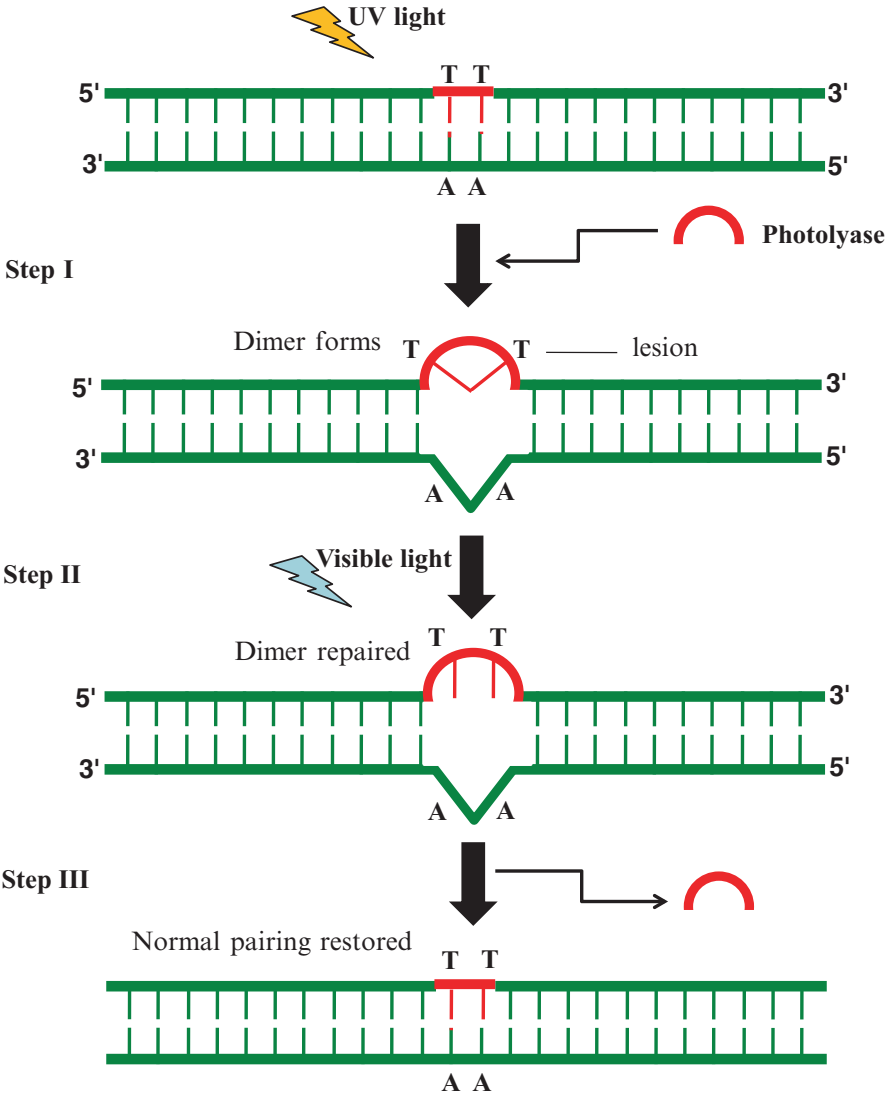

Fig. 7.2 Schematic representation of photoreactivation mechanism. The enzyme photolyase binds to the site of structural distortion in the DNA caused because of pyrimidine (thymine in this case) dimer (Step 1) and catalyzes the light (*blue*) activated reversion of the dimer to the monomeric form (Step 2). The enzyme is released from the DNA after the repair (Step 3)

in the CPD photolyase (Hidemal et al. 2000; Teranishi et al. 2004). In contrast, transgenic rice overexpressing CPD photolyase were demonstrated to be tolerant to growth inhibition caused by UV-B-induced damage and accumulated lower CPD lesions in leaves during growth under UV-B irradiation (Hidemal et al. 2007). Further, diurnal changes in CPD photolyase expression have been observed in

cucumber, suggesting regulation of the gene to prevent growth suppression by UV (Takahashi et al. 2002). In *Arabidopsis*, the overexpression of CPD photolyase resulted in a modest increase in biomass under UV-irradiated conditions (Kaiser et al. 2009). In addition, different ecotypes of *A. thaliana* have been demonstrated to possess variable UV-B response (Kalbina and Strid 2006).

Base Excision Repair

Only a minority of damaged bases in DNA are repaired by direct damage reversal; most of these are removed by excision repair (Lindahl and Wood 1999). Damaged bases that do not distort the helical structure of DNA are repaired by BER. Such damaged bases could be the result of spontaneous deamination or base loss or could be induced by IR, oxidative or methylating agents. In this multi-step repair pathway, specific DNA glycosylases excise the damaged base thereby creating an abasic (AP) site that is subsequently filled in by the correct DNA sequence (Lindahl and Wood 1999; Mol et al. 1995; Tuteja and Tuteja 2001). The repair mechanism has been classified into two sub-pathways: short-patch BER and long-patch BER. In short-patch BER, a glycosylase with specificity for a particular base-adduct slides along the minor grove of DNA helix and localizes to the site of the lesion where it removes the damaged base creating an AP site (Fromme et al. 2004). The abasic site is then recognized by an AP-endonuclease that nicks the DNA backbone on 5′-end of the AP site. Thereafter, the 5′-terminal deoxyribose-phosphate residue is excised by DNA polymerase β using its AP lyase activity (Matsumoto and Kim 1995). The missing base is then put in place by DNA polymerase β, and the nick is sealed by DNA ligase I or III with the help of XRCC1 (Fig. 7.3). In the case of long-patch BER, instead of the replacement of a single damaged base, a longer patch (2–10 nucleotides) is resynthesized by nick translation in conjunction with strand displacement in the 5′–3′ direction, generating a flap-like structure. The flap is removed by a flap endonuclease, FEN-1, with the aid of PCNA (Wu et al 1996; Klungland and Lindahl 1997; Fig. 7.3). The nick translation is catalyzed by DNA polymerase β or δ and the backbone is sealed by DNA ligase 1 (Fortini et al. 1998).

The study of BER in plants has revealed the presence of several homologs of different component proteins in *A. thaliana* and *O. sativa* (Kimura and Sakaguchi 2006). For example, glycosylases with specificity for particular modified bases have been reported (Santerre and Britt 1994; Garcia-Ortiz et al. 2001; Dany and Tissier 2001; Murphy and Gao 2001). Similarly, *Arabidopsis* XRCC1-like protein and rice FEN1 and DNA polymerase δ have been isolated (Martínez-Macías et al. 2013; Kimura et al. 2003; Uchiyama et al. 2002). However, plant homologs of DNA polymerase β and DNA ligase III have not yet been reported.

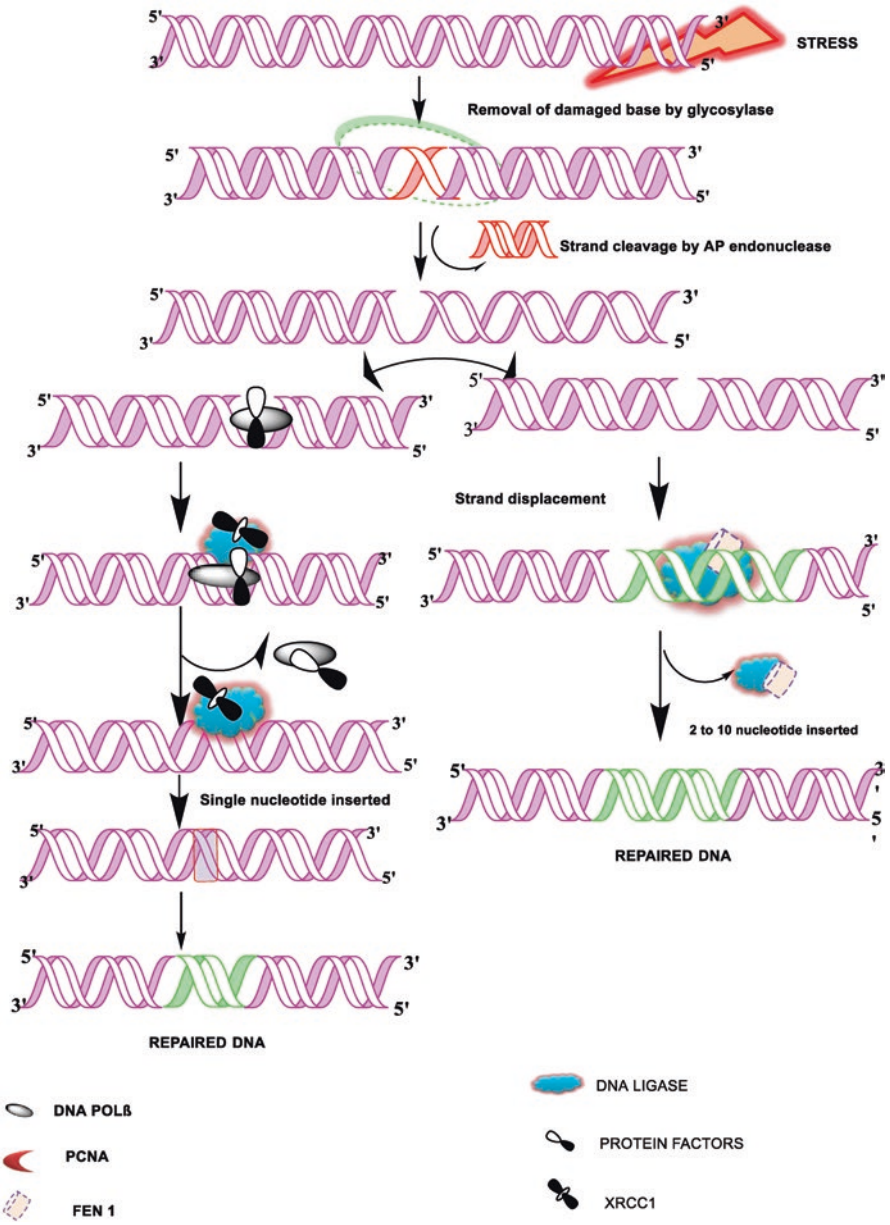

Fig. 7.3 Schematic representation of base excision repair pathway. Please refer to the text for explanation of the steps in the pathway

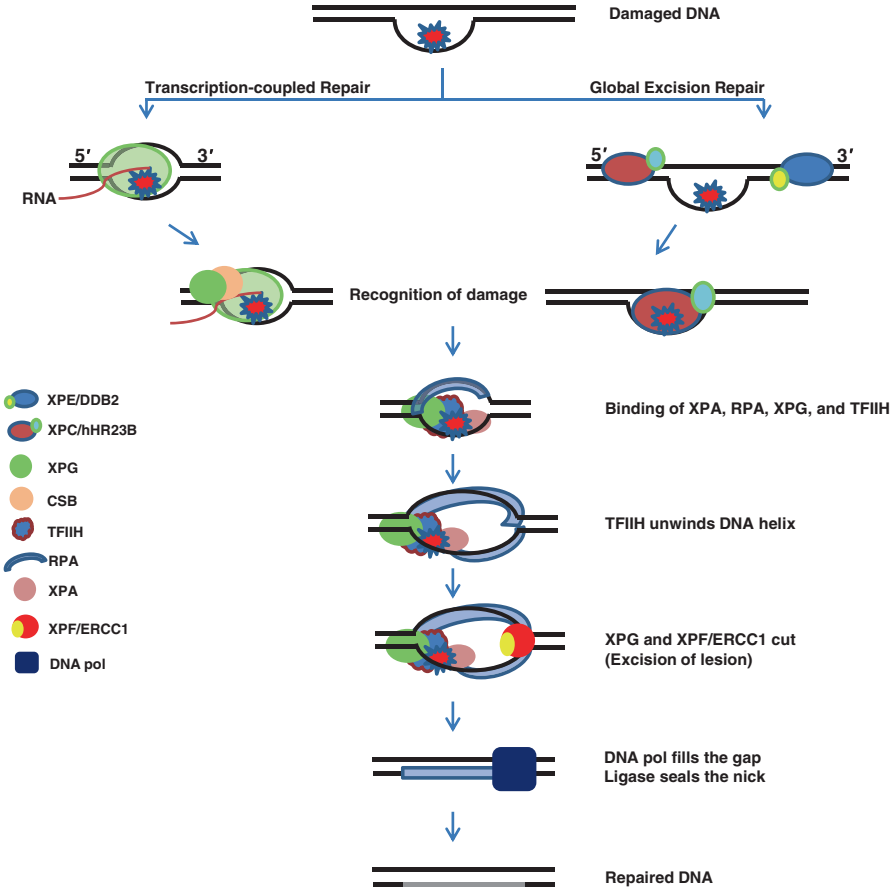

Fig. 7.4 Schematic representation of nucleotide excision repair. Please refer to the text for explanation of the steps in the pathway. (Adapted from Fuss and Cooper 2006)

Nucleotide Excision Repair

Lesions that cause large distortions in the helical structure of DNA are generally restored by nucleotide excision repair. The steps involved in this mechanism include recognition of the damaged site, opening of the double helix by the action of helicases and endonucleases, excision of a DNA segment carrying the lesion, and replacement of the segment using the template provided by the intact complementary strand followed by ligation of the nick (Costa et al. 2003; Fig. 7.4). The proteins involved in NER are sequentially assembled at the site of the lesion (Volker et al 2001). NER is classified into two distinct sub-pathways that differ in the DNA damage recognition: transcription coupled repair (TCR) and global genome repair (GGR). RNA polymerase II detects the lesion in TCR with the help of CSB and CSA. CSB has been demonstrated to alter DNA conformation and remodel

chromatin in an ATP dependent manner (Citterio et al. 2000). In GGR), XPC-hHR23B is the first factor to be involved in the lesion detection (Volker et al. 2001). In general, XPC-hHR23B complex is stabilized by hCEN2. Some other factors like XPE may aid in this recognition (Kusumoto et al. 2001). Subsequent to damage recognition other NER factors are recruited, which include TFIIH, XPD, XPA, XPB, RPA, XPF, and XPG (Evans et al. 1997). TFIIH is the basal transcription initiation factor that comprises nine different proteins (Winkler et al. 1998; Coin et al. 1999). XPD and XPB are ATP-dependent helicases that unwind the DNA helix in 5′–3′ and 3′–5′ direction, respectively (Schaeffer et al. 1994; Roy et al. 1994). The replication protein A (RPA) binds to DNA to provide complete opening of the helix around the lesion (Christians and Hanawalt 1993). XPA homodimer binds to RPA to form XPA2-RPA complex that provides proper three-dimensional orientation of the NER components required by excision of damage carrying DNA segment (Missura et al. 2001; Yang et al. 2002). With the DNA lesion recognized and helix around it unwound, XPG carries out structure-specific 3′-endonucleolytic activity (2–8 nucleotide away from the lesion) in conjunction with the 5′-endonucleolytic activity (15–24 nucleotide away from the lesion) of an XPF-ERCC1 complex. Thus, the lesion carrying segment of DNA is excised and is dissociated. Thereafter, the resultant gap is filled by DNA polymerase ε and δ using the 3′-end left by the XPF-ERCC1 incision as the primer (Hunting et al. 1991; Coverley et al. 1992). These polymerases require the presence of PCNA and RFC (Wood and Shivij 1997). Finally, the 5′-end of the newly synthesized DNA segment is sealed with the parent strand by DNA ligase I (Tomkinson and Levin 1997).

Most of the genes of NER pathway have been reported from *A. thaliana* (The *Arabidopsis* Genome Initiative 2000). Many genes with sequence similar to those of yeast and mammals have been cloned (Schultz and Quatrano 1997; Sturm and Lienhard 1998; Taylor et al. 1998; Costa et al. 2001; Liu et al. 2003; Dubest et al. 2004; Kimura et al. 2004). In addition, NER-related genes like PCNA, DNA polymerase δ, RPA, and CSB have been cloned from rice (Kimura and Sakaguchi 2006). The functional analysis of these genes and characterization of many mutants defective in some of them confirms the importance of NER in DNA damage repair in plants and the conservation of this pathway across the living kingdoms.

Conclusions

DNA damage response and repair pathways play a crucial role in maintenance of the integrity of genome and have been conserved to a great extent in various organisms. Many of these pathways have been thoroughly examined in mammals and yeast. Studies on plants have lagged behind in this regard. However, with the increasing genomic data available as a consequence of high-throughput sequencing efforts, homologs of different proteins involved in these pathways have been identified and functionally evaluated. Although most of the components are present, some crucial factors like p53, XRCC, DNA polymerase γ, DNA ligase III, etc. have been

conspicuously absent. Furthermore, some genes like CSB, RPA, PCNA, and FEN1 are present in multiple copies in plants. Some repair defects, for example, RAD50 knockout, which are lethal in animals, have been reported to have no physiological abnormalities in plants. Existence of novel plant-specific DDR components in plants has been alluded to in plants. The ongoing genetic, proteomic, and knock-out based strategies are expected to unveil novel DNA damage sensing and repair components in plants.

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Chapter 8
Plant Responses to Salinity Through an Antioxidative Metabolism and Proteomic Point of View

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Abstract Salt stress is one of the most damaging abiotic stresses because most crop plants are susceptible to salinity in different degrees. According to Food and Agriculture Organization of the United Nations (FAO), about 800 million Ha of land are affected by salinity around the world. In addition to the known components of osmotic stress and ion toxicity, salt stress is also manifested as an oxidative stress with all of these factors contributing to its deleterious effects. Although salinity-induced oxidative stress has been widely described, the effect of salinity on the antioxidative system and/or ROS generation in specific cell compartments has been less studied.

In recent years, high-throughput proteomic techniques have provided new ways to explore the complex network of plant salinity response in order to identify key elements for stress tolerance acquisition. However, from an overview of the available information about plant salinity responses it can be concluded that only a small number of the salt-inducible genes reported in the literature have been identified at the protein level. Most of the salt-responsive proteins identified in these studies correspond to the categories of amino acid metabolism, energy regulation, detoxification and redox regulation.

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The overexpression of genes encoding for different antioxidant enzymes is a common strategy to induce salt tolerance in crop plants. In this sense, the overexpression of H_2O_2-scavenging enzymes (APX, CAT), SOD, ASC-recycling enzymes or GSH-related enzymes resulted in increased salt tolerance in different plant species. In addition, some authors have used the co-expression of two or three genes encoding antioxidants to achieve salt tolerance in plants.

Introduction

The term "oxidative stress" is widely used in the scientific literature but it is rarely defined. Sies (1991) defined oxidative stress as a disturbance in the prooxidant/antioxidants balance in favour of the former, leading to a potential damaging (oxidative damage) to essential macromolecules and cell structures. The oxidative stress can result from an increased generation of reactive oxygen species (ROS) and/or an imbalance in the ROS-scavenging systems (antioxidant systems). In order to cope with ROS, plants have developed a complex arsenal of defences that include carotenoids, ascorbate, glutathione, tocopherols, anthocyanins and enzymes such as superoxide dismutase (SOD, EC1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), peroxidase (POX, EC. 1.11.1.7), as well as enzymes involved in the ascorbate-glutathione cycle (ASC-GSH cycle): ascorbate peroxidase (APX, EC 1.11.1.1), dehydroascorbate reductase (DHAR, EC1.8.5.1), monodehydroascorbate reductase (MDHAR, EC1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2) (Noctor and Foyer 1998).

Salt stress is one of the most damaging abiotic stresses affecting about 800 million Ha of land around the world (FAO). The physiological constraints imposed by salt stress include an osmotic stress but also an ion toxicity leading to a nutrient imbalance as well as a disruption of metabolism (Marschner 1995). Furthermore, and as previously reported, salt stress is also manifested as an oxidative stress at the subcellular level (Corpas et al. 1993; Hernández et al. 1993, 1995, 2001). All three factors can contribute to the negative effects produced by salinity in plants.

To cope with the deleterious effect of salinity, plants have evolved different physiological, biochemical and molecular mechanisms, including salt exclusion, control of ion uptake and translocation, ion compartmentation, synthesis of compatible solutes, morphological and anatomical modifications, membrane alteration, hormone profile changes and antioxidative metabolism responses (Hernández et al. 2000; Parida and Das 2005; Shabala and Munns 2012). For growth under saline conditions, plants have to achieve an osmotic adjustment in order to absorb water from the soil. The osmotic adjustment can be accomplished by the accumulation of inorganic ions and/or organic solutes (Ashraf 2004). In general, halophytes respond to salt stress by accumulating inorganic ions (particularly Na^+ and Cl^-). As a consequence of Na^+ and Cl^- accumulation, the absorption of other essentials ions, such as K^+ and Ca^{2+} is reduced. Both nutrients have a role in plant growth and development

as well as in the stomatal response, cellular turgor, cell wall and membrane stability, enzyme activation and cell signalling (Marschner 1995; Osakabe et al. 2014). On the other hand, glycophytes respond to salinity by partial salt exclusion as well as the biosynthesis of organic osmotic compounds (Ashraf 2004) such as nitrogen-containing compounds (proline, betaines and polyamines), alcohols (glycerol, mannitol, sorbitol, myo-inositol) and sugars (Kosová et al. 2013).

Salt-Induced Oxidative Stress at the Subcellular Level

It is well known that salt stress induces an oxidative stress at the subcellular level in salt-sensitive species (Hernández et al. 1993, 1995, Corpas et al. 1993; Gómez et al. 1999; Mittova et al. 2003a, 2004). However, few authors have studied the effect of salinity on the antioxidative system and/or the ROS generation in the different cell compartments. Most of these studies have been carried out in pea and tomato plants, but also other plant species such as wheat, poplar or the halophyte *Suaeda salsa* (See Tables 8.1 and 8.2) have been studied.

Mitochondria

In pioneering work, Hernández et al. (1993) described the effect of 70 mM NaCl on the activated oxygen metabolism in pea leaf mitochondria from two pea cultivars showing different sensitivity to salt stress, i.e. cv. Challis, susceptible to 70 mM NaCl, and cv. Granada, tolerant to the same NaCl level. In both pea cultivars NADH- and succinate-dependent superoxide ($O_2^{\bullet -}$) generation by sub-mitochondrial particles and lipid peroxidation in mitochondrial membranes were increased by the effect of salt stress, but changes were higher in the NaCl-sensitive pea cultivar than in the NaCl-tolerant cultivar. However, the latter displayed an increase in mitochondrial manganese-containing superoxide dismutase (Mn-SOD) isoenzyme, whereas a decrease in this enzyme occurred in mitochondria from the NaCl-sensitive pea cultivar (Hernández et al. 1993).

By using another NaCl-tolerant pea cultivar (cv. Puget), Gómez et al. (1999) studied the effect of different NaCl levels on the antioxidant systems of purified mitochondria. The Puget cultivar was tolerant to NaCl concentrations up to 90 mM, but it was susceptible to higher NaCl concentrations in terms of effect of NaCl of shots fresh and dry weight (Hernández et al. 1999). High salt stress (110 mM) increased mitochondrial Mn-SOD, ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR) activities, but a drop in monodehydroascorbate reductase (DHAR) activity was observed. Different oxidative stress biomarkers, such as H_2O_2, lipid peroxidation and carbonyl-proteins increased in purified mitochondria, confirming that in the presence of high NaCl levels (110 mM) the capacity to cope with the salt-induced generation of ROS was overcome in mitochondria (Gómez et al. 1999).

Table 8.1 Effect of salt stress on the activity of antioxidant enzymes in different cell compartments from salt-tolerant species

Plant species	NaCl treatment	Enzyme	Response	Cell compartment	References
Pisum sativum cv. Granada	70 mM	Mn-SOD	Increase	Leaf mitochondria	Hernández et al. 1993
Pisum sativum cv. Puget[a]	90 mM	APX, MDHAR, DHAR	Increase	Leaf mitochondria	Gómez et al., 1999
Lycopersicon pennellii	100 mM	Mn-SOD, APX, MDHAR, DHAR, GPX	Increase	Leaf mitochondria	Mittova et al. 2003a, 2003b
		GR	Decrease	Leaf mitochondria	Mittova et al. 2003a, 2003b
		Mn-SOD, APX	Increase	Root mitochondria	Mittova et al. 2004
		DHAR	Decrease	Root mitochondria	
Pisum sativum cv. Puget	90 mM	APX, DHAR, GR	Increase	Chloroplasts	Gómez et al., 1999
Pisum sativum cv. Granada	70 mM	Cu,Zn-SOD, APX	Increase	Chloroplasts	Hernández et al. 1995
Triticum durum Desf. cv. Ofanto	50 mM	stAPX, tAPX	Increase	Chloroplasts	Meneguzzo et al. 1998
		stMDHAR, stDHAR	Decrease	Chloroplasts	
Suaeda salsa	400 mM	tCu-Zn-SOD	Increase	Chloroplasts	Qiu-Fang et al. 2005
Pisum sativum cv. Puget	70–110 mM	tFe-SOD, sFe-SOD, tCuZn-SOD, sCuZn-SOD, sAPX, tAPX	Increase	Chloroplasts	Gómez et al., 2004
Pisum sativum cv. Granada	70 mM	CAT	Decrease	Leaf peroxisomes	Corpas et al. 1993
Lycopersicon pennellii	100 mM	SOD, APX, MDHAR, CAT	Increase	Leaf peroxisomes	Mittova et al. 2003a, 2003b
	100 mM	Mn-SOD, APX, MDHAR, CAT	Increase	Root peroxisomes	Mittova et al. 2004
Pisum sativum cv. Puget	90 mM	SOD	Increase	Leaf apoplast	Hernández et al. 2001
Triticum aestivam L. cv Gerek-79	250–500 mM	POX, SOD	Increase	Leaf apoplast	
Populus euphratica Oliv.	100–250 mM	SOD	Increase	Xylem sap	Wang et al. 2008
Brassica oleracea L.	80 mM	POX	Increase	Xylem sap	Fernández-García et al. 2011
Pisum sativum cv. Granada	70 mM	APX, MDHAR, DHAR, GR	Increase	Soluble fractions	Hernández et al. 2000

[a]cv Puget behaves as salt tolerant at NaCl concentration up to 90 mM

Table 8.2 Effect of salt stress on the activity of antioxidant enzymes in different cell compartment from salt-sensitive species

Plant species	NaCl treatment	Enzyme	Response	Cell compartment	References
Pisum sativum cv. Challis	70 mM	Mn-SOD	Decrease	Leaf mitochondria	Hernández et al. 1993
Pisum sativum cv. Puget[a]	110 mM	Mn-SOD, APX, MDHAR,	Increase	Leaf mitochondria	Gómez et al., 1999
		DHAR	Decrease		
Lycopersicon esculentum L.	100 mM	Mn-SOD, MDHAR, GR	Decrease	Leaf mitochondria	Mittova et al. 2003a, 2003b
		Mn-SOD, MDHAR	Decrease	Root mitochondria	Mittova et al. 2004
Pisum sativum cv. Puget	110 mM	Fe-SOD, Cu,Zn-SOD, APX, DHAR	Increase	Chloroplasts	Gómez et al., 1999
		MDHAR	Decrease		
Triticum durum Desf. cv. Adamello	50 mM	tAPX	Increase	Chloroplasts	Meneguzzo et al. 1998
		stAPX, stDHAR	Decrease	Chloroplasts	
Pisum sativum cv. Puget	70–110 mM	Fe-SOD, CuZn-SOD, APX, DHAR	Increase	Chloroplasts	Gómez et al., 2004
		MDHAR	Decrease		
Pisum sativum cv. Challis	70 mM	CAT	Decrease	Leaf peroxisomes	Corpas et al. 1993
Lycopersicon esculentum L.	100 mM	SOD, APX, MDHAR, DHAR	Decrease	Root peroxisomes	Mittova et al. 2004
Pisum sativum cv. Lincoln	90 mM	SOD	Decrease	Leaf apoplast	Hernández et al. 2001
		POX	Decrease		
Populus popularis cv. 35–44	100–250 mM	SOD, APX, GR	Increase	Xylem sap	Wang et al. 2008
Pisum sativum cv. Challis	70 mM	CuZn-SOD I	Decrease	Soluble fractions	Hernández et al. 2000
		DHAR	Increase		

[a]cv Puget behaves as salt sensitive at NaCl concentration over 110 mM

Table 8.3 Salt-tolerant transgenic plant species overexpressing different antioxidative enzymes

Plant species	NaCl (mM)[a]	Overexpressed gene(s)	Cell compartment	References
N. tabacum	300	APX	Chloroplast	Badawi et al. 2004a
N. tabacum	200–250	APX	Cytosol	Wang et al. 2005
N. tabacum	300	APX	Peroxisome	Li et al. 2009
A. thaliana	50–200	APX	Cytosol	Lu et al. 2007
A. thaliana	50–150	APX	Cytosol	Xu et al. 2008
P. domestica	100–150	APX	Cytosol	Díaz-Vivancos et al. 2013
N. tabacum	50–1000	CAT	ND	Al-Taweel et al. 2007
O. sativa	100–250	CAT	ND	Nagamiya et al. 2007
N. tabacum	300	MDHAR	Cytosol	Moriwaki et al. 2008
N. tabacum	300	MDHAR	Cytosol	Eltayeb et al. 2007
O. sativa	100–150	MDHAR	ND	Sultana et al. 2012
N. tabacum	100	DHAR	Chloroplast	Kwon et al. 2003
A. thaliana	100	DHAR	ND	Ushimaru et al. 2006
N. tabacum	200	DHAR	Chloroplast	Le Martret et al. 2011
S. lycopersicum	100	DHAR	Chloroplast, cytosol	Li et al. 2012
O. sativa	100	DHAR	ND	Kim et al. 2014
N. tabacum	100	GST	ND	Roxas et al. 2000
N. tabacum	250	GPX	Chloroplast, cytosol	Yoshimura et al. 2004
A. thaliana	50–150	GST	ND	Qi et al. 2010
N. tabacum	200	GST	Chloroplast	Le Martret et al. 2011
A. thaliana	50–150	Mn-SOD	ND	Wang et al. 2004
S. lycopersicum	200	Mn-SOD	ND	Wang et al. 2007
P. davidiana x P. bolleana	150	Mn-SOD	ND	Wang et al. 2010
O. sativa	100	Mn-SOD	ND	Chen et al. 2013
N. tabacum	300	Cu,Zn-SOD	Chloroplast	Badawi et al. 2004b
O. sativa	150	Cu,Zn-SOD	Cytosol	Prashanth et al. 2008
A. thaliana	50–150	Cu,Zn-SOD	Cytosol	Gill et al. 2010
P. domestica	100–150	Cu,Zn-SOD	Cytosol	Díaz-Vivancos et al. 2013
O. sativa	200	GST + CAT	ND	Zhao and Zhang 2006
B. rapa	50–200	Cu,Zn-SOD + CAT	Chloroplast	Tseng et al. 2007
N. tabacum	200	DHAR + GR	Chloroplast	Le Martret et al. 2011
N. tabacum	200	GST + GR	Chloroplast	Le Martret et al. 2011
G. hirsutum	200	SOD + CAT	Chloroplast	Luo et al. 2013
G. hirsutum	200	SOD + APX	Chloroplast	Luo et al. 2013
P. domestica	100	APX + Cu,Zn-SOD	Cytosol	Díaz-Vivancos et al. 2013
N. tabacum	100	Cu,Zn-SOD + APX + DHAR	Chloroplast	Lee et al. 2007

[a]NaCl treatment ranges show the lowest and highest concentrations tested
ND not defined

The effect of salt stress on antioxidative metabolism at the subcellular level was also studied in detail in leaves and roots from tomato plants (Mittova et al. 2002, 2003a, 2004). In these studies, the authors used a cultivated *Lycopersicon esculentum* (Lem) (now *Solanum lycopersicon*) as NaCl sensitive and its wild salt-tolerant relative *L. pennellii* (Lpa). As described in the earlier works carried out in pea plants, salt stress also induced an oxidative stress in mitochondria from the salt-sensitive tomato plants, as observed by increased H_2O_2 and lipid peroxidation levels (Mittova et al. 2003a), correlating with a drop in mitochondrial SOD, MDHAR and glutathione reductase (GR). However, this response was not observed in mitochondria from NaCl-tolerant plants, which resulted in an increase in mitochondrial SOD, APX, MDHAR, DHAR and glutathione-dependent peroxidase (GPX). These results were accompanied by a decrease in the redox state of ascorbate (ASC) and glutathione (GSH) in mitochondria from NaCl-sensitive plants, whereas an increase in these parameters was observed in NaCl-tolerant plants (Mittova et al. 2003a).

The information about the effect of salt stress on antioxidative metabolism as well as on ROS generation is very scarce in root mitochondria. Mittova et al. (2004) described a salt-induced oxidative stress in root mitochondria from NaCl-sensitive tomato as indicated by their increased H_2O_2 and lipid peroxidation levels. These authors attributed the increased H_2O_2 contents to the non-enzymatic reduction of superoxide anions by ASC and GSH rather than to its enzymatic generation. In fact, mitochondrial SOD activity dropped and the H_2O_2-scavenging enzymes remained at the same level than those observed in control plants. However, a decrease in these oxidative stress parameters was monitored in root mitochondria from NaCl-tolerant plants that correlated with the increase in SOD, APX and peroxidase (POX) activities as well as with increased ASC and GSH contents (Mittova et al. 2004).

Chloroplasts

It is known that salinity affects the photosynthetic process due to stomatal and non-stomatal limitations, including stomatal closure, a reduction in chlorophyll content, the inhibition of Calvin–Benson cycle enzymes¸ the alteration in the chlorophyll fluorescence parameters and the degradation of membrane-associated proteins in the photosynthetic apparatus suggesting inhibition of PSII electron transport (Parida and Das 2005; Stepien and Johnson 2009; Ikbal et al. 2014; Acosta-Motos et al. 2015a, b). The salt-induced stomatal closure can limit the CO_2 availability for the Calvin–Benson cycle, leading to a decrease of the final electron acceptor $NADP^+$, favouring the generation of ROS in the chloroplasts (Mehler reaction). The decrease in net photosynthesis and stomatal conductance resulting from short-term and long-term exposure to salinity has been reported by many authors. However, the reductions in these parameters have been found to be less marked in salt-tolerant than in salt-sensitive plants (Moradi and Ismail 2007). In the pea cultivars Challis and Granada, salt stress also produced an accumulation of H_2O_2 in chloroplasts, the increase being much higher in the salt-sensitive cultivar

than in tolerant plants (Hernández et al. 1995). As occurred in mitochondria, an increase of protective mechanisms against ROS generation took place in chloroplasts from NaCl-tolerant plants. In this sense, increases in ASC and APX and CuZn-SOD activity was described (Hernández et al. 1995). In a later work, using the NaCl-tolerant pea cultivar Puget, Gómez et al. (1999) studied the differential response of the SOD isozymes and the ASC-GSH cycle enzymes to increased NaCl levels in purified chloroplasts. In the presence of 110 mM NaCl, increases in chloroplastic Fe-SOD, CuZn-SOD II, APX and DHAR activities were recorded, but MDHAR decreased in the same conditions. Under severe salt stress, enhanced H_2O_2 and lipid peroxidation levels increased in chloroplasts, suggesting that the antioxidant machinery of chloroplasts cannot cope with the salt-induced generation of ROS (Gómez et al. 1999). A different response was observed in the two-recycling ASC enzymes in chloroplasts and mitochondria from cv. Puget, and this differential behaviour suggested that under saline conditions chloroplasts can reduce ASC via DHAR, whereas in mitochondria ASC recycling seems to be supported by MDHAR (Gómez et al. 1999).

The effect of salt stress on antioxidative metabolism was also studied in chloroplasts of tomato leaves from the above-mentioned cultivars (Mittova et al. 2002). The differential response to NaCl stress of the two tomato cultivars correlated with the activity of their chloroplastic antioxidant enzymes. The authors showed that salt-induced oxidative stress and damage was effectively alleviated in chloroplasts from NaCl-tolerant tomato by the selective up-regulation of different antioxidant enzymes (SOD, APX, GST, PHGPX, POXs). In addition, a reduction in lipooxygenase activity correlating with reduced levels of lipid peroxidation was observed (Mittova et al. 2002). In contrast, the NaCl-sensitive tomato failed to induce their chloroplastic antioxidant system. The authors concluded that the differential response of the chloroplastic antioxidant defences in both tomato plants (Lem and Lpa) can explain the increase in the oxidative stress parameters (H_2O_2 and lipid peroxidation) in NaCl-sensitive plants and its reduction in chloroplasts from NaCl-tolerant plants under saline conditions, and seems to be partially responsible for their overall salt-tolerance response (Mittova et al. 2002).

To cope with salt-induced oxidative stress not only is the induction of antioxidant mechanisms important, but also their intraorganellar location. For example, NaCl stress induced both stromal and thylakoidal Fe-SOD and CuZn-SOD isoenzymes in pea leaf chloroplast, but a different behaviour occurred for the chloroplastic APX isoenzymes: a significant increase in stromal APX took place in the presence of NaCl whereas a significant drop was reported for the thylakoidal APX (Gómez et al. 2004). By Western blot analysis, these authors observed that the increase in stromal and thylakoidal Fe-SOD activity was accompanied by enhanced protein content in both cases. It is known that Fe-SODs are H_2O_2-sensitive enzymes, and under NaCl stress H_2O_2 strongly increased in chloroplasts. However, it is probably that these Fe-SOD isoenzymes could present a partial resistance to H_2O_2. Increased thylakoidal Fe-SOD and CuZn-SOD can result in higher H_2O_2 contents near the thylakoidal membranes. In this situation, and due to the observed decrease in the ASC contents, the stromal APX activity could be not enough to remove the huge H_2O_2 increase

observed in chloroplasts under severe NaCl stress (Gómez et al. 2004). This response can produce an imbalance in the water–water cycle in pea chloroplast (Asada 1999), leading to an accumulation of H_2O_2 in the vicinity of the thylakoid membranes and diffusing away from to the stroma where it could be scavenged by the stromal APX, whose levels increased in the presence of 70 and 90 mM NaCl (Gómez et al. 2004). A similar response displayed by pea chloroplasts was reported for the stromal and thylacoidal APX isoenzymes in a NaCl-sensitive wheat cultivar (Meneguzzo et al. 1998). The increase in the thylakoidal-bound SOD and/or APX seem to be important to protect the photosynthetic machinery by directly scavenging the $O_2^{\bullet-}$ and H_2O_2 generated in the thylakoids (Sgherri et al. 2000). In the halophyte *S. salsa* L., NaCl stress (up to 400 mM) had no negative effect on plant growth, neither in net photosynthesis rate, nor in oxidative stress parameters, that correlated with a strong increase in chloroplastic Cu,Zn-SOD activity. Interestingly, most of the chloroplastic Cu,Zn-SOD activity corresponded to the thylakoidal enzyme, whose activity displayed a huge increase under high salinity conditions (Qiu-Fang et al. 2005).

Peroxisomes

As commented earlier the salt-induced stomatal closure can limit the CO_2 availability for the Calvin–Benson cycle, leading to a decrease of the final electron acceptor $NADP^+$, favouring the generation of ROS in the chloroplasts. Under this situation, photorespiration can prevent photo-oxidative damage by continuously recycling CO_2 for the chloroplast from the decarboxylation of glycine in the mitochondria and dissipates excess reducing equivalents (NADH, ATP), thereby preventing over-reduction of the photosynthetic electron chain (Fig. 8.1). However, photorespiration can lead to a high generation of H_2O_2 by the oxidation of glycolate by glycolate oxidase (Fig 8.1). If the generation of H_2O_2 is not controlled by the peroxisomal antioxidant mechanisms (catalase, ASC-GSH cycle, Jiménez et al. 1997), a NaCl-induced oxidative stress can be induced in the peroxisomes.

Information regarding the effect of NaCl on the peroxisomal metabolism is very scarce. The first work regarding the effect of NaCl stress on peroxisomal metabolism was carried out by the Del Rio team (Corpas et al. 1993). These authors also studied the effect of salt stress on the metabolism of activated oxygen and two key enzymes from the photorespiratory pathway (Fig. 8.1). In response to salinity, an increase in glycolate oxidase activity was observed in peroxisomes from NaCl-tolerant pea plants, whereas a decrease in hydroxypyruvate reductase was observed in peroxisomes from the NaCl-sensitive pea cultivar, suggesting the involvement of photorespiration in the response to salt stress (Corpas et al. 1993). Catalase (CAT) activity was significantly reduced by the effect of salt stress in both pea cultivars, whereas Mn-SOD activity did not exhibit any change (Corpas et al. 1993). In contrast to purified chloroplast, no ROS accumulation was observed in pea leaf peroxisomes. In addition, in spite of the decrease in CAT activity (an H_2O_2-scavenging enzyme) and the increase in glycolate oxidase (an H_2O_2-

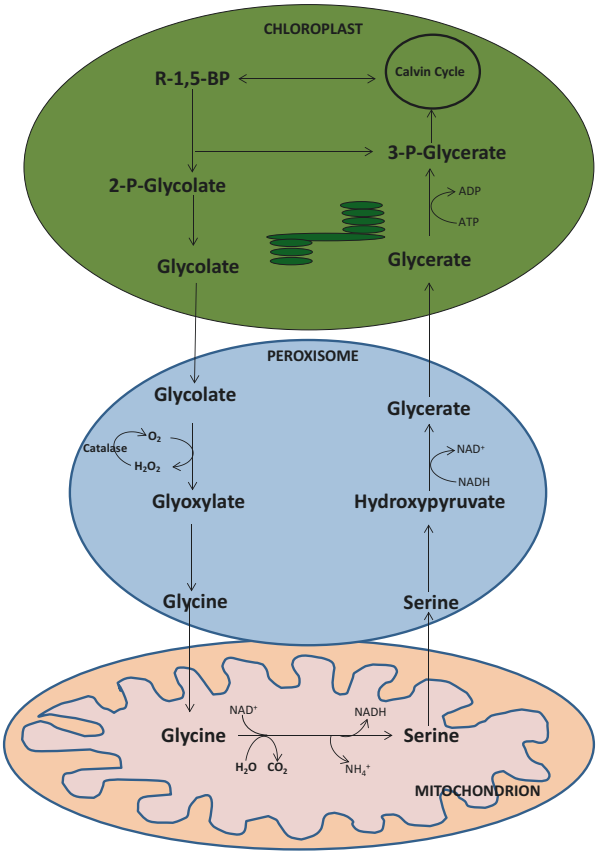

Fig. 8.1 Reactions of the photorespiration (or oxidative photosynthetic carbon C2) pathway that involves chloroplasts, peroxisomes and mitochondria. In order to simplify the picture, we obviate the transamination reactions to re-assimilate the molecule of ammonia released in the mitochondria. Modified from Taiz and Zeiger (2010)

producing enzyme) in peroxisomes, no H_2O_2 accumulation was observed. The lack of H_2O_2 accumulation in these cell compartments was explained by the possibility of diffusion out into the cytosol, inducing an oxidative stress also in this cell compartment (Hernández et al. 1993; Corpas et al. 1993). Another possibility suggested by Corpas et al. (1993) was that H_2O_2 could undergo a non-enzymatic reaction inside the peroxisome with some NaCl-induced metabolite such as glyoxylate. Moreover, the glycolate oxidase segment of photorespiration could be a molecular mechanism of NaCl tolerance (Corpas et al. 1993). In fact, one of the functions of photorespiration is to prevent photo-oxidative damage by continuously recycling CO_2 for the chloroplast from the decarboxylation of glycine in the mitochondria. In addition, glycerate, produced inside the peroxisome, can be imported into the chloroplast and enter the Calvin cycle. These mechanisms keep

the Calvin cycle working and prevent ROS generation in the electron transport chain, providing substrates for the chloroplast and decreasing the risk of photoinhibition under environmental stress conditions. This pathway also dissipates excess reducing equivalents (NADH), thereby preventing over-reduction of the photosynthetic electron chain (Halliwell and Gutteridge 2000).

As observed in pea peroxisomes, H_2O_2 levels were unchanged in tomato leaf peroxisomes, but in contrast salt stress increased lipid peroxidation in peroxisomes from tomato plants (Mittova et al. 2003a), although the increase was much higher in NaCl-sensitive plants (Lem) than in its wild salt-tolerant species *L. pennellii* (Lpa). However, a different response was observed in peroxisomes from both tomato cultivars. In this sense, NaCl stress induced SOD, APX, MDHAR and CAT in NaCl-tolerant plants, but reduced MDHAR, GR and CAT in salt-sensitive plants. This differential response was reflected in a drop in the redox state of ASC and GSH in the latter cultivar, whereas in peroxisomes from NaCl-tolerant plants, the redox state of ascorbate increased whereas the GSH/GSSG ratio was unchanged (Mittova et al. 2003a).

The effect of salt stress on the antioxidative metabolism of root peroxisomes was also studied in the same tomato plants (Mittova et al. 2004). In NaCl-sensitive plants no evidence of oxidative stress was observed under salt stress, but a reduction in peroxisomal SOD, APX, MDHAR and DHAR activities were recorded. Nevertheless, in the same conditions, NaCl-tolerant plants displayed lower H_2O_2 and lipid peroxidation levels, which correlated with increased peroxisomal SOD, APX, MDHAR and CAT activities (Mittova et al. 2004). According to these authors this response (decreased H_2O_2 levels under stress conditions) was partially as a result of the increases in H_2O_2-scavenging enzymes (APX and CAT) over the increase in SOD (an H_2O_2-producing enzyme).

Under salinity conditions, plant peroxisomes, in addition to the over-generation of ROS, are the main source of NO production (Corpas et al. 2009). These authors observed that under salinity conditions (100 mM NaCl) peroxisomal NO production increased nearly fivefold in roots. They also demonstrated that peroxisomes are required for NO accumulation in the cytosol, thereby participating in the production of peroxynitrite ($ONOO^-$), resulting in a nitrosative stress, indicated by an increase in protein Tyr nitration in salt-stressed plants (Valderrama et al. 2007; Corpas et al. 2009).

Changes Induced by NaCl on the Apoplastic Space

There is scarce information about the nature of the proteins present in the apoplastic space, and their responsiveness to environmental constraints, especially salt stress. Hernández et al. (2001) described for the first time the changes that occurred in the leaf apoplastic antioxidant defences in response to NaCl stress (90 mM) in two pea cultivars, cv. Lincoln, NaCl sensitive, and cv. Puget, tolerant to 90 mM NaCl. These authors demonstrated the presence of SOD, POX, ASC and GSH, in the leaf

apoplastic space. However, APX, MDHAR and GR seemed to be absent (Hernández et al. 2001). A NaCl-dependent increase in some oxidative stress parameters (H_2O_2, lipid peroxidation and CO proteins) was observed in the apoplastic space in both cultivars, but they were much higher in the NaCl-sensitive cultivar than in the NaCl-tolerant cultivar. This response correlated with higher ASC and GSH contents as well as SOD activity in the apoplastic space from the salt-tolerant cultivar (Hernández et al. 2001). The treatment of cv. Lincoln plants with 90 mM NaCl produced the appearance of necrotic leaf lesions localized initially on minor veins. The NaCl-oxidative stress was also observed as highly localized areas of ROS accumulation ($O_2^{\bullet-}$ and H_2O_2) in the same areas. However, ROS generation was less evident in the NaCl-tolerant cultivar (cv. Puget) (Hernández et al. 2001).

In a more recent work, Wang et al. (2008) studied the effect of increasing soil NaCl concentration on intracellular salt compartmentation and the antioxidant metabolism in xylem sap from two poplar species: the salt-tolerant *Populus euphratica* and the salt-sensitive *P. popularis* cv. 35–44. A difference between species was observed regarding ROS production in xylem sap. The production rates of $O_2^{\bullet-}$ and H_2O_2 in xylem sap from *P. euphratica* remained at control levels during the salt treatment. In contrast, both ROS contents significantly increased in the xylem sap of the NaCl-sensitive plants after 13 days of salt treatment. In this paper, the authors detected SOD, APX and GR activity in xylem sap from both species. In response to NaCl stress and in correlation with the ROS generation, a huge increase in SOD, APX and GR occurred in the xylem sap from NaCl-sensitive poplar plants, whereas in the salt-tolerant poplar, only an increase in SOD was observed. However, in spite of the dramatic increase in antioxidant enzymes in xylem sap from NaCl-sensitive poplar plants, salt stress caused an oxidative damage and leaf injury because of the low capacity of these plants for NaCl exclusion and vacuolar compartmentation in mesophyll cells (Wang et al. 2008).

The effect of NaCl on the activity of apoplastic antioxidant enzymes was also reported in leaves from two wheat cultivars, the salt-tolerant Gerek-79 and the salt-sensitive Bezostaya (Mutlu et al. 2009). These authors detected SOD, POX and surprisingly CAT activity in the apoplastic space of wheat leaves. However, no data concerning the potential of contamination of the apoplastic extract by symplastic component was provided. For this reason, the presence of CAT in the apoplastic fluid has to be taken with caution. In pea plants, Hernández et al. (2001) calculated the contamination of apoplastic extracts, which ranged from 0.86 to 1.03 % in control plants and from 1.54 to 2.38 % in 90 mM NacCl-treated plants. In the tolerant wheat plants, a NaCl-dependent increase was recorded for CAT, POX and SOD. In the sensitive cultivar also a salt-dependent increase in CAT was observed. However, POX activity dropped with the NaCl treatment whereas SOD decreased in the apoplastic extract from plants subjected to 250 mM NaCl but increased in the presence of 500 mM, although this response was less intense to that observed in the NaCl-tolerant wheat cultivar. These authors concluded that apoplastic CAT, POX and SOD play an important role in ROS scavenging in the apoplastic space and can be partly related to salt-induced oxidative stress tolerance in wheat (Mutlu et al. 2009).

In the xylem sap from *Brassica oleracea*, short-term NaCl stress induced a threefold reduction in H_2O_2 that remained at a lower concentration following long-term (7 days) salt stress, this response being paralleled with a dramatic increase in POX activity (Fernández-García et al. 2011). These authors detected glutathione in the xylem sap from *B. oleracea* plants, but the levels of both oxidized and reduced glutathione were very low in untreated plants. However, short-term NaCl treatment strongly increased the concentration of both reduced GSH and GSSG and their contents substantially decreased after 7 days of stress. Nevertheless, the GSH levels remained significantly higher in the xylem sap from salt-treated plants, leading to increased redox state of glutathione in salinized plants (Fernández-García et al. 2011).

Cytosol

The response of pea plants to NaCl challenge was examined in the cytosolic fraction. In this case, the effect of salinity on the ASC-GSH cycle enzymes and the non-enzymatic antioxidants ASC and GSH was also studied. In soluble fractions, enriched in cytosolic components, NaCl-tolerant plants again showed a better performance and increases in APX, MDHAR, DHAR and GR were produced in salinized plants. In contrast, NaCl-sensitive plants showed decreased CuZn-SOD I activity and no important changes in the ASC-GSH cycle enzymes. Total ASC and GSH contents decreased in both pea cultivars, but the decline was much higher in NaCl-sensitive plants (Hernández et al. 2000). Curiously, the basal levels of most of the enzymatic and non-enzymatic antioxidants examined in these studies were much higher in the NaCl-sensitive than in the NaCl-tolerant pea plants. Nevertheless, under salt stress conditions the situation was somewhat different and NaCl-tolerant plants induced enzymatic defences under NaCl stress. Interestingly, only the NaCl-tolerant plants exhibited an increase in the expression levels of mRNA encoding for some of the enzymes of antioxidative metabolism. In this sense, the transcript levels for mitochondrial Mn-SOD, chloroplastic Cu,Zn-SOD and phospholipid hydroperoxide glutathione peroxidase (PHGPX), cytosolic GR and APX were strongly induced only in the salt-tolerant pea cultivar, suggesting that the induction of antioxidant defences can be one component of the tolerance mechanism to long-term salt stress (Hernández et al. 2000).

The information about the effect of salinity on the ascorbate and glutathione contents at subcellular levels is very scarce. In a recent paper, Koffler et al. (2015) described the compartment-specific importance of the non-enzymatic antioxidants ascorbate and glutathione in the response to salt stress in Arabidopsis Col-0 (wild-type plants) and mutants *vtc2-1* (deficient in ascorbate) and *pad2-1* (deficient in glutathione). In this work, the authors described a higher NaCl tolerance in the wild-type plants that correlated with a strong increase in glutathione contents in mitochondria, chloroplasts and peroxisomes. The *pad2-1* mutant also showed a higher tolerance to salt stress than the ascorbate-deficient mutant, and this response was

also correlated with an increase in glutathione in all cell compartments, especially in the nuclei (up to 740 %). Conversely, the *vtc2-1* mutant showed a high sensitivity to NaCl stress and this response was parallel with a lower basal level of ascorbate as well as a decrease in ascorbate content in mitochondria, peroxisomes and cytosol (Koffler et al. 2015). As mentioned, salt stress strongly increased glutathione levels in the nuclei of the *pad2-1* mutants, but also a strong increase in the ascorbate content occurs in the nuclei of the *vtc2-1* mutants (up to 104 %) (Koffler et al. 2015). These authors suggested that the accumulation of ascorbate and/or glutathione in the nuclei could play an important role in the protection of DNA and redox-sensitive nuclear proteins from oxidation.

Emerging Trends in the Proteomics of the Salt Stress Response in Plants

In recent years, high-throughput proteomic techniques have provided new ways to explore the complex network of plant salinity response and to identify key elements for stress tolerance acquisition. Several authors have reported noticeable changes in protein abundance in response to salt stress, including citrus (Tanou et al. 2009), rice (Yan et al. 2005; Parker et al. 2006; Wang et al. 2008), soybean (Aghaei et al. 2009) or Arabidopsis (Jiang et al. 2007). However, from an overview of the available information regarding plant salinity responses, it can be concluded that only a minor number of the salt-inducible genes reported in the literature have been identified at the protein level. Most of the salt-responsive proteins identified in these studies correspond to the category of amino acid metabolism, energy regulation, detoxification and redox regulation. In these proteomic studies, the identified proteins are often subjected to hierarchical clustering to group proteins showing similar expression profiles. Understanding how the relative abundance of the different protein isoforms and the post-translational modifications of the proteins (PTMs) configure the salt tolerance mechanisms is a major challenge and will be a focus of salt stress research in the coming years. Along the following lines an overview of the latest advances in this field will be summarized.

Phosphoprotein Analysis During Salt Stress

Post-translational modifications (PTMs) are crucial mechanisms used by plants to modify their protein functions and thereby to face stressful environmental factors. Beyond identification of proteins, characterization of PTMs increases our understanding of the salt stress response and shed light to its underlying regulatory mechanisms. Among the different PTMs, phosphorylation is a key reversible modification regulating subcellular modification, protein function and cellular signalling (Mazzucotelli et al. 2008). With the latest advances of MS and MS-based

phosphopeptide enrichment techniques, high-throughput phosphoproteomic analysis has become ideal for the study of the dynamics of signalling networks. In a recent work, Hu et al. (2013) performed a comparative analysis of salt-responsive phosphoproteins in maize leaves using Ti(4+)--IMAC enrichment; they found 124 phosphopeptides from 92 phosphoproteins. Among them 72 were salt stress response proteins, and a further 35 had not previously been reported as responsive to salt in the literature. Gene ontology analyses revealed that cell signalling pathway members such as 14-3-3 and calmodulin proteins were overexpressed in response to salt stress. Moreover, multiple putative salt-responsive phosphoproteins were photosynthesis related.

During salt stress many kinase-mediated phosphorylation pathways become activated. Calcium-dependent protein kinases, mitogen-activated protein kinases (MAPK) and SNF1-related protein kinases among others are reported to regulate ABA signalling and the response to salt stress (Ouyang et al. 2010). In this sense, the Arabidopsis AtMKK2 has been identified as a key regulator of the cold- and salt-stress response (Teige et al. 2004). Plant SnRK2 kinase family is a core component of ABA signal transduction pathway and hyperosmotic stress responses. Nam et al. (2012) explored SnRK2 kinase function in salt response of rice roots by comparative proteomic analysis of OSRK1 transgenic rice. According to their results several metabolic pathways were constitutively activated by overexpression of OSRK1. Moreover, many proteins differentially expressed in OSRK1 transgenic rice shared homology to identified phosphoproteins, containing consensus SnRK2 phosphorylation sites.

Another strategy to detect phosphoproteins is the use of specific phosphoprotein staining following separation on 2-DE gel. By using this approach, Chitteti and Peng (2007) reported 31 salt stress differentially regulated proteins in rice root proteome; interestingly, many of these proteins did not change quantitatively, but changed in their phosphorylation status. In this sense, Jiang et al. (2007) reported 215 varying protein spots in salt-stressed Arabidopsis roots and found low correlation between protein expression and previous microarray results, which also emphasizes the need for combined transcriptomic and proteomic analyses and the importance of post-transcriptional regulation.

Crosstalk Between Reactive Oxygen Species Signalling and Salt Stress Protein Response

A common response to biotic and abiotic stresses is the generation and accumulation of ROS. Besides their harmful effects, ROS and in particular hydrogen peroxide (H_2O_2) may have a dual role in plant stress response; high concentrations of H_2O_2 are deleterious for the cell function whereas low concentrations may act as signal molecules upon a variety of biotic and abiotic stresses (Dat et al. 2000). A triggered accumulation of ROS has been directly correlated with induced cellular

changes upon salt stress (Hasegawa et al. 2000; Miller et al. 2010). Tanou et al. (2009) showed that pre-treatment of citrus plants with H_2O_2 enhanced the capacity of these plants to overcome salt stress, by a specific proteome reprogramming, that reduced the deleterious physiological effects of this stress, avoiding the accumulation of a large number of NaCl-responsive proteins. Witzel et al. (2009) studied the root proteome from two barley genotypes with contrasting levels of salinity tolerance. Their results emphasize the role of proteins involved in ROS detoxification during salinity stress, and identified potential candidates for increasing salt tolerance, including POX, glutathione-S-transferase (GST), lactoylglutathione lyase, SAM synthase and a carboxymethylenebutenolidase-like protein. In this sense, Song et al. (2011) reported a variety of responsive proteins involved in ROS detoxification in the apoplast of salt-stressed rice shoots; among them several peroxidases with roles in cell wall modifications were identified.

Other authors pointed out a protective role of nitric oxide (NO) against salt stress in maize at the proteomic level (Bai et al. 2011). In this work the authors described a G-protein signalling cascade leading to the formation of NO and subsequently to specific changes in the protein patterns and antioxidant enzymes activities, in addition to activation of defence proteins, energy metabolism and cell structure/division in salt-treated maize seedlings. Moreover, salt-stressed barley roots grown in hydroponics showed increased expression of proteins involved in ROS detoxification during salinity stress, including glutathione-based ROS scavenging proteins (Witzel et al. 2009).

An increasingly relevant ROS-dependent protein modification is protein carbonylation; this irreversible protein oxidation leads to the loss of protein function and finally to degradation processes of the oxidized proteins. The reaction between carbonyl groups and 2,4-dinitrophenylhydrazine (DNPH) results in the formation of hydrazones, which can be detected spectrophotometrically, or by the use of an antibody against DNPH-derivatized protein followed by immunoblotting and mass spectrometry. Consequently, several authors have used this redox approach in order to identify the carbonylated proteins under salinity conditions. Tanou et al. (2009, 2012) described an alleviation of salinity-induced protein carbonylation in citrus plants pre-treated with either H_2O_2 or sodium nitroprusside (SNP, a NO-releasing chemical), and the stimulation of S-nitrosylation. In addition, these authors showed that the accumulation of NaCl-responsive proteins is greatly reduced by H_2O_2 and SNP pre-treatments. Overall their results suggest an overlap between NO- and H_2O_2-signaling pathways in acclimation to salinity and highlight protein carbonylation as a key mechanism by which both •NO and H_2O_2 act as priming agents minimizing salt stress. In agreement with this, Hoque et al. (2008) reported a protective effect of both proline and glycinebetaine against NaCl-induced oxidative damage by reducing protein carbonylation, and triggering antioxidative metabolism. Conversely, Bolu and Polle (2004) determined a decrease in protein carbonylation contents in roots and shoots of a salt-sensitive poplar species under salinity conditions, finding highest concentrations in controls, intermediate in roots of low salinity-exposed plants and lowest in plants exposed to high salinity. The authors explain this unexpected result as a consequence of the increased protein degradation in the salt-stressed plants (Bolu and Polle 2004).

The Apoplast Proteome in Response to Salt Stress

The plant cell apoplast, which consists of the cell wall and the intercellular space, is involved in a variety of functions during plant growth and development as well as in plant defence responses to stress conditions (Pennell 1998). To test the role of apoplastic proteins in the initial phase of salt stress, 2-DE approaches have been used to identify apoplastic salt response proteins; Ramanjulu et al. (1999) identified increased apoplast protein content in response to salinity. Remarkably, many of the stress-induced proteins expressed under salinity conditions appear to be related to abiotic stress resistance in the tobacco leaf apoplast (Dani et al. 2005; Guo and Song 2009) and rice roots (Zhang et al. 2009). Moreover, Song et al. (2011) reported an increase in ROS detoxification proteins in the apoplast of salt-stressed rice shoots, including several peroxidases, indicating that plant apoplastic proteins may have an important role in salt stress response signal pathway. Other authors demonstrated an alleviation of salt stress by the expression of a stress-inducible RD22-like apoplastic protein from soybean, which belong to a protein family putatively associated with stress responses (Wang et al. 2012). Despite these promising findings, the involvement of the apoplast and apoplastic proteins in the perception of salt stress and thereby in the activation of a stress response is largely unrevealed and further research should address this question; in this sense the attention might be focused on the overlapping protein responses among salt stress and other abiotic stresses (Pennell 1998; Sierla et al. 2013; Xu and Brosché 2014).

Genetically Modified Antioxidant Machinery to Induce Salt Stress Tolerance

It has been traditionally accepted that developing a more efficient ROS scavenging capacity/system is a common strategy to induce salt tolerance in many plant species; some examples are maize (Azevedo-Neto et al. 2006), pea (Hernández et al. 2000) and tomato (Mittova et al. 2003b). In addition, in salt-sensitive plants the observed imbalance between antioxidant defences and increased ROS levels had been correlated with oxidative stress-induced damage (Foyer and Noctor 2000; Hernández et al. 2001; Ikbal et al. 2014). From this point of view, there are authors that correlated salt tolerance with a higher constitutive levels of some antioxidant enzymes (Gueta-Dahan et al. 1997; Hernandez et al. 2002; López-Climent et al. 2008), whereas other authors described a coordinated up-regulation of the antioxidative machinery as one of the mechanisms involved in the salt tolerance response (Gómez et al. 1999; Hernández et al. 2000, 2001; Mittova et al. 2003b). In spite of this traditional point of view, contradictory results have been also obtained, and increases in antioxidant enzymes do not always guarantee tolerance to a given stress, as an example, salt-sensitive loquat plants also displayed an increased APX, MDHAR and DHAR activities when exposed to salt stress (Hernandez et al. 2002).

Alleviation of salt-induced oxidative damage using genes encoding antioxidant enzymes has been widely used in the development of transgenic plants. Here we present a brief summary of the research carried out about this topic for the main antioxidant enzymes.

H_2O_2 Scavenging Enzymes (APX and CAT)

H_2O_2 production is pivotal for protecting cells from stress-induced oxidative damage. Most of the works correlating the overexpression of APX and CAT activities with salt stress tolerance suggest that the tight control of the H_2O_2 production displayed by transgenic plants is one of the main factors responsible of this response. It has been described that the overexpression of APX in chloroplasts of tobacco (*Nicotiana tabacum*) plants induced salt stress tolerance, as well as enhanced tolerance to other stress situations such as water deficit or exposure to paraquat (Badawi et al. 2004a). The overexpression of a cytosolic APX gene from pea (cyt*apx*) also minimized the deleterious effect of salt exposure (200 or 250 mM NaCl) in tobacco plants (Wang et al. 2005). More recently, transgenic tobacco plants overexpressing a *Populus* peroxisomal APX gene showed an increased root growth, when compared to non-transformed plants, which was associated with enhanced salt stress tolerance (Li et al. 2009). Moreover, these plants also exhibit tolerance to other abiotic stresses such as methyl viologen (MV) and drought (Li et al. 2009).

The salt-stress tolerance induced by APX has been also described in other plant species. For example, Arabidopsis plants overexpressing two rice cytosolic APXs genes (OsAPXa or OsAPXb) also exhibited increased salt tolerance, with the OsAPXb gene playing a more functional role in salt-stress tolerance in transgenic plants (Lu et al. 2007). Other authors also reported a salt tolerance response in transgenic Arabidopsis plants carrying a peroxisomal APX gene from barley (*Hordeum vulgare* L.) (Xu et al. 2008). Using a similar approach to that described by Wang et al. (2005), in vitro transgenic plum plants harbouring cyt*apx* from pea also exhibited tolerance to 100 and 150 mM NaCl (Diaz-Vivancos et al. 2013). In addition, these transgenic plum plantlets showed higher levels of non-enzymatic antioxidants (GSH and ASC) under control and salt condition than non-transformed plants (Diaz-Vivancos et al. 2013).

Regarding CAT activity, an increased salt stress tolerance has been described in tobacco plants and in two rice cultivars harbouring a gene coding for a catalase (katE) gene from *Escherichia coli* (Al-Taweel et al. 2007; Nagamiya et al. 2007; Moriwaki et al. 2008). In the case of rice plants, these transgenic plants were able to grow in the presence of 250 mM NaCl and complete the growth cycle to seed production in the presence of 100 mM NaCl (Nagamiya et al. 2007).

Ascorbate Recycling Enzymes (MDHAR and DHAR)

Ascorbic acid (ASC) is the main antioxidant in plants and it functions as a major redox buffer and as a cofactor for enzymes. It is involved in many pathways such as photosynthesis, hormone biosynthesis, regeneration of other antioxidants, defence responses, cell division and growth, and signal transduction (Gallie 2013). An important role for ASC in determining the level of tolerance to many environmental stresses has been suggested, including chilling, drought, salt and exposure to heavy metals (Gallie 2013), with its recycling to the reactive reduced form crucial for those stress responses.

Under salt stress condition, transgenic tobacco plants overexpressing *A. thaliana* MDHAR gene (AtMDAR1) in the cytosol showed higher MDHAR activity and reduced ASC levels as well as lower H_2O_2 levels than non-transformed control (Eltayeb et al. 2007). These transgenic tobacco plants exhibited an enhanced salt stress tolerance as well as enhanced tolerance to other abiotic stresses such as ozone and osmotic stress. In addition, the expression of MDHAR from acerola (*Malpighia glabra*) in tobacco plants conferred tolerance to 300 mM NaCl and minimized the oxidative stress (measured as a reduced lipid peroxidation and chlorophyll degradation) induced by the salt treatment (Eltelib et al. 2012). Other authors have reported that transgenic rice lines overexpressing a MDHAR from the mangrove plant *Acanthus ebracteatus* displayed better yield attributes than non-transformed rice plants (Sultana et al. 2012).

Regarding DHAR activity, transgenic tobacco seedlings, expressing a human DHAR gene in chloroplasts, were more tolerant to NaCl and to other abiotic stresses than non-transformed plants (Kwon et al. 2003). Ushimaru et al. (2006) described that the expression of a DHAR gene from rice enhanced salt tolerance (100 mM NaCl) in transgenic Arabidopsis plants. The overexpression of DHAR in chloroplasts of transgenic tobacco plants confers tolerance to 200 mM NaCl (Le Martret et al. 2011). In tomato plants the overexpression of potato cytosolic DHAR (*DHAR1*) or chloroplastic DHAR (*DHAR2*) led to salt stress and MV tolerance (Li et al. 2012). These transgenic tomato plants exhibited higher germination rate, plant fresh weight, seedling length and chlorophyll content than non-transformed plants under salt stress conditions (Li et al. 2012). More recently, it has been described that rice plants overexpressing the *OsDHAR1* gene are more tolerant to salt stress than non-transformed plants (Kim et al. 2014). These authors reported also increases in MDHAR, GR and APX (ASC-GSH cycle related enzymes) activities and high levels of reduced ASC in transgenic rice plants, suggesting that the adaptation of rice plants to salt stress is strongly related to the ASC pool and redox homoeostasis (Kim et al. 2014).

Glutathione-Related Enzymes (GR, GST and GPOX)

The complex antioxidant network of plant cells has the thiol tripeptide GSH at its centre to buffer ROS and facilitate cellular redox signalling, which controls growth, development and environmental stress responses (Diaz-Vivancos et al. 2010). Thus, GSH recycling is an important factor in terms of stress tolerance, and changes in GR activity have been positively associated with salt stress tolerance. In this sense, transgenic tobacco plants showing a decreased GR activity displayed an enhanced sensitivity to oxidative stress (Ding et al. 2009). However, in spite of the widely described changes in GR activity and expression in salt-treated plants, there is no information available regarding salt stress and GR-overexpressing plants.

GSTs have been traditionally associated with the metabolism of xenobiotics and secondary products via the formation of glutathione conjugates, thus protecting cells against oxidative stress (Edwards et al. 2000). GPXs are a family of enzymes that reduce hydroperoxides to the corresponding hydroxyl compounds using GSH, and they are considered to be the main enzymatic defence against oxidative membrane damage (Yoshimura et al. 2004). Roxas et al. (2000) reported that transgenic tobacco plants overexpressing a gene (Nt107) encoding an enzyme with both GST and GPX activity showed enhanced seedling growth under salt stress conditions, among other stressful conditions. Interestingly, these transgenic plants displayed high levels of MDHAR, ASC and GSH that were related with a reduced oxidative damage under stress conditions (Roxas et al. 2000). Moreover, the overexpression of a GPX from Chlamydomonas in the cytosol or chloroplast of tobacco plants led to an increased tolerance to 250 mM NaCl, as well as to MV and chilling stress, due to low malondialdehyde (MDA) content (biomarker of lipid peroxidation) and enhanced antioxidative system (Yoshimura et al. 2004). Arabidopsis transgenic plants harbouring a GST from *S. salsa* showed higher GST and GPX activities than non-transformed plants, this response being correlated with an enhanced Arabidopsis seedling salt tolerance (Qi et al. 2010). In addition, transgenic tobacco plants expressing GST in chloroplasts displayed tolerance to 200 mM NaCl (Le Martret et al. 2011).

However, other authors have reported a negative response in terms of salt stress tolerance in Nt107 overexpressing plants (Light et al. 2005). These authors reported that transgenic cotton (*Gossypium hirsutum* L.) plants expressing the tobacco Nt107 gene exhibited high GST whereas GPOX activity did not change under salt stress conditions, this response being insufficient to provide protection against salt-induced oxidative stress. These authors suggest that the expression of Nt107 may disrupt the endogenous antioxidant system in cotton (Light et al. 2005). In addition, using an Arabidopsis GSTU17 knockout mutant, Chen et al. (2012) suggested that AtGSTU17 functions as a negative component of the adaptive responses to drought and salt stresses.

Superoxide Dismutase

Superoxide dismutases (SODs) catalyze the breakdown of superoxide radicals into O_2 and H_2O_2 and are considered the first line of defence against ROS (Wang et al. 2007). Transformation of different plants species with genes coding for SODs has been a widely used approach in order to achieve salt stress tolerance.

Transgenic Arabidopsis plants overexpressing manganese SOD (Mn-SOD) showed enhanced salt stress tolerance (up to 150 mM NaCl). This response was associated with increases in other antioxidant enzymes such as Cu,Zn- and Fe-SODs, CAT and POX, as well as with low levels of lipid peroxidation (Wang et al. 2004). Moreover, the overexpression of Mn-SODs has been used in other plants species. Transformed tomato plants with increased expression of a heterologous Mn-SOD were more tolerant to salt stress (up to 200 mM NaCl) than non-transformed tomato plants (Wang et al. 2007). Poplar plants overexpressing a MnSOD gene from *Tamarix androssowii* also showed enhanced salt stress tolerance when compared with non-transformed plants (Wang et al. 2010). In rice, the introduction of a gene from the halophile *Natrinema altunense* (NaMn-SOD) also confers salt stress tolerance (Chen et al. 2013). These transgenic rice plants showed higher levels of photosynthesis, and total SOD and CAT activities, as well as lower lipid peroxidation levels than non-transformed plants (Chen et al. 2013).

The overexpression of Cu,Zn-SOD isoform has been also used to induce stress tolerance. In tobacco plants, the expression of a cytosolic Cu,Zn-SOD from *O. sativa* in chloroplasts led to an enhanced tolerance to salt, water and PEG stresses, via an increased chloroplast antioxidant system (Badawi et al. 2004b). Transformation of rice plants with a cytosolic Cu,Zn-SOD from the mangrove plant *Avicennia marina* also induce tolerance to 150 mM NaCl, as well as to MV exposure and drought (Prashanth et al. 2008). Gill et al. (2010) described that the overexpression of a cytosolic Cu,Zn-SOD from *Potentilla atrosanguinea* in Arabidopsis plants confers tolerance to salt stress, with transgenic plants displaying better germination rate, seedling development and growth under NaCl condition than non-transformed plants (Gill et al. 2010). In addition, cotyledonary explants from these transgenic Arabidopsis plants also showed an improved callus induction, shoot regeneration and salt stress tolerance (Shafi et al. 2015). In woody plants, a salt tolerance response has been described in in vitro plum plants overexpressing a cytosolic Cu/Zn-SOD from *Spinacia oleracea*. These transgenic plum plantlets also displayed an increase in non-enzymatic antioxidants (Diaz-Vivancos et al. 2013).

Coexpression of Antioxidant Enzymes

Due to complexity of the ROS scavenging pathways, some authors have tried the coexpression of antioxidant enzymes in order to achieve tolerance against environmental stresses. The combination of transgenes encoding ROS-scavenging

enzymes could have a synergistic effect on stress tolerance and could be a more appropriate strategy to enhance stress tolerance than the use of a single transgene (Faize et al. 2011).

Transformation of rice plants with both GST and CAT from *S. salsa* enhanced tolerance to salt stress (200 mM NaCl), these transgenic plants displaying lower H_2O_2 content and membrane damage than non-transformed plants (Zhao and Zhang 2006). In transgenic Chinese cabbage plants expressing the maize Cu/Zn-SOD and CAT genes in chloroplasts a salt stress tolerance response was also reported (Tseng et al. 2007). The coexpression of DHAR + GR and GST + GR in chloroplasts of tobacco plants also led to 200 mM NaCl tolerance (Le Martret et al. 2011).

More recently, it has been described that the overexpression in cotton chloroplasts of GhSOD1 + GhCAT1 or GhSOD1 + GhAPX1 from *G. hirsutum* enhanced salt stress tolerance (Luo et al. 2013). These authors showed that the best response in terms of stress tolerance was exhibited by the synergistic effect of GhSOD1 and GhCAT1 coexpression (Luo et al. 2013). In woody plants, Diaz-Vivancos et al. (2013) described an enhanced salt stress tolerance (100 mM NaCl) in transgenic plum plantlets carrying genes encoding cytosolic APX and Cu,Zn-SOD from *P. sativum* and *S. oleracea*, respectively.

Other groups have attempted the overexpression of three antioxidants genes to achieve stress tolerance. In this sense, transgenic tobacco plants carrying genes encoding for CuZn-SOD, APX and DHAR in chloroplasts were more tolerant to NaCl than non-transformed and CuZn-SOD + APX transgenic plants (Lee et al. 2007). These transgenic tobacco plants were also resistant to paraquat and showed high DHAR activity as well as high reduced ASC and oxidized glutathione contents. These authors suggest that the simultaneous expression of multiple antioxidant enzymes could be a more effective strategy to induce abiotic stress tolerance than single or double transformation (Lee et al. 2007).

Conclusions

Salt stress is one of the most important abiotic stress challenges affecting plant growth and productivity. The results described here show that cell metabolism under salt stress favours the formation of $O_2^{\bullet-}$ and H_2O_2 at subcellular level in higher plants demonstrating that chloroplast, mitochondria, peroxisomes as well as the cytosol and the apoplastic space can be a source of ROS generation under saline conditions. However, some studies indicate that ROS accumulation may be related with the induced cellular changes upon salt stress.

Obtaining salt-tolerant species is one of the goals for breeders, and probably, the use of transformed plants could improve the salt response in crop plants. In this way, transformed plants with enhanced antioxidant defences have been obtained in different laboratories and in most cases, these plants displayed a salt tolerance response.

The proteomic approach has shown that post-translational modifications can be crucial mechanisms used by plants to modify their protein functions and thereby to face stressful environmental factors.

The development of plant metabolomics techniques can provide valuable information about the effect of salt stress on the cell metabolism. In addition, these techniques can allow the discovery of new metabolites that can be used as markers to better understand the salt tolerance response and can help breeders to select new tolerant species.

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Chapter 9
Tolerance Response Mechanisms to Iron Deficiency Stress in Citrus Plants

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Abstract Iron chlorosis is one of the main abiotic stress factors in plants as it participates in some life-sustaining processes, e.g., respiration and photosynthesis, and is involved in electron transfer through Fe^{2+}/Fe^{3+} redox reactions.

Plants have developed adaptive mechanisms to increase both Fe-uptake and Fe-transport capacities under Fe-deficiency conditions. Characterizing the iron uptake system and its response to different chlorotic situations that compromise the availability of this element to cause Fe deficiency in plants is key to better understand Fe homeostasis regulation in citrus. Using Fe chlorosis-tolerant genotypes as rootstocks is a widely applied system to prevent Fe deficiency in fruit crops. So the comparative study of this response in different rootstocks and distinct chlorotic situations will help meet this objective.

Thus, the iron absorption ability of citrus increases when Fe is absent in the medium through the activation of element acquisition system components, which have been described in dicotyledonous and non-grass monocotyledonous species as Strategy I. Fe deficiency enhances the transcript abundance of genes *HA1*, *FRO2*, and *IRT1* that encode for FC-R and H^+-ATPase enzymes and the iron transporter, which regulate acidification, reduction, and Fe transport responses in citrus roots under Fe-deprived conditions. Fe deficiency also leads to organic acid accumulation in the root system, mainly citrate and malate, due to the activation of various enzymes from Krebs cycle. Activation of the dicarboxylate-tricarboxylate carrier plays a key role by shuttling these compounds between the cytosol and mitochondria. Consequently, organic acids are released to xylem sap (to promote long-distance Fe transport) or to the exterior (to acidify the rhizosphere and facilitate the solubilization and reduction of Fe^{3+} ions).

Presence of bicarbonate HCO_3^- in the medium induces iron chlorosis in citrus plants, which stunts growth and alters the photosynthetic apparatus in leaves because this ion HCO_3^- has a buffering effect on the acidification of the medium to inhibit iron absorption by roots and to lower the Fe concentration in plants. Consequently, citrus induces responses to Fe deficiency in roots. Bicarbonate hin-

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ders the transport of Fe from roots to shoots, and also blocks the mobilization of Fe from radicle cotyledons in seedlings, which are not Fe supplied. This last effect can be due to the NRAMP transporter being inhibited by bicarbonate, which is located in the tonoplast and regulates vacuolar Fe release.

Presence of relatively high concentrations of other micronutrients in the medium, like zinc and manganese, reduces Fe absorption. This is due to a competitive effect of these elements at the transport level across the plasma membrane as responses to Fe deficiency are activated by poor entry of Fe in root cells. Thus, reduced Fe translocation of cotyledons is due to blocked NRAMP transporter activity in the vacuole.

In conclusion, all responses to Fe deficiency in roots are metabolically intercorrelated and are involved in the absorption of this element by plants to solubilize Fe by rhizosphere acidification, to reduce ion Fe^{3+} to Fe^{2+} that is available to plants, and to activate iron transporters from the root plasma membrane. The level of citrus tolerance to iron chlorosis is determined by the level of response of the Fe acquisition system components in a chlorotic situation. Here, the role of enzyme FC-R and the capacity of the plants to accumulate Fe in their root apoplast are important.

Introduction

Iron (Fe) deficiency or Fe chlorosis is one of the most important nutritional disorders as it participates in some life-sustaining processes, such as respiration and photosynthesis, where it is involved in electron transfer through Fe^{2+}/Fe^{3+} redox reactions. Although Fe is relatively abundant in many cultivated soils, its acquisition by crop plants is often impaired by certain soil properties. Therefore, Fe deficiency is a widespread and very important agricultural problem in crops grown on calcareous soils, which occupy about 30 % of land surface (Chen and Barak 1982), and especially affects most orchards in the Mediterranean basin, where this soil type is extremely common (Jaegger et al. 2000). Fe chlorosis affects most vegetable and fruit crops—including citrus—some of much economic interest, which limits their performance and causes vast economic losses.

The most effective way to prevent Fe chlorosis is to use tolerant rootstocks. With citrus fruit, huge differences between species have been reported in their behavior to clorosant media; therefore, some are used as tolerant rootstocks to Fe deficiency (Forner and Alcaide 1994; Sudahono et al. 1994). Currently, however, availability of resistant rootstocks is extremely limited, so the commonly used technique to combat Fe chlorosis is to correct and/or prevent this deficiency by applying the soil of synthetic ferric chelates based on molecules of Fe-EDDHA and Fe-EDDHMA molecules which are more effective under alkaline conditions as they present high stability to pHs within the 7–9 range (Norvell 1991). Unfortunately, the high price of these chemical fertilizers restricts their application to only highly profitable crops and Fe-chlorosis treatment can represent up to 50 % of annual costs of fertilizers on farms (Sanz et al. 1992).

To understand and improve the control of this deficiency in citrus, the following actions are essential: identifying the components involved in Fe absorption; closely examining the biochemical and genetic mechanisms that are activated; closely regulating the signals involved in Fe homeostasis in this crop.

Location and Function of Fe in Plants

Iron is an essential micronutrient for plants because the ease with which it switches its oxidation state means that this ion is involved in many plant biochemical processes and is a cofactor in many enzymatic complexes.

Photosynthesis

Iron plays an essential role in photosynthesis as it participates in several steps in the biosynthetic pathway of chlorophyll, and it regulates the activity of the enzyme system to form protochlorophyll and other photosynthetic pigments (Marschner 1995).

Chlorophyll biosynthesis is a complex process. In the first phase, amino acid glutamic acid is converted into γ-aminolevulinic acid. From this point, several metabolic phases are needed for the formation of the tetrapyrrole four rings. Some chloroplast enzymes can insert Mg^{2+} in the center of the tetrapyrrole, which finally results in chlorophyll, or in Fe^{2+} to synthesize heme groups. Protoporphyrin IX can also be exported from the chloroplast to mitochondria, where it is used to produce cytochromes or as a substrate for the synthesis of phytochrome (Hans-Walter 1997). Thus, Fe deficiency reduces the tetrapyrrole products involved in the synthesis of photosynthetic pigments, particularly chlorophylls, and yellow pigments (carotenes and xanthophylls) predominate in Fe-deficient plants and are responsible for foliar yellowing.

Photosynthetic pigments are localized in the thylakoid membranes of the chloroplasts associated with proteins, whose function is to intercept and channel light energy to chemical energy during photosynthesis (Seibert 1993). Excess absorbed photons are not dissipated efficiently by photochemical processes (heat emission and fluorescence). Therefore, under Fe stress conditions, the photosynthesis rate lowers, while heat dissipates and fluorescence increases (Molassiotis et al. 2006). The inverse relationship between the fluorescence emissions of chlorophyll photochemical processes allows the use of the former as an indicator of a plant's photosynthetic efficiency.

Fe is also responsible for the morphology, structure, and maintenance of chloroplasts, but it does not appear to affect their number per leaf unit (Abadia 1992; Marschner 1995). Fe deficiency drastically reduces the number of ribosomes, and therefore diminishes protein synthesis and raises the concentration of amino acids

in chlorotic leaves, more in chloroplasts than in cytoplasm (Funkhouser and Price 1974). Reduced photosynthetic capacity under Fe-deficiency conditions results in low levels of sugar and starch. A markedly altered metabolism generally occurs, which results in poor physiological plant functioning (Abadia 1992).

Enzymatic Systems

Iron is present in plants and comprises a number of important enzymatic systems for their metabolism, which can be divided into hemic complexes (or hemoproteins) and non-hemic ones, depending on whether the ion Fe in the protein is attached to a porphyrin ring or not, respectively.

Hemics enzyme systems include:

- Cytochrome: the complex responsible for energy transport in redox systems of chloroplasts, mitochondria, and the redox chain of the nitrate reductase enzyme (Marschner 1995).
- Leghemoglobin: an enzyme involved in nitrogen fixation in legumes (Marschner 1995).
- Catalase: an enzyme involved in photorespiration and the Calvin cycle. It facilitates dismutation processes of H_2O_2 in H_2O and O_2 in chloroplasts (Römheld and Marschner 1991).
- Peroxidase: it promotes the elimination of H_2O_2 in chloroplasts and is involved in the polymerization of phenols to lignin biosynthesis in rhizodermis and root endodermis (Marschner 1995).
- Phytochrome: an essential sensing molecule in photomorphogenesis or plant development (Hans-Walter 1997).

Non-hemics enzyme systems are characterized by Fe being coordinated with a thiol group of cysteine or with inorganic S to form Fe-S bonds, or even both (Römheld and Marschner 1991). This group includes:

- Ferredoxine: an iron protein that acts as a terminal electron acceptor in a number of metabolic processes, such as photosynthesis and nitrate and sulfate reduction (Marschner 1995; Mengel and Kirkby 2001). Its high redox potential allows it to reduce substances like $NADP^+$, nitrate, oxygen, and sulfate.
- Aconitase: an enzyme that catalyzes the isomerization of citrate to isocitrate in the tricarboxylic acid cycle (Marschner 1995).
- Riboflavin: a molecule required for root growth because it reduces the amount of auxin (root growth inhibitor). The amount of riboflavin in Fe-deficient plants is about 200 times higher than in plants grown in media with a normal Fe supply (Welkie and Miller 1989).
- Superoxide dismutase: an enzyme that eliminates superoxide anion-free radicals. Their isoenzymes are common in chloroplasts, but are also found in mitochondria, peroxisomes, and the cytoplasm (Droillard and Paulin 1990).

- Aminolevulinic acid synthase and coproporphyrin oxidase: enzymes related to the synthesis of porphyrin.
- Xanthine oxidase: an enzyme that performs several functions in metabolic processes, such as photosynthesis, mitochondrial respiration, and N_2 fixation, and reduces SO_4^{2-} to SO_3^{2-}.
- Ribonucleotide reductase enzyme: an enzyme involved in RNA synthesis (Römheld and Marschner 1991). Here, Fe deficiency lowers the number of ribosomes in leaf cells, which means inhibition of cell division in severe cases of deficiency.
- Other enzymes: a group of lesser known enzymes in which Fe acts like either a redox-metal component or a connecting element between the enzyme and substrate. This is the case of lipoxygenase, which works by regulating lipid peroxidation. So it is involved in cellular senescence and disease resistance (Nagarathana et al. 1992).

Iron Reserves in Plants

Finally, the free iron pool (Fe which is not complexed with carbon compounds) may interact with oxygen to form superoxide anion (O_2^-), which damages cell membranes by degrading unsaturated lipid components (Peterson 1991). Plant cells limit this damage by storing excess Fe in an Fe-protein complex called phytoferritine, which is located mainly in leaves (Bienfait and Van der Mark 1983). This storage protein $[(FeO{\cdot}OH)_8\ (FeO{\cdot}OPO_3H_2)]$ is composed by 24 identical subunits that form a hollow sphere whose molecular mass is 480 kDa. The center of this area contains some 5400–6200 Fe^{3+} atoms in the form of a ferric-phosphate oxide complex. Phytoferritine is the main Fe reserve in plants and corresponds to 12–23 % of total Fe in plants on a dry matter basis.

Quantitatively, 65 % of Fe is located in leaves associated with proteins; of this amount, 35 % accumulates in the form of phytoferritine, 10 % is bound to hemeproteins, and 20 % is bound to non-hemic enzyme systems (Price 1968). In leaves, 80 % of Fe is located in the chloroplast (Tiffin 1966).

Factors That Affect Iron Chlorosis

Iron is the fourth most abundant element in the earth's layer after oxygen, silicon, and aluminum, and it represents 5 % of the earth crust's weight and 3.8 % of soil composition (Lindsay 1995). Therefore, Fe deficiency is not caused by insufficient total Fe content in soil, but is normally induced by some circumstantial condition which comprises how plants use this element. Iron chlorosis causes are usually related with certain soil factors although genotype, environmental conditions, and cultural management are likely to interact in this disorder.

Chemical Components in Soil

Mineral Soil Components

Iron takes an Fe^{2+} form in most primary soil minerals (olivine, hornblende, and biotite). In aerobic conditions, it precipitates mainly as Fe^{3+} oxides and hydroxides, so different compounds with a distinct composition and degree of crystallinity may coexist, but with distinct solubility. According to Lindsay (1995), the amount of solubilized Fe depends on the mineral: the more soluble it is, the higher the available Fe concentration is in soil solution. Fe minerals are classified according to their solubility as follows: amorphous Fe oxide ($Fe(OH)_3$) > soil ferrihydrite ($Fe(OH)_3$) > γ–lepidocrocite (FeOOH) > γ–maghemite (Fe_2O_3) > α–hematite (Fe_2O_3) > α–goethite (FeOOH), where goethite is the lowest soluble and, therefore, the main mineral in which Fe is present in soil.

Soil pH

The iron present in soil comes in two oxidation states: ferric (Fe^{3+}) and ferrous (Fe^{2+}). The predominant form under acid and non-oxidizing conditions is the soluble form (Fe^{2+}). However, Fe^{2+} is rapidly oxidized to the Fe^{3+} form in the presence of oxygen and alkaline medium, where it precipitates as oxides and hydroxides of Fe^{3+}, and as other intermediate forms, such as Fe $(OH)^{2+}$ or Fe $\left(OH\right)_2^+$, according to this reaction:

$$4\,Fe^{2+} + O_2 + 10H_2O \rightarrow 4Fe\left(OH\right)_3 + 8H^+$$

Hence, Fe solubility is closely related to soil pH. Increasing the pH in a single unit may diminish the Fe solubility of compounds by 1000 times, which lowers the soluble Fe concentration to values below 10^{-20} M for a pH value of 7.5 in the medium (Lindsay 1995).

Lime and Bicarbonate Contents in Soil

A marked alkaline reaction in calcareous soils impairs Fe solubilization from oxides and hydroxides (Lindsay 1995). Under these conditions, the free Fe concentration is lower than 10^{-20} M, while the Fe levels required by plants are higher, between 10^{-9} and 10^{-4} M (Guerinot and Yi 1994). Accordingly, this element becomes a deficient nutrient for plants.

High ion bicarbonate concentrations are normally generated in calcareous soils and are controlled by the following reaction:

$$CaCO_3 + 2H^+ \rightarrow Ca^{2+} + H^+ + HCO_3^-\left(ac\right) pK = -7.82$$

Consequently, most calcareous soils present a pH somewhere between 7.5 and 8.5 and can even reach pH values above 9 if soil has a high dissolved $NaHCO_3$ content. At these pH values, the soluble Fe concentration is quite low as the presence of bicarbonate may also be formed by ferric hydroxide (Lindsay 1995):

$$4Fe^{2+} + O_2 + 8HCO_3^- + 2H_2O \rightarrow 4Fe(OH)_3 + 8CO_2$$

Hence, Fe^{2+} ion content in calcareous soils of normal aeration is usually very low, and Fe predominates as ferric hydroxide, which is not only the thermodynamically most stable form of Fe present in soils, but also the least soluble and most difficult to be used by plants.

High HCO_3 levels in soil solution can directly induce Fe deficiency in plants by decoupling both its absorption and transport (Cinelli 1995). However, the mode of action of bicarbonate is not fully understood. It has been proposed that this ion may act by: (1) buffering soil solution and maintaining it within a range of values between 7.5 and 8.5, and markedly reducing Fe solubility and availability (Donnini et al. 2009); (2) inhibiting the induction of the FC-R enzyme in Fe-deficient plants (Romera et al. 1997; Alcantara et al. 2000); (3) preventing Fe transport from roots to stems (Deal and Alcantara 2002); (4) causing apoplast and xylem alkalinization of leaves, which reduces the Fe absorbed by mesophyll cells (Wegner and Zimmermann 2004).

Complex Formation

Fe has the ability to associate with certain natural compounds to form stable complexes and to facilitate element mobility in soil solution and plants acquiring it (Lindsay 1995). These molecules mainly result from the transformation of organic matter (humic and fulvic acids, amino acids, etc.), exudation of the root system (phytosiderophores), or secretion by certain microorganisms (siderophores). However, with excess organic matter, Fe can be fixed at slightly soluble humic compounds (Sanz-Encinas and Montañés 1997).

Presence of Compounds and/or Mineral Elements at High Concentrations

High sodium (Na^+) ion concentration in soil can displace Fe to chelating agents. Elevated levels of Na^+ and potassium and a low calcium level, degrade the soil structure by reducing its aeration and altering soil–water relationships (Loeppert et al. 1994). A high phosphate concentration in acid soils may precipitate Fe as insoluble Fe phosphates (Lindsay 1995).

Fe absorption is closely related to the absorption of other metals, and high Zn^{2+} and Mn^{2+} concentrations in the medium interfere with Fe^{2+} absorption in roots by lowering the Fe concentration in plants, which may even show Fe-deficiency symptoms (Izaguirre-Mayoral and Sinclair 2005; Adiloglu 2006; Aref 2011; Shanmugam

et al. 2011). This effect has been attributed to competition between ion absorption sites (Marschner 1995). In contrast, both Zn^{2+} and Mn^{2+} are more easily absorbed by Fe-deficient root plants (Alcantara et al. 1994; Korshunova et al. 1999; Eckhardt et al. 2001). High concentrations of other trace elements in the environment can cause structural and biochemical alterations by disrupting the affinity of transport systems for Fe^{2+} (Zaharieva and Römheld 2000).

Soils with a high content of heavy metals, plus their likely toxicity for plants, may affect the root absorption system by impairing the reduction, translocation, and use of the ion Fe by plants (Alcantara et al. 1994; Chang et al. 2003; Lucena et al. 2003).

Physical Components of Soil

Texture and Soil Structure

In sandy degraded soils with a low pH, ion Fe^{2+} is solubilized, washed by rain or irrigation, and moves to deeper soil layers. Under these conditions, the total Fe concentration that lies within reach of the root system is very low (Mengel and Kirkby 2001). Otherwise when soils have a high clay content, Fe^{3+} ions can be adsorbed by them and remain strongly attached, which hinders element assimilability (Kolesch et al. 1987).

Flooding

In highly humid and poorly aerated soils (reducing conditions), carbon dioxide (CO_2) accumulates because of the slow gas diffusion rate in water (Inskeep and Bloom 1986). Excess CO_2 leads to the appearance of ion bicarbonate, which concentrates in soil solution and the amount of Fe^{2+} ions reduces by half due to ferric hydroxide formation.

Excess moisture can also affect soil texture, which occurs with calcareous clayey soils, where soil is compacted and waterproofing of surface layers occurs. This leads to poor soil aeration (reducing conditions) and the bicarbonate ion accumulates. In addition, excess CO_2 with both high temperatures and waterlogged soils can directly influence plant as it may hamper the activity of the enzymes that are related to Fe uptake by plants, and consequently leads to Fe chlorosis (Zude-Sasse and Schaffer 2000; Martinez-Cuenca et al. 2015). Excess moisture also decreases the redox potential in acid soils so that the concentration of iron oxides lowers and causes excess Fe^{2+} in soil (Ponnamperuma 1972). Excessive uncontrolled Fe^{2+} absorption can lead to toxic ion concentrations in plant tissues, which implies Fe chlorosis symptoms, such as blackened root tips, inhibited root growth and necrotic leaf marks, commonly known as bronzing (Snowden and Wheeler 1995).

These effects are due to the oxidative stress induced by free radical generation caused by Fenton reactions (Briat 2002).

The additive combined effect of Fe deficiency and root anoxia affects the biochemical composition and structure of pea leaf chloroplasts (Ladygin 2004). The higher chl a/b ratio they observed in the leaves of flooded plants degraded Chl b more quickly than Chl a (Martinez-Cuenca et al. 2015). This is indicative of the advancing reduction of the light-harvesting chl a/b-protein complex in chloroplast membranes compared to the chl a-protein complexes of the reaction centers of PS I and II.

Environmental Factors

Low temperatures produce inhibited root growth, which affects Fe absorption. In bananas, maximum Fe absorption occurs above 37/30 °C (day/night temperature). When temperatures drop to 17/10 °C, the amount of Fe absorbed lowers by twofold or threefold (Lahav and Turner 1984). Moreover, high temperatures cause degradation phytosiderophores, which directly affect absorption in the Fe II strategy of plants (Bergmann 1992).

Factors Inherent to Plants and Their Culture

Genetic Variability

In the same plant species, both efficient varieties and inefficient varieties against Fe deficiency may exist. This happens with some genotypes of cucumber (Rabotti and Zocchi 2006), alfalfa (Donnini et al. 2009), pea (Jelali et al. 2010a), vine (Dell'Orto et al. 2000a; Ksouri et al. 2006), kiwi (Vizzotto et al. 1999), pear, quince, and olive (De la Guardia and Alcantara 2002) and peach (Gogorcena et al. 2004; Jimenez et al. 2008, 2011). Several authors have classified Citrus genotypes according to their physiological response to Fe-deficient conditions (Treeby and Uren 1993; Pestana et al. 2005; Castle et al. 2009; Gonzalez-Mas et al. 2009; Llosa et al. 2009). The susceptibility to Fe chlorosis of main genotypes for citrus in the Mediterranean basin has been recorded in Table 9.1.

Cultural Practices

Fe absorption can also lower if herbicides are abused because they incorporate Fe antagonists into its composition, overwatering, and organic amendments (Udoh and Nelson 1986; Yen et al. 1988; Marschner and Römheld 1994).

Table 9.1 Susceptibility to iron chlorosis of main *Citrus* genotypes used in the Mediterranean basin

Rootstocks	Botanical name	Degree of susceptibility	Reference
Carrizo citrange	*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.	Susceptible	Castle et al. 2009
Swingle citrumelo	*C. paradisi* × *P. trifoliata*	Highly susceptible	Castle et al. 2009
Cleopatra mandarin	*C. reshni* Hort. ex Tan.	Highly tolerant	Castle et al. 2009
Macrophylla	*C. macrophylla* Wester	Highly tolerant	Castle et al. 2009
Volkameriana	*C. volkameriana* Ten. & Pasq.	Tolerant	Castle et al. 2009
Forner-Alcaide 5	*C. reshni* × *P. trifoliata*	Tolerant	Llosa et al. 2009
Forner-Alcaide 13	*C. reshni* × *P. trifoliata*	Susceptible	Llosa et al. 2009
C-35	*C. sinensis* × *P. trifoliata*	Susceptible	Cimen et al. 2015
Poncirus trifoliata	*P. trifoliata*	Highly susceptible	Castle et al. 2009

Symptomatology of Iron Deficiency in Citrus

The symptomatology of Fe deficiency in Citrus, as in most crops, is manifested mainly in leaves; as they lose their bright green color, which evolves into shades ranging from light green to pale yellow, depending on the intensity of the damage caused. This fact is due to a drop in the concentration of photosynthetic pigments, mainly chlorophylls (Abadia and Abadia 1993). Leaf veins usually maintain the dark green color, which appear marked on a lighter background and resemble a mesh or reticle (Fig. 9.1b). This demonstrates the low mobility of Fe, even within the leaf, and its low distribution toward growing zones.

Iron deficiency also affects the development and growth of new plant organs. New shoots downsize as a result of the poor translocation of this element from adult leaves or other organs, and become progressively less vigorous, with shorter internodes and smaller leaves. When chlorosis is severe, damage turns into necrosis and causes premature defoliation of shoots, and even their progressive death, which always begins with their apical region. Root system growth can also be stunted (Bertoni et al. 1992).

The production of trees grown under low Fe conditions lowers quickly because of decreased fruit set (and, therefore, a smaller number of fruits) and their quality (Pestana et al. 2003). With severely deficiency levels, mature citrus fruits are usually smaller, their peel turns smoother and they lack color. Moreover, flavedo becomes yellowish-brown and does not acquire the reddish-orange color that normal fruits show. Fe deficiency also decreases total soluble solids content of juice. In addition, the tree's life cycle is shortened and its productive life is considerably shorter than it would be normally (Alvarez-Fernandez et al. 2005), which means the premature death of trees in extreme deficiency cases (Fig. 9.1a). All these effects have a severe economic impact on commercial plantations.

Fig. 9.1 Symptomatology of iron deficiency in *Citrus*. (**a**) Tree afection on field (*Left*, No symptoms; *Right*, Fe-chlorotic symptoms); (**b**) Gradual visual symptoms in leaves

Mechanisms of Response to Iron Deficiency in Citrus

Uptake System: Strategy I

Plants take Fe ions directly from soil solution by an active absorption process through roots (Uren 1984), mainly absorbed in the form of Fe^{2+}. Under normal Fe availability conditions, plants develop low-affinity transport systems to absorb the Fe they need to develop properly. However, high soluble Fe^{2+} concentrations in soil can actually cause toxicity, and if absorbed in large amounts, can react with oxygen and form free radicals, which damage cellular components such as DNA, proteins, lipids, etc. (Hell and Stephan 2003). Plants induce high-affinity transport systems under Fe-deficient conditions to improve Fe absorption in the root system (Andrews et al. 1999; Kaplan 2002).

So we can classify plants as "inefficient" ones, those that remain indifferent or develop low-intensity reaction responses to Fe deficiency. In contrast, "efficient" plants develop effective coping mechanisms to increase their ability to acquire Fe from soil solution (Mengel and Kirkby 2001). The degree of response within each group differs according to species, varieties, and genotypes (Brown and Jolley 1989; Shi et al. 1993). Efficient plants have adopted two different strategies depending on the way they acquire Fe (Marschner and Römheld 1994; Hell and Stephan 2003): they are divided into Strategy I plants (non-grass monocotyledonous and dicotyledonous species, including citrus; Fig. 9.2) and Strategy II plants (grasses).

Acidification of the Root Rhizosphere

One of the physiological mechanisms stimulated in the plants that develop Strategy I under Fe-deficiency conditions is the ability to acidify the rhizosphere (Marschner and Römheld 1994; Rabotti et al. 1995; Dell'Orto et al. 2000b; Rabotti and Zocchi 2006). In these plants, roots cause the pH of the solution in which they grow to drop

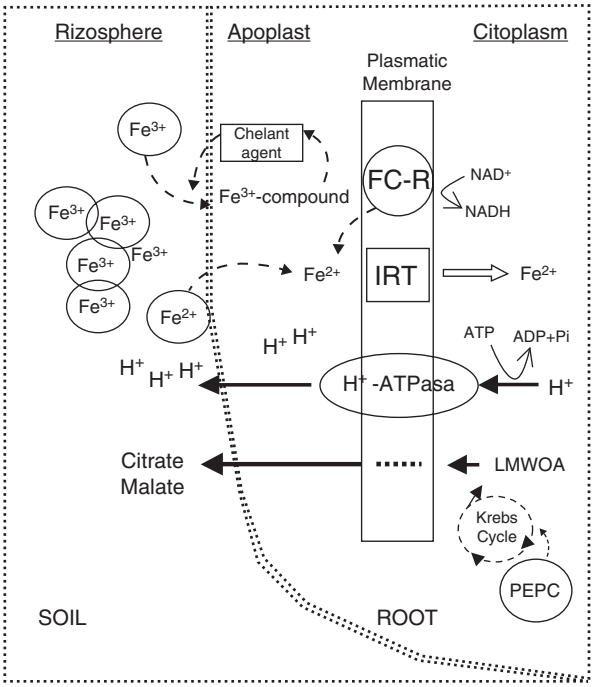

Fig. 9.2 Schematic drawing illustrating the model of Fe uptake system in *Citrus* roots (Modification of Hell and Stephan 2003). *FC-R* Ferric Chelate Reductase enzyme, *IRT* Iron transporter, *H⁺-ATPase* Proton-ATPase enzyme, *PEPC* Phosphoenolpyruvate carboxylase enzyme, *LMWOA* low-molecular-weight organic acids

given the activation of a proton (H^+) pump ATP-dependent enzyme called H^+-ATPase, which is located in the plasma membrane of subapical root cells (Susin et al. 1996). Studies have revealed a variable induction of this enzyme in Fe-deficient plants ranging from 18 to 100%, depending on the species (Susin et al. 1996; Schmidt et al. 1997; Rabotti and Zocchi 2006). In a comparative study carried out in citrus rootstocks, Treeby and Uren (1993) observed a significant increase in the H^+ efflux in response to Fe deficiency in more lime-tolerant rootstocks, rough lemon, Cleopatra mandarin, and sour orange, while it was not altered when measured in lesser tolerant ones, e.g., sweet orange, Carrizo citrange, and trifoliate orange.

Release of H^+ when Fe is lacking is linked to morphological changes in roots, such as proliferation of lateral roots and root hairs, thickening of apices, and increased transfer of cells in the rhizodermis. Rhizodermic cells are probably centers of the H^+ flux in Fe-deficient roots. Yet when Fe is supplied, these cells become smaller in number in 1 or 2 days, with the consequent reduced release of H^+ (Marschner and Römheld 1994). This ATPase has been biochemically characterized and its optimum pH "in vitro" is 6.6 (Michelet and Boutry 1995). Its mechanism of action is due to the creation of a phosphorylated intermediate, stable at an acid pH, which requires magnesium for it to work.

This root system response to Fe deficiency causes the following effects:

1. Acidification of the root environment in Fe-deficient plants, which reaches up to approximately 2 mm from roots, even in calcareous soils (Schaller 1987), and

promotes the dissolution of precipitated Fe forms on the ground (Rabotti et al. 1995).
2. Acidification of the root apoplast, which generates the same effect as that described above, but also at the intercellular level (Susin et al. 1996).
3. Improvement of the reduced Fe^{3+} form as: (a) it provides an optimum pH for the maximum activity of enzyme Ferric Chelate Reductase, FC-R (Susin et al. 1996); (b) it improves the affinity of enzyme FCR for its substrate by neutralizing the negative charge of ferric chelate and preventing repulsion in reduction centers (Cohen et al. 1997).
4. Generation of an electrochemical gradient across the membrane (negative inside the cell) that constitutes the driving force for Fe^{2+} absorption reduced by enzyme FC-R near roots (Zocchi and Cocucci 1990).
5. Induction of phosphoenolpyruvate carboxylase enzyme, PEPCase, by increasing CO_2 fixation and the subsequent synthesis of organic acids (Rabotti et al. 1995).

The H^+-ATPase enzyme is regulated by a large family of genes, which was first described in *Arabidopsis thaliana* (Santi and Schmidt 2009). At least one of them, the *AHA1* gene, is regulated in response to Fe deficiency. Similarly, the expression level of putative *HA1* gene is strongly induced in *Citrus* seedlings grown under Fe-chlorotic conditions (Martinez-Cuenca et al. 2013a, b; Fig. 9.3a). However, no differences in the constitutive expression of *HA1* and *HA2* genes have been detected in roots when comparing different genotypes (Martinez-Cuenca et al. 2013a). Enzymatic activity and H^+ excretion by roots into solution paralleled *HA1* transcripts levels (Fig. 9.3b). In cucumber roots, induction of gene *CsHA1* by Fe deficiency has also been observed, but no gene *CsHA2* overexpression was found (Santi et al. 2005; Santi and Schmidt 2009). Fe chlorosis-sensitive and -tolerant genotypes exhibit different abilities to acidify the rhizosphere in *Citrus*, actinidia, and pear plants (Vizzotto et al. 1999; Chouliaras et al. 2004a; Donnini et al. 2009). However, the lower level of induction of the these genes than the expected ones found between different *Citrus* genotypes can be explained by the existence of different isoforms of this gene, which are differently regulated under Fe-deficient conditions, as previously indicated in *Prunus* rootstocks by Gonzalo et al. (2011).

Induction of the Reducing Capacity of the Root System

Prior to Fe absorption, Strategy I plants need Fe^{3+} forms from soil solution to be reduced to assimilable Fe^{2+} forms. This reduction in Fe compounds occurs through an enzyme called Ferric Chelate-Reductase, FC-R (Bienfait 1988a), which is specific to the plasma membrane of surface cells from the subapical zone of roots (Cohen et al. 1997). This enzyme takes electrons from NADH and reduces Fe from high potential acceptors, such as ferricyanide (+360 mV) and synthetic iron chelates, with lower potentials between +100 and +250 mV (Nikolic 1998). FC-R reaches its maximum activity at a pH between 4 and 5 (Schmidt and Bartels 1997). The ability of roots to reduce Fe^{3+} from the medium increases under Fe-deficiency

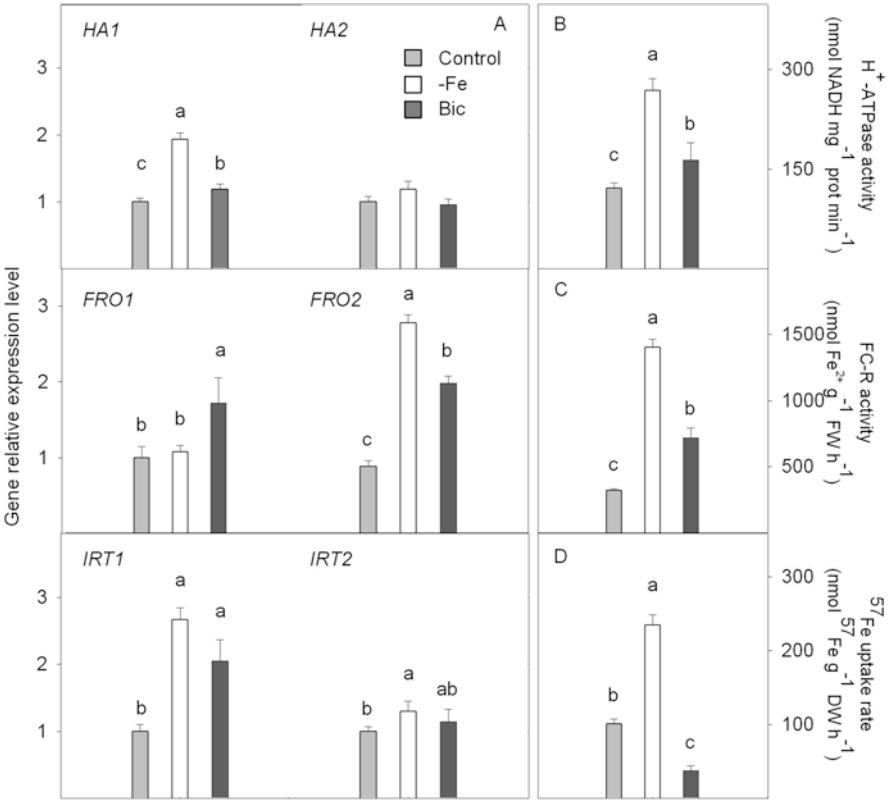

Fig. 9.3 (**a**) Relative expression of putative *HA1*, *HA2*, *FRO1*, *FRO2*, *IRT1*, and *IRT2* genes measured by real-time RT-PCR analysis, (**b**) H^+-ATPase activity, (**c**) Ferric-chelate reductase (FC-R) activity and (**d**) ^{57}Fe uptake in roots (Martinez-Cuenca et al. 2013a, b). All analysis were carried out in roots of *Citrus* seedlings grown in Fe-sufficient (Control), Fe-deficient (−Fe), and Fe-sufficient and bicarbonate (Bic) nutrient solutions. The values are the mean ± SE of three independent experiments ($n = 3$) run in triplicate. *Bars* with different letters indicate significant differences at $*p < 0.05$ using the LSD multiple range test (*ns* not significant). *Prot* protein, *FW* Fresh Weight, *DW* Dry Weight

conditions and can be 10–20 times greater than the control values (Susin et al. 1996). As mentioned above, Fe absence favors the thickening of apices and the appearance of root hairs, so the root surface increases as, consequently, do the potential reducing areas (Kramer et al. 1980).

Induction of enzyme FC-R as result of Fe deficiency or low levels of this element has been described by many authors in the plants that follow Strategy I response mechanisms (Fig. 9.3c). Moreover, the need of its activation for proper Fe absorption has been demonstrated (Susin et al. 1996; Yi and Guerinot 1996; Schmidt 2005; Jeong and Connolly 2009). The increased capacity of Fe reduction appears critical for determining the degree of tolerance of a genotype to Fe chlorosis. This has been described in herbaceous (Andaluz et al. 2009; M'sehli et al. 2009; Jelali et al. 2010a) and woody crops (Rombola et al. 2002; Gogorcena et al. 2004), including citrus

(Manthey et al. 1993; Treeby and Uren 1993; Rivera-Ortiz et al. 2007). Thus, the close relationship between the reduction capacity of enzyme FC-R in roots and the susceptibility of plants to Fe chlorosis can be used as selection criteria for tolerance to Fe chlorosis in new genotypes of plants (Dell'Orto et al. 2000a; Gogorcena et al. 2004; Castle et al. 2009; Llosa et al. 2009; Martinez-Cuenca et al. 2013a). Among rootstocks, Volkamer lemon plants obtained the highest Fe^{3+} reduction rate, *C. macrophylla*, C-35, and Carrizo citranges gave intermediate values, while Swingle citrumelo, Kinkoji, Rangpur, and sour orange achieved the lowest rates (Castle et al. 2009).

Some authors have stated that the presence of a minimum amount of Fe in media is an essential requirement to induce the enzyme in some plants, including citrus (Romera et al. 1996; Chouliaras et al. 2004a). This dependence may be due to the relationship of FC-R activity with ethylene metabolism (Romera et al. 1996). However, this is not true for some species like sugar beet, where FC-R activity is induced even when Fe is absent in the medium (Susin et al. 1996).

Recent works have isolated the genes related with FC-R enzyme expression, whose transcripts accumulate in response to Fe deficiency (Yi and Guerinot 1996). These genes belong to a superfamily that encodes for flavocytochromes, which transport electrons through membranes. For this purpose, they have binding sites for FAD and NAD(P)H, which act as electron donors to reduce external Fe^{3+} (Robinson et al. 1999; Waters et al. 2002). Apparently, *FRO* genes have different expression patterns, and therefore specific functions (Jeong and Connolly 2009). *AtFRO2* is induced in roots of *Arabidopsis* (Robinson et al. 1999), *PsFRO1* in roots, stems, nodules, and mesophyll cells of pea leaves (Waters et al. 2002), *FRO1* in roots and leaves of tomato (Robinson et al. 1999; Li et al. 2004), and *FRO2* in *M. truncatula* roots (Andaluz et al. 2009). This has also been observed in citrus, where the expression level of putative gene *FRO2* is strongly up-regulated in the tolerant genotype Cleopatra mandarine due to Fe starvation compared with the most sensitive Carrizo citrange and, consequently, to the induction of the Fe^{3+} reduction response (Martinez-Cuenca et al. 2013a). The significant differences in the levels of *FRO2* relative expression between genotypes grown under Fe-normal conditions suggests that chlorosis tolerance is also linked to a highly constitutive Fe-reducing capacity (Li et al. 2002). The increased gene *FRO2* expression with low Fe availability (Fig. 9.3a) may indicate that Fe-deficient plants develop a more efficient system to reduce this element, and gene *FRO2* isolation has potential implications for growing crops with higher nutritional quality and better growth in soils with low Fe availability (Robinson et al. 1999). However when Fe is supplied to the medium, protein degradation occurs and, therefore, prevents excessive Fe reduction, which could be toxic to plants (Schmidt 2005).

Fe^{2+} Transport into Root Cells

Reduced Fe is transported into the cell by a ferric transporter called IRT1. This protein is localized in the plasma membrane and functions primarily as a high-affinity carrier that regulates Fe^{2+} absorption into roots (Guerinot 2000; Cohen et al.

2004). Fox et al. (1996) reported increased Fe^{2+} absorption in *Pisum sativum* induced by Fe deficiency using a chelate enriched in ^{59}Fe, with increased activity of an Fe^{2+} transport protein in the plasmalemma. IRT belongs to the family of metal transporters ZIP, which are apparently not selective with the substrate and can transport metals of different natures. In fact, the initial findings originally complemented the assays done to restore growth of yeasts after alteration to the absorption of various nutrients (Fe, Zn, Mn). So although *AtIRT1* has been described as a high-affinity transporter carrier for Fe (Cohen et al. 2004), it also has other substrates, e.g., Zn, Mn, and Cd (Korshunova et al. 1999; Rogers et al. 2000).

The gene that encoded the expression of the IRT transporter was first identified in *Arabidopsis* (Guerinot 2000; Kim and Guerinot 2007), where the induction of *AtIRT1* and its homologous *AtIRT2* was reported in the epidermis of the roots of plants grown under Fe-deficient conditions (Vert et al. 2001, 2002). Both genes encode the major Fe transporters responsible for participating in processes of the high-affinity uptake of this metal (Vert et al. 2001, 2002, 2009). Orthologs to IRT have been found in many plant species, such as tomato (Eckhardt et al. 2001), pea (Cohen et al. 2004), cucumber (Waters et al. 2007), rice (Bughio et al. 2002), peanut (Ding et al. 2010), and tobacco (Enomoto et al. 2007). In Citrus, putative genes *IRT1* and *IRT2* appear to be regulated differently as a result of Fe starvation (Martinez-Cuenca et al. 2013a, b; Fig. 9.3a). The up-regulation of gene *IRT1* under these conditions, but not of gene *IRT2*, might indicate a key role of *IRT1* in the reaction of citrus rootstocks against Fe deficiency by increasing root Fe transport capacity. Moreover, in the presence of external Fe, both *IRT1* and *IRT2* expression levels differed between genotypes, which indicates distinct constitutive transport activity in *Citrus* plants (Martinez-Cuenca et al. 2013a). However, greater gene *IRT1* induction has been recorded for the apparently sensitive genotype Carrizo citrange compared to tolerant Cleopatra mandarin, but with a lower ^{57}Fe uptake rate. This indicates that the ability to increase Fe^{3+} reduction in response to Fe deficiency constitutes the limiting factor for Fe acquisition. Accordingly, Manthey et al. (1993) suggested that levels of ^{55}Fe uptake in Citrus seedlings showed good correlations with reduction rates.

Finally, protein is degraded if Fe supply is sufficient, which thus avoids the toxicity of the element (Schmidt 2005). Similarly to *FRO2* regulation, some authors have indicated that the presence of a minimum amount of Fe is apparently necessary to induce the expression of *IRT* genes (Vert et al. 2003). Furthermore, the expression of IRT in roots could be controlled by the release of signals from stems, even when the Fe levels outside are adequate, which indicates the nutritional status of Fe in plants (Grusak and Pezeshgi 1996). Hence, some plant hormones (ethylene, auxin) or their precursors have been proposed to be potential signals that regulate the response mechanisms generated by plants to Fe deficiency (Romera et al. 2005).

Biosynthesis of Low-Molecular-Weight Organic Acids (LMWOA)

Fe-deficient plants are characterized by the stimulation of the synthesis of organic acids in the cell. The response of organic acid metabolism induced by iron chlorosis has been studied in different species, which have demonstrated an increase in the

concentrations of mainly citrate and malate acids in different plant organs, such as roots leaves and xylem (Jones 1998; De Nisi and Zocchi 2000; Lopez-Millan et al. 2000, 2009; Abadia et al. 2002; Rombola et al. 2002; Jimenez et al. 2008, 2011; Jelali et al. 2010b). In *Citrus*, citrate and malate were the major LMWOA found in both the plant and xylem sap, at around 70 % and 20 %, respectively, of the total pool (Martinez-Cuenca et al. 2013c). These values fall within the same range as those found in other woody species, including *Prunus*, grapevine, and kiwi (Ollat et al. 2003; Jimenez et al. 2008, 2011). Moreover, Fe-deficiency conditions increased the concentration of citrate and malate acids in both plant fractions compared with normal Fe-supplied plants. Synthesis of organic acids under Fe-deficiency conditions could be involved in the following functions:

1. Stimulation of the exudation of acidificant compounds to mobilize Fe^{3+} from soil solution (Jelali et al. 2010b). Jones (1998) suggested that even at low citrate release rates, the amount of solubilized Fe^{3+} is sufficient to satisfy plant Fe needs.
2. Formation of complexes with Fe from soil to facilitate its absorption by the root system (Jones 1998).
3. Participation in the neutralization of cytoplasm pH to compensate the extrusion of protons carried out by enzyme H^+-ATPase (Landsberg 1981). H^+ flow causes cytoplasm alkalinization and activates CO_2 fixation, in association with PEPCase enzyme activity (Miller et al. 1990; Lopez-Millan et al. 2000). According to this theory, carboxylation of phosphoenolpyruvate to form organic acids (mainly citric and malic) returns cytosol pH to its initial value (Felle 1988).
4. Fe transport from the root system to other organs (long distance) of plants (Tiffin 1966; Pich et al. 1995) is mainly complexed with citrate. An increase in the concentration of citrate and malate in xylem sap of plants grown in the absence of Fe has been described (Lopez-Millan et al. 2001; Rombola et al. 2002; Martinez-Cuenca et al. 2013c). A significant proportion of the anions produced in roots forms part of the pool of compounds in xylem sap, likely loaded by protein FRD3 (Durret et al. 2007).

Organic acid biosynthesis takes place inside mitochondria. Pyruvate is the substrate prior to acid synthesis in Krebs cycle, and its synthesis occurs in the cytoplasm of the cell, and may occur via two routes: directly by the phosphorylation of phosphoenolpyruvate catalyzed by kinase pyruvate enzyme, or by the action of phosphoenolpyruvate carboxylase (PEPCase). This enzyme catalyzes the carboxylation of phosphoenolpyruvate to oxaloacetate, which can be subsequently reduced to malate by cytosolic malate dehydrogenase, cMDH (Chollet et al. 1996). Cytosolic malate is converted into pyruvate by the malic enzyme in either the cytosol or mitochondria, and its accumulation activates the synthesis of other acids from the cycle. Under Fe-deficient conditions, the activation of enzyme PEPCase has been demonstrated, as has therefore the described second route (Miller et al. 1990; Chollet et al. 1996; Jones 1998; De Nisi and Zocchi 2000; Abadia et al. 2002; Andaluz et al. 2002). PEPCase is, thus, the main enzyme involved in the supply of intermediate compounds of the tricarboxylic acid cycle (TCA) in non-photosynthetic tissues under Fe-deficient conditions (Chollet et al. 1996). Lack of Fe also stimulates the

activity of other enzymes related with Krebs cycle and the glycolytic pathway (Lopez-Millan et al. 2000, 2009; Jelali et al. 2010b; Vigani 2012), and in some cases, increased enzyme activities have been linked to the up-regulated expression of the corresponding genes (Thimm et al. 2001; Andaluz et al. 2009).

Anaplerotic fixation of carbon via PEPCase-cytosolic malate dehydrogenase (cMDH) onto malate plays a central role in the response of herbaceous plants to Fe deficiency and provides substrates for both the Krebs cycle and LMWOA long-distance transport (Zocchi 2006; Lopez-Millan et al. 2009). In *Citrus*, this carbon fixation also occurs in roots upon Fe shortage (Fig. 9.4), and is mediated by the up-regulation of putative gene *PEPC1* and higher enzyme PEPCase activity. Accordingly, enhanced PEPCase activity has also been reported in several woody species, such as grapevine (Ollat et al. 2003; Jimenez et al. 2008), kiwi (Rombola et al. 2002), pear and quince (Donnini et al. 2009), and peach (Jimenez et al. 2011). Up-regulation of

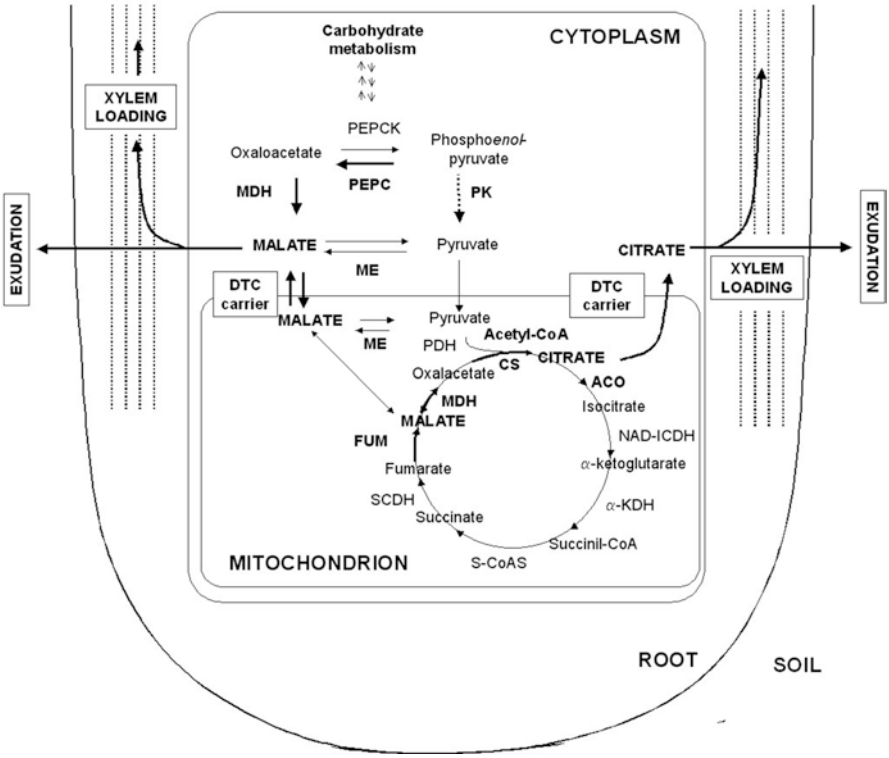

Fig. 9.4 Schematic drawing illustrating the activities regarding LMWOA metabolism in roots of *Citrus* seedlings grown under iron deficient conditions (Martinez-Cuenca et al. 2013c). Line simbology: induction [*thick line*], repression [*dotted line*], or no change [*fine line*]. *PEPCK* phospho*enol* pyruvate carboxyquinase, *PEPC* phospho*enol* pyruvate carboxylase, *PK* pyruvate kinase, *ME* malic enzyme, *PDH* pyruvate dehydrogenase complex, *CS* citrate synthase, *ACO* aconitase, *NAD-ICDH* NAD-isocitrate dehydrogenase, *α-KDH* α-ketoglutarate dehydrogenase, *SCoAS* succinyl-CoA synthase, *SCDH* succinate dehydrogenase, *FUM* fumarase, *DTC carrier* di-tricarboxylate carrier

PEPC transcripts by Fe deficiency have also been described for *Arabidopsis thaliana* and *Medicago truncatula* (Thimm et al. 2001; Andaluz et al. 2009). It has been hypothesized that cytoplasm alkalinization, associated with induced H^+ extrusion in Fe-deprived roots, could cause PEPCase enzyme activation (Abadia et al. 2002).

Regarding mitochondrial enzymes, activities of fumarase (FUM), mitochondrial malate dehydrogenase (mMDH), and citrate synthase (CS) have been reported to be enhanced in −Fe extracts of citrus roots vs. +Fe controls, whereas no significant differences between treatments were found for aconitase (ACO) activity. The induction of FUM, mMDH, and CS, at both the activity and transcription levels, indicates that in addition to anaplerotic carbon fixation in the cytoplasm, induction of some TCA enzymes occurs in the mitochondria of −Fe citrus roots. Similar increases in TCA enzymes, including CS, isocitrate dehydrogenase, FUM and mMDH, have been reported for numerous herbaceous species (Zocchi 2006; Vigani 2012), although none of these studies have involved purified mitochondria, and most of these TCA cycle enzymes are also present in the cytosol. Interestingly, mitochondrial ACO is not altered by Fe deficiency in Carrizo citrange at either the activity or transcript levels, which therefore has become a limiting step in the TCA cycle, and likely contributes to citrate accumulation in limited Fe-availability roots. This has also been observed in *Citrus* fruit vesicles when subjected to Fe shortage (Shlizerman et al. 2007). ACO is an Fe-containing enzyme. There are contradictory reports about the changes induced by Fe deficiency on its activity in herbaceous species, with increases reported in tomato and sugar beet (Li et al. 2008; Lopez-Millan et al. 2009), and decreases in cucumber (Donnini et al. 2010). These results generally support that the TCA could work in a noncyclic flux mode upon Fe deprivation, as previously proposed in herbaceous species (Lopez-Millan et al. 2000; Vigani 2012).

The mitochondrial membrane also has a family of transporters that facilitates the movement of metabolites between the interior and exterior of this organelle, and maintains communication with the cytosol (Vigani and Zocchi 2009; Vigani et al. 2012). Much progress has been recently made in identifying the members of a family of carrier proteins in *Arabidopsis thaliana*, where at least 60 putative genes have been described to encode their expression (Picault et al. 2004). A mitochondrial carrier (DTC) has been identified, which is capable of transporting both dicarboxylic acids (e.g., oxaglutarate, oxaloacetate, malate, succinate, maleate, and malonate) and tricarboxylic acids (e.g., citrate, isocitrate, cis-aconitic, and trans-aconitic). This carrier might play a key role in these plant metabolic functions, which require acid flow to or from mitochondria (Picault et al. 2002). The expression of this gene in citrus significantly increased (62%) in Fe-deprived roots compared to the controls. This up-regulation supports the increased transfer of LMWOAs, mainly malate and citrate, between mitochondria and the cytosol, and could also serve as a strategy to allow for the faster turnover of reducing equivalents (Martinez-Cuenca et al. 2013c). DTC transports a broad spectrum of di- and tri-carboxylates, and may accept the single protonated form of citrate ($H\text{-citrate}^{2-}$), as well as the unprotonated form of malate, $malate^{2-}$ (Picault et al. 2002). DTC protein accumulation has been reported to date only in Fe-deprived cucumber roots, while increased citrate export from mitochondria to the cytosol has been observed in several species under Fe deficiency (Vigani and Zocchi 2009).

Excretion of Low-Molecular-Weight Compounds

Plant roots are able to release a wide variety of organic compounds, including reducing sugars, amino acids, organic acids, and phenolic and flavins compounds, which can constitute up to 20 % of assimilated carbon. Type of root exudates is genetically determined, but some environmental factors could alter its composition: pH, temperature, soil type, light intensity, age, plant nutritional status, and presence of microorganisms (Jones 1998).

1. Phenols: Fe-deficient plants produce and excrete in nature different phenolic compounds (Marschner and Römheld 1994), such as caffeic, phenolic, chlorogenic, and p-coumaric acids (Hether et al. 1984). In *Citrus*, the release of phenolic compounds significantly increases in response to Fe deficiency in rough lemon and Cleopatra mandarin seedlings (Treeby and Uren 1993). The main role of phenols probably consists in inhibiting the degradation of organic acids, whose role in nutrition as chelating ferric Fe is very important.
2. Flavins: under Fe-deficiency conditions, some species accumulate and excrete flavins, such as riboflavin or riboflavin sulfates (Susin et al. 1994). These substances are capable of directly reducing Fe^{3+} compounds in the presence of electronic donors like NADH or NADPH (Gonzalez-Vallejo et al. 1998). Furthermore, riboflavin sulfates exert antimicrobial action and reduce the possible competition between microorganisms from soil and plants in Fe absorption (Susin et al. 1994).
3. Organic acids: these compounds can become the most abundant components excreted by the roots of plants grown under Fe-deficient conditions (Kraffczyk et al. 1984). Depending on their dissociation properties and the number of carboxylic groups, organic acids can vary their negative charge and, thus, their ability to form complexes with various metals from soil solution. Under Fe-deficient conditions, the most likely function of these acids is solubilization of the Fe^{3+} form. When soil pH is low, organic acids form very stable complexes with Fe^{3+} and favor its dissolution in soil solution (Jones 1998). In calcareous soils with a high pH, Fe mobilization by citrate and malate is slow because formed complexes are unstable. However, the combination of the acidifying power of enzyme H^+-ATPase in roots and the chelator power of organic acids (which increases with a lowering pH) could be a viable mechanism for mobilizing the Fe present in the rhizosphere (Jones 1998).

Increased LMWOA exudation due to Fe deficiency has been described in herbaceous species (Jelali et al. 2010b), and also recently in *Citrus* plants (Martinez-Cuenca et al. 2013c). These authors stated that the rate of citrate and malate exudation in Carrizo citrange roots grown under Fe-deficient conditions was 41 % and 75 % higher, respectively, than that measured in the control plants. Based on the differential exudation rates for both compounds, and also on the differential increases in xylem sap concentrations, it is tempting to speculate that increased citrate in xylem sap would in part aim to improve long-distance Fe transport, whereas malate may play a more significant role in root exudates and metabolic processes of root adaptation reprogramming.

Synergism of H^+-ATPase, FC-R, and PEPCase Enzymatic Activities in the Fe Absorption Mechanism in Roots

Activation of both acidifying and reductive capabilities in roots with Fe deficiency is considered a synergistic action of enzymes H^+-ATPase and FC-R to improve Fe acquisition in roots (Vizzotto et al. 1999; Dell'Orto et al. 2000b; Rabotti and Zocchi 2006; Donnini et al. 2009; M'sehli et al. 2009). The interactive effect of both enzymes has also been described in the cell. In particular, the rapid increase in FC-R activity, due to Fe deficiency, causes the oxidation of the NAD(P)H, primary electron donor for the reduction of Fe^{3+} compounds. When these protons accumulate in the cell, it helps lower the pH in the cytosol and, at the same time, induces enzyme H^+-ATPase activity (Rabotti et al. 1995; Rabotti and Zocchi 2006). The depolarization of the membrane electric potential has also been observed in cells of plants grown under Fe-deficient conditions (Zocchi and Cocucci 1990). This has been attributed to the transfer of transmembrane electrons, which are used by enzyme FC-R during Fe^{3+} reduction (electron acceptor). To ensure cell repolarization, extrusion of protons and other ions such as K^+ is activated (Alcantara et al. 1991).

Accumulation of organic acids under Fe chlorosis conditions seems to be involved in the cytoplasmic pH regulation mechanism (Landsberg 1981). The increment of intracellular pH as a result of H^+ extrusion leads to PEPCase activation and, therefore, to acid synthesis in Krebs cycle (Abadia et al. 2002). Thus, the cell achieves an ionic balance or homeostasis in the cytoplasm by neutralizing the alkalizing effect produced by enzyme H^+-ATPase.

However, some authors have described the individual action of some enzymes as a predominant factor in Fe absorption. Grusak et al. (1990) attributed it to reduction capacity, while Wey et al. (1997) suggested the release of H^+. The result of the synergy between the two enzymes is increased Fe absorption by plants. Similarly, the main trait that determines Fe-chlorosis tolerance in *Citrus* is likely the ability to boost Fe^{3+} reduction in response to Fe deficiency through enhanced *FRO2* gene expression (Martinez-Cuenca et al. 2013a).

Effect of Bicarbonate on Strategy I

Bicarbonate is widely accepted as one of the main factors to cause Fe chlorosis in Strategy I plants (Alcantara et al. 2000). Studies carried out in plants grown in a medium that contains bicarbonate have revealed a notable increase in FC-R activity in roots (Zuo et al. 2007; Donnini et al. 2009; Jelali et al. 2010b). However, this increase was not as marked as the levels found in the plants grown when Fe was completely lacking. Other reports carried out in *Citrus* and peach plants grown under high HCO_3^- conditions have observed reduced FC-R enzyme activity (Chouliaras et al. 2004a; Molassiotis et al. 2006). However, when *Citrus* plants were grown in nutrient solution without Fe, but with a very low Fe content or with the addition of HCO_3^-, the Fe reduction capacity markedly increased compared to the activity noted in the roots of plants with an adequate Fe supply (Chouliaras et al. 2004b). Bicarbonate

treatment in Fe-deficient plants, or with a low Fe concentration, increases the reducing capacity in the roots of "Newhall" orange grafted onto Citrange troyer rootstocks (Pestana et al. 2001), and also in *Citrus taiwanica* and *Citrus volkameriana* seedlings (Chouliaras et al. 2004a), which also seems to occur in cucumber, pea, tomato, and *Arabidopsis* (Lucena et al. 2007). Lucena et al. (2007) showed that this ion may induce chlorosis in plants by inhibiting the expression of the genes that control enzymes FC-R and H^+-ATPase, and ferric transporter, probably through an altered expression efficiency of certain transcription factor proteins.

Disruption of the acidification process by bicarbonate cannot be considered a cause of Fe uptake inhibition in plants that grow in nutrient solutions in which Fe is applied in a soluble chelated form (Martinez-Cuenca et al. 2013b). It is more likely that the ion present in the root apoplast neutralizes the H^+ pumped out of the cytosol, thus preventing a transmembrane electrochemical gradient, the driving force of Fe^{2+} transport (Zocchi and Cocucci 1990), and reducing Fe^{2+} uptake and transport in plants. The Fe-deficient conditions induced by this ion would result in not only the enhanced expression of some genes related with the Fe acquisition system (*IRT1*, *FRO1*, *FRO2*, *HA1*, and *PEPC1*), but also in the activity of the corresponding enzymes, which appears to constitute an adaptive mechanism of citrus roots to these soils (Fig. 9.3a). This response has also been observed in the presence of a source of Fe, like that present in cotyledons (Martinez-Cuenca et al. 2013d). Finally, it is likely that cytoplasm alkalinization associated with bicarbonate treatment could cause PEPCase enzyme activation to result in an increase in both the capacity to fix carbon from bicarbonate and the synthesis of organic acids to maintain the pH of the root cell cytoplasm (Abadia et al. 2002).

Influence of Micronutrients on Strategy I

Some authors have described that Strategy I responses to Fe deficiency not only exert action on Fe homeostasis in plants, but also on the absorption of other metal ions, such as Zn, Mn, or Cd (Izaguirre-Mayoral and Sinclair 2005; Shanmugam et al. 2011). As described above, micronutrients bioavailability is determined by soil pH, so when plants stimulate rhizosphere acidification to promote the solubilization of Fe^{3+} and Fe^{2+} ions, they also enhance the provision of Zn^{2+} and Mn^{2+} from different soil compounds (Lindsay 1995). Zn normally presents a +2 oxidation state, so unlike Fe, plants directly access it with no reduction. However, Mn can take several forms and its availability to roots depends on its oxidation state. Thus, oxidized forms Mn^{3+} and Mn^{4+} cannot be absorbed by roots. It has been suggested that enzyme FC-R can reduce Mn^{3+} under Fe-deficient conditions (Pittman 2005).

Finally, absorption of divalent metals by root cells appears to be regulated by members of the gene family of zinc and iron transporter proteins, ZIP (Grotz et al. 1998). ZIP transporters regulate Zn accumulation in yeast cells, where *ZRT1* and *ZRT2* are high- and low-affinity Zn transporters, respectively, which are regulated by changes in the cellular content of Zn. *ZIP3* and *ZIP1* are expressed mainly in the roots of plants subjected to Zn deficiency, which suggests they play a role in the

absorption of Zn^{2+} from soil solution (Clemens 2001). As previously reviewed, Fe^{2+} is transported into root cells by high-affinity transporter IRT1 (Vert et al. 2002). While the primary function of *AtIRT1* is Fe absorption, it can also carry other metals, including Zn^{2+} and Mn^{2+} (Korshunova et al. 1999). This was first studied in a mutant of *Arabidopsis*, in which IRT1 activity was suppressed. As expected, this mutant demonstrated that IRT1 was the main way to acquire Fe by roots of plants under Fe-deficient conditions (Vert et al. 2002). There is also a report of a marked reduction in Mn concentration compared to unmodified species, which indicates that IRT1 can also function as a carrier of Mn^{2+}, and definitely constitutes one of the main absorption routes of Mn^{2+} in roots of *Arabidopsis* plants grown under Fe-deficiency conditions. Other transporters from the ZIP family, such as *MtZIP4* and *MtZIP7* in *Medicago truncatula* (Lopez-Millan et al. 2004), and *LeIRT1* and *LeIRT2* in tomato (Eckhardt et al. 2001), also appear to possess the ability to transport Mn^{2+}. Moreover, *irt1* mutants exhibit lower levels of Zn, which indicates the ability of IRT1 to transport this element. However, IRT1 showed a high affinity for Fe transport and a low affinity for Zn when expressed in yeast (Cohen et al. 2004). *IRT2* has also been reported to have the ability to transport Zn when expressed in epidermal root cells of Fe-deficient *Arabidopsis* plants (Vert et al. 2001). In *Citrus*, the possibility of reducing root Fe uptake as a result of Zn^{2+} and Mn^{2+} antagonism may induce root responses to Fe-deficiency, particularly the activity of putative genes *FRO2*, *IRT1*, and *NRAMP3* (Martinez-Cuenca et al. 2013e). However, ionic antagonism might not be the main cause of these responses since (1) plants grown in a medium supplemented with Fe and with a high Zn or Mn concentration induced Strategy I responses to a greater extent than plants grown without Fe; (2) genes *FRO2* and *IRT1* also were stimulated even in plants grown with no external Fe.

Accordingly, it has been reported that the Fe^{3+} reduction capacity of roots decreases with Zn^{2+} or Mn^{2+} deficiency, which has been stimulated after the resupply of these ions (Sijmons and Bienfait 1986). In a later work, Romera et al. (1997) suggested that in Fe-deficient cucumber plants, the bicarbonate ion can inhibit the development of the reducing capability of Fe^{3+} from roots as a result of the reduced availability of certain ions required for this response; e.g., Zn^{2+} and Mn^{2+}. Later, this was partly confirmed by Bohorquez et al. (2001), who found that the reduction capacity of peach plants, which were grown at a low Fe concentration because of bicarbonate treatment, increased when they received an additional supply of Zn. A similar effect has been observed with the acidification response, where the removal of ions Zn^{2+} and Mn^{2+} from the nutrient solution blocked H^+ excretion from the roots of Fe-deficient bean plants (Sijmons and Bienfait 1986). These plants restarted root H^+ excretion when any of these metals were added to the medium.

Effect of Flooding on Strategy I

In citrus, the combined action of the disruption of Fe uptake and root anoxia caused the photosynthesis rate to further drop by a factor of 5.2 (Martinez-Cuenca et al. 2015). Accordingly, Fe deficiency and root anoxia developed different and

independent action mechanisms on the leaf chloroplast structure and function, and their effects were additive when both stresses occurred simultaneously. This effect has been previously described in pea-flooded plants (Ladygin 2004).

Regarding Fe-deficiency responses, the amount of H^+ that flooded plants released to the media diminished (Fig. 9.5b), which suggests inactivation of the plasma membrane H^+-ATPase enzyme from root epidermal cells. Disruption of H^+ extrusion to media by waterlogging prevents a transmembrane electrochemical gradient from being generated, which constitutes the driving force of Fe^{2+} uptake (Zocchi and Cocucci 1990). Under these conditions, mitochondrial respiration is inhibited and, although the energy flow is redirected through alternative anaerobic reactions, these processes (e.g., production of lactate, ethanol) lead to very modest energy harvests. Under such conditions, the drop in the cytoplasmic ATP level is sharp and H^+-pumps, which are major consumers of ATP, work at a very low activity rate, which results in a poorer H^+ export across the plasma membrane (Felle 2005).

Moreover, membrane depolarization likely disturbs, and even inverts, the inward driving force of cations, and the diffusion potential might actually promote ions to leak out of the cell. Fe may also be immobilized in the root apoplast, probably due to an enhanced apoplasmic pH (Kosegarten and Koyro 2001; Martinez-Cuenca et al. 2013a, b).

The effect of waterlogging treatment on Fe-deficient plants not only impairs the induction of putative gene *FRO2*, but also markedly lowers its expression level to similar values to those recorded in the control plants (Fig. 9.5a). This suggests that the expression of gene *FRO2* under waterlogging conditions is not regulated by the plant's Fe-nutritional state. It is noteworthy that other factors like lack of NADH from long-term oxygen depletion likely have an adverse effect on FC-R activity. As O_2 deficit enhances ROS production, and the reactions to cope with cellular oxidative damage (Mittler 2002) need to consume electron donors (Fukao et al. 2003), NADH availability is restricted for Fe-reduction activity in waterlogged plants.

However, there is evidence to suggest that induction of putative gene *IRT1* expression, and therefore of Fe-transport capacity, under waterlogged conditions is unable to improve Fe uptake by roots. So it would appear that the synergistic action of both H^+-ATPase and FC-R enzymes is the preferential regulator of the Fe acquisition system under waterlogging conditions. Impairment of the regulation of genes *HA1* and *FRO2* in waterlogging-stressed seedlings leads to the blockage of Fe uptake capacity, as supported by ^{57}Fe labeling experiments (Fig. 9.5d). ^{57}Fe uptake experiments have indicated that regardless of the plant's nutritional status, waterlogging markedly impairs Fe uptake by roots, and the inhibitory effects of anoxia on *HA1* and *FRO2* genes are major causes of this constraint, likely due to the limited energy supply from the anaerobic metabolism, which may not suffice to maintain the Fe uptake process (Kosegarten and Koyro 2001; Martinez-Cuenca et al. 2013a, b). Consequently, ^{57}Fe transport and distribution to aerial parts under flooding conditions are also blocked (Martinez-Cuenca et al. 2015).

It is worth mentioning the particular case of lime alkaline soils as they induce Fe-deficiency due to bicarbonate production as a result of oxygen depletion, thus hindering gas exchange and CO_2 accumulation (Zude-Sasse and Schaffer 2000). In

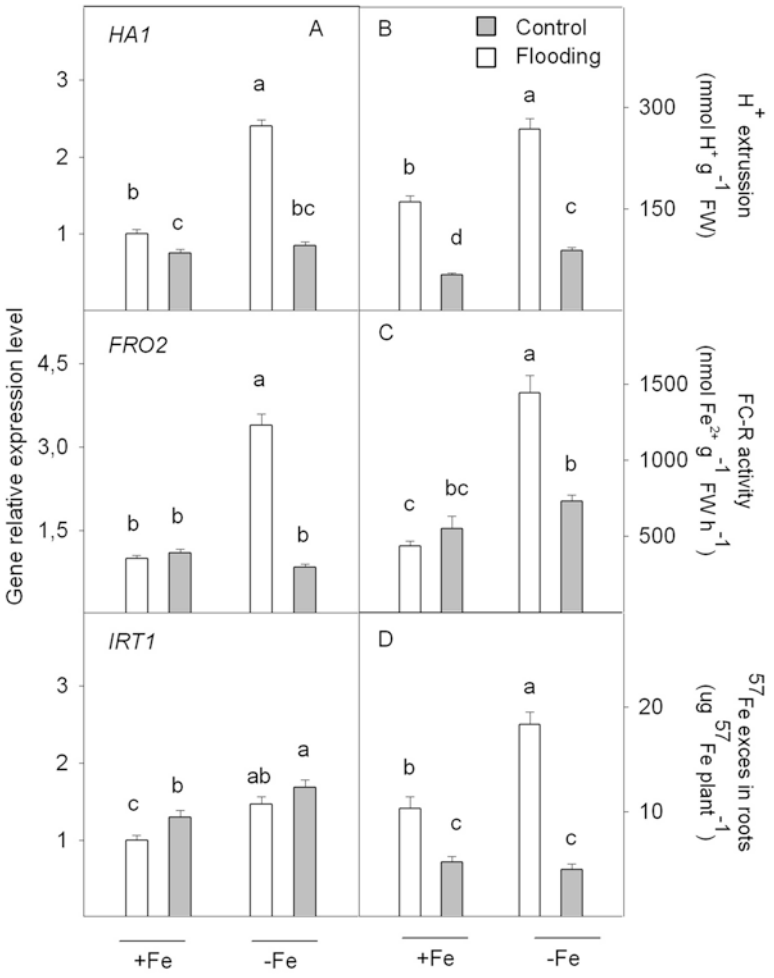

Fig. 9.5 (**a**) Relative expression of putative *HA1, FRO2*, and *IRT1* genes measured by real-time RT-PCR analysis, (**b**) H^+ extrusion, (**c**) Ferric-chelate reductase (FC-R) activity and (**d**) ^{57}Fe content of in excess (Martinez-Cuenca et al. 2015). All analysis were carried out in roots of *Citrus* seedlings grown for 21 days in Fe-sufficient (+Fe) or Fe-deficient (−Fe) nutrient solutions with the non-stressed (Control) or the flooding treatment (Flooding). The values are the mean ± SE of three independent experiments ($n = 3$) run in triplicate. *Bars* with different letters indicate significant differences at $^*p < 0.05$ using the LSD multiple range test (*ns* not significant). *Prot* protein, *FW* Fresh Weight

this case, a high bicarbonate level induces Fe deficiency in plants through buffering soil solution to inhibit FC-R induction, which enhances the alkalinization of xylem sap and the cell apoplast, and also prevents Fe uptake and transport from roots to shoots (Wegner and Zimmermann 2004; Donnini et al. 2009; Martinez-Cuenca et al. 2013b). However, the experiments by Martinez-Cuenca et al. (2015) were conducted in the absence of HCO_3^-, which indicates that inhibition of Fe uptake by flooding under field conditions is not due to only the presence of this ion.

Morphological Changes in the Root System

These changes include the formation of secondary roots and root hairs, as well as thickening of subapical zones, and stimulation of transfer cells formation (Kramer et al. 1980). Thus, roots of plants respond by increasing their contact surface under Fe-deficiency conditions.

Alteration to Protein Content

Various studies performed by electrophoresis and in vitro transcription of mRNA from roots have shown that when plants are grown under Fe-deficient conditions, the protein synthesis of different polypeptides alters (Bienfait 1988b; Rellan-Alvarez et al. 2010).

Iron Transport Throughout the Plant and Allocation in Different Plant Tissues

Long-Distance Iron Transport

The involvement of aerial parts and global iron status in the regulation of Fe-deficiency responses has been well documented (Grusak and Pezeshgi 1996; Vert et al. 2003). Changes in gene expression are probably the result of a complex signaling system of Fe uptake which involves not only roots, but also shoots. This is in agreement with a local induction from the root iron pool and its availability in the root apoplast, and through a systemic long-distance pathway that involves a shoot-borne signal which complementarily adjusts the nutrient uptake proteins to the demand of the whole plant (Vert et al. 2003).

After entering the root symplast, Fe is bound to chelating compounds and moves via intercellular connections into the stele along the diffusion gradient. This requires the Fe efflux from the symplast in the apoplastic space, but this mechanism is still not clearly understood. However, it is generally assumed that presence of Fe in the xylem is preferentially chelated as Fe^{3+}-citrate, which evidences the role of this molecule in long-distance Fe transport. Thus, TCA cycle activation and the release of organic acids, mainly citrate, to xylem sap is a response mechanism to Fe-deficiency in citrus plants (Martinez-Cuenca et al. 2013c; Fig. 9.4). In *Arabidopsis*, the citrate efflux into xylem vessels is linked to gene FRD3, which belongs to the multidrug and toxin efflux (MATE) family, and encodes a transmembrane protein of small molecule transporters (Durret et al. 2007).

Intercellular Iron Transport

Once inside the cell cytoplasm, free iron must be preserved from oxidation to avoid not only its precipitation, but also the formation of superoxide and hydroxyl radicals (Hell and Stephan 2003). This suggests that the Fe located in the cytoplasm is chelated to some type of compound to avoid these harmful effects in plants. Pich et al. (1995) have proposed nicotianamine (NA) to be the principal complexing agent for free Fe in cells. Fe transport from epidermal cells of roots to the xylem has been performed through the symplast as the Fe^{2+}-NA complex. Once it is there, it is probably oxidized to Fe^{3+} (Landsberg 1984; Hell and Stephan 2003) and transported over long distances to upper plant organs, bound mainly to citrate and other organic anions, such as malonate or malate (Tiffin 1966; Lopez-Millan et al. 2001; Martinez-Cuenca et al. 2013c), and even other organic substances (Cataldo et al. 1988).

Although Fe transport occurs mainly via the xylem pathway in the same direction as the mass flow created by plant transpiration, this element can also concentrate in growing organs, where transpiration intensity is lower due to incomplete xylem structure (Landsberg 1984). Remobilization of Fe from adult to young developing organs via the phloem pathway also depends on NA (Hell and Stephan 2003).

Subcellular Iron Transport

When Fe, mainly complexed as ferric citrate, reaches leaves, it must be reduced to enter cells. For this reduction, the existence of an FC-R enzyme located in the plasma membrane of leaf mesophyll cells has been proposed (Gonzalez-Vallejo et al. 1999; Larbi et al. 2001). This reductase uses Fe^{3+}-citrate chelate, or another Fe source, as a substrate, and the energy required for Fe^{3+} reduction comes from light (Bienfait and Scheffers 1992). After this reduction, Fe^{2+} enters leaf cells, as it does in roots. Some studies carried out in *Arabidopsis* have reported that genes *FRO* and *IRT* are also expressed not only in roots, but also in leaves (Baüer et al. 2004). Nevertheless, this is still a poorly studied aspect in citrus plants.

At the cellular level, plants possess homeostatic mechanisms that allow them to maintain the correct concentrations of essential metal ions in various cell compartments and to minimize damage from exposure to toxic concentrations (Clemens 2001). A complex network of transport processes, and highly regulated chelation and sequestration, allow the absorption, accumulation, displacement, and detoxification of metal ions to be controlled (Haydon and Cobbett 2007).

Thus, vacuoles are crucial compartments for Fe storage and sequestration in cells, particularly in seeds. In the membrane of this organelle, we find a family of proteins that is related with natural resistance mechanisms (NRAMP transporters) in plants (Supek et al. 1996). NRAMP transporters seem to be evolutionarily conserved in all living organisms (animals, fungi, bacteria, etc.), including plants, where NRAMP genes have been cloned and characterized in several species

(Bereczky et al. 2003; Kaiser et al. 2003). In *Citrus*, putative gene *NRAMP3* is induced under Fe-deficiency conditions (Martinez-Cuenca et al. 2013e), which indicates that NRAMP transporters likely play a key role in Fe homeostasis in this plant. Similar results have been obtained in *Arabidopsis* and tomato plants (Thomine et al. 2003; Lanquar et al. 2005). The subcellular localization of *AtNRAMP3* and *AtNRAMP4* in the tonoplast suggests that these transporters act by mobilizing vacuolar Fe deposits toward the cytoplasm (Thomine et al. 2003; Lanquar et al. 2005). As in other organisms, NRAMP transporters in plants are not specific to a single metal substrate. Thus *AtNRAMP3*, which can carry Fe^{2+}, but also Zn^{2+}, Mn^{2+}, and Cd^{2+}, plays a key role in intracellular metal homeostasis (Thomine et al. 2003; Xiao et al. 2008). Fe mobilization, in turn, influences other micronutrients because it has been observed that accumulation of Zn and Mn competes with Fe to bind to action sites. Therefore, dispute is more marked when there are high concentrations of the above cations, as opposed to low Fe levels in Fe-starved roots (Martinez-Cuenca et al. 2013e). Even under normal Fe-growing conditions, addition of large amounts of Zn^{2+} and Mn^{2+} results in a significant increase in the putative *NRAMP3* expression level; consequently, a drop in the Fe concentration in shoots might explain the induction of genes *FRO2* and *IRT1* in citrus plants.

It is worth noting the role of the NRAMP transporter in the mobilization of Fe from seeds, especially in plant germination under low Fe conditions (Thomine et al. 2003; Lanquar et al. 2005). Thus, a reduction in Fe displacement from the vacuole to the cytosol in citrus lemon seedlings grown in the presence of HCO_3^- has been observed, which is likely due to the inhibition of putative gene *NRAMP3* expression in cotyledons (Martinez-Cuenca et al. 2013d). Consequently, Fe-deficiency responses in plants were activated. To support this, Thomine et al. (2003) reported the overexpression of the *AtNRAMP3* down-regulated *FRO2* and *IRT1* genes that code for enzyme FC-R and the primary iron transporter, respectively.

Fe reaches its final destination in the chloroplast through active light-dependent transport into this cell organelle, where it participates in photosynthesis (Bughio et al. 1997). Any remaining Fe is stored in a nontoxic protein called phytoferritin, which can be released according to plant requirements (Briat and Lobreaux 1997). Another destination of this Fe might be the mitochondrion, which is the organelle where the proteins that include an Fe-S bond are generated (Kushnir et al. 2001). To our knowledge, no information about the molecular regulation of Fe in these organelles in citrus plants exists.

New Research Approaches and Perspectives

Iron Inactivation in Leaves

Citrus plants locate most total Fe in plants at the root level, and it is mainly trapped in the root apoplast (Martinez-Cuenca et al. 2013a, b). However, what proportion of absorbed Fe is retained at the root level and how much is transported to the canopy

remain unclear. In the canopy, it undergoes a second reduction before entering mesophyll cells. On many occasions, Fe content in chlorotic leaves is similar to, or even higher than, that found in green leaves. This phenomenon is known as the "Fe paradox" (Römheld 2000). Its regulation in this crop is still unclear, but will eventually explain the efficiency of Fe homeostasis in the whole plant.

Active Fe in leaves varies between *Citrus* rootstocks, and ranges from 600 μM in the most tolerant species to 125 μM in the most sensitive ones (Sudahono et al. 1994). This parameter and leaf chlorophyll concentrations are negatively related with nutrient solution pH in most genotypes. The rise in pH in the apoplast reported in Fe-chlorotic leaves is likely one of the main factors behind the inhibition of Fe moving into the cell (Lopez-Millan et al. 2001), which impairs FC-R enzyme activity, and consequently depresses Fe transport across the plasmalemma (Susin et al. 1996; Kosegarten et al. 1999). Thus the optimum pH value, approximately 5.0, has been stimulated in an intact leaf apoplast (Kosegarten et al. 1999), which likely comes close to the optimum pH for FC-R activity in leaves, ranging from 5.5 to 6.0 (Gonzalez-Vallejo et al. 2000). An alkaline apoplast pH may attain values of 6.3–7.0 and is able to depress Fe^{3+} reduction (Kosegarten et al. 1999). This theory matches experimental data that have shown chlorotic leaf regreening as a result of spraying acidic solutions on the canopy (Kosegarten and Koyro 2001). Other reports have suggested that an increment in the citrate:Fe ratio in the leaf apoplast under Fe-chlorosis conditions might reduce FC-R activity. Under these conditions, ferric citrate may form large citrate polymers as a result of increasing pH values, which may depress Fe^{3+}-citrate reduction (Lopez-Millan et al. 2000).

Remobilization and Redistribution of Iron in Shoots

It is widely assumed that Fe remobilization from older leaves to growing plant tissues takes place via phloem transport. The transpiration flow in xylem vessels is inefficient in developing organs, such as buds, young leaves, fruits, and storage organs, as the xylem structures in these organs are incomplete. Moreover, as pH in phloem sap is over 7.0, Fe needs to be bound to chelators to remain soluble. Studies have indicated the Fe transport protein ITP, which specifically binds Fe^{3+} as a Fe-chelator in the phloem. In addition, NA has been proposed to function in Fe transport in the phloem given its ability to form stable complexes with Fe^{2+} at a neutral and weakly alkaline pH (Von Wiren et al. 1999). To our knowledge, it is an unknown matter in *Citrus* plants.

Otherwise, isotopic dilution is a technique used to evaluate mineral distribution and/or redistribution in plants and has been widely employed in nitrogen, calcium, or carbon assays (Martinez-Alcantara et al. 2012). Traditionally, Fe labeling experiments have been carried out with radioactive isotopes, such as ^{59}Fe and ^{54}Fe, but their use is problematic because of the specific laboratory and personal training requirements involved (Fox et al. 1996). The combination of treatments with a stable ^{57}Fe isotope and an analysis by inductively high-resolution-coupled plasma

mass spectrometry (Q-ICP-MS) is a good and safe tool to determine Fe uptake and distribution in plants (Rodriguez-Castrillon et al. 2008). Hence, its use might clarify the mobilization of Fe from reserve organs during the development of young organs in different phenological states in citrus.

Conclusion

Significant advances have been made in recent years in the Fe uptake mechanism in citrus plants, particularly molecular studies into acidification, reduction, and transport responses in root systems, conducted in a wide range of chlorotic situations. However, more research is needed to improve our current knowledge about local- and long-range signaling in plants, how these events are integrated with Fe deficiency responses, and the mechanisms involved in Fe remobilization in plants. All together, they will enable us to fully understand the molecular basis of Fe homeostasis, transport, and remobilization in citrus plants, and to therefore improve their specificity, if necessary.

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Chapter 10
Brassinosteroids: Physiological Roles and its Signalling in Plants

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Abstract Brassinosteroids (BRs) are known to be naturally occurring polyhydroxylated plant steroids that showed diverse roles in regulation of various physiological and developmental processes in plants under both natural and stressful conditions. With the advancement of various omic approaches, informations available provide huge data to understand the processes responsible for BRs biosynthesis, transport, signalling, and metabolism pathways. Moreover, in the recent past BRs have shown the ability to cross talk with other phytohormones such as auxin, polyamines, cytokinin, ethylene, and jasmonic acid in regulating varied ranges of physiological and biochemical processes in plants. On the other hand, the exogenous application of BRs in agriculture to improve growth and yield under various stress conditions including drought, salinity, extreme temperatures, and heavy metal toxicity is of immense significance as these stresses severely hamper the normal metabolism of plants. The information available till date regarding BRs will definitely help in establishing various mechanisms which modulate various processes in plants and overcome the future challenges in agriculture.

Introduction

Growth and development of plants are regulated by both endogenous and exogenous factors. As endogenous factors, hormones play an indispensable role in regulating the developmental processes. Hormonal regulation of development is a complex process with interactions of various hormones at transcriptional, translational, and cellular levels (Chandler 2009). Among hormones, the well-recognized plant steroidal hormone brassinosteroid (BR) also plays pivotal role in promotion of cell expansion, cell elongation, cell division, and vascular differentiation, and

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provides protection against various abiotic and biotic stresses. Over the past decade, many of the plant hormone receptors, signalling components, and downstream transcriptional networks have been identified through genetic analyses. Similar progress in understanding the synthesis and transport of hormones has provided a more complete picture of the mechanisms controlling the distribution and abundance of these molecules. It is now possible to explore the more complex question of how a plant coordinates its growth with the simultaneous input of multiple stimuli by using insights into hormone functions and the plethora of signalling and synthetic mutants. The crosstalk between two hormones, at both physiological and molecular levels, is receiving substantial attention, bringing new understanding of how they are able to act either antagonistically or synergistically in a tissue-specific fashion to influence plant growth and development. This review focuses on our understanding of the molecular mechanisms that control physiological processes regulated by hormones particularly brassinosteroids and its signalling network to regulate various physiological processes in stress and stress-free conditions.

Brassinosteroids

Brassinosteroids (BRs) are a group of steroidal plant hormones that play pivotal roles in a wide range of developmental phenomenon in plants including cell division and cell elongation in stems and roots, photomorphogenesis, reproductive development, leaf senescence, and stress responses (Clouse and Sasse 1998). Till date, more than 70 BRs have been isolated from plants (Bajguz and Hayat 2009). BRs play vital roles in plant development and also promote tolerance to the wide range of abiotic stresses. Although much has been learned about their roles in plant development, however the mechanisms by which BRs control stress responses and regulate stress-triggered gene expression in plant are not fully dissected.

Biosynthesis of Brassinosteroids

Brassinosteroid is a group of naturally occurring polyhydroxylated steroidal phytohormones playing fundamental roles in normal plant growth and development of plants. These are C27, C28, or C29 steroids depending on their C-24 alkyl substituents (Sakurai et al. 1999; Yokota 1999; Khripach et al. 1999; Fujioka and Yokota 2003). Among all the BRs identified till date, brassinolide (BL) is the most biologically active compound and has been found in a large number of plant species (Kim et al. 2008). BL is a 28 carbon compound which possesses S-methyl group at the position C24 of the side chain of 5α-ergastane structure, which is the main focus of research on BRs. Other BRs are mainly intermediates of BL biosynthetic pathway or inactivated products that result from various catabolic reactions of BRs (Zhao and Li 2012). Detailed study of the biosynthesis of BL, a C28 BR, revealed that two

parallel pathways are operative viz., the early and late C-6 oxidation pathways (Fujioka et al. 1998). BR-specific biosynthetic precursor campesterol (CR) is initially converted to campestanol (CN) and then via early and/or late C-6 oxidation pathways to BL. Both early and late C-6 oxidation pathways are known as CN-dependent pathways as both these pathways start from CN as precursor. In crop plants like tomato and tobacco, the late C-6 oxidation pathway appears to be the predominant route because most of the endogenous BRs in these species comprise only members of the late C-6 oxidation pathway. In the early C-6 oxidation pathway, C-6 oxidation takes place before DWF4-mediated C-22 hydroxylation. In the early C-6 oxidation pathway, CN is mainly converted to 6-oxocampestanol (6-oxoCN) and then to cathasterone (CT), teasterone (TE), 3-dehydroteaserone (3DT), typhasterol (TY), and then to castasterone (CS), in order. In late C-6 oxidation pathway, C-22 hydroxylation takes place ahead of C-6 oxidation. Once CR is hydroxylated by DWF4, the intermediates are further modified and merged into the late C-6 oxidation pathway. CN is first hydroxylated at C-22 to form 6-deoxocathasterone (6-deoxoCT) and is then converted to corresponding intermediates like those in the early C-6 oxidation pathway but in C-6 deoxy forms (Zhao and Li 2012). The oxidation steps of 6-deoxoTY and 6-deoxoCS into TY and CS are catalyzed by the enzymes CYP85A1 and CYP85A2, respectively (Kim et al. 2005). However, in *Arabidopsis*, BR6ox at several points links the late C-6 oxidation pathway to the early C-6 oxidation pathway. DWF4 can also act on multiple biosynthetic intermediates in the upstream steps, and thus is CN independent. The pathways can branch at campesterol to establish an early C-22 hydroxylation pathway (Choe et al. 2001; Fujioka and Yokota 2003).

Signalling Pathway of Brassinosteroids

Brassinosteroids are the steroidal plant hormones which bind to plasma membrane-anchored BRI1 (BRASSINOSTEROID INSENSITIVE1), a leucine rich repeat (LRR) receptor-like kinase (RLK) receptor (Li and Chory 1997), to elicit signal cascade regulating the expression of genes through cytosolic and nuclear transcription kinases and phosphatases. Upon BRs perception, BRI1 rapidly releases BKI1 (BRI1 KINASE INHIBITOR1), a negative regulator at the C-terminal domain of BRI1, and activates its kinase activity by multiple autophosphorylations and sequential transphosphorylation of BRI1 with BAK1 (Li et al. 2002; Nam and Li 2002; Wang and Chory 2006). This isolated BKI1 enhances BRs signalling by degrading 14-3-3 proteins, responsible for the cytoplasmic retention of two master transcription factors (TFs), BZR1 (BRASSINAZOLE RESISTANT 1) and BES1 (BRI1-EMS SUPPRESSOR 1) of BRs signalling (Yin 2002; Ryu et al. 2010; Jaillais et al. 2011; Choudhary et al. 2012). Phosphorylation of BSKs (BRs signalling kinases) by activated BRI1 subsequently activates the BSU1 (BRI1 SUPPRESSOR 1) phosphatase. The activated BSU1 in turn deactivates BIN2 (BRASSINOSTEROID INSENSITIVE 2) through dephosphorylation (Clouse 2011; Tang et al. 2008;

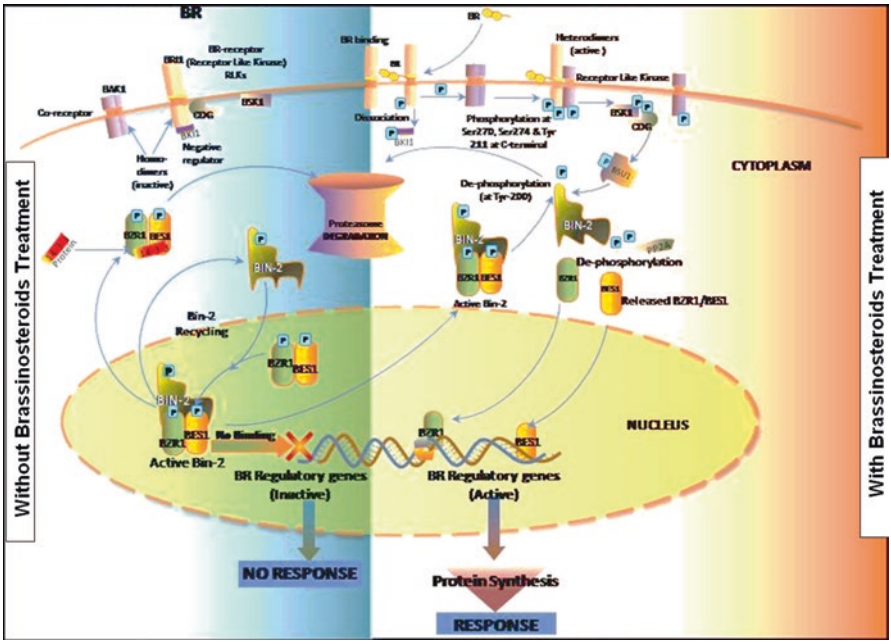

Fig. 10.1 Brassinosteroids signalling pathway in plants

Kim and Wang 2011). The inactivation of BIN2 relieves its suppression of the master TFs, BZR1 and BES1. The activated BZR1 and BES1 move into the nucleus to regulate BR-related gene expression directly or via an interaction with other TFs (Yan et al. 2009; Li 2010; Luo et al. 2010). In absence of BRs, brassinosteroid receptor (BRI1) remains as an inactive homodimer state owing to its interaction with the cytoplasmic domain, BKI1 (Gudesblat and Russinova 2011; Clouse 2011; Jaillais et al. 2011; Choudhary et al. 2012). The master TFs also remain attached with cytoplasmic 14-3-3 proteins (phosphopeptide-binding proteins highly conserved in all eukaryotes) (Gampala et al. 2007; See Fig. 10.1).

Physiological Roles of Brassinosteroids

Brassinosteroids are involved in the regulation of various developmental and physiological processes. They promote cell division, cell wall regeneration, cell expansion and elongation (Clouse and Sasse 1998) and interplay with auxins (Nemhauser et al. 2004). BRs are also necessary for the promotion of vascular differentiation (Cano-Delgado et al. 2004), pollen tube formation and elongation (Hewitt et al. 1985). Furthermore, BRs delayed senescence in BRs-deficient mutants, while it accelerated senescence in dying tissue that signifies the biological relevance of BRs

action (Clouse and Sasse 1998). BRs counteract abiotic stresses in plants (Sharma and Bhardwaj 2007; Sharma et al. 2008; Bajguz and Hayat 2009; Fariduddin et al. 2013) while it inhibit pathogen-associated molecular pattern (PAMP)-triggered immune signalling (Albrecht et al. 2012).

Brassinosteroids, Cell Expansion and Cell Division

Plant growth depends on both cell division and cell expansion where plant hormones, including brassinosteroids (BRs), are central to the control of these two cellular processes. BRs induce elongation of hypocotyls, epicotyls, and peduncles of dicots, as well as coleoptiles and mesocotyls of monocots (Cheon et al. 2010; Gonzalez-Garcia et al. 2011; Zhiponova et al. 2013). BRs-induced cell expansion is accompanied by the proton extrusion and hyperpolarization of cell membrane and markedly stimulates and accelerates the growth of cell cycle (Bajguz and Czerpak 1996). Plasticity of cell wall is increased when proton extrusion by H^+-ATPases acidifies the apoplast, thereby activating cell wall loosening enzymes. BRs are involved in the process of cell enlargement through their effects on gene expression and enzyme activity (Mussig and Altmann 1999). Studies for understanding the requirement of BRs in cell elongation have been revealed from molecular genetic approaches in mutants (Nicol et al. 1998; Azpiroz et al. 1998; Salchert et al. 1998; Munoz et al. 1998). Cellular analysis of root meristem cells, combined with assessment of molecular cell cycle markers and stem cell genes, revealed that brassinosteroids positively regulate the size of the root meristem (Hacham et al. 2011; Gonzalez-Garcia et al. 2011). BRs increase cell division by increasing the transcript levels of genes encoding cyclin-D3, a regulatory protein of the cell cycle. Cyclin-D3 is also regulated by cytokinins, and it may be significant that BRs can efficiently substitute for cytokinin in the growth of Arabidopsis callus and suspension cultures (Riou-Khamlichi et al. 1999; Hu et al. 2000). The role of cyclins and CDK genes has also been investigated in early fruit development of tomato (Joubès et al. 1999, 2000).

Brassinosteroids and Photosynthesis

The role of BRs in the regulation of photosynthesis and related attributes is a well-accepted phenomenon. The enhancement in the photosynthetic rate by the exogenous application of brassinosteroids has widely been demonstrated in various plant species (Hayat et al. 2010; Hola 2011). However, it is still not clear what exactly causes BRs-associated changes in photosynthesis and its related attributes. The most favored hypothesis is that BRs somehow enhance the efficiency of the photosynthetic carbon reduction cycle and by increasing the content of related enzymes. The aqueous solution of 28-homobrassinolide, applied to the foliage of wheat and mustard (Sairam 1994; Hayat et al. 2000, 2001a, b) or applied as seed soaking

treatment to mung bean (Fariduddin et al. 2003, 2004), and dialkylamino-ethylalkanoate or epibrassinolide, in association with GA3, to spinach enhanced the photosynthetic rate (Liang et al. 1998). Foliar spray of aqueous solution of BR to wheat and mustard (Braun and Wild 1984), epibrassinolide to seedlings of cucumber (Ding et al. 2009), and brassinolide to rice (Fujii et al. 1991) increased the rate of CO_2 assimilation. Likewise the foliar application of 24-epibrassinolide enhanced the light saturated net CO_2 assimilation rate and carboxylation rate of rubisco, thereby increasing the capacity of CO_2 assimilation in the Calvin cycle (Yu et al. 2004). However, the epicotyl of cucumber did not respond to epibrassinolide but the transport of the labeled (^{14}C) glucose towards the epicotyl was favored (Nakajima and Toyama 1999). Similarly, Hill activity in the foliage of *Vigna radiata* was favorably affected, when supplemented with aqueous solution of 28-homobrassinolide (Bhatia and Kaur 1997). In addition to this, Yu et al. (2004) revealed that EBL as foliar spray enhanced the rate of rubisco carboxylation, RuBP regeneration, and quantum yield of PSII. It is also reported that EBR recovered the loss of photosynthetic apparatus from cold stress (Jiang et al. 2012).

Effect of Brassinosteroids on Metabolic Enzymes

Carbonic anhydrase (CA), a zinc-containing enzyme, catalyzes the reversible conversion between carbon dioxide and bicarbonate ion. Plants store CO_2 as bicarbonate ions, which are converted back to carbon dioxide for photosynthesis by CA. In plants, a form of β-carbonic anhydrase is found that is the second most abundant soluble protein, other than RuBPcase, in C_3-chloroplast (Okabe et al. 1980; Reed and Graham 1981). It is a ubiquitous enzyme, containing zinc protein with a molecular weight of 180 kDa (Lawlor 1987a). It catalyzes the reversible interconversion of bicarbonates (HCO_3^-) and CO_2 (Sultemeyer et al. 1993). The rate of conversion of HCO_3^- to CO_2 is normally slow in alkaline conditions. However, CA activates the use of HCO_3^- in the production of CO_2 (Lawlor 1987a, b). In C_3 plants, CA has a close association with RuBPCase where it elevates the level of CO_2 at its active site (Badger and Price 1994). The soaking treatment of EBL to the seeds of *Vigna radiata* (Yusuf et al. 2012) or foliar treatment of HBL to *Brassica juncea* (Hayat et al. 2000, 2001b) significantly increased the activity of CA. Moreover, the seedlings of wheat and mung bean, raised from the grains treated with HBL, possessed high CA activity in their leaves (Hayat et al. 2001a; Fariduddin et al. 2003). Application of EBL also reduced the toxic effect of salinity and restored the activity of CA in *Cucumis sativus* (Fariduddin et al. 2013). Under various abiotic stress conditions BRs also restored the activity of nitrate reductase (NR) enzyme. It plays a pivotal role in the supply of nitrogen and in the growth and productivity of plants. The stress conditions like salinity inhibit the nitrate transport to shoot due to interference with nitrate uptake and xylem loading which is finally attributed to reduced NR activity in leaves (Anuradha and Rao 2003). On the application of BRs, the level of NR is increased in the plants of wheat (Hayat et al. 2001b), *Lens culinaris* (Hayat

and Ahmad 2003), pea (Shahid et al. 2011), *Vigna radiata* (Yusuf et al. 2012), and *Cucumis sativus* (Fariduddin et al. 2014b).

Role of Brassinosteroids in Ion Homeostasis

Ionic concentration in cell compartments and tissues is kept within the limits that are ideal for the cell performance. Homeostasis describes the ability of living organisms to regulate the concentration of mineral ions within a defined space despite their fluctuating concentrations in its surroundings. Ion homeostasis provides optimum conditions for enzyme activity, maintains turgor pressure of cells, and plays a vital role in cell signalling (Ashraf 1994, 2004; Munns et al. 2006). However, the salt stress adversely affects the ion homeostasis in cells. Any factor leading to the maintenance of ion homeostasis will result in salt tolerance of plants. BRs directly or indirectly help the plants to maintain ion homeostasis. BRs have been found to have positive effect on the activity of high affinity K^+ transporters and are associated with the reduction in Na^+ and enhancement in K^+ concentration thus result in the improvement of K^+/Na^+ ratio. Brassinosteroids have also been found to improve the Ca^{2+}/Na^+ and K^+/Na^+ ratios of the wheat cultivars by enhancing Ca^{2+} and K^+ uptake, and thus enhance salt tolerance (Qasim et al. 2006). Another mechanism which leads to the ion homeostasis is the activation of stress alleviating hormone like abscisic acid. Treatment with EBRs increased expression of various hormone marker genes in both wild-type and mutant seedlings. BRs exert antistress effect independently as well as through interactions with other hormones and share transcriptional targets with other hormones.

Brassinosteroids and Senescence

Senescence is a complex and highly regulated process that requires gene expression and involves the interactions of many signalling pathways. It is the final stage of plant development during which the plant reclaims the valuable cellular building blocks, deposited in leaves and other parts of the plant during growth (Arteca 1997; Buchanan-Wollaston 2007). Maintaining an efficient senescence process is essential for survival of plant and its future generations. Like other hormones (Rao et al. 2002), BRs also play a crucial role in regulating the processes leading to senescence. BRs accelerate senescence in the detached cotyledons of cucumber seedlings (Zhao et al. 1990) and leaves of mung bean seedlings (He et al. 1996) and wheat (Saglam-Çag 2007). However, BRs-deficient Arabidopsis mutants lacking bioactive brassinosteroids exhibited delayed senescence of chloroplast (Li et al. 1996).

Relationship of Brassinosteroids with Oxidative Stress and Antioxidant System

Like all aerobic organisms, plants have also well-developed metabolic pathways to utilize its energetic potential in the presence of oxygen (Navrot et al. 2007). One potentially damaging effect of this fact is the deleterious production of reactive oxygen species (ROS) that are produced during normal respiration, photosynthesis, and nitrogen fixation (Mittler et al. 2011). Oxidative stress develops when a shift in balance between ROS (oxidants) and antioxidant pathway occurs. Inhibition of the antioxidant pathway leads to oxidative stress and cellular injury in plants (Prasad 2004). Due to a highly transient nature and high chemical reactivity of ROS, a number of degradative changes occur quickly. This increased ROS level in plants causes oxidative damage to biomolecules such as lipids, proteins, and nucleic acid, thus altering the redox homeostasis (Gille and Sigler 1995) which leads to death of cells and tissue. Although, under normal growth conditions, the production of ROS in cell is very low, many stresses that disrupt the cellular homeostasis of cells enhance the production of ROS. These stresses include drought stress and desiccation, salt stress, chilling stress, heavy metals stress, ultraviolet radiation, ozone, mechanical stress, nutrient deprivation, and high light stress.

Moreover, when plants are subjected to stresses, a variety of ROS are generated, such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) (Gapper and Dolan 2006). However, the avoidance of the production of ROS would be considered to be the first line of defense against their harmful effects but this step only is rather limited, not avoided. Therefore, aerobic organisms, such as plants, contain systems which can control and detoxify ROS, thus limiting toxic effects (Gratao et al. 2006). The term antioxidant describes any compound that is able to detoxify ROS without undergoing conversion to a harmful radical. Antioxidant enzymes are involved in either processing ROS or catalyzing the reaction that takes place in an effort to reduce harmful radicals (Gratao et al. 2006). Although the antioxidant pathway has the same end goal, it consists of both enzymatic and nonenzymatic components. All the three nonenzymatic antioxidants namely ascorbate, glutathione, and α-tocopherol are responsible for detoxification of ROS (Sharma and Dubey 2005). In addition this, the major ROS scavenging enzymes involved in the antioxidant pathway in plants include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GOPX), ascorbate peroxidase (APX), monohydrate ascorbate reductase (MDHAR), glutathione reductase (GR), and glutathione peroxidase (GPX) (Ruley et al. 2004; Simonovicova et al. 2004). These enzymes combine with the nonenzymatic components to detoxify ROS to oxygen and water using ascorbate, glutathione, and nicotinamide adenine dinucleotide phosphate (NADPH) as electron donors (Ezaki et al. 2000). ROS undergo a series of oxidation/reduction reactions known as the Halliwell Asada pathway (Gratao et al. 2006).

BRs play pivotal role in plant growth and development and have been implicated in many physiological responses (Sasse 2003). However, little is known about the

role of BRs in the plant response to oxidative stress. It was shown that exogenous application of BRs modified antioxidant enzymes such as SOD, CAT, GPX, and APX and nonenzymatic antioxidants such as ascorbic acid, tocopherols, carotenoids, glutathione, etc. in plants under different stress conditions. Vitamins C, E, and glutathione react directly or via enzyme catalysis with OH, H_2O_2, or O_2, while carotenes directly operate as effective quenchers of reactive intermediary forms of oxygen (Vardhini and Rao 2003; Ozdemir et al. 2004). When maize (*Zea mays*) seedlings treated with brassinolide (BL) were subjected to water stress, the activities of SOD, CAT, APX, as well as ascorbic acid and carotenoids contents increased (Li et al. 1998). On the other hand, BRs enhanced the activity of CAT and reduced the activities of POX and ascorbic acid oxidase under osmotic stress conditions in sorghum (*Sorghum vulgare*) (Vardhini and Rao 2003) and also regulate secondary metabolism in tomato which might enhance tolerance to phenanthrene (Ahammed et al. 2013).

Rice seedlings exposed to saline stress and treated with BRs showed a significant increase in the activities of CAT, SOD, and GR and a slight increase in APX (Nunez et al. 2003). Study conducted on two lines of rice viz salt-sensitive and salt-resistant varieties in the presence of epibrassinolide (EBL) revealed the role of BRs in salt stress (Ozdemir et al. 2004). EBL treatment, at least in part, improved the tolerance of salt-sensitive seedlings to short-term salt stress. The difference in activities of antioxidant enzymes in salt-sensitive rice cultivar suggested that the higher salt stress resistance in sensitive seedlings induced by EBL could be due to higher activity of APX.

Chlorella vulgaris responded to heavy metals (cadmium, copper, and lead) by induction of several antioxidants, including several enzymatic antioxidant systems, and the synthesis of low molecular weight compounds. Treatment with brassinolide was effective in increasing the activity of antioxidant enzymes (CAT, GR, and APX) and the content of ascorbic acid, carotenoids, and glutathione (Bajguz and Hayat 2009). Treatment of EBL to tomato leaf disc exposed to high (40 °C) temperature had high activities of CAT, POX, and SOD (Mazorra et al. 2002).

It has been shown that the Arabidopsis mutant *det-2*, which is blocked in the biosynthetic pathway of BRs, had significantly thicker leaves, cuticle layers, and cell walls in the epidermal and mesophyll layers with increased stomatal density and compacted leaf structure and less intercellular space than the wild type when grown under normal oxygen conditions (Choe 2006). Addition of BL to the growth medium resulted in the development of leaves that were more similar in morphology to those of wild-type Columbia. It has been also demonstrated that *ATPA-2* and *ATP-24* genes encoding peroxidases were constitutively upregulated in the *det-2* Arabidopsis mutant (Goda et al. 2002). Furthermore, the oxidative stress-related genes encoding monodehydroascorbate reductase and thioredoxin, the cold and drought stress response genes *COR-47* and *COR-78*, and the heat stress-related genes hsp83, hsp70, hsf3, hsc70-3, and hsc70-G7 have been identified by microarray analysis of either BR-deficient or BR-treated plants (Müssig et al. 2002). The enhanced oxidative stress resistance in *det-2* plants was correlated with a constitutive increase in SOD activity and increased transcript levels of the defense gene

CAT. Therefore, a possible explanation for the fact that the *det-2* mutant exhibited an enhanced oxidative stress resistance is that the long-term BR deficiency in the *det-2* mutant results in a constant in vivo physiological stress that, in turn, activates the constitutive expression of some defense genes and, consequently, the activities of related antioxidant enzymes. It may be suggested that endogenous BRs in wild-type plants somehow act to repress the transcription or post-transcription activities of the defense genes to ensure the normal growth and development of plants. However, it is still unclear whether BRs directly or indirectly modulate the responses of plants to oxidative stress (Cao et al. 2005). Moreover, BRs induce stress tolerance by triggering the accumulation of apoplast H_2O_2, which subsequently upregulated the antioxidant system (Jiang et al. 2012).

Crosstalk of Brassinosteroids with Hormones

The concentration of the stress hormones like ABA and ethylene increases under stress conditions. ABA has been found to induce the expression of various genes involved in the defense system against biotic and abiotic stresses. Salt stress induces the synthesis of ABA by improving the level of ABA synthesizing enzymes, in root tip cells or parenchyma cells of vascular bundles (Koiwai et al. 2004). Roots are not very active in basic biosynthesis; however, they can synthesize ABA, PAs (Shevyakova et al. 2006), and various other secondary metabolites. These are essential for plant stress adaptations, as roots are the first plant organs which face the stress conditions like salinity stress, water deficit, and various other environmental stresses. ABA can induce the synthesis of PAs especially under water and salt stresses (Alcazar et al. 2006). Exogenous application of ABA was also useful in identifying the stress signalling pathways regulating PA metabolism under stress conditions. It was demonstrated that exogenous application of ABA upregulated the expression of *ADC2*, *SPDS1*, and *SPMS* at transcription level but did not affect *ADC1* and *ACL5* (Hanzawa et al. 2002; Urano et al. 2003; Alcazar et al. 2006). The involvement of ABA in the transcriptional regulation of PA metabolism was confirmed by expression profiling analysis in ABA-deficient (aba2) and ABA-sensitive (abi1) mutants (Vladimir and Shevyakova 2007). Recently, it is found that BRs can cross talk with numerous other hormones in regulating many developmental processes in plants. To date, many physiological and genetic studies have confirmed that BRs and ABA can co-regulate many developmental processes (Steber and McCourt 2001; Finkelstein et al. 2008). About 35 % of BRs-regulated genes are also regulated by ABA, indicating that ABA may regulate BRs signalling (Goda et al. 2008; Nemhauser et al. 2006). BRs also act synergistically with auxin to promote cell elongation, and mutants of either signalling pathway show similar developmental defects (Hardtke 2007; Teale et al. 2008). Another stress hormone which significantly rises under stress conditions is ethylene. Ethylene and polyamines are regulators of diverse physiological processes manifested during plant growth and development (Wang et al. 2002) and are also involved in severe stress responses.

It has been demonstrated that the main natural polyamines (Put, Spd, and Spm) closely interact with ethylene in the regulatory processes (Galston et al. 1997; Kaur-Sawhney et al. 2003). Both PAs and ethylene share a common biosynthetic precursor, S-Adenosyl-methionine (SAM). This might be the ethylene-induced downregulation of polyamine synthesis and the mutual inhibition of their biosynthesis, which became the basis for the competition between major PAs and ethylene (Evans and Malmberg 1989; Galston et al. 1997). Since the PAs and ethylene are antagonistic in function but their biosynthesis is regulated in coordination with physiological progressions. Along with competitive interrelations between major PAs (Spd and Spm) and ethylene, which could be established under stress conditions, the interaction between cadaverine and ethylene may be rather evolved as synergistic. This permits a fresh insight into the problem of compensatory reactions maintaining PA homeostasis required for plant survival under stress conditions. Cadaverine accumulation retards cell wall expansion and supply of H_2O_2 for suberization and lignification and thus reduces cell wall permeability for salts (Kuznetsov et al. 2007) and also induces the expression of Cu/Zn-SOD gene in roots (Aronova et al. 2005; Kuznetsov et al. 2007).

Possible Mode of Mechanism of Brassinosteroids Action

The mechanism of action of BRs has been an attractive target for researchers since the elucidation of the structure of BL, but for many reasons only recently some significant progress in this direction has been achieved. Although this problem is still rather far from its final solution, however, some important data have brought us closer to the understanding of the mode of regulatory action of BRs.

Considering higher variability of the physiological effects of BRs, it is believed that more than one molecular mechanisms of their action exist. Two main aspects of the primary mechanism have to be considered first: an effect of BR on the biosynthesis of enzymes via an effect on genome expression and secondly the effect of BR on membranes. The former is responsible for slow reactions of plants to the exogenous hormone, and the latter for the quick reactions. It is well documented that steroids function as a signalling molecule both in animals and plants and BRs in plants are perceived by a cell surface receptor kinase, BRASSINOSTEROID-INSENSITIVE 1 (BRI1).

BL, the most active brassinosteroid in bioassays, binds to the extracellular domain of BRI1 receptor. BRI1 is a plasma membrane-localized leucine-rich repeat (LRR)-receptor serine/threonine (S/T) kinase. The LRR-receptor kinases constitute the largest receptor class predicted in the Arabidopsis genome, with over 230 family members. This family has a conserved domain structure, composed of N-terminal extracellular domain with multiple tandem (adjacent) LRR motifs, a single transmembrane domain, and a cytoplasmic kinase domain with specificity towards serine and threonine residues. In case of BRI1, the number of LRRs is 25. BRI1 also has a unique feature that is required for BR binding, a stretch of amino acids called as

island domain that interrupts the LRRs between LRRs 21 and 22 (Kinoshita et al. 2005). This domain plus the flanking LRR22 compose the minimum binding site for BRs. BL binding to BRI1 triggers the interaction between BRI1 and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1). BRI1 is phosphorylated at multiple sites along with its intracellular domain, some of which have been shown to regulate receptor activity. The BL signal is then transmitted to the cytoplasm by an unknown mechanism where it inhibits BRASSINOSTEROID-INSENSITIVE 2 (BIN2), which is a negative regulator of BR biosynthetic pathway. BIN2 is a protein kinase that interacts with and phosphorylates two nearly identical transcription factors, BRI 1-EMS SUPPRESSOR 1 (BES1) and BRASSINAZOLE RESISTANT 1 (BZR1), negatively regulating their activities. BRI 1 SUPPRESSOR 1 (BSU1) dephosphorylates BES1 and BZR1 to counteract the effect of BIN2. BRs regulate the expression of hundreds of genes. A significant portion of the unregulated genes is predicted to play a role in growth processes. BES1 binding activity and expression level of its target genes are enhanced synergistically by BES1-INTERACTING MYC-LIKE1 (BIM1). B1M1 is another transcription factor that dimerizes with BES1 and increases its activity. Genes that are downregulated by BR include several BR biosynthetic genes. BZR1 binds to specific elements in their promoters to repress their activity. The genes repression by BZR1 represents a negative feedback loop for the regulation of growth by BR (Wang et al. 2006; Kim and Wang 2010; See Fig. 10.2).

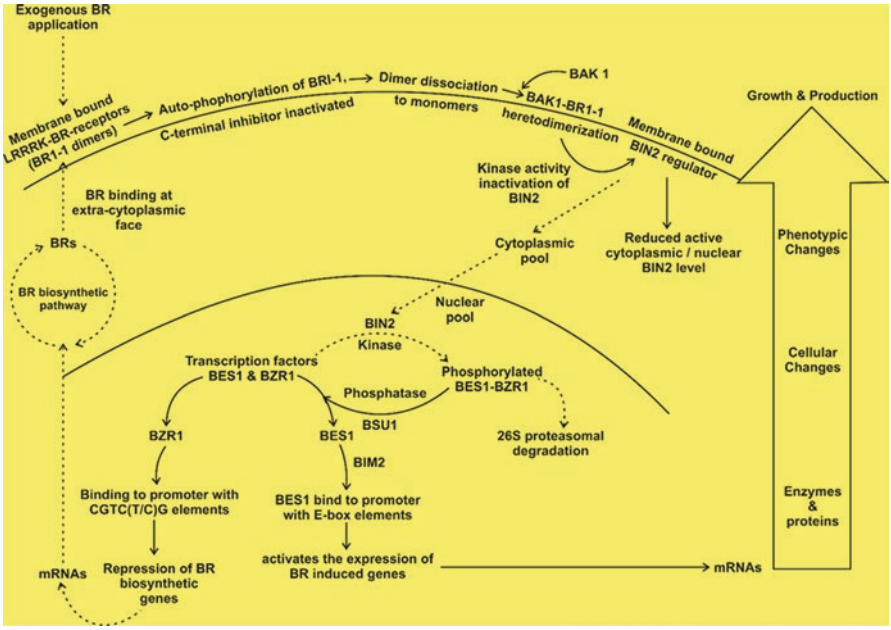

Fig. 10.2 Possible mechanism of brassinosteroids action

Concluding Remarks and Future Prospective

The end of the twentieth and particularly the beginning of the twenty-first centuries brought unquestionable evidence that BRs have the ability to improve yield quantity and quality of various crop species and also protect plants against various kinds of stresses. However, although several attempts to resolve the actual relationship between these phytohormones and the functioning of various parts of photosynthetic apparatus have been made, our knowledge on the mode of BRs' action in the regulation of photosynthetic processes is still far from being complete. So far, it seems that the main site of BRs' impact on photosynthesis is probably the photosynthetic carbon reduction cycle and that these compounds could perhaps somehow affect the activation state of rubisco, the main CO_2 fixing enzyme. We can also speculate about the possible effect of BRs on the activity of carbonic anhydrase which modulates the ratio of inorganic carbon species (CO_2/HCO_3^-) and thus improves the availability of CO_2 for rubisco.

What are the challenges that we are expected to face in order to elucidate the relationship between BRs and photosynthesis? First, a detailed examination of the participation of these phytohormones in the development of photosynthetically active chloroplasts is surely needed. Second, an analysis of the role of BRs in photosynthetic electron transfer, aimed at the dissection of components other than photosystem II, should be made, and at least some data on this topic is currently available. Third, the determination of BRs' influence on all enzymes participating in photosynthetic CO_2 fixation (not only in plants with C_3 pathway of carbon fixation but also C_4 and perhaps CAM pathway as well) would not be amiss to further clarify the doubt whether this part of photosynthetic process really serves as the main target for the action of these hormones. Fourth, a more comprehensive BR structure-photosynthetic activity studies could perhaps provide some reasons why some photosynthetic characteristics (e.g., photosynthetic pigments' content) seem to be affected only by specific types of BRs. Fifth, a question why the effect of BRs on photosynthesis is more pronounced in plants subjected to some unfavorable environmental factor demands an answer as well. Sixth, a more frequent utilization of modern methods of molecular and cell biology including various "omics" technologies should enhance our knowledge on BRs' role in the regulation of photosynthesis at the cellular level. New information on the interconnection between BRs, photosynthesis, and light signalling is also necessary to improve our understanding of the complex network of regulating pathways involving this phytohormones.

A major research priority in improving our understanding of the molecular mechanisms of BRs–other hormones crosstalk in regulating growth and development is the identification of transcriptional networks that regulate the synthesis of proteins that act as developmental modulators. The growing numbers of high quality and publically available genome-wide transcript data sets inform the exploration of signalling-induced gene expression changes. Such data sets are available for auxin and ethylene individually, but the limited number of data sets that explore the response to both hormones limits understanding of the transcriptional crosstalk

between these two hormones. Furthermore, these data sets were generated using diverse growth media, light conditions, and hormone concentrations, making comparison between data sets almost impossible. As more data sets use mutants altered in transcription factors or signalling proteins to identify the downstream networks of transcription factors, consider the expression patterns of individual cell and tissue types, and explore expression changes with high temporal resolution, a clearer picture of hormone signalling will emerge. Finally, the inclusion of growth and developmental data and localization of important protein products to parallel the transcriptional responses will inform the interpretation of the biological significance of these data sets. The increasing availability and decreasing costs of next generation sequencing approaches and developing collaborations between biologists and computer scientists are likely to lead to insights into the molecular basis of crosstalk between BRs and other hormones in both model and crop plants.

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Chapter 11
Trehalose: Metabolism and Role in Stress Signaling in Plants

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Abstract Trehalose is a nonreducing disaccharide composed of two glucose residues bound by an α-α linkage. First reported in 1832 in rye following ergot infection, trehalose has since been detected in a wide range of organisms, including bacteria, fungi, invertebrates, and plants. It has role in plant response to various environmental stresses such as cold and salinity, and in regulation of stomatal conductance and water-use efficiency. Trehalose is a potential signal metabolite in plant interactions with pathogenic or symbiotic microorganisms and herbivorous insects. Recently it has been found that trehalose metabolism is also crucial for normal plant growth and development. In plants, as in other eukaryotes and many prokaryotes, trehalose is synthesized via a phosphorylated intermediate, trehalose 6-phosphate (T6P). In plants, trehalose 6-phosphate (T6P), the intermediate of trehalose biosynthesis, is thought to be a signal of sucrose status and current studies point towards the role of T6P as a regulatory molecule, especially in sugar influx and metabolism. A meta-analysis reveals that the levels of T6P changes are in parallel with sucrose, which is the major product of photosynthesis and the main transport sugar in plants. The intermediate T6P is now confirmed to act as a sensor for available sucrose, hereby directly influencing the type of response to the changing environmental conditions. This is possible because T6P and/or trehalose or their biosynthetic enzymes are part of complex interaction networks with other crucial hormone and sugar-induced signaling pathways, which may function at different developmental stages.

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Because of its effect on plant growth and development, modification of trehalose biosynthesis, either at the level of T6P synthesis, T6P hydrolysis, or trehalose hydrolysis, has been utilized to try to improve crop yield and biomass. It has been shown that alteration of the amounts of either T6P and/or trehalose results not only in increased stress tolerance, but also in many unexpected phenotypic alterations.

The present review discusses the trehalose metabolism in plants and its role in plant signaling.

Introduction

Trehalose is a disaccharide that was considered to be rare in plants but now appears to be ubiquitous. Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranoside) is a nonreducing disaccharide sugar made up of two glucose units joined by an α,α-1,1 linkage and is common in nature. Understanding of the trehalose pathway in the last decade has gathered pace in the context of its metabolism and role in cellular signaling. Evidences suggest trehalose 6-phosphate (T6P) is a strong and essential sugar signal in plants integrating metabolism with development (Olivier et al. 2010; Liam et al. 2013; Delorge et al. 2014a, b). There is considerable genetic propagation of the pathway, which is under extreme selection pressure, imitating its biological importance likely with specific plant functions (Liam et al. 2013).

Trehalose Metabolism in Plants

Plants have only one pathway for trehalose biosynthesis, a two-step process that involves trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) that synthesize and, subsequently, dephosphorylate the intermediate T6P (trehalose-6-phosphate). In *A. thaliana*, there are 11 TPS or TPS-like genes and 10 TPP genes (Leyman et al. 2001; Eastmond and Graham 2003). Interestingly, there is only one trehalase-encoding gene (TRE), which hydrolyzes trehalose into two glucose molecules (Delorge et al. 2014a, b). For trehalose biosynthesis, OtsA–OtsB pathway is the widespread pathway found in all prokaryotes and eukaryotes, and is the only pathway for trehalose biosynthesis in plants (Paul et al. 2008). The biosynthesis and degradation of trehalose is similar to that of sucrose (Fig. 11.1). Trehalose is synthesized from UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase (TPS), resulting in trehalose-6-phosphate (T6p), which is subsequently dephosphorylated into trehalose by trehalose-6-phosphate phosphatase (TPP) (Cabib and Leloir 1958).

TPSs comprise two distinct classes and eleven genes in *A. thaliana* (Table 11.1). Among the class I TPSs (TPS1–4 in *A. thaliana*), only one (TPS1) shows demonstrable TPS activity. Class II TPSs (AtTPS5–11) have a synthase and a phosphatase

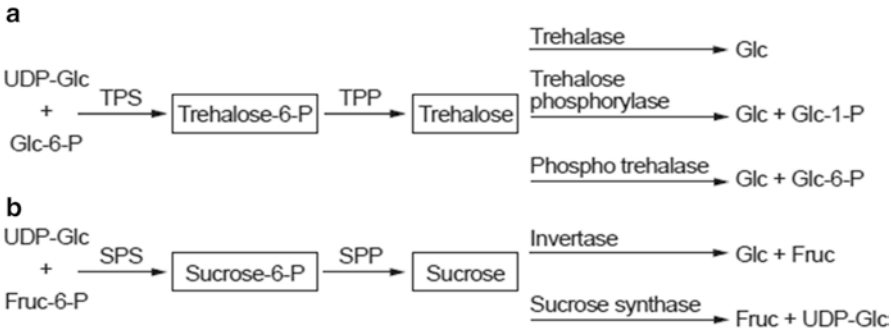

Fig. 11.1 Comparison between enzymatic reactions involved in the biosynthesis and degradation of (**a**) trehalose and (**b**) sucrose pathways in plants (Paul et al. 2008)

Table 11.1 Trehalose pathway genes of *Arabidopsis thaliana* (Paul et al. 2008)

Trehalose phosphate synthase		Trehalose phosphate phosphatase	
Gene	Locus	Gene	Locus
Class I		Class I	
TPS1	At1g78580	*TPP1 C*	At1g22210
TPS2	At1g16980	*TPP2 D*	At1g35910
TPS3	At1g17000	*TPP3 B*	At1g78090
TPS4	At4g27550	*TPP4 E*	At2g22190
Class II		*TPP5 F*	At4g12430
TPS5	At4g17770	*TPP6 G*	At4g22590
TPS6	At1g68020	*TPP7 H*	At4g39770
TPS7	At1g06410	Class II	
TPS8	At1g70290	*TPP8 I*	At5g10100
TPS9	At1g23870	*TPP9 A*	At5g51460
TPS10	At1g60140	*TPP10 J*	At5g65140
TPS11	At2g18700	***Trehalase***	At4g24040

Both class I and class II TPS proteins contain glycosyltransferase domain analogous to the TPS enzymes from yeast (*Saccharomyces cerevisiae*; ScTPS1) and *Escherichia coli* (otsA) (Lunn et al. 2014)

domain, with less well-conserved active sites compared with class I TPS, lacking both TPS and TPP activity (Harthill et al. 2006; Vogel et al. 2001). Class II TPSs are expressed in the whole plant with parallel expression pattern of TPS8 and TPS11 (Paul et al. 2008). Further, class II TPSs are highly regulated by sugars, light, starvation, cytokinin, and diurnal rhythms (Brenner et al. 2005; Thimm et al. 2004; Schluepmann et al. 2004a, b, c; Zimmerman et al. 2005).

A. thaliana TPP with two distinct classes with ten genes has conserved amino acid motif, characteristic of the active sites of enzymes of 2-haloacid dehalogenase

superfamily (Burroughs et al. 2006), and is under strong purifying selection (Avonce et al. 2006).

Moreover, class I and class II TPS proteins have a C-terminal region resembling the phosphatase domain of yeast TPP (encoded by the ScTPS2 gene) (Bell et al. 1998; Leyman et al. 2001).

Both the classes of TPS gene are represented in the genomes of nonflowering plants [*Selaginella moellendorffii* (Hieron. in Engl. & Prantl.) and *Physcomitrella patens* (Hedw.) Bruch & Schimp. (B.S.G.)] and chlorophyte algae (*Ostreococcus* species), suggesting that the gene families that encode the enzymes of trehalose metabolism are very ancient, predating the divergence of the chlorophyte and streptophyte lineages (Lunn 2007).

In *A. thaliana*, AtTPS1 transcripts detected in most parts of the plant show a little variation in abundance between organs or at different developmental stages (Schmid et al. 2005). However, the expression is not ubiquitous, as revealed by in situ hybridization AtTPS1 transcripts are localized in specific zones of shoot apex. In *A. thaliana* tps1 null mutants, AtTPS1 is essential for vegetative growth, embryogenesis, and flowering (Gómez et al. 2010; Gómez et al. 2006). In *Arabidopsis*, AtTPS1 is expressed in all tissues, but is highly upregulated during embryo maturation, suggesting its role in embryo maturation (Eastmond et al. 2002).

T6P regulates the sucrose level in plant, as a genetically induced change in T6P causes a reciprocal change in sucrose (Lunn et al. 2014). Thus, T6P acts as part of a homeostatic mechanism to control the sucrose level in plant cells, ensuring that it does not drop too low or rise too high, similar to the control of blood glucose levels in animals by insulin and glucagon (Lunn et al. 2014). *A. thaliana* shoot apex shows a parallel change in sucrose and T6P levels in both source (Sulpice et al. 2014) and sink organs. Thus, T6P may be a part of regulatory network, regulating the provision of sucrose for its export in source organs, and its use in growing sink organs (Lunn et al. 2014). In *A. thaliana* seedlings, the response of T6P to exogenous sucrose is inhibited by cycloheximide but remains insensitive to cordycepin, showing its dependence on de novo protein synthesis and not on de novo transcription (Yadav et al. 2014), and hence suggesting that the T6P leads to the stimulation of starch synthesis by sugars via redox modulation of AGPase (Kolbe et al. 2005).

T6P level is controlled by the relative activities of TPS, TPP, and trehalase (Eastmond and Graham 2003). Trehalose is hydrolyzed by the enzyme trehalase. This enzyme is ubiquitous in higher plants (Aeschbacher et al. 1999). In plants, the trehalose is accumulated in presence of validamycin A (a specific trehalase inhibitor), suggesting the trehalase as a sole route for trehalose breakdown (Muller et al. 2001). In *Arabidopsis* and soybean (*Glycine max*), single-copy trehalase genes have been identified and functionally characterized (Muller et al. 2001). The trehalose breakdown may be regulated by the level of trehalose (Eastmond and Graham 2003). Trehalase induction has been reported in Arabidopsis plants infected with *Plasmodiophora brassicae*, a trehalose-producing pathogen (Brodmann et al. 2002).

T6P is also important for normal plant development (Ponnu et al. 2011). During embryo development, T6P plays an important role in controlling the cell wall biosynthesis, cell cycle activity, and cellular metabolism (Gómez et al. 2006).

Fig. 11.2 *Arabidopsis thaliana* plants defective in trehalose-6-phosphate synthesis are late flowering. Wild-type (Col-0) and homozygous tps1 mutant carrying a chemically inducible TPS1 rescue construct (GVG::TPS1), flowering late compared to wild-type control (Ponnu et al. 2011)

In later plant development, TPS1 is essential for timely flower induction. The tps1 mutant plants rescued through embryogenesis by dexamethasone-inducible TPS1 expression completely failed to flower, unless the expression of TPS1 was induced (Fig. 11.2).

Role of Trehalose in Plant Stress

In many organisms, trehalose is used as a stress response but its precise role is not clear yet although some data indicate that trehalose plays an important protective role during abiotic stress. In contrast, some metabolism caused due to trehalose mutations exhibit growth aberrations having deleterious effects on plant physiology. Trehalose, a nonreducing disaccharide consisting of two units of glucose (-D-glucopyranosyl-1,1--D-glucopyranoside), is widely spread in a variety of organisms: bacteria, yeast, fungi, lower and higher plants, as well as insects and other invertebrates (Elbein et al. 2003). In insects, trehalose is mainly present in blood and serves as source of energy during flight (Elbein et al. 2003). During periods of drought, plasma membrane in anhydrobiotic organisms is preserved by trehalose which is known to accumulate in higher concentration so that they could survive from complete dehydration (Crowe et al. 1984). In yeast, trehalose plays an important role in osmotic stress tolerance (Hounsa et al. 1998), heat and desiccation tolerance. Due to osmotic stress, bacteria are also able to accumulate trehalose (Styrvold and Strom 1991). There are two steps by which trehalose is synthesized in microorganisms. First, trehalose-6-phosphate is formed from glucose-6-phosphate and UDP-glucose catalyzed by enzyme trehalose 6-phosphate synthase (TPS) and then trehalose 6-phosphate phosphatase (TPP) catalyzes the dephosphorylation of trehalose-6-phosphate to

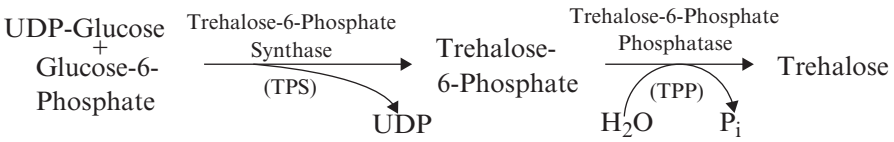

Fig. 11.3 Trehalose pathway through the production of the intermediate trehalose-6-phosphate

trehalose (Fig. 11.3). In yeast, both TPS (TPS1) and TPP (TPS2) are part of a complex that contains two other regulatory subunits, TPS3 and TSL1 (Bell et al. 1998). There are few reports that confirm that trehalose is present in higher vascular plants. Most of those referred to a few desiccation-tolerant plants (Drennan et al. 1993; Bianchi et al. 1993; Albini et al. 1994). However, reports are emerging in multiple species about transcripts encoding putative trehalose biosynthetic genes. For example, eleven TPSs and ten TPPs are present in *Arabidopsis*, and rice genome was also found to contain nine TPSs and nine TPPs. Even though in some of the plants, trehalose biosynthesis genes have been isolated and functionally characterized, but still we are far apart to understand the role and the impact of trehalose/trehalose-6-phosphate on plant growth and development.

To face multiple stresses throughout their life cycle normal physiology, plant growth and development are severely affected (Mahajan and Tuteja 2005; Agrios 2005; Hirt and Shinozaki 2004). There are two types of stresses that are categorized as abiotic and biotic. Abiotic stress includes both physical and chemical environmental factors such as cold, heat, wind, drought, oxidation, or radiation. Biotic stress is caused by biological agents like bacteria, fungi, and insects (Mahajan and Tuteja 2005). Stress does not affect plant survival, but can alter physiological process to varying degree ranging from collapsing of important functions to complete tissue damage (Agrios 2005; Hirt and Shinozaki 2004). To overcome the damage, large set of defense mechanisms operate in plant some are constitutive and others are expressed only when specific stress signals are perceived. The events that occur in plants in response to both categories of stress consist of (1) Changing of signal transduction pathway (Chinnusamy et al. 2007; Bari and Jones 2009), (2) induction of the downstream gene expression (Chinnusamy et al. 2007), (3) making of proteins that are related to pathogenesis (Van Loon et al. 2006), and (4) aggregation of metabolites such as sugars, proline (Moore et al. 2009), or phytoalexins that are antimicrobial molecules (Hammerschmidt 1999). From its physical and chemical properties as well as its role in stress management in fungi and bacteria (Purvis et al. 2005; Ni and Yu 2007) there is no clear evidence whether trehalose or its precursor trehalose-6-phosphate (T6P) is involved in plant stress response. Recently abnormal amount of trehalose was characterized as the sweet mutant of *A.thaliana* (up to four times) than wild type (Veyres et al. 2008). This mutant exhibits higher constitutive expression of genes involved in response to abiotic stress. It also developed similar aberrations in other plants that overexpress gene responsible for biosynthesis of trehalose.

In order to understand how trehalose may react, several studies were conducted where trehalose was added to seedling or adult plants. During exogenous application of trehalose to plants it was shown to induce both abiotic and biotic stress-related genes (Schluepmann et al 2004b). When the concentration of trehalose was used in range of 30 mM instead of 100 mM together with 1 % sucrose, abiotic stress genes showed more downregulation than that of upregulated ones (Bates et al. 2012). These results can also be justified by using of different DNA microarray providers, also it has been linked to transcriptional factor bZ1P11 (leucine zipper motif), and those plants that have been overexpressed by sbZ1P11 showed insensitivity towards supplied trehalose (Delatte et al. 2011). External application of trehalose is useful when the plants are facing salt stress. When the rice plants were supplied with flow level of trehalose (110 mM), root cells preserve their integrity and it induced rapid cell division (Garcia et al. 1997). The protective role of trehalose could also be explained by conservation of ion pumps which helps to keep out sodium from chloroplast. Trehalose also plays an important role in preserving lipid bilayer integrity and functioning of enzyme during stress condition (Garcia et al. 1997). Apart from these functions it harbors many antioxidants including phenolics and flavonoids; however during drought stress the level of seed oil and content of oleic and linoleic acid was severely affected; however, exogenous application of trehalose (30 mM)-Tween 20 solution improved growth, seed composition, and level of antioxidant activity in maize plants (Ali et al. 2012). In *Papaver dubium*, a significant correlation has been observed between sucrose content and drought tolerance in seeds and pollens (Hoekstra and Van Roekel 1988; Olivier et al. 2010). For long-distance transport this sugar acts as carbon and energy source carrier and for this reason sucrose is more important due to higher free energy upon hydrolysis. Thus these results question the role of trehalose in stress protection in higher plants (Delorge et al. 2014a, b).

Manipulation of Trehalose Metabolism

Tobacco, the first transgenic plant to accumulate trehalose, was obtained by expressing *E. coli* OtsA or ScTPS1 gene from yeast. The transgenic plants obtained showed drought resistance (Holmström et al. 1996; Pilon-Smits et al. 1998). Expression of TPS1 gene in tomato and potato also displayed drought tolerance (Yeo et al. 2000; Cortina and Culianez-Macia 2005). Morphological and growth abnormalities were found in all such cases mostly due to accumulation of T6P. In order to avoid pleiotropic effects of T6P, a gene construct comprising translational fusion of OtsA and OtsB was expressed in rice (Garg et al. 2002). The resulting plants displayed normal phenotype and were tolerant to drought, cold, and salinity. In Arabidopsis, a yeast chimeric gene coding for TPS and carboxy terminal region of TPP was expressed. The plants obtained did not show any morphological alterations and were tolerant to drought, salinity, freezing, and heat (Miranda et al. 2007). Alfalfa plants showing tolerance to multiple abiotic stresses were obtained by expressing bifunctional

TPS–TPP enzyme, stunted plants were obtained when constitutive 35S promoter was used, and when RD29A a stress inducible promoter was used plants with larger size and increased biomass were obtained (Suárez et al. 2009).

Overexpression of homologous AtTPS1 gene in transgenic *Arabidopsis* showed drought tolerance. Overexpression of AtTPS1 in other plants did not evoke any abnormalities, except for delay in flowering by 1 week, and opposite of this was observed in transgenic plants expressing TPS gene from *E. coli* and *S. cerevisiae*. Drought tolerance in transgenic plants was obtained due to changes in expression of important signaling and stress response gene. However low quantity of trehalose accumulated in transgenic plants cannot explain its role as osmoprotectant (Avonce et al. 2004, 2005). In order to obtain stress-tolerant plants by introducing trehalose biosynthetic gene into crops, many attempts have been made by different research groups. The first trait was drought tolerance obtained by constitutive overexpression of the yeast ScTPS1 gene (promoter 35S) in tobacco (Romero et al. 1997) and AtTPS1 gene in *A. thaliana* (Avonce et al. 2004). Resulting transgenic lines accumulated trehalose up to levels of 170 mg/g FW, respectively. An increase in T6P content (3 mg^{1}FW versus 0.75 mg^{1}FW in wild type) was seen in transgenic *A. thaliana* (Avonce et al. 2004). Stress inducible and chloroplast targeted expression of plastid TPS1 in tobacco and expression of bifunctional fusion gene OtsA-OtsB in rice and tobacco respectively resulted in improved drought tolerance (Garg et al. 2002; Karim et al. 2007). These transgenic lines of tobacco and rice accumulated trehalose (50 mg g^{-1}FW and 200 mg g^{-1}FW). By using transgenic strategies related to trehalose metabolism, other resistant characters in transgenic tomato (S*olanum lycopersicum*) by overexpressing ScTPS1 gene have been obtained and are more salt, drought, and oxidative stress resistant (Cortina and Culianez-Macia 2005). In *A. thaliana*, constitutive or stress inducible expression of bifunctional ScTPS1and ScTPP2 gene resulted in improved freezing and heat stress tolerance, leading to weak but significant accumulation of trehalose (up to 40 mg g^{1} FW) (Miranda et al. 2007). Overexpressing the *E. coli* trehalose synthesis gene (Ots A and Ots B) in rice conferred tolerance to salt and low temperature stress. The resulting plants displayed trehalose accumulation (increased threefold to tenfold with respect to control non-transgenic) and also showed stronger photosynthetic activity and global accumulation of carbohydrates (Garg et al. 2002). *A.thaliana* sweeti mutant is of special interest regarding modification of soluble carbohydrate which suggests that stress tolerance phenotype in trehalose genetically engineered plants could be partly due to modulation of sugar sensing and carbohydrate metabolism (Veyres et al. 2008). Sweeti plant and plants genetically engineered for trehalose synthesis show similar physiological tolerances like (1) overexpression of stress-related genes (2) hyper-accumulation of carbohydrate, and (3) hyper-accumulation of trehalose and T6P. As sweeti protein and trehalose biosynthetic enzymes do not show any homology, suggesting the idea that that trehalose and/or T6P could be responsible for the stress-tolerant phenotype of this mutant. Alterations in plant development like severe dwarfism is the characteristic shared by sweeti and some trehalose genetically engineered plants (Veyres et al. 2008). Growth alterations in tobacco are caused due to constitutive overexpression of the

yeast ScTPS1 gene (Romero et al. 1997; Cortina and Culianez-Macia 2005), which causes stunt growth form in tobacco and abnormal root development in tomato. Such developmental alterations might suggest interaction between trehalose and general carbohydrate metabolism (Paul et al. 2008). It has been shown that in *A. thaliana* when grown in trehalose containing medium leads to expression of ApL3 gene involved in starch synthesis. Manipulating stress tolerance by engineering trehalose biosynthesis is a delicate process as there is a close correlation between primary carbohydrate metabolism absolutely necessary to normal plant development and trehalose-related metabolism.

Trehalose-6-Phosphate as a Signaling Molecule

A very crucial and intricate sugar metabolic and signaling network is integrated through a number of internal regulators and environmental wrong doings to administrate and sustain plant growth and survival (Sheen 2014; Delorge et al. 2014a, b). Although a plethora of sugar signals have surfaced as critical regulators from embryogenesis to senescence, considerable number of evidences suggests that trehalose 6-phosphate (T6P) is a potential player and a requisite sugar signal in plants that integrates metabolism with development (Yuan et al. 2013; Caldana et al. 2013). There is considerable genetic proliferation of the pathway, which is under purifying selection pressure, reflecting its biological importance with likely plant specific functions. Trehalose and T6P synthesis in plants determines UDPG and G6P pool size without upsetting any other function of the trehalose pathway (Caldana et al. 2013). It was suggested from the TPP co-expression analysis of *Arabidopsis* multigene family members that TPPs are actively involved in a number of signaling networks and metabolic processes (Smeekens et al. 2010; Li et al. 2011; Delorge et al. 2014a, b). It is very difficult to quantify T6P levels under normal conditions of growth in plants (Delatte et al. 2011). Constitutive TPS1 expression which mediates T6P synthesis reflects the availability of sucrose, UDPG, and hexose phosphates feeding in this pool. Thus T6P has all the features to qualify as a signaling molecule (Liam et al. 2013). The role of sugars as signaling molecule has been widely reported (Thevelein and Hohmann 1995; Eveland and Jackson 2013). It has been found to be difficult to discriminate between sucrose and glucose as signaling molecules, in relation to metabolic signaling, as sucrose is impulsively converted into glucose. Both glucose and sucrose, however, are clearly proficient to deliver their own specific information. Glucose signaling occurs via conserved hexokinase (Thevelein and Hohmann 1995; Eveland and Jackson 2013). It also behaves as a molecular signal that integrates senescence (Acevedo-Hernández et al. 2005). Rapid communication of metabolic prestige is triggered by the low abundance and dynamic response of T6P that reflects pool size of UDPG, G6P, and sucrose in turn paving the way for diverse and explicit kind of signaling to that of other sugars (Dobrenel et al. 2013; Delorge et al. 2014a, b).

It has been elucidated that HXK2 activity in the yeast is inhabited by T6P; it is probably that glycolysis is regulated through this metabolite by moderating the flow of phosphorylated sugars towards this pathway (Thevelein and Hohmann 1995; Blázquez et al. 1993). As a signal activating development, trehalose is the key player as is evident from *Arabidopsis* tps1 mutants that display an embryo-lethal phenotype (Eastmond et al. 2002). Reduction of sucrose supply, but not T6p addition, results in rescuing embryonic development in vitro conceivably due to lack of transport into the cell of this molecule. It was also revealed during the studies that these tsp1 mutant plastids accumulate large starch granules persistent during development of seed, as compared to those of wild-type plastids were transient starch accumulation occurs (Delorge et al. 2014a, b). Ample number of studies demonstrates that AtTPS1 enzyme in *Arabidopsis* is among the signaling molecules (Avonce et al. 2004). Overexpression of AtTPS1 gene aggravates ABA and glucose insensitivity in Arabidopsis seedlings (Avonce et al. 2004, 2005). Studies have shown that the presence of glucose inhibits the expression of AtTPS1 in wild plants. It is, however, found that this repression is overcome by HXK1 knockout, suggesting that AtTPS participates in the sugar signaling mediated by the HXK1 sugar sensor (Rolland et al. 2006). It was however shown that the expression of *E. coli* OtsA (bacterial TPS) gene in Arabidopsis was not able to hinder the repression caused by glucose (Schluepmann et al. 2003). This opposing condition could be explained after comparison of OtsA and AtTPS presumed protein sequence, whereas AtTPS1 possesses amino and carboxy terminal ends, in addition to the catalytic domain that is homologous to OtsA. It was thus found that the amino-terminal end may have a regulatory function, because when deleted the enzymatic activity of AtTPS1 is increased by upto 40 times (Van Dijck et al. 2002). In addition, phosphorylated class II plant TPS is known to interact with 14-3-3 phosphoprotein, resulting in signaling cascades that activate SnRK1 (Harthill et al. 2006; Liam et al. 2013).

Another evidence for trehalose as a signaling molecule comes from the studies on Arabidopsis where it was found that overexpression of AtTPS1 using the 35S promoter led to a small increase in not only trehalose and trehalose-6-P levels but also *abscisic acid-insensitive* (ABI) 4 gene expression (Avonce et al. 2004). ABI4 encodes a transcription factor that is homologous to APETELA2 (Ap2) domain proteins. AtTPS1 regulates the transcription of ABI4 which in turn acts as a transcriptional repressor of photosynthesis genes (Avonce et al. 2005; Acevedo-Hernández et al. 2005; Delorge et al. 2014a, b).

Future Perspective

The recent advance in trehalose metabolism in higher plants has a great impact on plant metabolism, physiology, and development researches. Trehalose metabolism is under tight regulated not only by developmental stage of the plant but also

by environmental stresses as well. Studies of individual trehalose biosynthesis genes will help us to precisely assess their specific roles in not only abiotic stress context but also in signaling as well. Such approach will help us to devise new approaches to develop plants that are stress tolerant and better equipped to face environmental adversity.

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Chapter 12
The Role of Ethylene and Other Signals in the Regulation of Fe Deficiency Responses by Dicot Plants

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Abstract Iron (Fe) is abundant but its availability for plants is low specially on calcareous soils. To facilitate its acquisition, dicot (Strategy I) plants switch on several morphological and physiological changes in their roots, known as Fe responses. Once plants acquire enough Fe, the responses are switched off. Their regulation is not totally known but since the 1990s different results have supported a role for the plant hormone ethylene in such a process. Most of those results have been based on morphological and physiological studies and have been previously reviewed. Although the role of ethylene in the regulation of morphological Fe responses has been generally accepted, its role in the regulation of physiological Fe responses has been more controversial. In this review, we discuss the most recent results supporting a role for ethylene in the regulation of physiological Fe responses, most of them based on transcriptomic, proteomic, molecular and genetic analyses. In addition, we review results suggesting a role, either as activators or suppressors of physiological Fe responses, of other hormones and non-hormonal substances, such as auxin, nitric oxide, glutathione, and phloem Fe. As conclusion, we propose a Working Model that integrates both positive and negative signals in the regulation of physiological Fe responses. As positive signals, ethylene and nitric oxide would act at the end of the signalling cascade leading to the activation of physiological Fe responses while phloem Fe could play an important role as repressor.

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Abbreviations

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
BRs	Brassinosteroids
CKs	Cytokinins
DMHDA	Dimethylhexadecylamine
ET	Ethylene
GAs	Gibberellins
GSH	Glutathione
GSNO	*S*-nitrosoglutathione
JA	Jasmonic acid
NA	Nicotianamine
NICs	Nitrosyl iron complexes
NO	Nitric oxide
PAs	Polyamines
SAM	*S*-adenosylmethionine
TFs	Transcription factors

Introduction

Iron (Fe) participates in many essential plant functions, such as photosynthesis, respiration and nitrogen metabolism (Marschner 1995). It is abundant in most soils but its availability is low, specially in calcareous soils (more than 30 % of arable soils), where its solubility is very low and is frequent the incidence of Fe chlorosis (Lindsay 1995). According to the World Health Organization, the most common nutritional disorder in humans is Fe deficiency, with over 30 % of the world's population affected (http://www.who.int/nutrition/topics/ida/en/). One of the most important factors causing Fe chlorosis in calcareous soils is bicarbonate while hypoxia, due to flooding or other causes, can aggravate its negative effects (García et al. 2014). To acquire Fe from soils, plants have developed different strategies. Today, two main strategies are considered: the Strategy I, present in non-graminaceous plants (dicots and non-grasses monocots, etc.) and the Strategy II, present in graminaceous plants (Römheld and Marschner 1986; Ivanov et al. 2012; Kobayashi and Nishizawa 2012). When plants suffer from Fe deficiency, they switch on several morphological and physiological changes, known as Fe deficiency stress responses (hereafter named Fe responses), which are switched off once plants acquire enough Fe. The characteristics of Fe acquisition and Fe responses in Strategy I plants are described below while those of Strategy II plants are not considered in this review (for recent information about Strategy II plants, the reader is referred to Kobayashi and Nishizawa 2012). So, from now on, this review is devoted to dicot (Strategy I) plants.

The regulation of Fe responses is not totally known and several questions are still matter of discussion: (1) which part(s) of the plant perceive the Fe deficiency? the roots? the shoots? both? (2) once perceived the Fe deficiency, how is this perception transmitted into the activation of Fe responses? (3) are there positive and/or negative signals involved in the regulation of Fe responses? (4) which hormones and non-hormonal substances are involved in the regulation of Fe responses? In which order do they act? To answer these questions, several experimental approaches have been employed since the 1980s, such as isolated roots, detopped plants, split-root experiments, mutants altered in the regulation of Fe responses, reciprocally grafted plants between wt and mutants altered in the regulation of Fe responses, exogenous application of hormones and their inhibitors, and hormone mutants (Romera and Alcántara 2004; Romera et al. 2007). In the last years, new methodological approaches, based on transcriptomic, proteomic, molecular and genetic analyses, are providing new tools to look deeper into the regulation of Fe responses.

Since the first studies about this topic, several hormones and signalling substances have been implicated in such a regulation. To our knowledge, the first hormones involved in this process were auxin (Landsberg 1984; Römheld and Marschner 1986) and ethylene (Romera and Alcántara 1994), which are tightly interrelated (Romera et al. 2011; Muday et al. 2012). The role of ethylene on the regulation of Fe responses at the morphological and physiological level has been previously reviewed (Romera and Alcántara 2004; Romera et al. 2007). However, its role at the molecular level was not considered since at the time the above reviews were written very few molecular data were available. For this reason, a new review about the role of ethylene in Fe responses, taking into account the new data available, is opportune. Most of these new data are based on transcriptomic, proteomic, molecular and genetic analyses, and can serve to update and integrate the role of ethylene on Fe responses in a more complex framework.

Besides the above reason, there are at least two other reasons to justify this review. First, in the last years several other hormones and signalling substances have been proposed to participate in either activation or suppression of Fe responses (Brumbarova et al. 2015). Among the ones proposed for activation are auxin (Landsberg 1984; Römheld and Marschner 1986), NO (nitric oxide; Graziano and Lamattina 2007) and GAs (gibberellins; Matsuoka et al. 2014). Among the ones proposed for suppression are CKs (cytokinins; Séguéla et al. 2008), JA (jasmonic acid; Maurer et al. 2011) and BRs (brassinosteroids; Wang et al. 2012). ABA (abscisic acid) has been proposed as activator as well as suppressor of Fe responses (Séguéla et al. 2008; Lei et al. 2014). After these different proposals, it is necessary to clarify the specific role of each hormone and signalling substance on the regulation of Fe responses and the possible interactions of ethylene with them (see section "Participation of Other Hormones and Signalling Substances, Besides Ethylene, in the Regulation of Physiological Fe Responses").

Second, in the last years evidence has been accumulating showing that ethylene is also involved in the regulation of plant responses to many other nutrient deficiencies, such as P deficiency, K deficiency and others (reviewed in Iqbal et al. 2013b; García et al. 2015). This leads to the following question: How can a unique hormone

regulate so many responses? A possible explanation is that ethylene could act through different signalling pathways in the different deficiencies and/or in conjunction with other signals conferring specificity to the responses. Some of these possible Fe-related signals are discussed below (see section "Participation of Non-hormonal Substances in the Regulation of Physiological Fe Responses").

Fe Responses in Strategy I Plants

The main characteristic of Strategy I plants is the necessity of reducing Fe^{3+}, the most abundant in soils, to Fe^{2+}, which is the one preferentially absorbed. The Fe^{3+} reduction is mediated by a ferric reductase while the Fe^{2+} uptake is mediated by a Fe^{2+} transporter (Ivanov et al. 2012; Kobayashi and Nishizawa 2012; Brumbarova et al. 2015). The genes encoding the ferric reductase and the Fe^{2+} transporter were first cloned in *Arabidopsis* [*AtIRT1* (Iron-Regulated Transporter): Eide et al. 1996; *AtFRO2* (Ferric Reductase Oxidase): Robinson et al. 1999], and subsequent homologs have been cloned in other plant species, such as pea, tomato and cucumber (Waters et al. 2007; García et al. 2010). Under Fe-deficient conditions, Strategy I plants induce morphological and physiological changes in their roots, known as Fe responses, which favour Fe acquisition. Among the physiological responses are: enhanced ferric reductase activity (due to higher expression of the *FRO* gene); enhanced Fe^{2+} uptake capacity (due to higher expression of the *IRT* gene); acidification of the rhizosphere [due to higher expression of *HA* (H^+-ATPases) genes]; release of riboflavin and phenolic compounds; and enhanced production of organic acids, like citric acid. Additionally to their transcriptional control, most of the above mentioned genes are also subjected to post-transcriptional control (Connolly et al. 2003; Haruta et al. 2010; Barberon et al. 2011; Brumbarova et al. 2015). Among the morphological responses are: development of root hairs and transfer cells (Landsberg 1986; Römheld and Marschner 1986; Ivanov et al. 2012). Both physiological and morphological responses are located in the subapical regions of the roots (Römheld and Marschner 1986).

In the last years, the knowledge of the mechanisms involved in Fe acquisition by Strategy I plants has increased considerably. Now, it is known that many Fe acquisition genes, that facilitate Fe uptake, are induced by Fe deficiency in roots. Moreover, some of them [such as *FRO* (reductase), *IRT* (Fe transporter) and *HA* (acidification) genes] are activated by bHLH TFs (transcription factors), induced themselves by Fe deficiency too (Wang et al. 2007; Ivanov et al. 2012; Kobayashi and Nishizawa 2012; Brumbarova et al. 2015). Among these bHLH TFs, FER in tomato and its homolog FIT in Arabidopsis (bHLH29, **F**ER-LIKE **I**RON DEFICIENCY-INDUCED **T**RANSCRIPTION FACTOR; formerly named FRU or FIT1; Bauer et al. 2007) are considered the master regulators of Fe acquisition from the medium, since their knockout mutants are very chlorotic and lack the ability to activate most Fe responses in roots (Brown et al. 1971; Ling et al. 2002; Colangelo and Guerinot 2004; Jakoby et al. 2004). Other bHLH TFs involved in Fe acquisition in Arabidopsis

are bHLH38, bHLH39, bHLH100 and bHLH101, which seem to be redundant and need to interact with FIT for most of their functions (Yuan et al. 2008; Wang et al. 2013). All these bHLH TFs are induced in both shoots and roots under Fe deficiency except FIT, which is solely induced in roots (Wang et al. 2007). FIT is also subjected to post-transcriptional control (Lingam et al. 2011; Meiser et al. 2011).

Besides genes related to Fe acquisition from the medium, there are many other genes that are induced under Fe deficiency in roots and/or leaves, and that are important for regulation of Fe responses and Fe homeostasis (Colangelo and Guerinot 2004; García et al. 2010; Long et al. 2010; Ivanov et al. 2012; Palmer et al. 2013). Some of these genes are TFs, such as *POPEYE* (Long et al. 2010), *MYB72* and *MYB10* (Colangelo and Guerinot 2004; Palmer et al. 2013). POPEYE (PYE) is also a bHLH TF (bHLH47) that has been involved in the regulation of a subset of Fe-response genes related to the vasculature (Long et al. 2010). MYB72 and MYB10 are MYB TFs that have redundant functions and are involved in the Fe deficiency-induced up-regulation of *NAS4* (NicotianAmine Synthase 4; Palmer et al. 2013) and phenolic secretion (Zamioudis et al. 2014). NAS are enzymes (in Arabidopsis there are 4 *NAS* genes) involved in the synthesis of NA (nicotianamine), a chelating agent implied in the long-distance transport of Fe (and other metals) and that facilitates the transport of Fe through the phloem to sink organs (Klatte et al. 2009; Schuler et al. 2012). Other genes induced under Fe deficiency that are very important for regulation of Fe responses and Fe homeostasis are *FRD3* (Ferric Reductase Defective; Rogers and Guerinot 2002), *OPT3* (OligoPeptide Transporter; Stacey et al. 2008) and *NAS* (Klatte et al. 2009). The loss-of-function mutants related to these genes [Arabidopsis *frd3*, *opt3-2* and *nas4x*, and tomato *chloronerva* (related to *NAS*)] show constitutive activation of Fe responses, even when grown under Fe-sufficient conditions (Rogers and Guerinot 2002; Stacey et al. 2008; Klatte et al. 2009; García et al. 2013). The FRD3 protein belongs to the multidrug and toxin efflux (MATE) family and has been involved in the loading of citrate into the xylem, which is necessary for Fe translocation from roots to shoots (Rogers and Guerinot 2002; Roschzttardtz et al. 2011). The OPT3 protein belongs to the oligopeptide transporter family and has been involved in the transport of Fe through the phloem (Stacey et al. 2008). Very recently, Zhai et al. (2014) have shown that OPT3 indeed transports Fe^{2+} ions into the phloem. Finally, NAS proteins are involved in the synthesis of NA (see above).

Ethylene Synthesis and Signalling

Ethylene is a gaseous plant hormone involved in many aspects of plant life, including seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening (Romera and Alcántara 2004). It also plays a very important role in the responses of plants to both biotic and abiotic stresses (Romera and Alcántara 2004; Sauter et al. 2013). Among the latter ones, ethylene has been involved in the responses to many nutrient deficiencies, such as Fe deficiency,

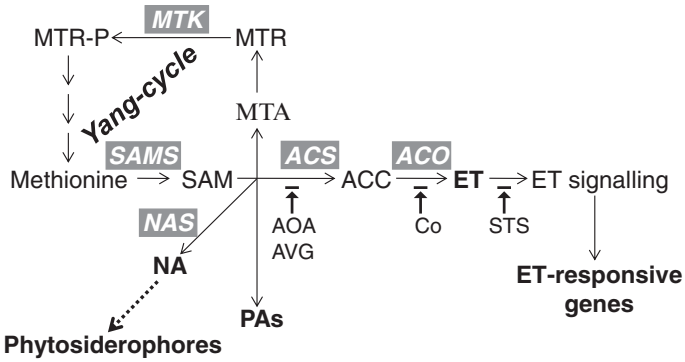

Fig. 12.1 Schematic pathway of ethylene biosynthesis. It is also depicted the steps at which several ethylene inhibitors act and the pathways of NA, phytosiderophores and PAs biosynthesis. (*Dotted arrow*: only in Strategy II plants; ⊤: inhibition). *ACC* 1-aminocyclo-propane-1-carboxylic, *ET* ethylene, *MTA* 5′-methylthioadenosine, *MTR* 5′-methylthioribose, *MTR-P* 5′-methylthioribose-1-phosphate, *NA* nicotianamine, *PAs* polyamines, *SAM* *S*-adenosylmethionine. Enzymes: *ACS* ACC synthase, *ACO* ACC oxidase, *MTK* methylthioribose kinase, *NAS* nicotianamine synthase, *SAMS* SAM synthetase. Ethylene inhibitors: *AOA* *O*-(Carboxymethyl)hydroxylamine hemihydrochloride, formerly aminooxyacetic acid; *AVG* (*S*)-*trans*-2-Amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride, formerly aminoethoxyvinylglycine; *Co* cobalt; and *STS* silver thiosulfate

P deficiency, K deficiency and others (reviewed by Lynch and Brown 1997; Romera and Alcántara 2004; Iqbal et al. 2013b; García et al. 2015). Ethylene is synthesized from methionine via the pathway shown in Fig. 12.1, which also includes some of the inhibitors of ethylene synthesis and action (Romera and Alcántara 2004; Sauter et al. 2013). Several enzymes participate in this pathway, such as SAMS (SAM Synthetase) that catalyses the formation of SAM (*S*-adenosylmethionine) from methionine; ACS (ACC Synthase) that catalyses the formation of ACC (1-aminocyclo propane-1-carboxylic acid) from SAM; and ACO (ACC Oxidase) that catalyses the formation of ethylene from ACC. All of them have several isoforms in Arabidopsis (García et al. 2010). Besides ethylene, SAM is also precursor for the synthesis of NA and PAs (polyamines; Fig. 12.1; Sauter et al. 2013). The synthesis of these three compounds generates a common by-product, MTA (5′-methylthioadenosine), that is detoxified through the Yang cycle (Fig. 12.1; Sauter et al. 2013). This cycle also serves for the replenishment of the substrate methionine, being MTK (MethylThioribose Kinase) one of the enzymes involved (Fig. 12.1; Sauter et al. 2013). NAS enzymes participate in the synthesis of NA, which is the precursor of phytosiderophores (Strategy II plants) but is also important as chelating agent of Fe and other metals for both Strategy I and Strategy II plants (Fig. 12.1; Klatte et al. 2009; Kobayashi and Nishizawa 2012; Schuler et al. 2012).

The mode of action of ethylene (ET) is not fully understood, but based on epistatic analyses of different Arabidopsis ethylene mutants, a signalling pathway for ethylene has been proposed, in which CTR1 (Constitutive Triple Response1; a kinase)

acts downstream of ET receptors (ETR1, ETR2, ERS1, ERS2 and EIN4 in Arabidopsis) and EIN2 (Ethylene Insensitive2; it is localized to the ER membrane) and EIN3/EIL1 (Ethylene Insensitive3/EIN3-Like1; both are TFs) act downstream of CTR1 (see also Fig. 12.3; Ju et al. 2012; Ji and Guo 2013):

ET → ET receptors ⊣ CTR1 ⊣ EIN2 → EIN3/EIL1 → → ET responses

In the absence of ethylene, the kinase CTR1 phosphorylates EIN2, preventing the cleavage and translocation of the EIN2 C-terminal fragment into the nucleus. In the presence of ethylene, CTR1 is inactivated, resulting in dephosphorylation of EIN2. As a consequence, the EIN2 C-terminal fragment is cleaved and transported into the nucleus where it participates in EIN3 stabilization and downstream gene activation (Ju et al. 2012; Ji and Guo 2013). The ethylene signalling pathway is far more complex than the one depicted above, since there are also ERF (Ethylene Response Factors) TFs acting downstream of EIN3/EIL1 and this pathway presents branches and cross-talks with other hormones (Cho and Yoo 2009; Zhao and Guo 2011). In the last years, evidence has been accumulating to suggest that there are alternative ethylene signal transduction pathways to the conventional one including CTR1 and EIN2 (Shakeel et al. 2013).

Role of Ethylene in the Regulation of Fe Responses

In 1994, our group published a pioneering paper (Romera and Alcántara 1994) showing for the first time that ethylene could be involved in the regulation of Fe responses. In this work, based on the use of ethylene inhibitors and precursors, it was proposed that Fe deficiency would cause an enhanced production of ethylene by Strategy I plants and that ethylene would then trigger the activation of both morphological and physiological Fe responses. This hypothesis relating ethylene to Fe responses has been further confirmed by different data obtained at morphological, physiological and molecular level. In the following paragraphs, recent data are described along with some others already presented in previous reviews (Romera and Alcántara 2004; Romera et al. 2007).

Physiological and Morphological Studies Supporting a Role for Ethylene

The evidence for the involvement of ethylene in the regulation of physiological Fe responses comes from results obtained with ethylene inhibitors and precursors, and from results related to ethylene production. The addition of ethylene inhibitors to Fe-deficient plants inhibits the induction of most of their physiological Fe responses, such as ferric reductase activity, Fe^{2+} (Cd^{2+}) uptake capacity, acidification and flavin

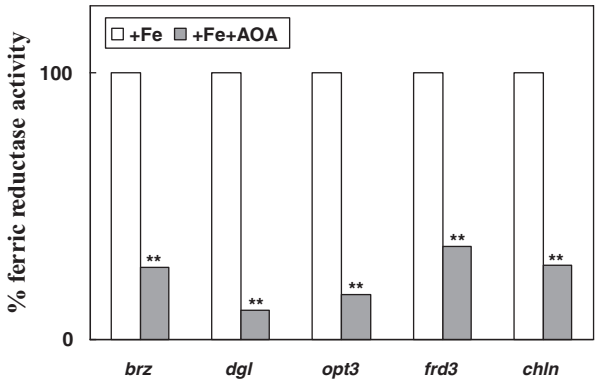

Fig. 12.2 Effect of the ethylene inhibitor AOA on the ferric reductase activity of several constitutive Fe response mutants. The *brz* and *dgl* pea mutants, the *opt3* and *frd3* Arabidopsis mutants, and the *chln* tomato mutant were grown in Fe-sufficient conditions (+Fe). During the last 24 h, AOA (20 μM for pea and tomato and 50 μM for Arabidopsis) was added to the nutrient solution of half of the +Fe plants (+Fe+AOA). After that, ferric reductase activity was determined and results expressed as percentage of +Fe plants. Experiments were repeated at least twice with similar results. Within each mutant, the two *asterisks* indicate that the AOA-treated plants show statistically significant differences ($P \leq 0.01$) from the untreated ones

excretion (Romera and Alcántara 2004; Lucena et al. 2006; Waters et al. 2007; Romera et al. 2007). It is worth mentioning that the ferric reductase activity is also drastically inhibited by ethylene inhibitors in a number of mutants that show constitutive Fe responses, like the pea *brz* (*bronze*) and *dgl* mutants, the Arabidopsis *opt3-2* and *frd3* mutants and the tomato *chln* (*chloronerva*) mutant (Fig. 12.2; Romera and Alcántara 2004).

In contrast to ethylene inhibitors, the addition of ethylene or ethylene precursors (ACC or the ethylene releasing substance ethephon) to plants grown with low levels of Fe induces some physiological Fe responses, such as enhanced ferric reductase activity, located in the subapical regions of the roots where formation of root hairs is also induced (Romera and Alcántara 2004; Lucena et al. 2006; Waters et al. 2007; Romera et al. 2007; García et al. 2010, 2011).

According to the hypothesis proposed by Romera and Alcántara (1994), Fe deficiency would cause an enhanced production of ethylene by Strategy I plants. This proposal has been further confirmed by different data showing that Fe-deficient roots from several Strategy I plants produce more ethylene than Fe-sufficient ones (Romera and Alcántara 2004; Romera et al. 2007; Zuchi et al. 2009; Wang et al. 2012; Li et al. 2014). Moreover, it has been shown that Fe-efficient cultivars of pea (Kabir et al. 2012) and *Medicago truncatula* (Li et al. 2014) produce more ethylene than the Fe-inefficient ones. Additionally, Wu et al. (2011) have found that Fe-deficient roots from rice (a Strategy II plant species that presents some characteristics of Strategy I plants) produce more ethylene than Fe-sufficient ones.

The role of ethylene on the development of morphological Fe responses has been supported by results obtained with ethylene inhibitors and precursors but also by results obtained with ethylene mutants. Among these results (reviewed in Romera and Alcántara 2004; Romera et al. 2007) are the following: (a) ethylene inhibitors block the development of subapical root hairs in Fe-deficient roots of Strategy I plants; (b) both ethylene and ethylene precursors (ACC or ethephon) induce the formation of subapical root hairs and transfer cells in Fe-sufficient plants; and (c) some ethylene-insensitive mutants, like Arabidopsis *etr1* and *ein2*, soybean *etr1* and *Medicago truncatula sickle*, do not develop subapical root hairs either under Fe deficiency or upon ACC treatment.

In conclusion, the evidence obtained by using ethylene inhibitors and precursors, and ethylene determination, suggests that ethylene is involved in the regulation of several physiological and morphological Fe responses, such as ferric reductase activity, Fe^{2+} transporter, acidification, flavin excretion, subapical root hairs and transfer cells (Romera and Alcántara 2004; Romera et al. 2007). Additionally, the results obtained with ethylene mutants suggest that morphological and physiological Fe responses are not regulated by ethylene in the same way: ferric reductase activity is induced in the Arabidopsis *etr1* and *ein2* mutants either under Fe deficiency or upon ACC treatment, while they do not develop subapical root hairs in any case (Schmidt et al. 2000; Romera and Alcántara 2004; Lucena et al. 2006; Romera et al. 2007; García et al. 2010, 2015). These latter results may be explained by taking into account that ethylene could act through alternative signal transduction pathways to the one including CTR1 and EIN2 (Shakeel et al. 2013; Lucena et al. 2015).

Molecular Studies Supporting a Role for Ethylene

The identification of genes involved in Fe acquisition from the medium, such as those encoding Fe^{2+} transporters and ferric reductases, as well as those coding for key TFs controlling them, opened the way for the study of Fe responses at the molecular level. The genes encoding the ferric reductase and the Fe^{2+} transporter were first cloned in *Arabidopsis* in the late nineties (see section "Fe Responses in Strategy I Plants"). The master regulators FER in tomato and FIT in Arabidopsis were cloned later on (*SlFER*: Ling et al. 2002; *AtFIT*: Colangelo and Guerinot 2004; Jakoby et al. 2004). The first paper relating ethylene to the regulation of these genes was the one by Lucena et al. (2006). In this work, and in the one by Waters et al. (2007), it was shown that ethylene activated the expression of *AtFIT* and *SlFER* and, consequently, of their target genes, *IRT1* and *FRO* (Table 12.1). Additionally, it was shown that ethylene activated the expression of the H^+-ATPase gene *CsHA1*, related to the acidification response in cucumber (Table 12.1). Later on, the role of ethylene has been extended to a wider number of genes involved in Fe acquisition and homeostasis in Arabidopsis, such as *AtbHLH38*, *AtbHLH39*, *AtMYB72*, *AtNAS*, *AtFRD3* and others (Table 12.1; Lucena et al. 2015). Besides Strategy I plants, Wu et al. (2011) have found that ethylene is also involved in the

Table 12.1 Genes related to Fe acquisition and homeostasis up-regulated by ethylene in roots of dicot plants

Name	Function	Plant species	References
Genes coding for transcription factors			
FER	Master regulator	Tomato	Lucena et al. (2006)
FIT	Master regulator	Arabidopsis	Lucena et al. (2006)
			García et al. (2010)
			Lingam et al. (2011)
bHLH038	TF (interacts with FIT)	Arabidopsis	García et al. (2010)
bHLH039	TF (interacts with FIT)	Arabidopsis	García et al. (2010)
MYB72	TF	Arabidopsis	García et al. (2010)
Genes involved in Fe acquisition			
FRO2/1	Ferric reductase	Arabidopsis	Lucena et al. (2006)
		Tomato	Waters et al. (2007)
		Cucumber	García et al. (2010)
			Lingam et al. (2011)
IRT1	Iron transporter	Arabidopsis	Lucena et al. (2006)
		Tomato	Waters et al. (2007)
		Cucumber	García et al. (2010)
			Lingam et al. (2011)
CsHA1	H^+-ATPase	Cucumber	Lucena et al. (2006) and Waters et al. (2007)
Genes involved in Fe translocation and compartmentalization			
FRD3	Xylem Fe transport	Arabidopsis	García et al. (2010)
NAS	NA synthesis	Arabidopsis	García et al. (2010)
CCCL	Vacuolar Fe transport	Arabidopsis	García et al. (2010)

activation of some Fe-related genes, such as *OsITR1*, *OsNAS1*, *OsNAS2* and *OsIRO2* (a homolog of *AtbHLH38* and *AtbHLH39*; Matsuoka et al. 2014), in rice (Strategy II plant, see above).

More recently, it has been shown that EIN3 and EIL1, two TFs in the ethylene signalling pathway, interact with MED25 (Mediator; it facilitates RNA polymerase II interactions with DNA binding regulators) that, at the same time, interacts with MED16 to form a complex involved in the transcription of *AtFIT* (Yang et al. 2014; Zhang et al. 2014a). Previously, it was shown, by using a yeast two-hybrid assay and bimolecular fluorescence complementation, that EIN3 and EIL1 interact with FIT and participate in its post-transcriptional regulation (Lingam et al. 2011). All these results, and the previous ones, strongly suggest that ethylene plays a role at both transcriptional and post-transcriptional level in the regulation of physiological Fe responses.

In addition to the results supporting a role for ethylene in the up-regulation of a wide number of Fe-related genes, in the last years evidence has been accumulating showing that many genes involved in ethylene synthesis and signalling are up-regulated under Fe deficiency in several Strategy I plants (see Table 12.2, Figs. 12.1 and 12.3) and in rice (Strategy II plant, see above; Zheng et al. 2009; Wu et al. 2011; Lucena et al. 2015).

Table 12.2 Genes involved in ethylene synthesis and signalling up-regulated by Fe deficiency in roots of dicot plants

Name	Function	Plant species	References
Ethylene synthesis			
AtSAM1	SAM synthetase	Arabidopsis	Buckhout and Thimm (2003)
			García et al. (2010)
			Yang et al. (2010)
AtSAM2	SAM synthetase	Arabidopsis	García et al. (2010)
AtACS4	ACC synthase	Arabidopsis	García et al. (2010)
AtACS6	ACC synthase	Arabidopsis	García et al. (2010)
AtACS9	ACC synthase	Arabidopsis	García et al. (2010)
AtACO	ACC oxidase	Arabidopsis	Buckhout and Thimm (2003)
AtACO1	ACC oxidase	Arabidopsis	García et al. (2010)
AtACO2	ACC oxidase	Arabidopsis	García et al. (2010)
AtMTK	Methylthioribose kinase	Arabidopsis	García et al. (2010)
			Yang et al. (2010)
SlMTK	Methylthioribose kinase	Tomato	Zamboni et al. (2012)
Ethylene signalling			
AtETR1	ET-receptor	Arabidopsis	García et al. (2010)
AtCTR1	ET-signalling	Arabidopsis	García et al. (2010)
AtEIN2	ET-signalling	Arabidopsis	García et al. (2010)
AtEIN3	ET-TF	Arabidopsis	García et al. (2010)
AtEIL1	ET-TF	Arabidopsis	García et al. (2010)
Gm-c1028-8658	ET-receptor	Soybean	O'Rourke et al. (2007)
Gm-c1004-7092	ET-TF	Soybean	O'Rourke et al. (2007)
Gm-c1009-2900	ET-TF	Soybean	O'Rourke et al. (2007)

At the proteomic level, several studies also show a significant increase in the SAMS protein, involved in ethylene synthesis (Fig. 12.1), under Fe deficiency (reviewed by López-Millán et al. 2013). All these results are in agreement with the higher levels of ethylene production found in Fe-deficient roots (see section "Physiological and Morphological Studies Supporting a Role for Ethylene") and also support a role for ethylene in the regulation of Fe responses.

Participation of Other Hormones and Signalling Substances, Besides Ethylene, in the Regulation of Physiological Fe Responses

The influence of other hormones and signalling substances, besides ethylene, in the regulation of Fe responses has been studying since the 1980s, with the pioneering works of Landsberg suggesting a role for auxin in such a regulation

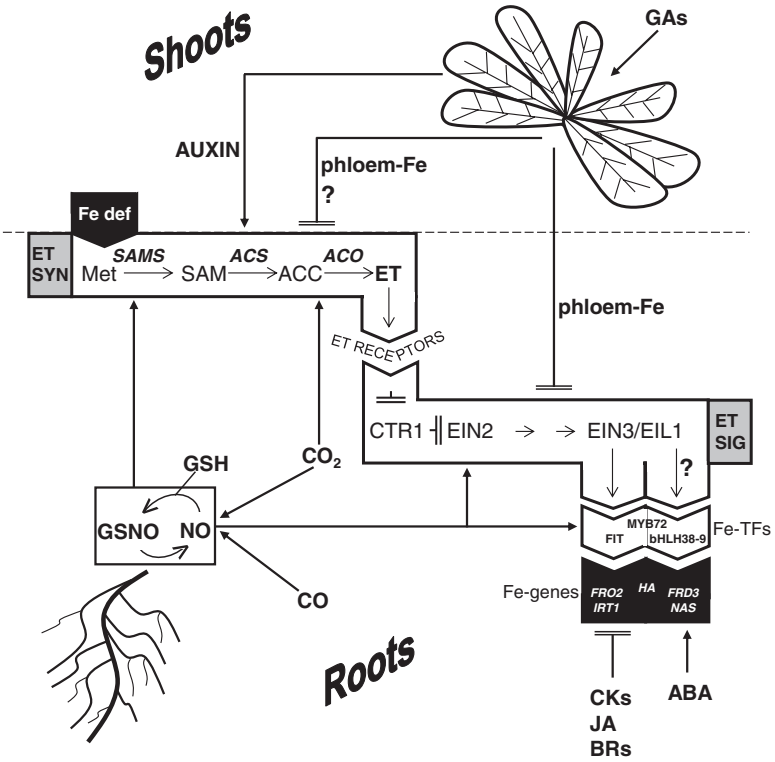

Fig. 12.3 Working model to explain the role of hormones and non-hormonal substances in the regulation of physiological Fe responses in Arabidopsis. Fe deficiency up-regulates the expression of genes involved in ethylene synthesis (ET SYN) and signalling (ET SIG) (see Table 12.2). As a consequence, there is an increase of ethylene production and action, which is mediated through ET-TFs such as EIN3/EIL1. These ET-TFs (and perhaps others) participate in the activation of FIT and other Fe-TFs, that up-regulate the expression of Fe-genes, such as *FRO2* and *IRT1* (see Table 12.1). Several other hormones and non-hormonal substances could affect the regulation of Fe-genes by acting, either positively or negatively, at different steps of ethylene synthesis and/or signalling. Additionally, some hormones, such as ABA, can participate in the regulation of small groups of Fe-genes. Hormones and signalling substances: *ABA* abscisic acid, *BRs* brassinosteroids, *CKs* cytokinins, *ET* ethylene, *GAs* gibberellins, *JA* jasmonic acid, *NO* nitric oxide. Non-hormonal substances: *CO* carbon monoxide, CO_2 carbon dioxide, *GSH* glutathione, phloem Fe. (*Arrow*: promotion; ⊤: inhibition). For further details, see section "Working Model to Explain the Regulation of Physiological Fe Responses"

(Landsberg 1984). In the last years, and after the proposal of Graziano and Lamattina (2007) suggesting a role for NO in the activation of Fe responses, several other hormones and signalling substances have come into play (Fig. 12.3), some of them as activators of Fe responses [GAs (Matsuoka et al. 2014)], some of them as repressors [CKs (Séguéla et al. 2008), JA (Maurer et al. 2011), BRs (Wang et al. 2012)], and some of them as activators and/or repressors [ABA (Séguéla et al. 2008; Lei et al. 2014)].

The involvement of several hormones and signalling substances in the same process is not strange since someones can influence the production of others and there are intricate cross-talks among them. Moreover, some hormones and signalling substances can affect a whole process and others only a part of such a process. The question is to determine which hormones and signalling substances act upstream and which ones downstream, and to establish the exact relationships among them.

The role of auxin, ethylene and NO as activators of Fe responses, and their possible interactions, has been reviewed by Romera et al. (2011) and studied in some recent works (i.e. Blum et al. 2014). Romera et al. (2011) suggested, based on the results presented by Chen et al. (2010) and Romera et al. (2007), that NO and ethylene could act downstream of auxin in the regulation of some Fe responses. This view is now supported by recent results presented by several authors. Yang et al. (2014) have shown that EIN3 and EIL1, two TFs in the ethylene signalling pathway, are directly involved in *AtFIT* transcription. On the other hand, EIN3/EIL1 (Lingam et al. 2011) and NO (Meiser et al. 2011) have been involved in the post-transcriptional regulation of FIT. These results clearly suggest that ethylene/NO act downstream of auxin in the regulation of, at least, the Fe responses controlled by FIT (Fig. 12.3). Since auxin can enhance both ethylene and NO production, it is possible that the positive effects of auxin in the activation of some physiological Fe responses could be mediated through ethylene/NO (Fig. 12.3; Romera et al. 2011).

To place ethylene and NO in the signalling cascade leading to the activation of Fe responses is more complicated, since NO could act at many different steps: i.e. NO can modify the activity of many proteins involved in such signalling cascade through *S*-nitrosylation (Lindermayr et al. 2006; Darbani et al. 2013). In fact, NO (or perhaps GSNO; see section "Participation of Non-hormonal Substances in the Regulation of Physiological Fe Responses") has already been involved in the post-transcriptional regulation of SAMS (Lindermayr et al. 2006) and FIT (Meiser et al. 2011). Besides this, García et al. (2011) found that NO up-regulates the expression of many genes involved in ethylene synthesis and signalling, which indicates that NO can affect both ethylene production and action (Fig. 12.3). In summary (Fig. 12.3), NO could participate in Fe deficiency signalling by affecting SAMS (Lindermayr et al. 2006); by affecting the expression of ethylene synthesis and signalling genes (García et al. 2011); and by affecting the post-translational regulation of FIT (Meiser et al. 2011).

On the other hand, García et al. (2011) showed that ACC can enhance NO production in the subapical regions of the roots of several Strategy I plants. Similar results showing a mutual influence of ethylene and NO have been presented by Lin et al. (2013). Taken together, all these results clearly imply that each hormone (ethylene, NO) can influence the production of the other.

Very recently, ABA and GAs have also been involved in the activation of some physiological Fe responses (Lei et al. 2014; Matsuoka et al. 2014). Although ABA can induce subapical root hairs (Landsberg 1996), several results have related ABA to the inhibition of physiological Fe responses (Landsberg 1986; Séguéla et al. 2008; Lei et al. 2014). Now, Lei et al. (2014) have shown that ABA, which increases in Fe-deficient roots, can activate the expression of some Fe-related genes, such as *AtFRD3* and *AtNAS2*, involved in Fe translocation (see section "Fe Responses in

Strategy I Plants"). In relation to GAs, Matsuoka et al. (2014) have found that the application of GAs to shoots, but not to roots, positively affects the expression of *AtbHLH38*, *AtbHLH39*, *AtFRO2* and *AtIRT1* in roots. Additionally, these authors did not find increased expression of GA biosynthetic genes in Fe-deficient roots. Taken together, these results suggest that ABA could regulate a subset of genes related to Fe translocation while GAs could cause metabolic changes in shoots that may contribute to activate the expression of some Fe-related genes in roots (Fig. 12.3; Lei et al. 2014; Matsuoka et al. 2014).

By contrast to the hormones involved in the activation of Fe responses (auxin, ethylene, NO, ABA and GAs), several other hormones have been involved in their suppression (CKs, JA and BRs). CKs and JA suppress the expression of the Fe acquisition genes *AtFRO2*, *AtIRT1* and *AtFIT*, in both Fe-sufficient and Fe-deficient wt Arabidopsis plants, and also in the *fit* mutant (Séguéla et al. 2008; Maurer et al. 2011). Similarly, BRs suppress the expression of the Fe acquisition genes *CsFRO1* and *CsIRT1* in Fe-deficient cucumber plants (Wang et al. 2012). The way CKs suppress Fe responses is unknown but it is possible that they could negatively interact with ethylene and/or NO. The negative interaction between CKs and ethylene has been described in some processes (Wojtania and Wegrzynowicz-Lesiak 2012). On the other hand, CKs can also interact with NO. High levels of CKs suppress the action of NO most likely through direct interaction between them, leading to the reduction of endogenous NO levels (Liu et al. 2013). In relation to JA, the way it suppresses Fe responses is not clear but it is known that JA can repress ethylene signalling by affecting EIN3 (Zhang et al. 2014 b), one of the TFs involved in FIT activation (Lingam et al. 2011; Yang et al. 2014). Similarly to CKs and JA, the way BRs suppress Fe responses is unknown but Wang et al. (2012) found that BRs diminished ethylene production in Fe-deficient cucumber roots.

In conclusion, among the hormones that are activators of Fe responses, auxin and GAs probably act upstream of ethylene and NO; ethylene and NO would simultaneously act at the end of the Fe deficiency signalling cascade; and ABA could regulate a subset of genes related to Fe translocation (Fig. 12.3). In relation to the hormones that suppress Fe responses (CKs, JA, BRs), it is still unknown their mode of action but possibly they could negatively interact with ethylene, which deserves further research.

Participation of Non-hormonal Substances in the Regulation of Physiological Fe Responses

Besides hormones, several other substances have been involved in the regulation of Fe responses, some of them as activators [CO (carbon monoxide), CO_2 (carbon dioxide), DMHDA (dimethylhexadecylamine), GSH (glutathione); Jin et al. 2009; Kong et al. 2010; Koen et al. 2012; Orozco-Mosqueda et al. 2013] and some of them as repressors (phloem Fe; Maas et al. 1988; García et al. 2013).

The expression of several Fe acquisition genes, such as *AtIRT1*, *AtFRO2* and *AtFIT1*, was up-regulated by CO exposure in Fe-deficient Arabidopsis seedlings

(Kong et al. 2010). Similarly, some Fe responses, such as enhanced ferric reductase activity, acidification, subapical root hairs and the expression of *SlFER*, *SlFRO1* and *SlIRT1*, were greater in tomato plants grown under elevated CO_2 than in the ones grown in the ambient CO_2 (Jin et al. 2009). In both cases, the effects of CO and CO_2 could be mediated through NO (Fig. 12.3) since CO and CO_2 exposure induced NO accumulation in roots and their activating effects were blocked by the NO scavenger cPTIO (Jin et al. 2009; Kong et al. 2010). In addition to their action through NO, both CO and CO_2 could also act through ethylene (Fig. 12.3). The promoting effect of CO on the development of subapical root hairs was blocked by ethylene inhibitors, such as AVG or Ag^+ (Fig. 12.1; Guo et al. 2009). In the same way, CO_2 could increase ethylene production by affecting ACO activity (Finlayson and Reid 1994). In conclusion, the activating role of CO and CO_2 on Fe responses could be mediated through ethylene/NO (Fig. 12.3).

Very recently, Orozco-Mosqueda et al. (2013) have shown that DMHDA (a volatile, organic compound produced by the plant growth-promoting rhizobacteria *Arthrobacter agilis*) caused higher rhizosphere acidification and enhanced ferric reductase activity in *Medicago truncatula* plants. To our knowledge, it is not known whether this compound affects ethylene/NO (also volatile compounds) metabolism or action but these results open the way to study the role of rhizobacteria in the Fe nutrition of plants.

GSH is another substance involved in the up-regulation of some Fe acquisition genes, such as *AtFRO2* and *AtIRT1*, in *Arabidopsis* plants (Koen et al. 2012; Shanmugam et al. 2015). Additionally, GSH has been associated with Fe-mediated tolerance to Zn excess (Shanmugam et al. 2012) and with internal Fe availability (Ramírez et al. 2013) in *Arabidopsis* plants. GSH is a tripeptide (γ-glutamylcysteinyl glycine) involved in responses of plants to abiotic stresses, that usually increases in leaves and roots affected by Fe deficiency (Zaharieva and Abadia 2003; Kabir et al. 2013). The way GSH up-regulates the expression of Fe acquisition genes is not known but several results suggest the existence of possible interactions between GSH and ethylene/NO. GSH and ethylene can interact because both compounds need S for their synthesis (Iqbal et al. 2013a). On the other hand, GSH and NO can interact to generate GSNO (*S*-nitrosoglutathione), a nitrosothiol compound (Corpas et al. 2013), that enhances the expression of several Fe acquisition genes (Fig. 12.3; García et al. 2010, 2011; Koen et al. 2012). Very recently, Shanmugam et al. (2015) have presented data suggesting that GSH acts upstream of NO but downstream of long-distance signals. Finally, Fe, NO and GSH can interact to form NICs (nitrosyl iron complexes), which are considered intracellular NO reservoirs as well as vehicles of NO and Fe throughout cells and organs (Lewandowska et al. 2011; Ramírez et al. 2011; Corpas et al. 2013; Darbani et al. 2013).

In addition to the non-hormonal substances described above as activators of Fe responses (CO, CO_2, DMHDA and GSH), several results support a role for phloem Fe as repressor of Fe responses (Maas et al. 1988; Lucena et al. 2006; García et al. 2013). As example, auxin, ethylene and NO can up-regulate the expression of Fe acquisition genes in plants grown with low levels of Fe (or without Fe) but have almost no effect in plants grown with high levels of Fe (Lucena et al. 2006; Graziano

and Lamattina 2007; Chen et al. 2010; García et al. 2011). This suggests that the induction of physiological Fe responses does not depend only on hormones (auxin, ethylene and NO), that would act as activators, but also on Fe, that would act as inhibitor. The question arises as to how Fe acts to repress physiological Fe responses and which pool of Fe (i.e. root, phloem) is monitored by the plant to mediate this negative control. The existence of mutants that show constitutive activation of physiological Fe responses when grown under Fe-sufficient conditions, and accumulate high levels of Fe in roots (*frd3*, *opt3-2*, *nas4x*, *dgl*, *brz, chln*), suggests that total Fe in roots is not the repressor (García et al. 2013). An alternative possibility is to consider that the Fe that acts as repressor is the Fe recirculating back from leaves to roots through the phloem, as proposed by Maas et al. (1988). This suggestion has been supported by results presented by Lucena et al. (2006) and, very recently, by several other authors. Lucena et al. (2006) showed that constitutive Fe responses in the Arabidopsis *frd3* mutant, unable to translocate Fe from roots to shoots, could be suppressed by foliar application of Fe. On the other hand, García et al. (2013) have found that foliar application of Fe to Fe-deficient *Arabidopsis* wt Columbia plants drastically inhibited their physiological Fe responses in roots while it did not inhibit when applied to the *opt3-2* mutant. The *Arabidopsis opt3-2* mutant harbours a T-DNA insertion in the *AtOPT3* promoter resulting in reduced *AtOPT3* expression (Stacey et al. 2008) and, very recently, it has been shown that OPT3 is a transporter involved in the loading of Fe^{2+} ions into the phloem (Zhai et al. 2014). Accordingly, knock-down of *OPT3* in the *opt3-2* mutant results in plants incapable of loading Fe into the phloem (either from leaves or from leaves treated with Fe) for the long-distance transport to the roots, where it would repress physiological Fe responses. In the same way, Schuler et al. (2012) have found that in the *Arabidopsis nas4x-2* mutant, that also shows constitutive activation of Fe responses, the lack of NA impedes the transport of Fe out of the phloem and consequently phloem Fe cannot exert its repressing action in roots. In support of the existence of repressors for the regulation of Fe responses, it should be noted that Meiser et al. (2011) found that *AtFIT* expression was up-regulated in Fe-sufficient roots upon cycloheximide treatment, which suggests the action of a short-lived repressor eliminated by this treatment. Taken together, the results described above provide evidence that phloem Fe participates in the regulation of physiological Fe responses in roots.

However, to consider phloem Fe as the only key factor involved in the regulation of physiological Fe responses is not sufficient to fully explain some experimental results, such as the up-regulation of Fe acquisition genes caused by some substances (ethylene, auxin, NO, CO, CO_2, etc.) or the repression caused by others (CKs, JA, BRs; see sections "Role of Ethylene in the Regulation of Fe Responses", "Participation of Other Hormones and Signalling Substances, Besides Ethylene, in the Regulation of Physiological Fe Responses", and "Participation of Non-hormonal Substances in the Regulation of Physiological Fe Responses"). Thus, it is necessary to integrate the signals that activate physiological Fe responses and those that repress them in a more complex framework (see section "Working Model to Explain the Regulation of Physiological Fe Responses").

Working Model to Explain the Regulation of Physiological Fe Responses

The integration of positive and negative signals in the regulation of Fe acquisition genes was first addressed by Lucena et al. (2006), which proposed a model that implicated both phloem Fe and ethylene in such a regulation. Recently, this model has been extended to more Fe-related genes and, besides ethylene, NO and auxin have also been included as positive signals (García et al. 2011; Romera et al. 2011). According to this model, auxin/ethylene/NO would act as activators of the expression of Fe acquisition genes, while phloem Fe would act to repress their expression (Fig. 12.3; García et al. 2011; Romera et al. 2011). All the results obtained so far suggest that auxin acts upstream of ethylene/NO (see section "Participation of Other Hormones and Signalling Substances, Besides Ethylene, in the Regulation of Physiological Fe Responses"). Moreover, several results suggest that auxin comes from shoots to roots (Landsberg 1984; Bacaicoa et al. 2011; Wu et al. 2012), which also supports its role upstream of ethylene/NO (Fig. 12.3). In relation to phloem Fe, it seems clear that it exerts a negative effect on the regulation of physiological Fe responses (see section "Participation of Non-hormonal Substances in the Regulation of Physiological Fe Responses"; García et al. 2013; Zhai et al. 2014). The way phloem Fe represses Fe responses is not totally known but several results suggest that it could negatively affect ethylene/NO action. García et al. (2013) found that both ACC and GSNO up-regulated the expression of Fe acquisition genes when applied to Fe-deficient plants but not when were simultaneously applied with foliar Fe. This suggests that phloem Fe, or a signal derived from it, could block ethylene/NO action (Fig. 12.3). On the other hand, neither the Arabidopsis ethylene constitutive mutant *ctr1* nor the ethylene overproducer mutants *eto* show constitutive expression of Fe responses (Schmidt et al. 2000; García et al. 2014). These results also indicate that Fe (presumably phloem Fe) blocks the up-regulation of Fe responses downstream of ethylene production (Fig. 12.3). However, this does not discard a possible negative effect of phloem Fe on ethylene production too (Fig. 12.3), which deserves further research.

In this review, the model integrating positive and negative signals in the regulation of Fe acquisition genes has been completed by taking into account the positive effects of the hormones ABA and GAs, and the negative effects of JA, CKs and BRs. As previously discussed, ABA could regulate a subset of genes related to Fe translocation and GAs probably act upstream of ethylene/NO (Fig. **12.3**; Lei et al. 2014; Matsuoka et al. 2014). In relation to the hormones that suppress Fe responses (CKs, JA, BRs), it is still unknown their mode of action (Séguéla et al. 2008; Maurer et al. 2011; Wang et al. 2012) but possibly they could negatively interact with ethylene (see section "Participation of Other Hormones and Signalling Substances, Besides Ethylene, in the Regulation of Physiological Fe Responses").

In relation to the non-hormonal substances implicated on the regulation of Fe responses, the positive effects of CO and CO_2 could be mediated through NO/ethylene (Fig. 12.3; Finlayson and Reid 1994; Jin et al. 2009; Kong et al. 2010).

The effects of GSH could be mediated through NO due to the formation of GSNO (Fig. 12.3; Corpas et al. 2013). However, the effects of GSH on Fe responses could be more complex because this substance can participate in many other processes related to Fe transport and homeostasis (see section "Participation of Non-hormonal Substances in the Regulation of Physiological Fe Responses").

Concluding Remarks and Future Perspectives

In 1994, we proposed that ethylene was involved in the regulation of both morphological and physiological Fe responses (Romera and Alcántara 1994). After that proposal, the participation of ethylene in the regulation of morphological Fe responses was easily accepted by other researchers. However, the acceptation of the role of ethylene on the regulation of physiological Fe responses has been more controversial (Romera et al. 2007). Possibly, this controversy may be related to its great complexity. Now, there is physiological and molecular evidence that ethylene participates in the regulation of physiological Fe responses (see section "Role of Ethylene in the Regulation of Fe Responses") but it is also evident that other hormones and substances are involved (see sections "Participation of Other Hormones and Signalling Substances, Besides Ethylene, in the Regulation of Physiological Fe Responses" and "Participation of Non-hormonal Substances in the Regulation of Physiological Fe Responses"). Considering all the hormones and substances involved, the results obtained so far indicate that ethylene/NO would act as activators of the physiological Fe responses downstream of the other hormones and substances while phloem Fe would act as repressor of ethylene/NO action and, probably, ethylene/NO production (Fig. 12.3).

The Working Model presented in Fig. 12.3 intends to place ethylene, NO and other hormones and substances in the framework of the regulation of physiological Fe responses but there are still many questions to solve. As examples, we would like to point out some of them. It is necessary to study whether additional key genes, such as *POPEYE*, *bHLH100* and *bHLH101*, are also regulated by ethylene/NO. It is necessary to know which steps of ethylene synthesis and signalling are affected by NO, possibly through *S*-nitrosylation. In relation to this point, an important goal would be to decipher the exact interrelationship between NO and GSNO, to know the specific role of each one in the *S*-nitrosylation processes and, consequently, on the regulation of Fe responses (Fig. 12.3). Related to this, it would be also interesting to study the role of NICs, formed through the interaction of NO, GSH and Fe, in the long-distance transport of Fe and NO, and to look further into the characteristics of phloem Fe: How is Fe transported through the phloem? How is Fe unloaded out of the phloem to exert its repressive role in roots? Which is the role of NA in that process? Which steps of ethylene synthesis and/or signalling are affected by phloem Fe (or by the signal derived from it)? Finally, it is necessary to better understand the relationship of ethylene/NO with the other hormones involved in the activation or suppression of Fe responses.

A general conclusion of this review is that ethylene acts as a coordinator of the activation of most Fe responses, although for this it needs to act in conjunction with other hormonal and non-hormonal signals. From these other signals, we consider of great interest the role of NO, tightly interrelated to ethylene, and phloem Fe, because it could confer specificity to Fe responses. It should be taken into account that ethylene has also been involved in the regulation of responses to other nutrient deficiencies (Iqbal et al. 2013b; García et al. 2015). From this point of view, the participation of phloem Fe in the regulation of physiological Fe responses provides a way for shoots to inform roots of their Fe status and serves to integrate the role of shoots and roots in the regulation of Fe responses. The better knowledge of this integration could contribute to obtain more Fe-efficient genotypes and to better understand the interaction scion–rootstock, frequent in fruit trees.

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Chapter 13
Leaf Senescence in Plants: Nutrient Remobilization and Gene Regulation

Maryam Sarwat

Abstract In order to study the development of a plant, the best organs are leaves as they exhibit the life history which is reproducible. Leaves are also useful as experimental materials. As leaves grow, they encounter various changes in their development, physiology, and metabolism which ultimately ends up in senescence and death. Senescence is the process of degradation of the nutrients produced during the life time of a leaf and its further redistribution to the developing parts of the plant. Over the years, the studies have shown leaf senescence to be a complex molecular phenomenon which is regulated at the transcriptional, posttranscriptional, and post-translational level.

Introduction

Senescence is an age-dependent process of degradation and degeneration. It affects the cells, organs, or the entire organism and culminates into death (Lim et al. 2007a). Although leaf senescence seems to be a process of wear and tear, it's a highly regulated phenomenon (Sarwat et al. 2013). All the transitions in the cellular metabolism and degradation of cellular structures occur in an orderly manner. A very good representation of leaf senescence is the changes which occur in leaf chloroplast can be seen as the distinct changes occurring due to degradation of chloroplast. The senescence in mitochondria and nucleus occurs at the last. The metabolic changes that take place during leaf senescence include hydrolysis of lipids, proteins, nucleic acids, and pigments which are synthesized during the growth phase (Watanabe et al. 2013). The degradation of these macromolecules is very essential to make them as

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transportable nutrients which are then remobilized to young and expanding organs of the plants. The breakdown of proteins leads to transportable nitrogen (Buchanan-Wollaston 1997). The specific types of proteases involved in this protein breakdown are not very well known, but two proteases cysteine proteases and aspartic proteases have shown their role in this phenomenon. In addition to the proteases, various nucleases (Taylor et al. 1993) and lipases (Ryu and Wang 1995) for degradation of nucleic acids and lipids also play their role during senescence. The genes encoding these nucleases and lipases are collectively termed as senescence-associated genes (SAGs) (Sarwat et al. 2013). These SAGs express only after initiation of the senescence process and do not participate in its induction. When a leaf gets matured, it sends signal(s) for reduced photosynthesis, senescence induction and mobilization of resources to the growing organs of the plant. In annual plants, the products of this hydrolysis then transferred to the developing seeds and fruits. In trees, these hydrolyzed products are stored in stems and roots which are later utilized for development of new leaves, flowers, etc. The spring flowers take their nutrients from senescing autumn leaves and this inevitable cycle goes on. It helps in optimization of resource utilization by the plant. In the process of nutrient mobilization, first the macromolecules get degraded and the metabolites get converted into transportable forms. This process is a highly controlled and complex phenomenon regulated by various genes. Thus senescence is an inevitable orderly process in the life cycle of an organism, which ensures its fitness and survival. Various endogenous and exogenous environmental factors decide the onset, progression, and completion of senescence (Fig. 13.1). There are intricate pathways for the regulation of senescence (Buchanan-Wollaston et al. 2005; Lim et al. 2007a). How these pathways are linked to the

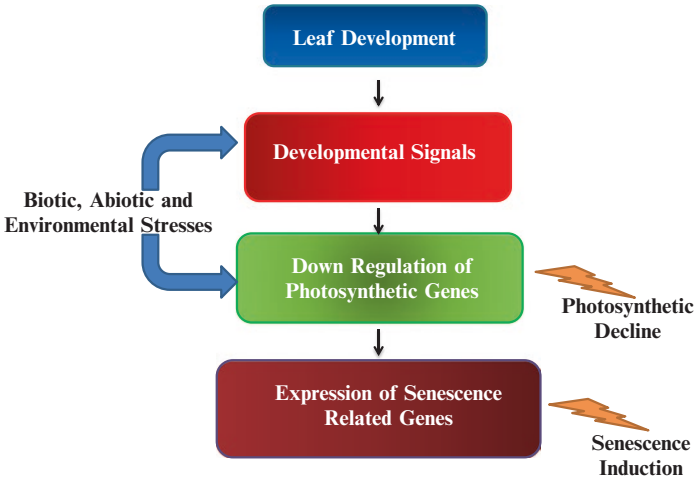

Fig. 13.1 Initiation of leaf senescence by downregulation of photosynthetic genes. Biotic/abiotic stress factors and other environmental factors may directly or indirectly cause changes in the developmental signal leading to induction of senescence

environmental factors is a matter of extensive research. The molecular changes taking place during senescence is also a matter of research. Further, how these senescence cycles are related to the productivity and fitness of the plants. The research in the past decade has shown multilayered regulation of leaf senescence.

Signals for the Onset of Leaf Senescence

Initiation of leaf senescence takes place through perception of senescence signals and their processing by the help of expression of SAGs. Involvement of various hormones and other environmental cues have been reported (Fig. 13.2). The level of cytokinins has found to be reduced in senescing leaves, so it may be a possible hormonal signal which may lead to either induction or enhancement of leaf senescence. Similarly, exogenous application of cytokinins or their overproduction in transgenic plants significantly retards senescence (Sarwat et al. 2013). Ethylene is a well-known hormone for fruit ripening, and its inhibition by antisense technology causes decreased fruit ripening. As fruit ripening is a type of programmed cell death (PCD), ethylene can also be involved in senescence induction and a common signaling pathway for senescence and PCD has been suggested (Gan and Amasino 1995).

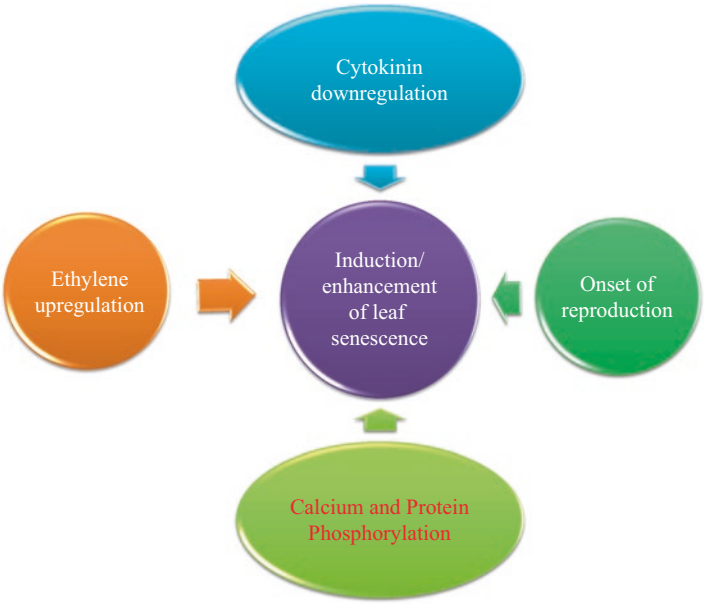

Fig. 13.2 Possible signals for initiating leaf senescence

Other types of signals may involve the development of reproductive organs in plants (Hayati et al. 1995). Other authors have discussed the involvement of calcium signaling and protein phosphorylation in inducing senescence (McCabe et al. 1997).

Regulation of Plant Leaf Senescence

The regulation of leaf senescence starts from the onset and proceeds through the progression and completion of senescence. Various genes are found to be involved during these processes. They are collectively known as SAGs (Buchanan-Wollaston et al. 2005; Pourtau et al. 2006; van der Graaff et al. 2006; Breeze et al. 2011). The role of various transcription factors in regulating these SAGs has also been studied. These transcription factors get activated at different time points and cause age-dependent expression of these SAGs. Most of these transcription factors can be divided into two families NAC and WRKY. These transcription factors get activated by various stress responses, thus proving that senescence is a cumulative response of various environmental cues and developmental signals.

WRKY transcription factor family has shown its potential in regulating leaf senescence. WRKY53 targets various SAGs (e.g., pathogen-related genes, stress-related genes, and transcription factor genes) and exerts its positive effect on leaf senescence (Miao et al. 2004). When WRKY53 binds with EPITHIOSPECIFYING SENESCENCE REGULATOR (ESR), its DNA-binding activity gets inhibited (Fig. 13.3a) (Miao and Zentgraf 2007). The transcription factors WRKY54 and WRKY70 also have weak roles in leaf senescence, when expressed alone (as *wrky54* and *wrky70*), but the double mutant of *wrky54 wrky70* showed clearly early-senescence (Besseau et al. 2012). This indicates that there might be partly redundant functions of WRKY54 and WRKY70 in regulating leaf senescence. But both have them have a clear cooperative function in the event. Analyses of the putative promoter regions of WRKY genes revealed several cis-element-like sequences for both senescence and stress signaling pathways (Rinerson et al. 2015), Thus, showing involvement of WRKY genes in both type of pathways.

Scarpeci et al. (2013) have shown the negative senescence regulators WRKY54 and WRKY70 and the positive senescence factor WRKY53 interact with the transcription factor WRKY30, whose involvement in ROS signaling has been suggested earlier. Therefore, it can be postulated that the negative senescence regulators exert their effect with the positive senescence factor. The positive regulation of leaf senescence is further substantiated by the finding of one of its downstream target WRKY22, which is found to be a positive regulator of dark-induced leaf senescence (Zhou et al. 2011). Overexpression of *WRKY22* induces leaf senescence, and *wrky22*-knockout plants show delayed leaf senescence.

The knockout mutants and overexpressing transgenic plants have shown the changes in leaf phenotype of the senescing plants. Researchers have unraveled the upstream regulators and downstream targets of these transcription factors which has further thrown light on the signaling networks controlling leaf senescence

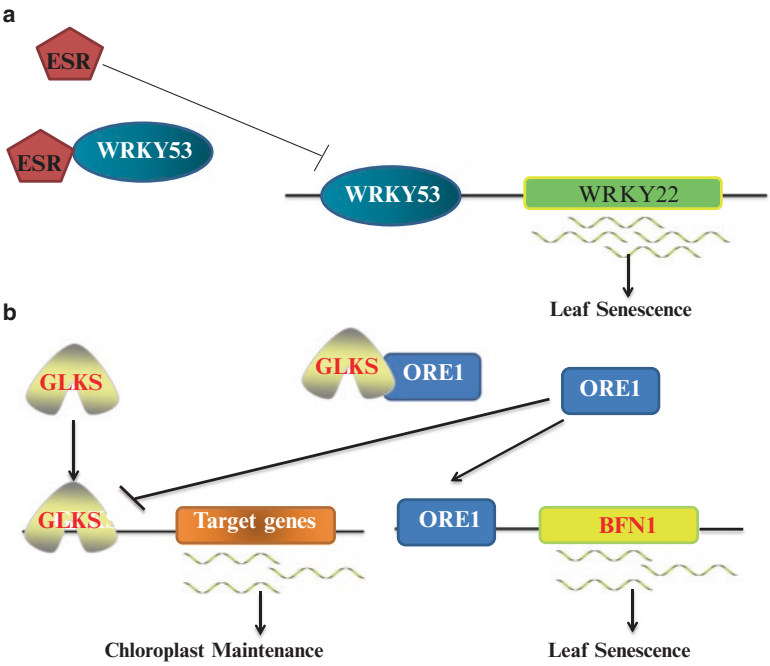

Fig. 13.3 Transcriptional regulation of leaf senescence by (**a**) WRKY53 and (**b**) ORE1, the well-known positive regulators of senescence. As a transcription factor, WRKY53 regulates the expression of many SAGs and stress-related genes (transcriptional regulation). It is also known that WRKY53 is inactivated by interacting with ESR. ORE1 controls the expression of many SAGs including BFN1 (transcriptional regulation). ORE1 also modulates the activity of GLKs through protein–protein interactions

(Guo and Gan 2006; Zhang and Gan 2012; Hickman et al. 2013). One of such transcription factor is NAC-LIKE transcription factor, which is ACTIVATED BY AP3/PI (AtNAP/ANAC029). It is an important senescence-regulating NAC transcription factor in *Arabidopsis*.

Overexpression of *NAP* in young leaves induces precocious senescence while knockout mutants of *NAP* cause delayed leaf senescence (Guo and Gan 2006). NAP binds directly to the promoter of *SAG113*. It is a negative regulator of the abscisic acid (ABA) pathway which causes inhibition of stomatal closure, ultimately leading to leaf senescence (Zhang and Gan 2012).

Another NAC transcription factor is ORESARA1 (ORE1, ANAC092). It is a positive regulator of leaf senescence in *Arabidopsis* (Kim et al. 2009; Balazadeh et al. 2010). During leaf aging, *ORE1* expression increases by ETHYLENE INSENSITIVE 2 (EIN2). It is negatively regulated by the microRNA164 (miR164). ORE1 controls the expression of 170 genes. Out of these, 78 known SAGs (Balazadeh et al. 2010). A direct target of ORE1 is BIFUNCTIONAL NUCLEASE1 (BFN1) (Matallana-Ramirez et al. 2013), suggesting that ORE1 might regulate the

nucleic acid degradation by activating the expression of *BFN1* during leaf senescence (Fig. 13.3b). The proteins which are being regulated by ORE1 have exhibited a new regulatory mechanism controlled by ORE1 (Rauf et al. 2013). ORE1 interacts with the G2-like transcription factors GLK1 and GLK2. These are important transcription factors for the development and maintenance of chloroplast. When the leaves are young, there is maximum expression of GLKs, which induces various target genes (e.g., photosynthesis-related genes). When the leaves get older, the expression of ORE1 is increased which causes reduced expression of GLK and the transcription activity.

Another NAC transcription factor is JUNGBRUNNEN1 (JUB1, ANAC042). It is activated by H_2O_2 NAC and induces the expression of *DREB2A* and other ROS-responsive genes (Wu et al. 2012). Its overexpression causes delayed leaf senescence in *Arabidopsis. These* plants also show tolerance to various abiotic stresses. Whereas, its knockout plants show fast senescence and have reduced abiotic stress tolerance. Thus showing JUB1 as a negative regulator of leaf senescence, possibly by lowering cellular H_2O_2 levels.

In various crop plants, the NAC transcription factors are shown to be as central regulators of leaf senescence. A NAC transcription factor NAM-B1 has shown to promote senescence by facilitating nutrient remobilization from leaves to grains in ancestral wild wheat (*Triticum turgidum* L. ssp. *dicoccoides*). Due to this nutrient remobilization, the protein, zinc, and iron content of the grain has shown to be improved (Uauy et al. 2006; Waters et al. 2009). Overexpression of two NAC transcription factors from *Brassica napus* (NAC2 and NAC5) (Zhong et al. 2012) and a NAC transcription factor from bamboo (NAC1) (Chen et al. 2012) in *Arabidopsis* has shown to regulate leaf senescence.

Other transcription factors involved in the regulation of leaf senescence are ABI3/VP1 (RAV), *Arabidopsis* R-R-type MYB-like transcription factor, C-REPEAT/DEHYDRATION RESPONSIVE ELEMENT BINDING FACTOR 2 (CBF2), etc. RAV1 has been found to be a positive regulator of leaf senescence (Woo et al. 2010). Another positive regulator of leaf senescence is *MYBL*. Its overexpression causes acceleration in leaf senescence (Zhang et al. 2011). Whereas, CBF2 overexpression delays leaf senescence, suggesting it to be a negative regulator of leaf senescence (Sharabi-Schwager et al. 2010). Other transcription factors involved in hormone signaling have also exhibited their roles in regulating leaf senescence. The AUXIN RESPONSE FACTOR 2 (ARF2) shows an important role in regulating auxin-mediated leaf senescence (Lim et al. 2010), and SIGNAL RESPONSIVE 1 (SR1) shows regulation of ethylene-induced senescence (Nie et al. 2012). The results suggested that ethylene receptors negatively regulate ethylene-controlled PCD in the lace plant. Rantong et al. (2015) showed the ethylene receptors AmERS1a and AmERS1c regulate ethylene-induced programmed cell death during leaf morphogenesis of the lace plant *Aponogeton madagascariensis*. Ethylene is a well-known senescing hormone, and now Jasmonic acid (JA) is also emerging as a key player in regulating plant senescence (Kim et al. 2015).

Another novel Arabidopsis transcription factor is Arabidopsis thaliana activating factor1 (ATAF1). It is activated by abscisic acid (ABA) and hydrogen peroxide and

acts as a novel upstream regulator of senescence. ATAF1 directly binds to the promoters of the two TFs GOLDEN2-LIKE1 (GLK1) and ORESARA1 (Arabidopsis NAC092) which are key players for chloroplast maintenance and thus promotes senescence. ATAF1 activates ORESARA1 and represses GLK1 expression, thus changing the physiology towards the progression of senescence. ATAF1 direct effects the ABA homeostasis genes and thus involved in ABA- and hydrogen peroxide-induced senescence. Thus, ATAF1 couples stress-related signaling with senescence-related signaling (Garapati et al. 2015).

A number of gene regulatory networks are shown to be involved in leaf senescence. A network model developed by Breeze et al. (2011) on the basis of temporal expression profiling of SAGs during *Arabidopsis* leaf senescence predicts the effects of ORE1 on the expression of its various downstream targets and on several stress-related transcription factors. Hickman et al. (2013) have used high-throughput yeast one-hybrid assays and time course gene expression data to predict a gene regulatory network model that involves ANAC019, ANAC055, and ANAC072. More information can be obtained through co-regulated pathways and promoter motif analysis of genes involved in leaf senescence and stress responses. Such exercises are helpful for identification of potential upstream regulatory genes and new downstream genes. Other approaches like combine chromatin immunoprecipitation sequencing (ChIP-seq), gene expression profiling, and computational analyses will further be helpful in the elucidation of complex gene networks regulating the senescence-associated signaling pathways and their further interlinking with each other.

Regulation of the chromatin structure through histone modification and chromatin-remodeling enzymes are important in regulating leaf senescence in eukaryotes. A recent study through ChIP-seq and gene expression analysis by Brusslan et al. (2012, 2015) revealed that in comparison to young leaves, the old leaves show upregulation of genes with increased levels of histone H3 trimethyl lysine 4 (H3K4me3), whereas downregulation of genes shows decreased levels of this histone during leaf senescence. H3K4me3 is a proved marker of actively transcribed chromatin. An inactive histone marker histone H3 trimethyl lysine 27 (H3K27me3), its found that genes that lose this marker in old leaves are upregulated during leaf senescence. Thus, it is proved that histone methylation plays important role in the regulation of gene expression during leaf senescence.

Histone methylation is linked with the regulation of leaf senescence, and this association is shown by histone methyltransferase SU(VAR)3-9 HOMOLOG 2 (SUVH2) (Ay et al. 2009). Overexpression of *SUVH2* inhibits the transcripts of *WRKY53* and its targets, causing delayed leaf senescence. HISTONE DEACETYLASE 3 (HDA6) is a well-known factor regulating leaf senescence (Wu et al. 2008). It affects leaf senescence and flowering time and the response of plant for jasmonic acid (JA) treatment. In the absence of HDA6, leaf senescence is delayed as shown by its loss-of-function mutant. HDA6 is also involved in the downregulation of the expression of several SAGs. ORE7 (ESCAROLA) represents another example for leaf senescence regulation at the chromatin level (Lim et al. 2007a, b). Overexpression of this gene causes delayed leaf senescence.

Studies have proved that dynamic chromatin modifications are essential during aging as they cause alteration in gene expression patterns, thus controlling leaf senescence. Chromatin-mediated gene regulation is an evolutionarily conserved mechanism in plants and animals for controlling aging as well as senescence.

Noncoding RNAs (ncRNAs), such as small-interfering RNAs (siRNAs) and miRNAs, play important roles in the posttranscriptional modification of mRNAs. The importance of miRNAs in controlling cellular senescence and aging in animals is very well studied. Lin-4 (the first miRNA to be identified) has shown to regulate the life span of the nematode *Caenorhabditus elegans* (Boehm and Slack 2005). Other studies by de Lencastre et al. (2010) and Grillari et al. (2010) have shown miR-71 and miR-17-92 as regulators of cellular senescence and aging in *C. elegans* and mammals, respectively.

Research on the role of small ncRNAs, including miRNA and trans-acting siRNA (tasiRNA), in regulating senescence in plants is emerging. Kim et al. (2009) have described the role of miR164 in negative regulation of its target ORE1 which is a positive regulator of leaf senescence. When the leaf ages, the miR164 expression gradually decreases, through the activation of EIN2, and this in turn upregulates the *ORE1* expression. All these three factors *EIN2*, *ORE1*, and miR164 work as a part of a trifurcate feed-forward pathway for age-dependent leaf senescence. The TCP (TEOSINTE BRANCHED/CYCLOIDEA/PCF) transcription factors which are the coordinators of leaf growth and senescence also play their role in this trifurcate feed-forward pathway. *TCP4* overexpressing plants exhibit premature leaf senescence (Schommer et al. 2008), *TCP4* is a target of *miR319*, *and* miR319-overexpressing plants show delayed leaf senescence (Schommer et al. 2008). All these reports show miRNAs as important leaf senescence regulators in plants. miR164 is involved in another pathway promoting leaf senescence. It involves EIN3, miR164, and a NAC (NAM, ATAF, and CUC) transcription factor ORE1/NAC2. Ethylene insensitive 3 (EIN3) is another key transcription factor in the ethylene signaling. They are involved in regulating chlorophyll degradation by affecting chlorophyll catabolic genes (CCGs). It was studied that in *ein3 eil1* double mutant that ethylene cannot have the same induction of three major CCGs, NYE1, NYC1, and PAO. Further, EIN3 directly binds to the promoters of NYE1, NYC1, and PAO in Arabidopsis protoplasts and causes induction of their promoter activity. And thus acts as a positive regulator of CCG expression during ethylene-mediated chlorophyll degradation. ORE1 is a downstream target of EIN3 and also activates the expression of NYE1, NYC1, and PAO by directly binding to their promoters (Fig. 13.4). ORE1 also promotes ethylene production by activating the expression of ACS2, a major ethylene biosynthesis gene (Fig. 13.4). All this shows that EIN3, ORE1, and CCGs are a part of a feed-forward loop that causes regulation of ethylene-mediated chlorophyll degradation during leaf senescence in Arabidopsis (Qiu et al. 2015).

TasiRNAs also regulate leaf senescence posttranscriptionally (Fig. 13.5). These are plant-specific endogenous small RNAs. miR390 stimulates the synthesis of the tasiRNAs generated from *TAS3*. They work through their targets. One of their targets is *ARF2* which is a positive regulator of leaf senescence (Lim et al. 2010; Marin et al. 2010). Thus, in this way miR390 promotes leaf senescence by modulating *ARF2*.

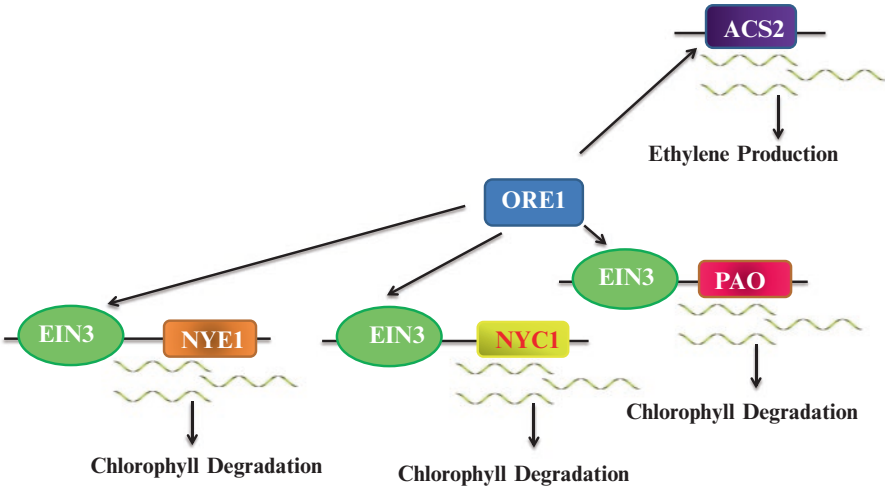

Fig. 13.4 Regulation of chlorophyll degradation by Ethylene insensitive 3 (EIN3) by affecting chlorophyll catabolic genes (CCGs); NYE1, NYC1, and PAO. EIN3 directly binds to the promoters of NYE1, NYC1, and PAO in Arabidopsis protoplasts and causes induction of their promoter activity during ethylene-mediated chlorophyll degradation. ORE1 is a downstream target of EIN3 and also activates the expression of NYE1, NYC1, and PAO by directly binding to their promoters. ORE1 also promotes ethylene production by activating the expression of ACS2, a major ethylene biosynthesis gene

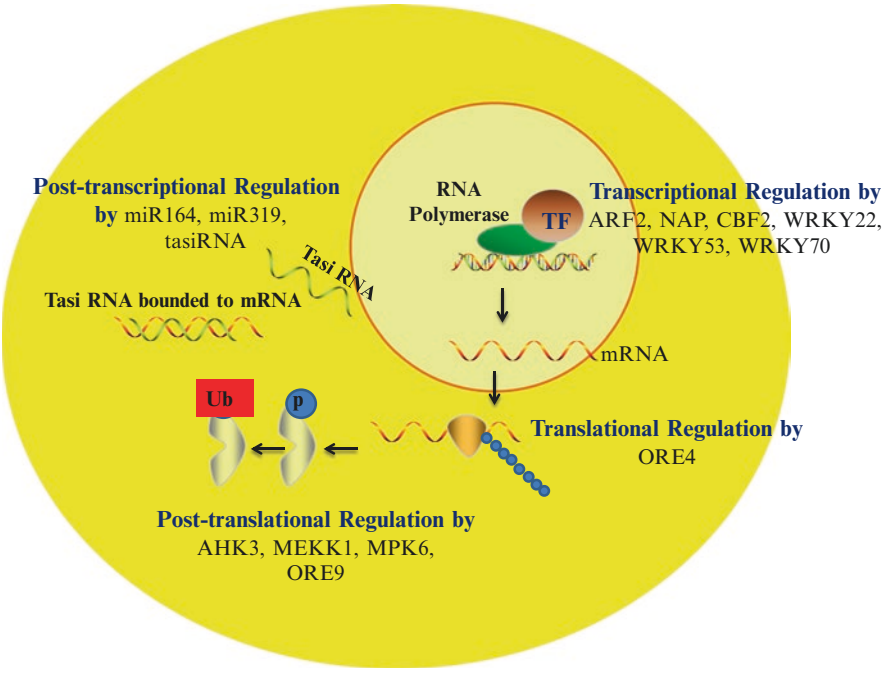

Fig. 13.5 Regulation of leaf senescence at transcriptional, posttranscriptional, translational, and posttranslational level. Expression of senescence-associated genes (SAGs) due to activation and/or suppression of diverse transcription factors (TFs). Posttranscriptional regulation through tasiRNAs and miRNAs (not shown in figure). Posttranslational modifications by phosphorylation (P) and ubiquitylation (Ub)

These studies indicate that posttranscriptional regulation has a much diversified role in the biological processes and the regulation of leaf senescence involves multifarious posttranscriptional mechanisms. Not much is known about the translational regulation of leaf senescence. It will be interesting to throw light on this important aspect, and few studies are discussed here. Woo et al. (2002) studied the *Arabidopsis* plants having mutation in the *PLASTID RIBOSOMAL SMALL SUBUNIT PROTEIN 17* (*PRPS17*) gene (in *ore4* plants), and they showed delayed leaf senescence. The reduced expression of PRPS17 causes reduction in the translation rate of the chloroplast, and hence reduced senescence. Studies on *C. elegans* and *Drosophila* have also revealed relationship between the protein synthesis and aging. Further, increased life span has been observed when there is reduction in mRNA translation (Arquier et al. 2005; Hansen et al. 2007; Syntichaki et al. 2007).

Suzuki and Makino (2013) have observed that during leaf senescence in rice there is a translational control of the synthesis of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) subunits. In higher plants, Rubisco is encoded by RBCS family of nuclear genes as well as the RBCL plastid genes (Dean et al. 1989). There is a need of highly coordinated regulation of the expression of these two Rubisco components, as they are encoded in different gene compartments and later assembled to form the holoenzyme. As aging progresses in a leaf, the mRNA levels of RBCS nuclear genes gradually decrease but it has been observed that mRNA levels of RBCLs plastid genes are lower than that in RBCS. The translation rate of the two mRNA species substantiates the above difference, thus suggesting a mechanism of expression of Rubisco subunits at the translational level.

The posttranslational regulation of control of leaf senescence can be at two stages, protein phosphorylation and protein ubiquitylation (Fig. 13.5). Expression of numerous protein kinases and phosphatases has shown to be up- or downregulated during plant aging (Breeze et al. 2011). Various kinases and phosphatases have shown their role in modulating leaf senescence. For example, MAP KINASE KINASE 9 (MKK9) and MAP KINASE 6 (MPK6) from the MAP kinase family. The expression of *MKK9* is upregulated during leaf senescence in *Arabidopsis* (Zhou et al. 2009). The loss-of-function mutant of this gene shows delayed leaf senescence, and its overexpression induces leaf senescence. The *mpk6* loss-of-function mutant also exhibits delayed leaf senescence. Another important role of *MPK6* is shown by the loss-of-function mutants of *MPK6* which partially suppresses the effect produced by the overexpression of *MKK9*. It indicates that MKK9 functions upstream of MPK6 in regulating leaf senescence. Another member of MAPK pathway is the transcription factor WRKY53. Its phosphorylation by MEKK1 increases its DNA-binding capacity for its target promoters (Miao et al. 2007). The roles of the MAPK cascade involving MEKK1–MKK1/2–MPK4 in biotic stress signaling have been earlier shown (Pitzschke et al. 2009). It seems, MEKK1 coordinates the biotic stress response by inducing senescence taking the help of WRKY53.

Arabidopsis histidine kinase 3 (AHK3) is another example showing the role of protein phosphorylation in the regulation of leaf senescence. It is a cytokinin receptor having histidine kinase activity. Cytokinins are well-known senescence-retarding

hormones, but the molecular mechanism of cytokinin-mediated retardation in plant senescence is still unclear. Some light have been thrown by a gain-of-function mutant *ore12-1* of *AHK3*. This mutant showed delay in leaf senescence as the transgenic overexpression of *AHK3* (Kim et al. 2006). Further investigation shows AHK3 phosphorylates ARR2, which is a type-B *Arabidopsis* response regulator, in the presence of cytokinin (Kim et al. 2006). Overexpression of *ARR2* causes delayed leaf senescence. But, when the AHK3 phosphorylation site is mutated, the overexpression fails to delay the leaf senescence. Suggesting the role of phosphorylation by AHK3 in controlling *Arabidopsis* leaf senescence.

Role of receptor-like protein kinases was also identified in regulating leaf senescence. The probable mechanism can be through plant hormone signaling (Lee et al. 2011). RECEPTOR PROTEIN KINASE 1 (RPK1) is a membrane-bound receptor kinase and has shown its role in ABA-mediated and age-dependent leaf senescence. Loss-of-function mutations in *RPK1* cause delay in ABA-induced and age-dependent leaf senescence (Lee et al. 2011). Senescence-associated receptor-like kinase from Soybean (GmSARK) and *Arabidopsis* (AtSARK) are positive regulators of leaf senescence (Xu et al. 2011). The overexpression of GmSARK in *Arabidopsis* shows altered responses for ethylene, auxin, and cytokinin. The overexpression of its *Arabidopsis* homolog AtSARK causes premature leaf senescence. This can be reverted back by the inhibition of auxin transport or through ethylene signaling. The mechanism of action of *SARK* in regulating leaf senescence can be through hormonal responses or by affecting phosphorylation of their target proteins.

UBP1-associated protein 2a (UBA2a), UBA2b, and UBA2c are three RNA-binding proteins (RBPs) from *Arabidopsis thaliana*. They are homologs of *Vicia faba* abscisic-acid-activated protein kinase-interacting protein 1 (VfAKIP1) and contain two RNA-recognition motif (RRM) domains each. They play important roles in wounding response and leaf senescence. Potato (Solanum tuberosum) also has seven AKIP1-like RBPs. StUBA2a/b and StUBA2c are the homologs of VfAKIP1 and Arabidopsis UBA2s. The constitutive expression of StUBA2a/b induces early leaf senescence in Arabidopsis. Through domain deletion studies of StUBA2s, it was revealed that the first RRM domain is crucial for its effect on Arabidopsis senescence. There is increased in the expression of pathogen-related genes (PR) and a senescence-associated gene (SAG13) (Na et al. 2015). The correlation between PR proteins and senescence is still unclear. There is one hypothesis that senescing leaves may be relatively prone to the attack of pathogens, and these PR protein might protect the plants against infection during senescence.

Another important regulation of leaf senescence is at the stage of ubiquitylation. Various ubiquitin-dependent degradation pathways are shown to be associated with leaf senescence. The E3 Ub-ligases cause poly-ubiquitylation of proteins in plants. These proteins then selectively degraded by the 26S proteasomes.

The *Arabidopsis ore9* mutant causes delayed leaf senescence (Oh et al. 1997; Woo et al. 2001). The ORE9 or MORE AXILLARY GROWTH 2 (MAX2) is also involved in photomorphogenesis (Shen et al. 2007), branching (Stirnberg et al. 2002), and signaling by strigolactone (Gomez-Roldan et al. 2008; Umehara et al. 2008) and karrikin (Nelson et al. 2011). ORE9 is found to be an F-box protein

which forms an SCF (S-phase kinase-associated protein 1 (SKP1)/Cullin/F-box protein) E3 ligase complex SCF^{ORE9}. This complex might target negative regulators of leaf senescence.

Another ligase involved in leaf senescence is HECT domain E3 ubiquitin-protein ligase UPL5. Overexpression of *UPL5* causes decreased expression of WRKY53, suggesting that UPL5 causes WRKY53 degradation through its ubiquitin-ligase activity (Miao and Zentgraf 2010). Further, the phenotype of *UPL5*-knockout plants and plants overexpressing WRKY53 is similar. Further supporting that UPL5 regulates leaf senescence by degrading WRKY53. Senescence-associated ubiquitin ligase 1 (SAUL1) is another ubiquitin-ligase involved in leaf senescence (Raab et al. 2009). It is a plant U-box-armadillo E3 ubiquitin-ligase and a negative regulator of plant senescence. The *saul1* loss-of-function mutants display early senescence under low-light conditions. The expression of key senescence regulators like *ORE1*, *WRKY53*, and *WRKY6* is also found to be altered in *saul1* mutants (Vogelmann et al. 2012). These studies suggest the importance of protein ubiquitylation in regulating leaf senescence.

Conclusions and Future Perspectives

Through leaves, we can easily understand the genetic processes associated with aging, senescence, and death of a plant. Leaf senescence is controlled by highly regulated mechanisms at transcription, translation, posttranslation, and ubiquitylation. We have yet to explore a lot to understand the molecular network of leaf senescence. Various genes and multiple signaling pathways are involved during the course of leaf senescence. In addition to these endogenous signals, various environmental cues also affect the onset, progression, and culmination of leaf senescence. Senescence is a continuous time-dependent dynamic process which involves transitions in the cellular physiology and metabolism of a plant. So, it has to be understood in a completely different perspective. Senescence involves interaction at cell to cell and organ to organ level. For fully understanding this complex process of leaf senescence multiple "omics" approaches can be utilized. For example, total RNA transcriptome, proteome, metabolome, and phenome should be studied at various time points. These approaches substantiated with computational modeling will be helpful in understanding leaf senescence and death. Other approaches can be protein and genetic interactions or protein localization maps. Senescence is mostly studied at the later and final stages, but it has to be studied as a part of plant lifecycle and is linked to all the previous stages of plants life. The leaf senescence should be studied not an individual component but as transitions of molecular networks, of network modules and their interactions, because the cellular processes are controlled by molecular networks consisting of complex interactions between DNA, RNA, proteins, and metabolites. Other aspects to be studied in association

with senescence are the coordinated regulation of overall developmental and physiological states of the entire plant with the senescing leaf. Further, how the leaf senescence is associated with plant productivity and the other aspects of nutrient mobilization is yet to be explored.

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Chapter 14
Role of Phytohormones and miRNAs in Nitrogen and Sulphur Deficiency Stress Signaling in Plants

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Abstract Nitrogen (N) and sulphur (S) are essential elements for plant development as they are key components of other cellular constituents such as nucleic acids, proteins, chlorophyll, and phytohormones. In the last few years, small regulatory RNAs have been investigated for their roles in post-transcriptional or translational gene regulation. MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are the two major groups of small RNAs in plants. A wide range of physiological processes are regulated by Plant miRNAs, such as flowering time, leaf morphogenesis, nodule development, and adaption to abiotic stresses. In the present chapter, roles of different miRNAs and hormones involved in different pathways of plant development and growth are described under the limitation of nitrogen and sulphur nutrition.

Nitrogen and Sulphur Nutrition in Plants

Nitrogen (N) is an essential element for plant development as it is a key component of other cellular constituents such as nucleic acids, proteins, chlorophyll, and phytohormones (Hawkesford et al. 2012). Two main phases of N uptake and usage during life cycle of plants have been well-known. The first phase is during the vegetative stage where N is taken up, stored, and assimilated into amino acids or other nitrogenous compounds. The second phase is the remobilization of assimilated N at senescence, where these compounds will be released and remobilized to reproductive organs to support the developing seeds (Kant et al. 2011). Plants take up N mainly in the forms of nitrate (NO_3^-), ammonium (NH_4^+), or urea from the soil; however, NO_3^- is a preferred form for most arable plants (Crawford and Forde 2002). Synergistic association of legumes, actinorhizal plants and several C4 grasses with symbiotic microorganisms can produce NH_4^+ that fix atmospheric N_2 via bacterial enzyme nitrogenase (Andrews et al. 2013). The N uptake process from the

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soil can be conducted either directly by roots or indirectly via mycorrhizal fungi (Andrews et al. 2013).

While NO_3^- enter plant cells by facilitation of nitrate transporters, NH_4^+ from external source is taken up by ammonium transporters (AMT) (Crawford and Forde 2002). Upon entering plant cells, NO_3^- is converted to nitrite, then to NH_4^+ and finally to amino acids through the action of nitrate reductase, nitrite reductase, and glutamine synthase (Crawford and Forde 2002). During reproductive growth, there are three pathways to release the nitrogenous compounds: the chloroplast degradation pathway; the vacuolar and autophagic pathway; and the ubiquintin-26S proteasome pathway (Liu et al. 2008). After degradation, the released amino acids will be loaded into phloem and remobilized to the developing seeds by facilitation of amino acid transporters completing the plant N cycle (Kant et al. 2011).

Two types of N uptake systems are well-defined in plants, i.e., high affinity transport system (HATS) and low affinity transport system (LATS), which functions at low external N concentrations (1 μm–1 mM) and high external N concentrations (>1 mM), respectively (Kraiser et al. 2011). Studies on the molecular mechanism of N uptake and translocation revealed the involvement of a number of genes for these processes (Masclaux Daubresse et al. 2010; Kant et al. 2011; Xu et al. 2012; Krapp et al. 2014). Four members of nitrate transporter families involved in the NO_3^- uptake process are described in *Arabidopsis thaliana,* i.e., nitrate transporter 1/peptide (NPD), nitrate transporter 2 (NRT2), the chloride channel (CLC), and slow anion channel-associated homologs (SLAC/SLAH) (Krapp et al. 2014). NPD is a recent nomenclature of nitrate transporter 1 (NRT1) family recently proposed by Léran et al. (2014) since NRT1 transporters have been reported to transport NO_3^- and other substrates such as auxin, ABA, and glucosinolates. However, here the original gene names as these were initially named are referred.

Sulphur (S) is one of the six macroelements that are essential for proper plant growth and development. It forms part of amino acids and proteins. It plays an important role in redox control of cellular processes (reviewed in Foyer and Noctor 2009) and in plant defence mechanisms (reviewed in Rausch and Wachter 2005; Noctor 2006). Sulphur is mainly taken by plant in the form of sulphate from the soil. within the plant, sulphate either remains in the roots or is transported via the xylem to the leaves, the main sites of sulphate reduction and assimilation, or remains in the roots (Rennenberg 1984; Brunold 1990) via the phloem part of the sulphate from the leaves are remobilized back to the roots (Hartmann et al. 2000; Herschbach and Rennenberg 2001). Furthermore, main storage for the sulphate is vacuoles (Leustek and Saito 1999) and it can be mobilized from these storage compartments if required (Bell et al. 1994, 1995). All these processes within the plant are controlled by sulphate transporters (SULTRs) for sulphate distribution (Buchner et al. 2004a, b; Hawkesford and De Kok 2006).

Sulphur depletion has been known to induce enhanced sulphate uptake and assimilation capacity, which are regulated strongly at the transcriptional level (Bolchi et al. 1999; Lee and Leustek 1999; Vidmar et al. 1999; Takahashi et al. 2000; Nikiforova et al. 2003; Buchner et al. 2004a, b; Nocito et al. 2006; Parmar et al. 2007). This response results in enhanced expression of enzymes involved in

sulphate assimilation such as ATP sulphurylase (ATPS) and adenosine 5-phosphosulphate (APS) reductase (APR) and several sulphate transporters (Bolchi et al. 1999; Vidmar et al. 1999; Takahashi et al. 2000; Hirai et al. 2003; Nikiforova et al. 2003; Buchner et al. 2004a; Kataoka et al. 2004; Maruyama-Nakashita et al. 2004a, 2006; Parmar et al. 2007).

The expression of different ATPS isoforms was regulated differently upon S depletion. Expression of the *Arabidopsis* ATPS, AtAPS3, increased during S depletion (Liang et al. 2010; Kawashima et al. 2011), while results for AtAPS2, the putatively cytosolic isoform (Hatzfeld et al. 2000; Rotte and Leustek 2000), were inconsistent between different studies (Logan et al. 1996; Takahashi et al. 1997; Kawashima et al. 2011). AtAPS1, AtAPS3, and AtAPS4 were shown to be post-transcriptionally regulated by microRNA395 (miR395), and the expression of miR395 was shown to be induced upon S depletion (Jones Rhoades and Bartel 2004; Kawashima et al. 2009, 2011; Liang et al. 2010). Beside these changes in gene expression, S depletion is also accompanied by changes in metabolite concentrations such as decreasing levels of glutathione, which were shown to repress sulphate uptake and assimilation (Rennenberg et al. 1988, 1989; Herschbach and Rennenberg 1991, 1994; Bolchi et al. 1999; Lappartient et al. 1999; Vidmar et al. 1999, 2000; Vauclare et al. 2002; Hartmann et al. 2004).

Sulphur starvation activates mechanism for increasing acquisition from soil in plants. Reduction in sulfur content results in the retardation of sulfate assimilation; elevated amounts of serine; decreased glutathione and cysteine, *O*-acetylserine, and tryptophan (Nikiforova et al. 2003); reduced amounts of chlorophyll, RNA, and total protein; raised photorespiration; decreased lipids; and nitrogen imbalance. However, the deduction in sulfate uptake leads to reduced assimilation activity when plants cannot acquire ample sulfate (Hirai et al. 2003, 2004; Takahashi et al. 1997, 2000) and affects many different metabolic processes. Gradually, the decreased plant tissue sulfur content arises from finite supplies of sulfur in plants (Blake-Kalff et al. 1998; Kutz et al. 2002; Lencioni et al. 1997; Nikiforova et al. 2003; Prosser et al. 2001). Overall, these changes result in reduction in rate of metabolism and growth (Nikiforova 2005).

From past few years, small regulatory RNAs have gathered attention because of their main roles in post-transcriptional or translational gene regulation (Baldwin et al. 2001; Bari et al. 2006; Blake-Kalff et al. 1998; Bloom and Finazzo 1986; Burleigh and Harrison 1999). The two major groups of small RNAs in plants mainly serve as negative regulators of gene expressionare microRNAs (miRNAs) and small interfering RNAs (siRNAs). miRNAs are processed from hairpin precursors by the ribonuclease III-like enzyme Dicer Dicer-like1 (DCL1) or DCL4. Unlike miRNAs, siRNAs are generated from perfectly complementary, long, double-stranded RNAs by RNA-dependent RNA polymerase 6 (RDR6)/RDR2.

In the present chapter, roles of different miRNAs and hormones involved in different pathways of plant development and growth are described under nitrogen and sulphur deficiencies.

Phytohormone in N Stress

It is already known that there is an interaction between N signaling and phytohormone activity. Recently, genetic studies have given ample evidence that ABA, auxin, and CKs are involved in nitrogen signaling.

Auxin

In *Arabidopsis*, miR167 target two Auxin Response Factors (ARF transcription factors), *ARF6* and *ARF8,* which regulate development of reproductive organs (Wu et al. 2006). Further, it was shown that lateral roots are regulated by *ARF8*, where its expression was induced in lateral root cap cells and in pericycle under N limiting conditions (Gifford et al. 2008). In case of induction or suppression of gene expression in response to the plant phytohormone auxin, ARF proteins bind to auxin-responsive *cis*-acting promoter elements (Hagen and Guilfoyle 2002; Liscum and Reed 2002). It is reported that miR167 targets *IAA-Ala Resistant3* (*IAR3*), whose protein hydrolyses the inactive auxin derivative indole-3-acetic acid alanine and releases bioactive auxin, which lead to root development during high osmotic stress (Kinoshita et al. 2012). Over-expression of miR167 resulted in plant morphologies identical to *arf6* and *arf8* mutant phenotypes (Wu et al. 2006). Therefore, lower expression of miR167 might lift its inhibition on auxin transcription factors under N deficiency which could in turn ignite lateral root growth (Liang et al. 2012).

In *Arabidopsis*, *ARF16* play role in modulation of root cap cell formation, while *ARF17* act as a regulator of *GH3*-like early auxin response genes (Mallory et al. 2005; Wang et al. 2005). In contrary, *ARF16* and *ARF17* were down-regulated, while miR160 was induced under N limitation (Liang et al. 2012). Studies have shown that miR160-over-expressed transgenic plants had more developed lateral roots which signify that induced expression of miR160 via mediation of *ARF16*and *ARF17* under N deficiency might promote lateral root growth (Liang et al. 2012). Further study showed that spatial regulatory complex of three miRNAs, miR160, miR167, and miR171, coordinated modulation of the root system under N starvation (Liang et al. 2012). Root growth was elevated by enhanced expression of miR160 and miR171 and reduced expression of miR167 under N deficiency.

The *NAC* gene family encoding transcription factors plays multiple roles in the developmental processes of plants. There are three gene families under *NAC*s; *NAM* (No Apical Meristem), *ATAF* (*Arabidopsis* Transcription Activation Factor), and *CUC* (CUP-shaped Cotyledon) (Olsen et al. 2005). A *NAC* locus has been reported to hasten senescence and enhance nutrient remobilization from leaves to the developing grains in wheat (Uauy et al. 2006). Under N limiting conditions, miR164 is up-regulated in maize leaf (Xu et al. 2011). This could conclude the role of miR164 in modulating both root and shoot development under N limitation adaptation (Fig. 14.1).

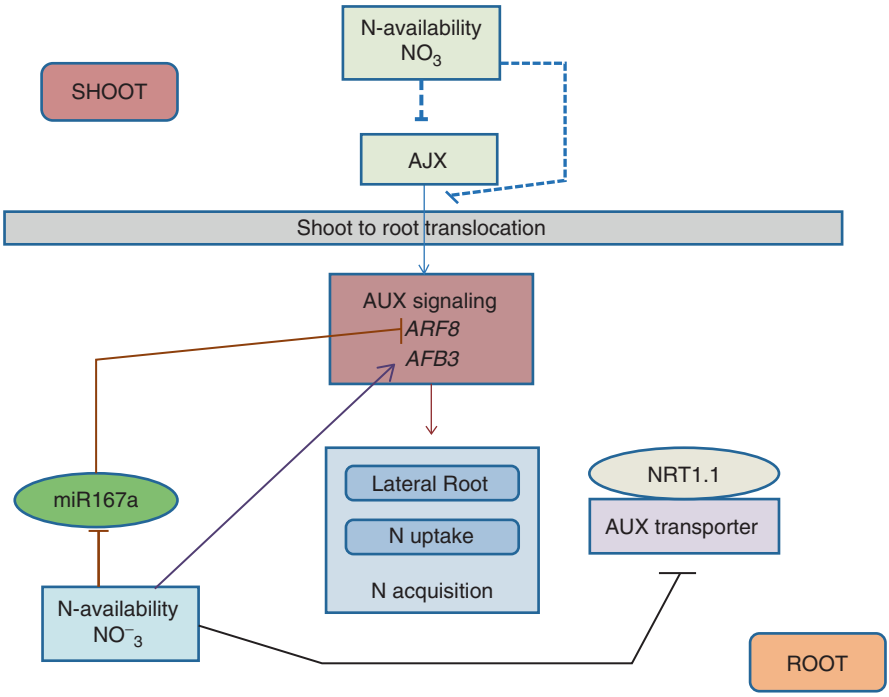

Fig. 14.1 Schematic representation of the interaction between nitrogen and phytohormone auxin. *Arrows* represent positive interactions and *blunted lines* represent inhibitory interaction. *Solid lines* represent defined interactions; *dashed lines* indicate presumed interactions

Cytokinin

Cytokinins (CK) are a class of phytohormones that play essential role in plant growth and development (Mok and Mok 2001; Sakakibara et al. 2006), including nitrogen signaling. Nitrogen supplementation causes an enhancement in CK content in xylem sap (also in roots and shoots) of maize (Takei et al. 2001), which indicates that CKs function as a root-to-shoot long-distance signal of nitrogen supplement (Sakakibara et al. 2006). A similar correlation has been reported in *Arabidopsis* (Takei et al. 2004). Studies in barley (Samuelson and Larsson 1993), tobacco (Singh et al. 1992), and *Urtica dioica* (Wagner and Beck 1993) indicated that the CK–nitrogen link the nitrogen supply. Moreover, *Arabidopsis* seedlings grown on high concentrations of nitrate (10 mM) contained higher levels of CKs than those grown on low nitrate which showed that CKs were not just a nitrogen supplement signal but were also involved as a nitrogen status signal. Recent evidence indicates that CKs also act as a local signal or as a shoot-to-root long-distance signal (Miyawaki et al. 2004; Matsumoto Kitano et al. 2008).

CKs translocated from the shoot or produced locally within roots may signal for the presence of ample nitrogen. Microarray analyses have shown that exogenous

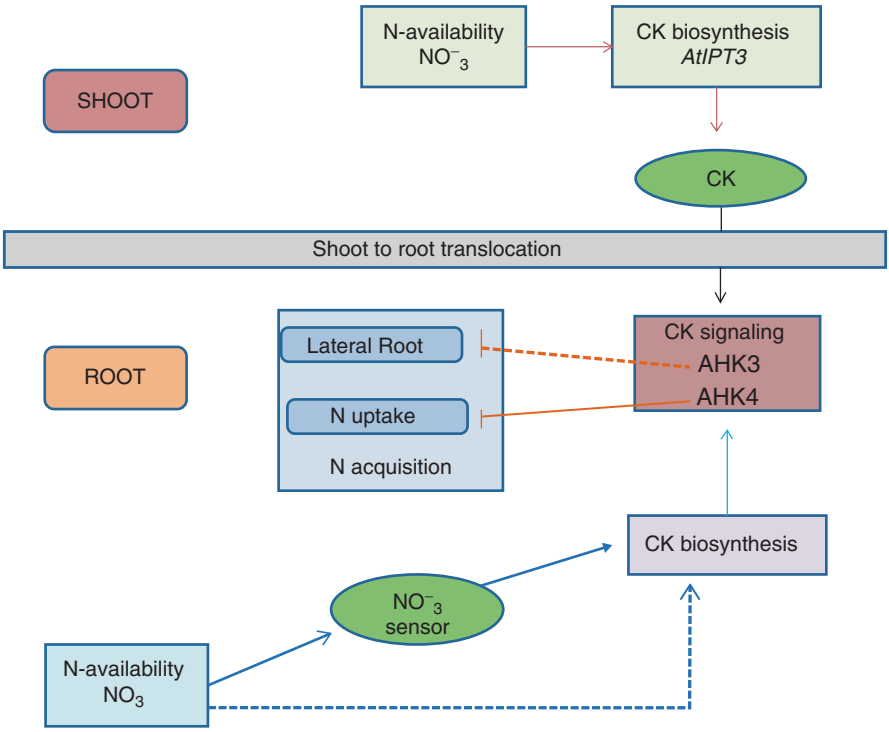

Fig. 14.2 Schematic representation of the interaction between nitrogen and phytohormone cytokinin. *Arrows* represent positive interactions and *blunted lines* represent inhibitory interaction. *Solid lines* represent defined interactions; *dashed lines* indicate presumed interactions

CK application intimidate two AtNRT2 genes (AtNRT2.1andAtNRT2.3), three ammonium transporter genes, three amino acid transporter genes, and a urea transporter gene in *Arabidopsis* (Brenner et al. 2005; Kiba et al. 2005; Sakakibara et al. 2006; Yokoyama et al. 2007). AtNRT1 and AtNRT2expression level correlate well with low- and high-affinity nitrate transport activity, respectively (Forde 2000; Okamoto et al. 2003), inferring that CK repression of the AtNRT genes results in a reduction in nitrate uptake activity (Fig. 14.2).

Abscisic Acid

Abscisic acid (ABA) is a stress hormone which is generally involved in abiotic and biotic stress responses. However, there are various evidences linking ABA levels and nitrogen status in several plant species (Radin et al. 1982; Peuke et al. 1994; Brewitz et al. 1995; Wilkinson and Davies 2002), but there is no compulsion to have correlation between the two. For example, in *Arabidopsis*, there is no statistically noticeable difference in ABA contents between seedlings grown under the high N and low N conditions. Thus, it is still unclear whether changes in ABA content are

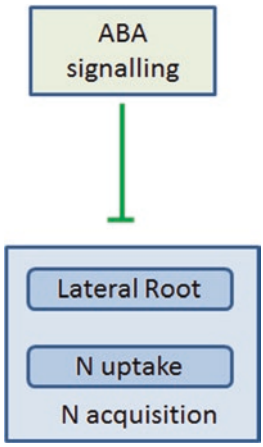

Fig. 14.3 Schematic representation of the interaction between nitrogen and phytohormone abscisic acid. *Blunted lines* represent inhibitory interaction

relevant to nitrogen signaling, but involvement of ABA in nitrogen signaling is becoming increasingly evident.

Reports on the involvement of ABA in lateral root development in response to high nitrate supply in *Arabidopsis* provided genetic evidence. A set of mutants identified that depend on their capacity to produce lateral roots in the presence of ABA (*labi* mutants) depicts reduced sensitivity to the inhibitory effects of high nitrate (Zhang et al. 2007). Signora et al. (2001) clearly showed that ABA-insensitive mutants (*abi4-1*, *abi4-2*, and *abi5-1*) and ABA-deficient mutants (*aba1-1*, *aba2-3*, *aba2-4*, and *aba3-2*) are less sensitive to the inhibitory effects of high nitrate (Fig. 14.3).

Adaptation Under Low N

Under N limiting conditions, miRNAs can be up- or down-regulated. In various crop species such as maize (Xu et al. 2011; Trevisan et al. 2012; Zhao et al. 2013), rice (Cai et al. 2012; Yan et al. 2014), soybean (Wang et al. 2013), and *Arabidopsis* (Pant et al. 2009; Liang et al. 2012) expression profiles of different miRNA families have been observed. Mediation of the expression of their target genes changes in the expression pattern of these miRNAs results in plant adaptive responses to N limitation in the soil (Zeng et al. 2014).

MicroRNA, **miR169a**, is the only candidate reported so far, regulating the expression of key target N transporters under N limiting conditions. There are 14 members under the MiR169 family in *Arabidopsis*; among these miR169a contributes the most to the total miR169 level (Zhao et al. 2011). In *Arabidopsis*, NFY (Nuclear Factor Y) is a ubiquitous transcription factor consisting of 3 subunits A, B, and C which is targeted by miR169; some of its subunits bind to promoter regions and regulate expression of the nitrate transporters AtNRT2.1 and AtNRT1.1 (Zhao et al. 2011). Up-regulation of NFYA5, a target of miR169, reportedly increases drought tolerance by stimulating expression of several antioxidant genes (Li et al. 2008).

Furthermore, over-expression of MIR169a inhibited expression of NFYA transcripts. These over-expresser transgenic plants accumulate less N, which resulted in leaf yellowing compared to the wildtype (WT) plants, and were especially hypersensitive to N starvation (Zhao et al. 2011). miR169 was strongly down-regulated under N deficiency and its target NFYA family members were strongly induced in root and shoot tissues (Zhao et al. 2011). The hypersensitivity of MIR169a over-expresser plants was linked with down-regulation of nitrate transporter genes AtNRT2.1 and AtNRT1.1, suggesting the regulatory role of miR169 in N uptake and remobilization.

NF-Y transcription factors present in almost all eukaryotes, but the biological roles of most of the NF-Y family members in plants are poorly understood. The other members of NFYA family are also potential targets of miR169, including NFYA1, NFYA2, NFYA3, NFYA8, NFYA9, and NFYA10 (Jones Rhoades and Bartel 2004). It was found that all the members of the NFYA family were expressed in both roots and shoots *Arabidopsis* plants as described by Siefers et al. (2009), in contrast to the tissue-specific expression patterns. With the exception of NFYA1 in the roots, the different transcripts that accumulated in the roots vs. shoots, the expression of members of the NFYA family also increased in N-limited roots and shoots, which was in truce with the reduction of miR169 expression in N-limited roots and shoots of *Arabidopsis*. These results suggested miR169 cleaved its target NFYA mRNA and this cleavage was directly related to the N status of the *Arabidopsis* plants.

Production of Antioxidant

It is a secondary metabolite protecting plants from photo-inhibition damage under abiotic stresses including N limitation. It has been reported that miRNAs are involved in the production of antioxidants (Kandlbinder et al. 2004; Shin et al. 2005; Liang et al. 2012). In *Arabidopsis*, miR826 target the AOP2 gene, which encodes 2-oxoglutarate-dependent dioxygenase and linked with glucosinolate biosynthesis (Liang et al. 2012). A recent study reported that under N limiting conditions, AOP2 transcripts were significantly repressed while miR826 was strongly induced (He et al. 2014). In addition, expression of genes of two transporters induced and these are the nitrate transporter (NRT2.1) and ammonium transporter (AMT1.5) (He et al. 2014). MiR826 over-expresser *Arabidopsis* transgenic plants showed enhanced tolerance under N limiting conditions, and less glucosinolate and anthocyanin contents, had higher biomass, more primary and lateral roots and increased chlorophyll (He et al. 2014).

It was reported that the expression of miR398 in plants is inhibited by N deficiency (Pant et al. 2009; Liang et al. 2012). MiR398 is a conserved miRNA in *Arabidopsis*, rice, Lotus, and Medicago (Sunkar and Zhu 2004). Transcripts of multiple genes targeted by this miRNA are: cytosolic CSD1, chloroplastic CSD2, COX5b-1, and CCS1. Cu/Zn superoxide dismutase (SOD) is an important radical scavenger that protects plants from oxidative stress damage encoded by CSD1 and

CSD2 (Sunkar et al. 2006; Jagadeeswaran et al. 2009). A subunit of the mitochondrial cytochrome C oxidase is encoded by COX5b-1 and the copper chaperone for SOD encoded by CCSI (Beauclair et al. 2010; Zhu et al. 2011). It was reported that the tolerance to oxidative stress is induced by high light conferred by Over-expression of CSD2 (Sunkar et al. 2006). The down-regulation of miR398 might reduce its control, as suggested, on these target antioxidant genes and therefore indirectly provide protection to the photosynthetic machinery from reactive oxygen species (ROS) generated from N deficiency (Kandlbinder et al. 2004; Shin et al. 2005).

Production of Anthocyanin

Anthocyanins have been shown to protect cells from high-light damage by absorbing blue-green and ultraviolet light, thereby protecting the tissues from photo-inhibition, or high-light stress. In plants, miR156 targets transcripts of the Squamosa Promoter Binding Protein Like (SPL) family of transcription factors whose expressions were mutually linked with anthocyanin biosynthesis (Gou et al. 2011). The expression of SLP is repressed by over-expression of miR156, concomitantly with an enhanced production of anthocyanin in *Arabidopsis*. The amassing of anthocyanin and reduction of photosynthesis are adaptive responses of plants which protect them from photo-inhibition damage under N limiting condition (Diaz et al. 2006; Peng et al. 2008). Thus, increased expression of miR156 might have resulted in higher levels of anthocyanin production conferring better protection of plants during N starvation (Liang et al. 2012).

Alteration of Flowering Time

Flowering time in plants have long been known to be controlled by microRNAs (Yamaguchi and Abe 2012; Spanudakis and Jackson 2014). MiR156 was shown to regulate flowering, vegetative phase changes, fertility, and leaf formation via intervening of the SPL genes (Wu and Poethig 2006; Wang et al. 2008, 2009; Wu et al. 2009; Xing et al. 2010). A prolonged juvenile phase, stunted growth, altered biomass production, and delayed flowering result in transgenic plants over-expressing miR156 (Wu and Poethig 2006; Xie et al. 2006; Chuck et al. 2007a; Zhang et al. 2011b; Fu et al. 2012; Shikata et al. 2012).

The AP2-like family of transcription factors including TOE1 and TOE2 is targeted by MiR172 and controls flowering time and floral organ identity in maize and *Arabidopsis* (Aukerman and Sakai 2003; Chen 2004; Chuck et al. 2007b; Zhao et al. 2007). miR172 over-expressers were shown to promote flowering in *Arabidopsis* as in contrast to miR156 (Aukerman and Sakai 2003; Chen 2004; Jung et al. 2011). There is only half of the normal transcript level of miR172 in over-expressed 35S::miR156a transgenic plants, whereas 35S::MIM156 transgenic plants had more than double the

miR172 level (Wu et al. 2009). Further research revealed expression of miR172 regulated by miR156 via mediation of the transcription factors SPL9 and SPL10 (Wu et al. 2009). The changes in the expression pattern of miR156 and miR172 under N deficiency (Liang et al. 2012) will lead to alteration of flowering time, since N starvation is known to induce early flowering in plants (Vidal et al. 2014).

Phytohormones in S Stress

The instant effects noticed in metabolic profiles of plants subjected to sulfur limitation are reducing amounts of the first organic sulfur-containing compounds on the sulfur assimilation pathway. Depletion of sulfate in growth medium resulted in quick drop down of internal sulfur, succeeded by a rapid reduction in cysteine (the first organic molecule into which inorganic sulfur is incorporated) and its derivative glutathione (Hirai et al. 2003; Nikiforova et al. 2003). Red signal on the biochemical pathway to cysteine results in the accumulation of its constant biosynthetic precursor *O*-acetyl-L-serine (OAS) as well as the instant OAS precursor serine and in the succeeding re-channeling of the metabolic flow to glycine and tryptophan (Hirai et al. 2003; Nikiforova et al. 2003, 2005a). All these effects may be regarded as being tided by the metabolic flow and therefore do not involve additional regulators for their accumulation.

The activation of glucosinolate catabolism is a next set of the response events in *Arabidopsis*. Glucosinolates are sulfur-rich compounds stored in the vacuoles. Sulfate is released and may be reassimilated into the essential sulfur-containing compounds, in their catabolism. Internal glucosinolate concentrations are reduced and the genes of glucosinolate catabolism are turn on, when sulfur is depleted in the growth medium (Hirai et al. 2004, 2005; Nikiforova et al. 2003, 2005a). Activation of glucosinolate catabolism is included in the processes that cannot be expounded without ambiguity by flux modification because of changed concentrations of sulfur-containing compounds; therefore, this process initiates with myrosinase-catalyzed hydrolysis reaction (Bones and Rossiter 1996; Rask et al. 2000), which needs the topside regulation of myrosinase and/or myrosinase-encoding genes. This specific cause–effect association between modified internal sulfur and activation of myrosinase-encoding genes remains vague yet.

The causal connection with enhanced lateral root formation provided by Surplus auxin, which is triggered by the activities of auxin-induced genes (Casimiro et al. 2003), can be observed under sulfur limitation (Nikiforova et al. 2004). Resulting in increment of root-to-shoot mass ratio (Nikiforova et al. 2003) and enhanced lateral roots in sulfur starved plants are further indirect argument pro excess auxin. With the presumption-based level of evidence, processes of glucosinolate catabolism and increased lateral root formation are observed when excess auxin is added as a connecting node into reasoning model. For the primary state of the limited sulfur, progress of the increased lateral roots can be regarded as the end-point physiological reaction.

The accumulation of auxin has not been shown in sulfur-depletion experiments; however, it is based on the various lines of affirmation including (a) excess tryptophan (Nikiforova et al. 2003), (b) downregulated glucosinolate biosynthesis (Hirai et al. 2004) and activated glucosinolate catabolism, and (c) strong over-expression of nitrilases, first shown by Kutz et al. (2002); the general positive change in auxin flux and/or auxin level can be presumed for the early stage of sulfur starvation in *Arabidopsis*.

The cause–effect link between the two auxiliary programs described above are poor and to a great extent vague. Recent understanding shows, relatively late in the time trajectory of the first state, the excess auxin leads to changes in free Ca^{2+} levels, which causes subsequently calmodulin activation. Activated calmodulin was linked in the direct cause-to-effect relationship with the *IAA28* gene of auxin, in the gene–metabolite correlation network of sulfur stress response (Nikiforova et al. 2005b). This gene acts as an inhibitor of the activity of auxin-induced genes (Rogg et al. 2001) and thus may cause down-regulation of the auxin response reporter *DR5*::GUS and repression of lateral root formation by sulfur depletion, observed by Dan et al. (2007).

In the process of plant adaptation to low sulfur, the internal lipid content is strongly decreased; this is indicated in the metabolic profiles of the plants subjected to the long-term sulfur starvation (Nikiforova et al. 2005a). Reduced amounts of the sulfur-containing molecule, *S*-adenosyl-methionine (SAM), result in the decreased chlorophyll content, which is required in biosynthesis of SAM, and elevate photorespiration. These factors provide cause–effect association to decreased photosynthesis, leading to shortcoming in energy assimilation, which in turn contribute to general decrease of metabolism. Inadequate sulfur supply results in imbalance with nitrogen, being further affirmed by the changes in tetrahydrofolate, a central cofactor in C_1 metabolism that links photorespiration, sulfur assimilation, and discard of imbalanced nitrogen. A dense network of coordination is formed by the mutual influences between these processes (Nikiforova et al. 2005a), which is depicted as a crucial part of the bio-reasoning model.

Sulphur (S)

Auxin

Till recently, available data obtained from the analysis of the transcriptional rejoinder of plants to sulfur deficiency shows potential regulatory connection between auxins and sulfur signaling. Therefore, it was observed that NIT3 (NITRILASE 3) is a gene which encodes a main enzyme in the synthesis of auxin (Nikiforova et al. 2003); in scanty sulfur conditions, its expression induced. Nevertheless, in sulfur-deprived seedlings such induction did not link with enhanced accumulation of auxins, but on the confliction, with a deduction in auxin levels or sensitivity (Dan et al. 2007; Kutz et al. 2002). Furthermore, transcription of several members of the Auxin/Indole-3-Acetic Acid (Aux/IAA) gene family (Nikiforova et al. 2003) has switch on under sulfur deprivation. Given that, it is valid to consider that sulfur

deficiency initiates auxin repression as the Aux/IAA proteins are repressors of the auxin signaling (Guilfoyle and Hagen 2007). On the other hand, it is advocated that sulfur–auxin interaction is bidirectional (Dan et al. 2007) because auxin treatment inhibited expression of a low-sulfur-responsive gene encoding a putative thioglucosidase, which possibly releases thiol groups from glucosinolates. However, these hormones only regulate a subset of the plant responses to sulfur starvation shown by low-sulfur-induced expression of SULTR1;2 and APR2 which was not pretented by auxins.

Cytokinin

It was known that CK downregulate the gene expression of high-affinity sulfate transporters and these are SULTR1;1 and SULTR1;2 (Maruyama-Nakashita et al. 2004); for this repressing effect of CK,CRE (CK receptor) is responsible. In spite of this, CK is also intricated in upregulation of APR and SULTR2;1 genes under S deficiency in *Arabidopsis* (Ohkama et al. 2002). Nevertheless, the information related to CK effect on S assimilation is lean as contrast to the N and P.

Jasmonic Acid

JA is also known as a possible component of signalling in leaves. Under sulfur deficiency, genes involved in JA biosynthesis are upregulated (Hirai et al. 2003; Jost et al. 2005). These genes which are upregulated include 12-oxophytodienoate reductase 1 and lipoxygenase (Hirai et al. 2003; Maruyama-Nakashita et al. 2003; Nikiforova et al. 2003). The expression of genes intricated in sulfate assimilation and GSH synthesis might also be regulated by JA (Jost et al. 2005; Xiang and Oliver 1998). Moreover, the activity of sulfur assimilation enzymes like serine acetyltransferase (SAT) and APR (Jost et al. 2005) are regulated by JA. Although JA plays role as a regulator of sulfur metabolism, its levels in plants are not well-specified under S-deficient conditions.

Abscisic Acid (ABA)

ABA is known to affect sulfur homeostasis by enhancing the levels of glutathione (GSH), an intercessor in sulfur metabolism which plays a critical role in maintaining the redox state of plants (Jiang and Zhang 2001; Shao et al. 2007). Thus, ROS levels control by ABA has been entreated to explain its role in protecting plants against oxidative conditions caused by various stress situations, including nutritional deficiencies. However, detailed study has not yet been done.

Sulfur Limitation1 (Role in S Limitation)

SLIM1 is related to a group of ethylene-insensitive3-like (EIL)-family transcription factors (Maruyama Nakashita et al. 2006). Moreover, its function is considered to be determined by the sulfur response (Maruyama Nakashita et al. 2006) and can be differentiated from other members of EIL family like EIN3 and its homologues, EIL1 and EIL2, that primarily conciliate the ethylene response (Guo and Ecker 2004). It is known as a key transcriptional regulator of sulfate uptake determined in *Arabidopsis* from a genetic screen for mutants interrupted in the sulfur-limitation response, using GFP (Green Florescent Protein) regulated by promoter as a reporter which is sulfur starvation–inducible *SULTR1;2* (Maruyama Nakashita et al. 2006). Sulfate transporters and enzymes for glucosinolate biosynthesis are regulated oppositely by SLIM1 in response to sulfur limitation stipulated by transcriptome analysis of a *slim1* mutant (Maruyama Nakashita et al. 2006). SLIM1 as an upstream coordinator globally controls the balance of sulfur utilization. Among all the genes controlled by sulfur limitation, SLIM1 does not regulate the *APR* transcript levels; it seems as an exception. Therefore, independent regulatory mechanisms have to be presumed for *APR*. SLIM1's ability to control the expression of the SULTR1;2 sulfate transporter signifies its function for SULTR1;2, the major sulfate uptake facilitator in *Arabidopsis* roots. SLIM1 was needed for the limited S-responsive activation of SULTR1;2 transcripts, which lead to a prodigious elevation of high-affinity sulfate uptake activities that finally contribute to the prolongation of plant growth under S limitation.

MicroRNA Under S-Deficient Response

miR395 mainly plays role under S limitation. miR395 plays main role in regulation of sulphate assimilation; its expression was inspected under different external sulphate concentrations. It was anticipated to target the mRNAs of several enzymes involved in S assimilation pathway, i.e., APS1, APS3, and APS4 encoding APS (Jones Rhoades and Bartel 2004). 5′-RACE analyses established the targeting of miR395 to the mRNAs of APS1 and APS4 (Jones Rhoades and Bartel 2004; Allen et al. 2005).

Many miRNAs are leaned to target multiple mRNAs in the same gene family, which include miR395 targeting the APS gene family. APS activity has been founded in the cytosol and in plastids in spinach (Lunn et al. 1990; Renosto et al. 1993) and *Arabidopsis* (Rotte and Leustek 2000) and different isoforms of APS are present. In *Arabidopsis*, four genes (APS1, APS2, APS3 and APS4) encode isoforms of APSs. APS1, APS3, and APS4 were found to be plastid-localized, but APS2 isoform may be present in cytosol (Hatzfeld et al. 2000). Moreover, the genes, plastid-localized APSs, are targeted by miR395 rather than the cytosolic APS2. The primary pathway of S assimilation includes the formation of cysteine in which most

of the sulphate is reduced and assimilated into cysteine by APSs in the plastid, whereas cytosolic APS activity was responsible for the synthesis of sulphate esters, such as glucosinolates by sulphation reaction (Rotte and Leustek 2000). This finding concluded that miR395 is intricated in the Regulation of sulphate (S) assimilation and allocation. It was found that the mRNA level of APS1 was decreased under low sulphate supply, but the expression of miR395 was induced (Jones Rhoades and Bartel 2004), which concurred with the negative regulation of miRNAs. Moreover, the down-regulation of APS by up-regulated miR395 under low-sulphate conditions disputed with earlier reports of elevated APS activity and transcript level of APS1 and APS3 in sulphate-deficient roots of *Arabidopsis* or *Brassica napus* (Lappartient and Touraine 1996; Takahashi et al. 1997; Lappartient et al. 1999).

The changes in the transcript level of APS1, APS3, and APS4 by sulphate limitation are marginal (0.79- to 1.16-fold); it was specified by examination of microarray data by Genevestigator expression analysis (Zimmermann et al. 2004). The observation that the amount of APS4 mRNA in leaves and roots was not amended by changes in sulphate status is again bracing the described finding (Hatzfeld et al. 2000). Nevertheless, in shoots of sulphate-starved *Arabidopsis* plants, APS1 mRNA was reduced in level about twofold (Takahashi et al. 1997). Whether different experimental conditions such as sulphate concentration, duration of treatment, or growth environment cause these inconsistency remains to be clear. In addition to targeting APS genes, miR395 also targets AST68 (AtSULTR2;1, At5g10180), encoding a low affinity sulphate transporter, which was again experimentally confirmed by 5′-RACE analysis (Allen et al. 2005). miR395 appears to regulate two different groups of genes that function cooperatively in the same metabolic pathway, such as sulphate translocation and assimilation; therefore this finding is unique for miRNA-mediated gene regulation. SULTR2;1 is a low-affinity sulphate transporter localized in the leaves and roots vascular tissues, which recommended that this low affinity transporter may be involved in the internal translocation of sulphate from roots to shoots (Takahashi et al. 1997, 2000). Under S deficiency, large amounts of SULTR2;1 mRNA quickly accumulated in roots (Takahashi et al. 1997, 2000). In disparity, it was found that the accumulation of SULTR2;1 mRNA in leaves was not changed by short-term sulphate starvation, but it declined after continuous starvation (Takahashi et al. 2000). The reduced APS1 mRNA level as well as the reduced SULTR2;1 mRNA in the shoots previously mentioned may somehow be linked with the elevated miR395 expression, which was noticed after prolong withdrawal of sulphate. Although the miR395 expression was detected in whole plants (Jones Rhoades and Bartel 2004), the cleavage of APS1 and SULTR2;1 was guided by miR395,which might take place specially in the shoots (Table 14.1).

Detailed analyses of the spatial and temporal expression patterns of miR395, together with AtSULTR2;1 and APS genes, under sulphate deprivation will provide a clearer picture. In addition, whether changes in the steady state mRNA level of APS1 and SULTR2;1 are a consequence of transcriptional and/or post-transcriptional regulation may offer another line of evidence for miR395-dependent regulation.

Table 14.1 Represent miRNA family and their function in presence and absence of N

miRNA family	Target gene or protein	Description of function	Involvement under low N	
			Plant tissue and plant specie (references)	
156	SQUAOMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors	Shoot development delayed vegetative phase change	R (+)	Maize (Zhao et al. 2012)
157	SQUAOMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors		R (+)	Arabidopsis (Liang et al. 2012)
159	MYB, TCB transcription factors	Plant development	R (+)	Maize (Zhao et al. 2012)
160	Auxin response factors	Reduce auxin responsive activities and vegetative growth	R (+)	Maize (Xu et al. 2011)
		Lateral and adventitious root development, signal transduction	R (+)	Arabidopsis (Liang et al. 2012)
162	Dicer like protein	Flower development	R (+)	Maize (Zhao et al. 2012)
164	NAC transcription factors	Accelerate senescence, N remobilization	L (+), R (−), S (−)	Maize (Zhao et al. 2012, Xu et al. 2011)
166	HD-ZIP transcription factors	Shoot development	R(−)	Maize (Trevisan et al. 2012)
167	Auxin responsive factors	Enhance auxin responsive activity, lateral root outgrowth, reduce fertility, impaired reproductive organ development	R(−)	Maize (Xu et al. 2011)
168	ARGONAUTE 1	Homeostasis and feedback regulation on miRNAs	R(−)	Maize (Xu et al. 2011)
169	HAP2 transcription factors CAAT binding factors/ NFYA	Nitrogen homeostasis, Stress response	R (−), S (−), L (−)	Maize (Zhao et al. 2012, Xu et al. 2011, Trevisan et al. 2012, Zhao et al. 2013)
		Nitrogen homeostasis, N uptake		
		Antioxidant	R (−), S (−)	Arabidopsis (Liang et al. 2012, Pant et al. 2009)
			R (−), S (−)	Arabidopsis (Zhao et al. 2011)
			R (−), S (−)	Arabidopsis (Wang et al. 2013)

(continued)

Table 14.1 (continued)

miRNA family	Target gene or protein	Description of function	Involvement under low N: Plant tissue and plant specie (references)	
171	SCARE CROW-like transcription factors	Root development	R (+)	Arabidopsis (Liang et al. 2012)
			R (+), S (+)	Maize (Liang et al. 2012)
			R (−), S (−)	Soybean (Wang et al. 2013)
172	AP2-like transcription factors	Ethylene-responsive pathway, N-remobilization	L (+), S (+)	Maize (Xu et al. 2012, Xu et al. 2011)
		Flower development	R (−)	Arabidopsis (Liang et al. 2012)
393	Auxin receptor	Root development, defence response	R (+)	Maize (Zhao et al. 2012)
395	ATP sulfurylase, sulphate transporter	Sulphate homeostasis	R (−)	Arabidopsis (Liang et al. 2012)
			R (−)	Maize (Zhao et al. 2012, Xu et al. 2011)
396	Growth-regulating factors	Leaf development	R (−)	Maize (Zhao et al. 2012)
			R (+/−), S (+/−)	Soybean (Wang et al. 2013)
397	Laccases	Reduce root growth, copper homeostasis	L (−), S (−), R (−)	Maize (Zhao et al. 2012, Xu et al. 2011)
			R (−)	Arabidopsis (Liang et al. 2012)
			R (−), S (−)	Soybean (Wang et al. 2013)
398	COX5b-1, CCS1	Copper homeostasis, oxidative stress	R (−), SD (−)	Arabidopsis (Liang et al. 2012, Pant et al. 2009)
	COX	Enhanced to produce ATP under stress	L (−), S (−)	Maize (Zhao et al. 2012, Xu et al. 2011)
			R (−), S (−)	Soybean (Wang et al. 2013)
399	Ubiquitine conjugase E2/ UBC24	Phosphate homeostasis, uptake and translocation	L(−), R(−)	Maize (Zhao et al. 2012, Xu et al. 2011)
			R(−)	Arabidopsis (Liang et al. 2012)
408	PLANTA-CYANIN LACCASE	Enhance electron carrier activity, copper homeostasis	L(−), R(−)	Maize (Zhao et al. 2012, Xu et al. 2011, Trevisan et al. 2012)

Plant tissue: *R*, root; *L*, leaf; *S*, shoot; *SD*, seedling; (+), up; (−), down. *Source*: Nguyen GN et al. (2015)

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