Dharmendra K. Gupta
José M. Palma
Francisco J. Corpas *Editors*

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This book is dedicated in the memory of ***Prof. Emeritus Kozi Asada****, Kyoto University, Japan, for his large contribution in the field of plant ROS, who passed away on 15th December, 2013, and also to* ***Prof. Paul Bolwell****, Royal Holloway, University of London, UK, for his long contribution in plant oxidative burst in response to pathogens who also passed away on 13th of April, 2012.*

Preface

In plants as well as in all aerobic organisms, reactive oxygen species (ROS) are produced commonly as a by-product of aerobic metabolism. It depends on the formation and nature of ROS; some are toxic and easily destroyed/detoxified by several enzymatic and nonenzymatic mechanisms in the plant cells. However, lately, the role of ROS as second messengers participating in signaling processes under normal and certain stress conditions was postulated (Foyer and Noctor 2003). Environmental stresses such as heat, cold, drought, salinity, heavy metal toxicity, ozone, and ultraviolet radiation as well as pathogens/contagion attack lead to enhanced generation of ROS in plants due to disruption of cellular homeostasis. When the increment of ROS in plant cells rapidly increased and the scavenging systems of ROS do not operate properly, a situation of oxidative stress and oxidative injury occurs. The toxicity caused by heavy metals leads to intervention with metabolism and other biological activities through the generation of ROS such as superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxide molecule (H_2O_2). Under certain conditions which involve the presence of transition metal ions, basically Cu^{2+} and Fe^{3+}, H_2O_2 may be reduced to $^{\bullet}OH$ radicals by superoxide and generates oxidative damage to the plants. One of the major consequences of heavy metals action in the cell is the enhanced generation of ROS which usually damage the cellular components such as membranes, nucleic acids, chloroplast pigments, and alteration in enzymatic and non-enzymatic antioxidants (Gupta et al. 2013a). Stress-induced increases in ROS level can cause different degree of oxidation of cell components and a gross change in redox status. Thus, an oxidative outburst as a consequence of stress is reflected in the levels of ROS molecules ($O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$), which are biochemically connected through metabolic reactions (Halliwell and Gutteridge 2007).

ROS generation is evident in chloroplast, mitochondria, peroxisomes, plasma membrane, and apoplast adjacent to membrane. In green plants, chloroplast is the most important among the organelles in respect of ROS generation as O_2 is continuously provided through the water autolysis and readily available inside the organelle. Several reports showed that ROS induction can take place in response to

cadmium stress in pepper (*Capsicum annum* L.) (León et al. 2002) and *Arabidopsis thaliana* (Remans et al. 2010), Pb and As stress in *Zea mays* and in *A. thaliana* (Gupta et al. 2009, 2013b), Cd and Cu stress in pea (*Pisum sativum*) and *A. thaliana* (Palma et al. 1987; Remans et al. 2010), Ni stress in wheat (*Triticum aestivum*) (Hao et al. 2006), and Zn stress in Brassica (Feigl et al. 2014). Since now there is no evidence that cytoplasmic phytochelatins (PCs) have a role in prevention of ROS induction at plasma membrane or associated ROS formation at apoplast. However, it is acceptable that cell-wall-associated peroxidase catalyzes formation of membrane-permeable H_2O_2 in apoplast and then makes it possible to interact with cytosolic PCs and other thiol peptides.

Since last three decades, it's indeed a big boom in the field of ROS and its role/function in plants. The main purpose of the book is to provide detailed and comprehensive knowledge to the academicians and researchers who are interested in the field of oxidative damage caused by stresses in plants with special reference to the metabolism of ROS and site of production of ROS in plant systems. Other key features of this book are ROS signaling, ROS and disease resistance, redox regulation, and antioxidant defense during stresses, heavy metal-induced oxidative stresses, and heavy metal toxicity and detoxification mechanism. Some chapters are also focusing on hormones/polyphenols as antioxidants and the future of transgenic plants in antioxidative defense. The functional interaction between ROS and the reactive nitrogen species (RNS) is also addressed in this volume. In the nutshell, the information compiled in this book will bring very deep knowledge and advancement in the field of ROS and oxidative damages caused by stresses in current years in plant sciences.

Dr. Dharmendra K. Gupta, Prof. José M. Palma, and Dr. Francisco J. Corpas personally thank the authors for contributing their valuable time, knowledge, and enthusiasm to bring this book into the present shape.

Hannover, Germany — Dr. Dharmendra K. Gupta
Granada, Spain — Prof. José M. Palma
Dr. Francisco J. Corpas

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Production Sites of Reactive Oxygen Species (ROS) in Organelles from Plant Cells

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Abstract Reactive oxygen species (ROS) have been considered for a long time as undesirable by-product of the cellular metabolism, but recently the role of ROS in molecular signaling processes has been reported. Consequently, the cell must keep a fragile equilibrium between ROS production and the antioxidant defenses that protect cells in vivo against potential damages (oxidative stress) and, alternatively, allow the inter- and intra-cell communications. This equilibrium may become disturbed under different array of adverse conditions by an excessive generation of ROS or by an impaired antioxidant defenses. Plant cells have a compartmentalization of ROS production in the different organelles including chloroplasts,

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mitochondria, or peroxisomes, and they also have a complex battery of antioxidant enzymes usually close to the site of ROS production. Cell compartmentalization has been demonstrated to be an additional mechanism of cellular ROS modulation for signaling purposes. This chapter will provide a general overview of the main system of ROS production/regulation in plant cells.

Keywords Reactive oxygen species • Chloroplasts • Mitochondria • Peroxisomes

1 Introduction

Reactive oxygen species (ROS) is a term which includes radical and non-radical oxygen species formed by the partial reduction of oxygen. The main ROS mostly investigated are superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) as radicals molecules, and hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3), and hypochlorous acid (HClO) as non-radical. Under normal conditions, these molecules are produced in many metabolic pathways as normal by-product, being the respective electron transport chains present in chloroplasts and mitochondria the main sources of these ROS (Halliwell 2006; del Río 2015). However, the presence of free metals, such as iron, copper, and manganese, released from metalloprotein complexes can also contribute to ROS production. Plant cells enclose a wide range of enzymatic and nonenzymatic antioxidant systems which usually are nearby the ROS production site being an excellent mechanism to avoid the undesirable potential negative effects of ROS (oxidative stress) but also to modulate their signaling role.

In parallel, plant cells contain a series of ROS-scavenging nonenzymatic antioxidants such as ascorbic acid, glutathione (GSH), carotenoids, and others, as well as a wide battery of enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), peroxiredoxin (Prx), and the ascorbate–glutathione cycle. All these latter elements have multiple isozymes located in all cell compartments which provide a highly efficient system for detoxifying ROS. The main goal of this chapter is to offer a general overview of the main system of ROS production/scavenging in the principal plant organelles.

2 Chloroplasts

Due to their abundance and diversity of pigments, chloroplasts are the cell organelles more susceptible to be attacked by ROS. These photosynthetic compartments are also great sources of ROS production, including basically $O_2^{\bullet -}$ and singlet oxygen ($^1O_2$). Chloroplasts harbor in thylakoids the key elements to fully carry out

the photosynthesis, with the structures involved in the light-dependent phase being mainly responsible for the ROS generation (Tripathy and Oelmüller 2012). Complementarily, these organelles contain powerful antioxidant systems to counterbalance the ROS production under normal conditions.

2.1 Production of Reactive Oxygen Species

The major site of superoxide radical's production is linked to the photosystem I (PSI). Under illumination conditions, O_2 is continuously provided by the water autolysis performed in the PSII as indicated in reaction [1], so light would favor the superoxide radical formation reaction [2] at the PSI location. There, under excessive reduced ferredoxin and low NADP availability, the autoxidation of this iron–sulfur protein occurs with the formation of $O_2^{\bullet -}$, as depicted in reaction [3].

Reaction [1] $2H_2O \rightarrow 4e^- + O_2 + 4H^+$
Reaction [2] $2O_2 + 2e^- \rightarrow 2O_2^{\bullet -}$
Reaction [3] $Fdred + O_2 \rightarrow Fdox + O_2^{\bullet -}$

If the conditions persist, the reduced ferredoxin is able to react with superoxide radicals to form hydrogen peroxide, and this is what Mehler (1951) found when he performed his experiments with illuminated chloroplasts (reaction 4).

Reaction [4] $Fdred + O_2^{\bullet -} + 2H^+ \rightarrow Fdox + H_2O_2$

Asada and colleagues (1974) corroborated later that all the H_2O_2 formation attributable to chloroplasts was a consequence of the disproportionation of superoxide radicals previously formed. It has been found that the H_2O_2 photo produced via $O_2^{\bullet -}$ accumulates in thylakoids, whereas in intact chloroplasts this ROS does not accumulate (Asada 2006). The steady-state level of H_2O_2 in chloroplasts was determined to be about 0.5 μM, with increases under stress conditions up to 1–15 μM.

The direct production of $O_2^{\bullet -}$ to a lower extent at the level of the PSI was also reported, and it was postulated that, when the NADP availability lowers and the Calvin–Benson cycle does not operate properly, the ferredoxin autoxidation takes place initially and afterwards the direct formation of superoxide radicals from the PSI (Halliwell and Gutteridge 2007). Simultaneously, another source of superoxide radicals is also associated to PSII, for instance, through the autoxidation of PSII electronic acceptors and mostly at the level of the plastoquinone (Gupta and Igamberdiev 2015). The superoxide radical's production in chloroplasts is promoted above the normal conditions under certain circumstances, basically stress situations which proceed with stomata closure. Then, the CO_2 availability decreases and the photosynthetic carbon reductive pathway (Calvin–Benson cycle) is somehow impaired, with the concomitant lower provision of NADP for the thylakoid-linked ferredoxin-NADP reductase. Accordingly, reduced ferredoxin accumulates

and develops the scenario described above. Overall, the rate of $O_2^{\bullet -}$ production in isolated chloroplasts was initially reported to be about 30 μmol mg^{-1} Chl h^{-1} (Asada 1992). Later, it was probed to that the superoxide radical's generation increased from 240 to 720 μM s^{-1} under stress conditions (Polle 2001).

Singlet oxygen is produced at the PSII (P680) by excitation of oxygen of the ground (triplet) state $^3O_2$ till singlet state ($^1O_2$), as indicated in reaction [5].

Reaction [5] $^3O_2 + {}^3P680^*$ (excited P680) $\rightarrow {}^1O_2 + {}^3P680$

Under intense illumination conditions and/or low CO_2 assimilation rate undergone due to environmental stresses or certain physiological conditions, electrons from chlorophyll are excited to a higher energy layer, and this energy excess is transferred to oxygen, thus generating singlet oxygen responsible for photodynamic damages such as bleaching of leaves (Telfer et al. 1994; Hideg et al. 1998; Asada 2006). Additionally, it has been also found that biosynthetic and catabolic intermediates of chlorophyll are photosensitizers which generate singlet oxygen (Wagner et al. 2004; Pruzinska et al. 2005). Although $^1O_2$ is rapidly quenched by water, its lifetime and diffusion distance from the generation site are very short. So, the distance among the generation and the target sites of $^1O_2$ is a critical factor to evaluate the biological effect of this ROS (Asada 2006).

Many herbicides, including methyl viologen (paraquat), diquat, DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], atrazine, and others base their mechanism of action by promoting the generation of ROS. Thus, cationic herbicides such as methyl viologen trigger the formation of superoxide radicals at the level of PSI; other polar compounds like DCMU uncouple the electron fluxes at the PSII level with excitation of chlorophyll and the energy excess of excited chlorophyll being transferred toward the formation of $^1O_2$. It has been demonstrated that many plants (tobacco, tomato, potato, and alfalfa, among others) transfected with additional *SOD* genes showed reduced damage symptoms after being subjected to diverse herbicides.

2.2 ROS Scavenging Systems

Chloroplasts contain a battery of scavengers that not only protect chloroplasts from the direct effects of ROS but also relax the electron excess stress. Thus, a series of antioxidant enzymes and small molecules regulate the endogenous ROS levels, thus allowing a coordinated response under stress conditions (Foyer et al. 1991, 1994; Gill and Tuteia 2010). Chloroplastic membranes are rich of carotenoids (provitamin A) and α-tocopherol (vitamin E), two powerful $^1O_2$ scavengers, so this ROS with high ability to diffuse in hydrophobic environments can be promptly removed by these antioxidants, although ascorbate can also be an active scavenger of this species.

Carotenoids, mainly β-carotene, besides working as complementary light-absorbing pigments, can dissipate the photodynamic effect directly and indirectly. Hence, the energy excess accumulated in the triplet state of chlorophyll as consequence of intense illumination can be transferred to carotenoids which move up to their triplet state. These excited carotenoids go back to their ground state by dissipating their excess energy as heat. On the other hand carotenoids are able to counterbalance the production of $^1O_2$ promoted by the triplet-state chlorophyll. Again, excited carotenoids, as consequence of their interaction with $^1O_2$, dissipate their higher energy as heat rendering the ground-state pigments. Up to 11 molecule of β-carotene have been assigned to the PSII reaction center and antenna subunit complex (Asada 2006). Xanthophylls, a series of molecules framed within the carotenoids group, are also involved in the antioxidant metabolism in a stroma–lumen interaction. This mechanism implies to violaxanthin, antheraxanthin, and zeaxanthin which are interconverted one in another by epoxidation/de-epoxidation reactions, thus giving rise to the so-called xanthophylls cycle (Adams and Demmig-Adams 1992; Demmig-Adams and Adams 2006). The epoxidation pathway (zeaxanthin–antheraxanthin–violaxanthin), carried out at neutral pH under low light in the stroma, depends on the provision of NADPH, whereas the de-epoxidation is achieved in the lumen at acid pH (around 5, high light) with the participation of ascorbate which is converted into dehydroascorbate (Adams and Demmig-Adams 1992; Demmig-Adams and Adams 2006).

Alpha-tocopherol is another molecule which can quench $^1O_2$, although its effectiveness regarding β-carotene is much lower, about two orders of magnitude. After the reaction of α-tocopherol with $^1O_2$, α-tocopherylquinone is formed (Halliwell and Gutteridge 2007), and this can regenerate again α-tocopherol by the reaction with ascorbate. As a result of this reaction chain, monodehydroascorbate is formed, and this is integrated within the enzymatic pathways displayed below (Fig. 1). Tocopherols are also involved in suppressing the lipid peroxidation of thylakoids by trapping lipid radicals (Muller et al. 2006).

From all antioxidant molecules, ascorbate seems to be the most versatile since this compound not only scavenges all types of ROS by itself but also participates in the ascorbate–glutathione cycle (see below) and in the regeneration of other antioxidants as reported above for α-tocopherol. Thus, a very significant role in the chloroplast redox homeostasis is attributed to ascorbate. In fact, chloroplasts are the main cellular pool of ascorbate in spite that this antioxidant is synthesized in mitochondria (Foyer et al. 1991; Smirnoff 2001).

The presence of several superoxide dismutases (SOD; EC 1.15.1.1) has been reported in chloroplasts (Hayakawa et al. 1984; Grace 1990). SODs are a class of metalloenzymes with different nature depending on the heavy metal located in the active site of the protein which catalyze the reaction [6]:

Reaction [6]: $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$

Three main SOD types have been described in plants: copper–zinc-, iron-, and manganese-containing superoxide dismutases (CuZn–SODs, Fe–SODs, and Mn–SODs, respectively; Rodríguez-Serrano et al. 2007). Chloroplasts commonly house

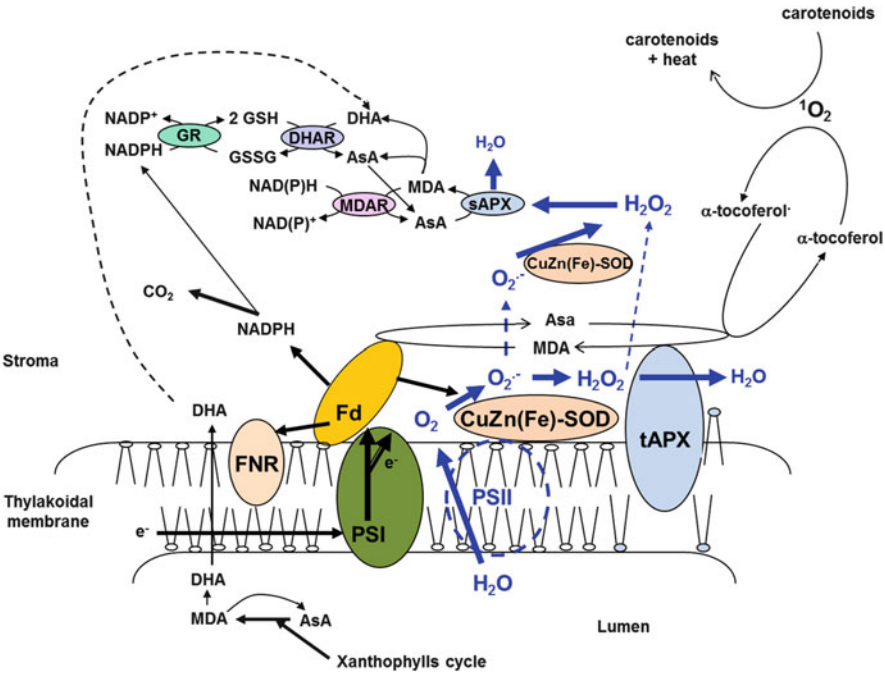

Fig. 1 Integrated model of production and scavenging of reactive oxygen species in chloroplasts. Electrons in PSI are usually "sailing" toward the PSI-linked ferredoxin (Fd) and by action of the NADP–ferredoxin reductase (FNR), NADPH is formed which can be used in the photosynthetic carbon fixation. Subsidiary, superoxide radicals ($O_2^{\bullet-}$) can be generated continuously in the presence of O_2 provided by PSII after H_2O photolysis. $O_2^{\bullet-}$ is then dismutated either by the thylakoid-linked superoxide dismutase (both CuZn–SOD and Fe–SOD) or the soluble forms of these isozymes. The H_2O_2 generated by the action of SOD is decomposed by either the ascorbate peroxidase bound to thylakoid membranes (tAPX) or the soluble isozyme (sAPX), using ascorbate (AsA) as reducing source. sAPX is integrated within the chloroplastic ascorbate–glutathione cycle (AGC) which implies the participation of the monodehydroascorbate reductase (MDAR), the dehydroascorbate reductase (DAR), and glutathione reductase (GR). This redox pathway is involved in the removal of H_2O_2 with expenses of NADPH. AsA could also be used to regenerate α-tocopherol from α-tocopherylquinone (α-tocopheryl radical), after this lipophilic antioxidant has been used as a singlet oxygen ($^1O_2$) quencher. $^1O_2$ can be also scavenged by carotenoids with excess energy being dissipated as heat. Throughout these processes, monodehydroascorbate (MDA) is formed, and this radical can be used to regenerate ascorbate in the stroma by either direct action of reduced Fd or through the AGC. MDA is also produced at the chloroplastic lumen in the xanthophylls cycle. MDA dismutates into ascorbate and dehydroascorbate which can migrate through the thylakoid membrane and be coupled to the stroma AGC. As depicted as blue arrows, a water–water cycle occurs, with consume of water in the lumen and production in the stroma side

CuZn–SOD and Fe–SOD isozymes, although the presence of one Mn–SOD has been reported in chromoplasts from pepper fruits (Martí et al. 2009). Both SOD isoenzyme types have been reported to be attached to the thylakoids near the PSI where $O_2^{\bullet-}$ is produced but also soluble in the stroma (Asada 2006; Mittova et al. 2015) (Fig. 1).

H_2O_2 is mainly removed by the action of the ascorbate peroxidase (reaction [7]; APX; EC 1.11.1.11) which, like SODs, is located either attached to the thylakoid membrane (tAPX) or soluble in the stroma (sAPX) (Yoshimura et al. 1999; Shigeoka et al. 2002; Maruta et al. 2010). In thylakoids, APX is in the vicinity of PSI so the flux of electrons through PSI, SODs, and tAPX forms a thylakoidal scavenging system which functions as the first defense against ROS, with the participation of reduced ferredoxin which directly provides electrons to monodehydroascorbate to regenerate ascorbate (reaction [8]; Fig. 1).

Reaction [7] $H_2O_2 + AsA \rightarrow 2H_2O + 2MDA$
Reaction [8] $2MDA + 2Fdred \rightarrow 2Asa + 2Fdox$

The sAPX is integrated within the ascorbate–glutathione cycle, also called Foyer–Halliwell–Asada cycle, where the enzymes monodehydroascorbate reductase (MDAR; EC 1.6.5.4), dehydroascorbate reductase (DAR; EC 1.8.5.1), and glutathione reductase (GR; EC 1.6.4.2) are involved in the H_2O_2 scavenging associated to the NADPH expense (Corpas and Barroso) (Fig. 1).

Overall, as the result of the series of reactions which involved the formation (reactions 1 and 2) and scavenging (reactions 6, 7 and 8) of ROS in chloroplasts renders the final stoichiometry given in reaction [9]:

Reaction [9] $2H_2O + O_2 \rightarrow O_2 + 2H_2O$

which allows introducing the concept water–water cycle proposed by Professor Kozi Asada (1999) as a unique pathway located in chloroplasts involving the dynamics of oxygen in these organelles and integrating a network of molecules which goes beyond the simple ROS-antioxidant pair.

Peroxiredoxins and thioredoxins are also systems involved in the detoxification of hydrogen peroxide in chloroplasts. Peroxiredoxins are thiol-based peroxidases which may utilize the reducing power provided through thioredoxins to scavenge H_2O_2(Puerto-Galán 2013). Thioredoxins are crucial for the chloroplast redox network, mediating environmental signals to the organelle proteins. Thus, chloroplast thioredoxins have been found to be very versatile and to control the structure and function of proteins by reducing disulfide bridges in the redox active site of a protein (Schürman and Jacquot 2000; Nikkanen, and Rintamaki 2014). A thioredoxin system which gains electrons from the PSI-linked ferredoxin and involves a ferredoxin–thioredoxin reductase has been found. Besides, a thioredoxin that uses NADPH as the reducing source through a NADPH–thioredoxin reductase has been reported (Nikkanen and Rintamaki 2014). Finally, a more complex system where the reducing power from NADPH is successively transferred following the sequence thioredoxin reductase, thioredoxin, and peroxiredoxin to reduce H_2O_2 up to water has been displayed (Dietz 2003). The possibility that this latter system may function as a water–water cycle under certain conditions was already proposed by Asada (2006).

3 Mitochondria

In mammalian cells, mitochondria are the major cell loci for ROS production. In plants, mitochondria constitute one of the main ROS production sites due to unavoidable impairments of the electron transport chain (ETC) responsible of the aerobic respiration which is located at the inner mitochondrial membrane. A short review of the ROS metabolism, both generation and scavenging involved systems, will be given in this chapter, although a wider view of this subject will be displayed in chapter "What Do the Mitochondrial Antioxidant and Redox Systems Have to Say Under Salinity, Drought and Extreme Temperature?" (F. Sevilla and colleagues).

Similarly to what happened in chloroplasts, the first reports on ROS in mitochondria in the mid-1960s revealed that these organelles were able to produce H_2O_2 (Hinkle et al. 1967). Years later, the demonstration of $O_2^{\bullet -}$ generation by submitochondrial particles bearing diverse ETC complexes (Loschen and Azzi 1975), along with the discovery of the presence of SOD activity in the organelle, led to conclude that the original ROS formed in mitochondria were superoxide radicals. About 2–5 % of the consumed O_2 in mitochondria is derived toward the formation of this species. By further research and thanks to the use of inhibitors of the ETC, namely, rotenone and antimycin, it was found that the $O_2^{\bullet -}$ production sites reside in complex I and complex III (Fig. 2a) (Møller 2001; Sweetlove and Foyer 2004;

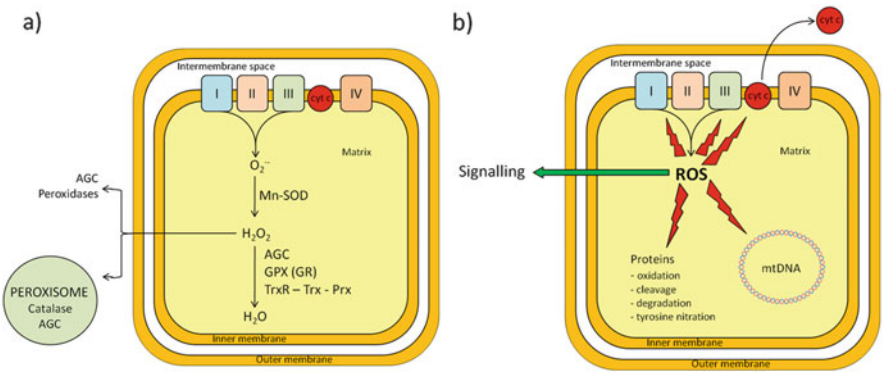

Fig. 2 Production and effects of ROS in mitochondria. (**a**) ROS production in mitochondria. Superoxide radicals ($O_2^{\bullet -}$) are generated at the complexes I and III from the electron transport chain located in the inner membrane. Mn–SOD disproportionates $O_2^{\bullet -}$ into H_2O_2 which, in turn, is removed by the ascorbate–glutathione (AGC) cycle enzymes in plants and in animal cells by a glutathione peroxidase (GPX) and a system involving thioredoxin (Trx), peroxiredoxin (Prx), and a thioredoxin reductase (TrxR). H_2O_2 can also come out of the mitochondria and be either scavenged in the cytosol by soluble peroxidases and the cytosolic AGC or driven to peroxisomes where catalase and AGC decompose it. (**b**) Effects of ROS on mitochondrial macromolecules. Under controlled conditions, ROS produced in mitochondria participates in signaling processes. However, when ROS generation exceeds the scavenging systems, ROS may attack mitochondrial DNA and trigger mutations, promote oxidation, cleavage and degradation or nitration of proteins, and favor the release of cytochrome *c* from the organelle membranes toward the cytosol, as it occurs in apoptosis

Gupta and Igamberdiev 2015). Rotenone inhibits the electron transfer from complex I (NADH–ubiquinone oxidoreductase) to ubiquinone, whereas antimycin binds to complex III (ubiquinol–cytochrome *c* oxidoreductase), thus avoiding this complex capturing electrons from the previous ETC components. A more precise study of the mitochondrial localization of $O_2^{\bullet -}$ production reported that this event develops in two ubiquinone pools: one associated to complex I and the other one linked to complex III (Raha and Robinson 2000; Popov 2015).

According to the mechanism of action of complexes I and III and the position of the respective ubiquinone pools in mammalian cells, it was postulated that $O_2^{\bullet -}$ generated in complex I was disposed of at the matrix of the organelle, whereas complex III dropped this ROS to the intermembrane space (Raha and Robinson 2000; Murphy 2009). In the matrix, $O_2^{\bullet -}$ dismutates by the action of a Mn–SOD (Fig. 2a), characteristic of mitochondria (del Río et al. 2002; Rodríguez-Serrano et al. 2007; Palma et al. 2013), and, in animal cells, the resulting H_2O_2 is detoxified by a selenium-dependent glutathione peroxidase (SeGPX) which, in turn, is coupled to a GR for the continuous provision of reduced glutathione (GSH). However, very few references have reported the presence of a CuZn–SOD in the intermembrane space, and this eventuality is far to be still consensed by the scientific community. H_2O_2 from the matrix can be pumped off to the cytosol through the mitochondrial membranes and then scavenged by diverse detoxifying systems such as peroxidases and the ascorbate–glutathione cycle or enters the peroxisomes, where catalase/ascorbate–glutathione cycle would decompose it. A thioredoxin–peroxiredoxin system located in the matrix could also remove H_2O_2 with the participation of a thioredoxin reductase which would utilize NADPH, provided by a NADP-dependent isocitrate dehydrogenase as electron donor (Murphy 2009). In plants, the presence of all enzyme components of the AGC in mitochondria has been demonstrated (Jiménez et al. 1997), and the participation of this pathway to remove H_2O_2 in this compartment is the most accepted issue for plant biologists (Fig. 2a) (Mittova et al. 2015). The necessary NADPH for the action of the GR is a common metabolite in plant mitochondria (Møller 2001). Alternative oxidase (AOX) has been reported to be activated when the reduction level of ubiquinone increases, so this is a dissipating mechanism which is also useful to prevent the overproduction of superoxide radicals (Maxwell et al. 1999; Rhoads et al. 2006; Gupta and Igamberdiev 2015).

Under certain stress conditions where H_2O_2 production overtakes the scavenging barriers and in the presence of transition metals, basically Fe^{3+} and Cu^{2+}, $^{\bullet}OH$ radicals can be formed in a Fenton-type reaction. Hydroxyl radicals could then be able to attack the mitochondrial genome provoking mutations in many of the ETC components which are encoded by the mitochondrial DNA (Fig. 2b) (Raha and Robinson 2000; Murphy 2009). ROS also damage proteins by diverse mechanisms which include oxidation, cleavage, and degradation of backbones and tyrosine nitration (Gupta and Igamberdiev 2015). Overall, ROS are important molecules to promote redox signaling events in mitochondria (Møller and Sweetlove 2010; Hebelstrup and Møller 2015), but under mitochondrial dysfunction, the overproduction of ROS under stress conditions and senescence ROS may lead to

apoptosis (programmed cell death, PCD) and necrosis. PCD is characterized by the release of cytochrome *c* from the inner mitochondrial membrane to the cytosol as a consequence of the damage (lipid peroxidation) undergone in membranes by ROS attack (Fig. 2b) (Murphy 2009).

3.1 Ascorbate Biosynthesis

A very important event in the antioxidant balance is the synthesis of ascorbate. This antioxidant molecule is synthesized by the great majority of phyla, excepting primates, rodents, and some others. Human cells lack the last enzyme of the ascorbate synthesis, the L-gulono-lactone oxidase, that makes human beings strictly dependent on an external ascorbate source, mainly fruits and vegetables. In plants, although several alternative pathways have been described, the main last step of the ascorbate biosynthesis is catalyzed by the L-galactono-lactone dehydrogenase (GalLDH), an enzyme which oxidizes L-galactono-lactone to ascorbic acid without the participation of any redox cofactor (Smirnoff 2001; Valpuesta and Botella 2004). GalLDH has been reported to be located in the inner mitochondrial membrane, neighbor to the ETC, and providing the electrons from the L-galactono-lactone to the terminal oxidase of complex IV (Bartoli et al. 2000). Thus, an interesting issue as a source of the investigation in plant antioxidant arises: ascorbate is synthesized in mitochondria but the major pool of this antioxidant is found in chloroplasts. The presence of ascorbate in other organelles suggest a very complex mechanism by which the ascorbate biosynthesis is triggered under certain stress conditions and how this important molecule is addressed to the diversity of organelles, mainly chloroplasts.

4 Plasma Membrane

Plant membrane-bound NADPH oxidase (NOX), also called respiratory burst oxidase homologue (RBOH), has the capacity to transfer electrons from intracellular NADPH across the plasma membrane to molecular oxygen in the apoplast site and generate $O_2^{\bullet -}$ which can then dismutate through different mechanisms to H_2O_2. *RBOH* genes belong to a multigenic family with 10 members in *Arabidopsis thaliana* (*RBOHA-RBOHJ*) and 9 in rice (*Oryza sativa*) but also with five groups of orthologous sequences (Torres et al. 2002; Sagi and Fluhr 2006; O'Brien et al. 2012; Skelly and Loake 2013).

The plant Rboh protein has two main components: (i) membrane-bound respiratory burst oxidase homologue (Rboh) with a molecular weight between 105 and 112 kDa (being homologue of $gp91^{phox}$ from mammalian phagocyte NAPDH oxidase) and (ii) its cytosolic regulator Rop (Rho-like protein) which is a Rac homologue of plants. Thus, the integral plasma membrane protein is composed of

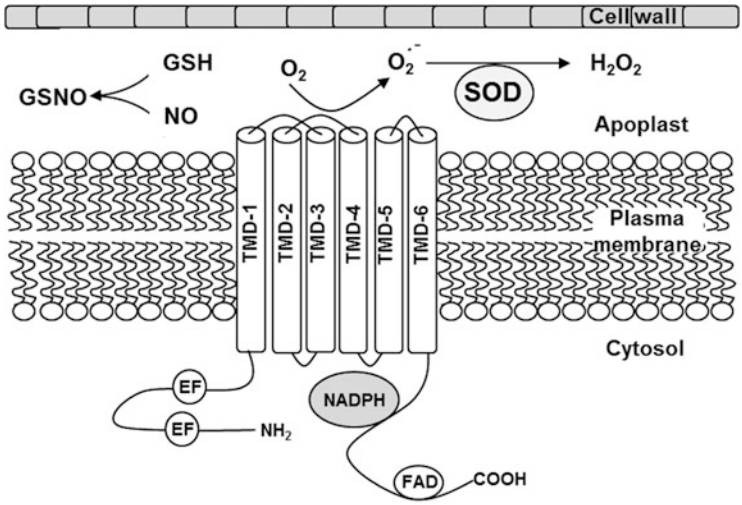

Fig. 3 Simple model of the structure and localization of the components of the plant membrane-bound respiratory burst oxidase homologues (RBOH) protein and other antioxidant elements. EF hand domains, FAD flavin adenine dinucleotide, GSH glutathione, GSNO S-nitrosoglutathione, NADPH reduced form of the nicotinamide adenine dinucleotide phosphate, NO nitric oxide, SOD superoxide dismutase, TMD-1 to TMD-6 transmembrane domains

six transmembrane domains (TMD-1 to TMD-6) connected by five loops (loops A–E) where TMD-3 and TMD-5 contain pairs of His residues required to bind two heme groups, C-terminal FAD and NADPH hydrophilic domains, and two N-terminal calcium-binding (EF-hand) motifs and some phosphorylation target sites (Yoshie et al. 2005; Marino et al. 2012) (Fig. 3). Besides this complex structure, there are also regulatory components involving phosphorylation and Ca^{2+} (Ogasawara et al. 2008) such as calcium-dependent protein kinases (CDPKs are Ser/Thr protein kinases that include a Ca^{2+}-binding calmodulin-like domain) (Kobayashi et al. 2007), Ca^{2+}/CaM-dependent protein kinase (CCaMK) (Shi et al. 2012), and Rop (Wong et al. 2007). Moreover, new mechanisms of regulation have been reported including phosphatidic acid binding (Zhang et al. 2009) and *S*-nitrosylation, which are posttranslational protein modifications mediated by nitric oxide-derived molecules (Corpas et al. 2015). Thus, in the *Arabidopsis* Rboh isoform D (AtRBOHD), the *S*-nitrosylation of Cys 890, thus abolishing the ability to generate $O_2^{\bullet-}$ (Yun et al. 2011), provides a clear interrelationship between reactive oxygen and nitrogen species.

Rboh is involved in many plant processes including cell growth (Foreman et al. 2003), plant development, stomatal closure (Shi et al. 2012), pollen tube growth (Kaya et al. 2014), symbiotic interactions (Marino et al. 2012; Kaur et al. 2014), abiotic stress, and pathogen response (Wojtaszek 1997; Torres et al. 2002; Daudi et al. 2012; Siddique et al. 2014). However, the number of Rboh isozymes which are differentially expressed suggests a certain grade of specialization for each one. For example, in *Arabidopsis thaliana* which has

10 genes, the focus has been pointed toward *AtRbohB*, *AtRbohC*, *AtRbohD*, and *AtRbohF*, especially *AtRbohD*, because it is constitutively and ubiquitously expressed (Kadota et al. 2014); however, the information about the other six *Rboh* genes is very scarce.

On the other hand, the apoplast space seems to be more complex than we could expect because it contains other elements such as SOD (Streller et al. 1997; Vanacker et al. 1998; Kukavica et al. 2005), the antioxidant glutathione (GSH) (Vanacker et al. 1999; Pignocchi and Foyer 2003), and nitric oxide (Stöhr and Ullrich 2002; Bethke et al. 2004). Thus, the SOD must regulate the H_2O_2 production during the dismutation of $O_2^{\bullet -}$ generated by Rboh being a mechanism of regulation of signaling between cells mediated by H_2O_2. Moreover, GSH and NO can interact to form *S*-nitrosoglutathione (GSNO), which is also recognized as a signaling molecule (Corpas et al. 2013), and can mediate the posttranslational modifications of proteins affecting their activities such as it occurs to ascorbate peroxidase (Begara-Morales et al. 2014).

Besides the mechanism of the local production of $O_2^{\bullet -}$ by Rboh, it has been proposed that after some stimuli (i.e., pathogens) and the generation of a local burst of ROS mediated by Rboh in an specific cells, there is a cascade of cell-to-cell communication events that carries a systemic signal over long distances throughout different tissues of the plants (see chapter "ROS as Key Players of Abiotic Stress Responses in Plants" of this book by Suzuki for deeper discussion) which opens a new perspective of the Rboh functions (Marino et al. 2012; Kaur et al. 2014).

5 Peroxisomes

Unlike other subcellular compartments, peroxisome is a single membrane-bounded compartment with a diverse range of specific metabolic functions depending on the tissue localization, the plant developmental step, and the environmental conditions (del Río et al. 2002; Mano and Nishimura 2005; Palma et al. 2009; Hu et al. 2012; Baker and Paudyal 2014). Among the principal functions of peroxisomes in plant cells, the fatty acid β-oxidation, the glyoxylate cycle, the photorespiration cycle, the metabolism of ureides, and the metabolism of reactive oxygen and nitrogen species (ROS and RNS) can be included, being the peroxisomal characteristic enzymes catalase and H_2O_2-generating flavin oxidases, which reflects a prominent oxidative metabolism. Table 1 summarizes the main peroxisomal ROS-producing systems and the involved enzymes.

5.1 H_2O_2-Producing System

Peroxisomal H_2O_2 generation is considered a side product of diverse pathways where peroxisomes are involved; however, the capacity to go through membranes

Table 1 Summary of the main ROS-producing systems and involved enzymes identified in peroxisomes from higher plants

Pathway	Peroxisomal enzyme	Reaction
H_2O_2-producing system		
β-oxidation	Acyl CoA oxidase (EC:1.3.3.6)	Acyl-CoA → *trans*-2-enoyl-CoA + H_2O_2
Photorespiration	Glycolate oxidase (EC 1.1.3.15)	Glycolate + O_2 → glyoxylate + H_2O_2
Sulphite detoxification	Sulfite oxidase (EC 1.8.3.1)	Sulfite + O_2+ H_2O → sulfate + H_2O_2
ROS metabolism	Superoxide dismutase (EC 1.15.11)	$O_2^{\bullet-}$ + $O_2^{\bullet-}$ + H+ → H_2O_2 + O_2
Purine metabolism	Urate oxidase (EC 1.7.3.3)	Uric acid + O_2 + H_2O → 5-hydroxyisourate+ H_2O_2 → allantoin + CO_2
Sarcosine metabolism	Sarcosine oxidase (EC 1.5.3.1)	Sarcosine + O_2 + H_2O → glycine + formaldehyde + H_2O_2 and L-pipecolate → Δ^1-piperideine-6-carboxylate + H_2O_2
Polyamine catabolism	Polyamine oxidase (EC 1.5.3.3)	Spermine + O_2 + H_2O → spermidine + 3-aminopropanal + H_2O_2
Superoxide-generating system		
Purine metabolism	Xanthine oxidase (EC 1.1.3.22)	Xanthine + O_2→ uric acid + $O_2^{\bullet-}$
Peroxisomal membrane polypeptides	PMP32 (membrane monodehydroascorbate reductase)	NADH + PMP32 → $O_2^{\bullet-}$

involves the capacity of this molecule to be used as a signal. Thus, peroxisomal fatty acid β-oxidation allows the breakdown of these molecules to acetyl-CoA and the subsequent conversion of acetyl-CoA to succinate via the glyoxylate cycle. In the β-oxidation pathway, the enzyme acyl-CoA oxidase catalyzes the conversion of acyl-CoA into trans-2-enyl-CoA with the concomitant generation of H_2O_2 (Arent et al. 2008). This pathway has a relevant physiological function because it allows the conversion of triacylglyceride pools in seedlings, the turnover of membrane lipids during senescence or starvation situation, as well the synthesis of fatty acid-derived hormones such as indole acetic acid (IAA), jasmonic acid (JA), and salicylic acid (SA) which consequently are involved in stress response and growth regulation (Poirier et al. 2006; Delker et al. 2007; Baker and Paudyal 2014). Photorespiration involves the light-dependent uptake of O_2 and release of CO_2 during the metabolism of phosphoglycolate, the two-carbon by-product by the oxygenase activity of Rubisco. This pathway involves several organelles (chloroplasts, mitochondria, and peroxisomes) with the peroxisomal glycolate oxidase generating H_2O_2.

There are other peroxisomal H_2O_2-producing enzymes but the available information on their function is still scarce. Thus, sulfite oxidase (SO) catalyzes the conversion of sulfite to sulfate with the concomitant generation of H_2O_2(Hänsch et al. 2006). It has been reported that low concentrations of sulfite inhibit catalase activity (Veljovic-Jovanovic et al. 1998), which could therefore be a means of regulating both enzymes. Sarcosine, also known as *N*-methylglycine, is an intermediate and by-product of glycine synthesis and degradation which also generates H_2O_2. The enzyme responsible is the sarcosine oxidase (SOX) which is a 46-kDa monomer that covalently attaches FAD molecule. Moreover, the SOX activity also catalyzes the conversion of L-pipecolate to Δ^1-piperideine-6-carboxylate plus H_2O_2 being a side branch of lysine catabolism (Goyer et al. 2004). In Arabidopsis, among the family of polyamine oxidases (PAO), it has been identified a peroxisomal isoform (AtPAO4) which is involved in polyamine catabolism especially in roots (Kamada-Nobusada et al. 2008; Planas-Portell et al. 2013).

5.2 Superoxide-Generating System

Xanthine oxidoreductase (XOR) is an FAD-, molybdenum-, iron-, and sulfur-containing hydroxylase enzyme that catalyzes the conversion of the purines hypoxanthine and xanthine into uric acid with the concomitant formation of either NADH or $O_2^{\bullet -}$ and plays an important role in nucleic acid degradation in all organisms (Harrison 2002). The enzyme is a homodimer, and each subunit contains one molybdenum atom, one FAD group, and two Fe_2S_2 centers. The molybdenum cofactor (Moco) present in XOR is also shared by other key enzymes that catalyze basic reactions in carbon, nitrogen, and sulfur metabolism, such as aldehyde oxidase, nitrate reductase, and sulfite oxidase (Schwarz and Mendel 2006). XOR exists in two interconvertible forms: an NAD-dependent dehydrogenase or xanthine dehydrogenase (XDH; EC 1.1.1.204), which can be converted into an oxygen-dependent oxidase or xanthine oxidase (XOD; EC 1.1.3.22). The presence of XOD activity in peroxisomes has been reported in different plant species (Sandalio et al. 1988; del Río et al. 1989; Mateos et al. 2003). More recently, additional biochemical and immunological results demonstrate the presence of XOR in leaf peroxisomes, showing that the XOD form, which generates superoxide radicals, is the predominant form in these oxidative organelles being differentially modulated under cadmium-induced oxidative stress (Corpas et al. 2008).

On the other hand, the peroxisomal membrane is another potential source of ROS, specifically $O_2^{\bullet -}$, through the existence of a small electron transport chain using NADH as electron donor. This is composed of a flavoprotein NADH:ferricyanide reductase of about 32 kDa and a cytochrome b (López-Huertas et al. 1999). The identity of the membrane protein of 32 kDa seems to be the enzyme monodehydroascorbate reductase (MDAR) since this enzyme has been described to be present in both matrix and membrane polypeptide of peroxisomes (Leterrier et al. 2005; Lisenbee et al. 2005). Additionally, using NADPH it was found a peroxisomal

membrane of 29 kDa that had the capacity to generate $O_2^{\bullet -}$ and to reduce cytochrome *c* (López-Huertas et al. 1999). The identity of this protein is not clear, but it could be related to the family of NADPH:cytochrome P450 reductase (López-Huertas et al. 1999).

5.3 *Peroxisomal Antioxidant Systems*

Besides the presence of catalase, a well-characterized peroxisomal antioxidant enzyme which keeps the H_2O_2 under control (Palma et al. 2013), there is another complementary antioxidant systems to regulate the content of $O_2^{\bullet -}$ and H_2O_2 in these organelles (Corpas et al. 2001).

In the case of the H_2O_2, plant peroxisomes enclose a particular ascorbate–glutathione cycle (Jiménez et al. 1997; Reumann and Corpas 2010) since its components have a special distribution with some membrane-bound enzymes such as the APX (Bunkelmann and Trelease 1996; Corpas and Trelease 1998) and the MDAR (Leterrier et al. 2005; Lisenbee et al. 2005) and others located in the matrix, such as the GR (Romero-Puertas et al. 2006) and also the DAR (Fig. 4). This peroxisomal system has been described to participate in the mechanism of response to different processes including growth (Narendra et al. 2006), leaf

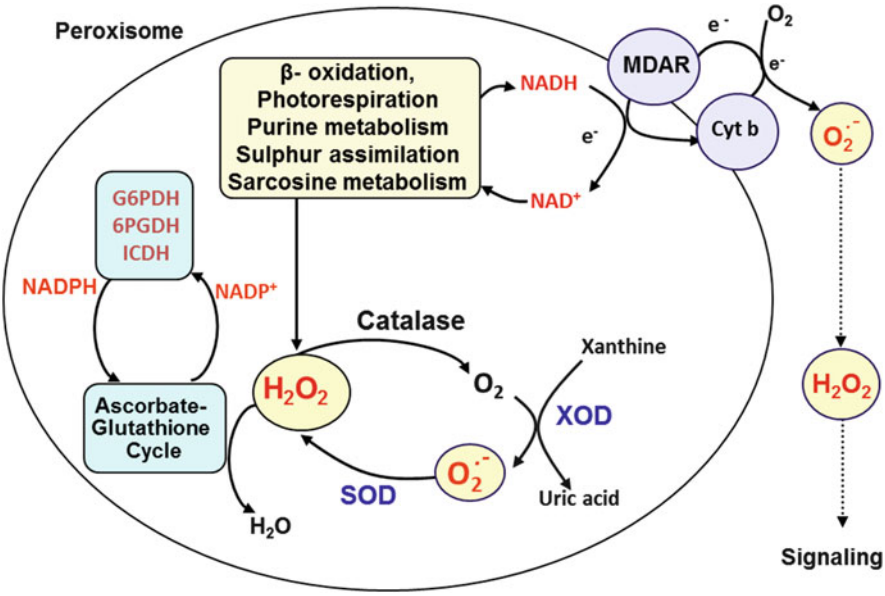

Fig. 4 Model of ROS production in plant peroxisomes. APX ascorbate peroxidase, G6PDH glucose-6-phosphate dehydrogenase, 6PGDH 6-phosphogluconate dehydrogenase, ICDH NADP–isocitrate dehydrogenase, MDAR monodehydroascorbate reductase, XOD xanthine oxidase

senescence (Jiménez et al. 1998; Palma et al. 2006), fruit ripening (Mateos et al. 2003), or heavy metal stress (Leterrier et al. 2005).

In animal cells, peroxisomes have been reported to contain exclusively a CuZn–SOD; however, in plant peroxisomes, it can be found, depending on the tissue and/or plant species and the three types of SOD isozymes, located in the matrix and/or in the membrane. Although the presence of either a CuZn–SOD or a Mn–SOD is the most common issue (del Río et al. 1983; Corpas and Trelease 1998; del Río et al. 2002), there are other cases where the presence of a Mn–SOD plus a CuZn–SOD (del Río et al. 2002) or a Fe–SOD has been demonstrated (Droillard and Paulin 1990).

Additionally, during the last decade, new components related with the peroxisomal metabolism of ROS have been discovered such as a closer family of molecules designated as reactive nitrogen species (RNS) (Corpas et al. 2013). All this indicates that peroxisomes enclose and complex nitro-oxidative apparatus characterized by a relevant flexibility which can adapt to fluctuating conditions.

6 Conclusions

In comparison to animal cells, higher plants have a most complex and active ROS metabolism under optimal environmental conditions which is in part consequence of the photosynthesis and photorespiration processes. ROS are obligated site products of many physiological pathways which are present in all cell compartments, including chloroplasts, mitochondria, plasma membrane, and peroxisomes. Although ROS have been considered as toxic molecules, this concept has changed because under a controlled production ROS are part of the mechanism of signaling or defense. This control is achieved by cellular complex of antioxidative systems which usually are close to the different sites of ROS production at subcellular level. However, under adverse environmental and/or certain physiological conditions, the cellular equilibrium between ROS production and scavenging could be broken and overcome the defense battery, which can provoke oxidative damage with fatal consequences for the normal cell functions. Future research is needed to get deeper knowledge and to decipher new mechanisms of regulation to keep under control the ROS production and their signaling implications in combination with RNS.

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What Do the Plant Mitochondrial Antioxidant and Redox Systems Have to Say Under Salinity, Drought, and Extreme Temperature?

F. Sevilla, A. Jiménez, and J.J. Lázaro

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Abstract Mitochondria are ubiquitous organelles with a notable oxidative metabolism. They are a significant site of reactive oxygen species (ROS) production in plant cells, including superoxide ($O_2^{\bullet -}$) and H_2O_2. In addition to ROS, there are compelling indications that nitric oxide (NO) can be generated in this organelle by both reductive and oxidative pathways. ROS and reactive nitrogen species (RNS) play a key role in signaling but they can also be deleterious via oxidation of cell components when overproduced as a consequence of adverse conditions. The high production of ROS obligates mitochondria to be provided with a set of

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ROS-scavenging mechanisms. The first line of plant mitochondrial antioxidants is composed of superoxide dismutase and the enzymes of the ascorbate–glutathione cycle, which are not only able to scavenge ROS but also to repair cell damage and possibly serve as redox sensors. Besides direct control by antioxidants, mitochondrial ROS production is tightly controlled by multiple redundant systems affecting inner membrane potential such as NADPH-dependent dehydrogenases, alternative oxidase (AOX), and uncoupling proteins. In addition, the presence of specific protein families responsible for dithiol/disulfide exchange reactions such as the thioredoxin (Trx), peroxiredoxin (Prx), and sulfiredoxin (Srx) families in the mitochondria has been recently reported. These proteins are critically important under some abiotic stress conditions by controlling the cellular redox status of thiol groups of cysteinyl residues as well as acting as antioxidant defense mechanisms. Here, we summarize the insights of the involvement of this Trx–Prx–Srx system and the ASC–GSH cycle components in plant tolerance to abiotic stress, more specifically in salinity, drought, and extreme temperatures, as some of the most important unfavorable environmental conditions for plant yield and growth. In the plant response to stress, it seems that not only the antioxidant but also the redox systems are emerging as key components functioning in both redox sensing and signal transduction pathways.

Keywords Abiotic stress • Antioxidants • Drought • Extreme temperatures • Mitochondria • Redox proteins • RNS • ROS • Salinity • Signaling

1 Introduction

Abiotic stress is defined as an environmental condition that reduces growth and yield below optimum levels, and in fact, environmental factors limit crop production by about 60 % (Boyer 1982). Plant responses to abiotic stresses are dynamic and complex and are dependent on the species, tissue, or organ. In addition, the application time, the level, and the duration of stress can have a significant effect on the complexity of the response. To survive under abiotic stress conditions, plants have developed a complex signaling network involving interactions and cross talk with many molecular pathways in which intracellular organelle signals and their interactions during stressful conditions represent one of the primary defense responses (Agrawal et al. 2011; del Río 2011). One of the most common molecular responses and one of the earliest signals in many abiotic stresses involve reactive oxygen species (ROS) and reactive nitrogen species (RNS), which modify enzyme activity and are involved in gene regulation. For instance, heavy metals, extreme temperatures (both heat and cold) or drought, and salinity are known to induce the production of ROS/RNS that play important roles in plant growth and development as well as in sensing environmental cues (Sandalio et al. 2001; Camejo et al. 2013; Foyer and Noctor 2013). However, ROS can also cause loss of redox homeostasis in

tissues, resulting in oxidative injuries in plants (Rodríguez-Serrano et al. 2009; Lázaro et al. 2013). In green tissues, although the main sources of ROS are chloroplasts and peroxisomes, mitochondria account for the total production generating ROS as a product of respiration (Moller 2001). The exposure to abiotic stress conditions causes a deregulation, an overflow, or even a disruption of electron transport chains (ETC) in mitochondria and chloroplasts, giving rise to ROS accumulation. Singlet oxygen ($^1O_2$), the hydroxyl radical ($^{\bullet}OH$), the superoxide radical ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) are all strongly oxidizing compounds and therefore potentially harmful for cell integrity. Among them, H_2O_2 is the most stable ROS formed in the reaction of $^1O_2$ with $O_2^{\bullet-}$ and as a product of spontaneous dismutation of $O_2^{\bullet-}$ (Foyer and Noctor 2009; Lázaro et al. 2013).

Mitochondria are central organelles in setting the cellular redox balance and homeostasis (Noctor et al. 2007). Changes in ROS levels due to the perturbation of the respiratory complex I have been proposed to trigger a mitochondrial retrograde signal (Rhoads and Subbaiah 2007) in which alternative oxidase (AOX) is one of the main components (Maxwell et al. 1999). Redox signals are probably transduced by oxidation of proteins such as ROS-activated transcription factors and kinases (Mittler et al. 2011). Moreover, changes in the thiol status of proteins are also probably involved in redox signaling under abiotic stress (Sevilla et al. 2015). Other molecules, including the antioxidants ascorbate (ASC) and glutathione (GSH), lipids, and organic acids, are modified by ROS, with implications for their signaling functions (Bartoli et al. 2004; Farmer and Mueller 2013). Similar to ROS, nitric oxide (NO) and NO-related molecules such as *S*-nitrosoglutathione (GSNO) also act as possible signals. These molecules regulate proteins, genes, and phytohormone signaling during environmental stress (del Río et al. 2002; Yu et al. 2014). The ROS detoxification system is essential for protecting organelles from the oxidative damage that can occur under abiotic stress conditions and transduction of the signal is considered as a primary response of defense (Camejo et al. 2013; Romero-Puertas et al. 2013).

In this context, the analysis of changes in gene expression and proteins or metabolites of a given cell compartment is a useful tool for understanding the effect of a specific stress situation and the response exerted by the different defense systems. Plant mitochondria contain a battery of nonenzymatic and enzymatic compounds able to modulate the ROS/RNS content produced during the normal metabolism and increased under stress conditions (Gómez et al. 1999; Mittler et al. 2004; Camejo et al. 2013). The first line of defense is Mn-SOD, which catalyzes the dismutation of $O_2^{\bullet-}$ to molecular oxygen and H_2O_2, and the ascorbate–glutathione (ASC–GSH) cycle. This pathway is in charge of the detoxification of H_2O_2, using NADPH as a reductant as well as the antioxidants ascorbate and glutathione (Jiménez et al. 1997, 1998a) and specific enzymes involved in the regeneration of the reduced forms of these antioxidants. Other enzymes involved in H_2O_2 and other hydroperoxides' detoxification are peroxiredoxins (Prxs), which, together with thioredoxin (Trx), thioredoxin reductase (TR), sulfiredoxin (Srx), and NADPH, constitute a system that ensures not only redox homeostasis in the

mitochondria but also signaling during normal and stress conditions (Pulido et al. 2009; Lázaro et al. 2013).

In this chapter, we review current knowledge on the involvement of plant mitochondria in abiotic stress, more specifically, in the response to salt, drought, and extreme temperature conditions. Special attention is paid to the ascorbate–glutathione cycle, AOX, and the Trxs/Prx/Srx response under these environmental conditions.

2 Mitochondria as Central Organelles in Stress

Mitochondria are highly dynamic, metabolically active cell organelles that are characterized by their involvement in processes like the tricarboxylic acid cycle and the production of ATP during oxidative phosphorylation. Mitochondria also have an important role in cellular proliferation, development, growth, and programmed cell death (Rhoads and Subbaiah 2007; Mittler et al. 2011). From a functional point of view, plant mitochondria differ from their animal counterparts by playing additional roles, as they house enzymes involved in vitamin biosynthesis and some enzymes of the photorespiratory pathway in photosynthetic tissues, as well as some additional alternative complexes which play an important role when the cells are exposed to metabolic dysfunction and/or stress (Rasmusson and Moller 1991; Ribas-Carbó et al. 2005a).

In the past two decades, it has become clear that mitochondria have developed mechanisms to communicate their biosynthetic and bioenergetic fitness to the rest of the cells, meaning that they also have signaling functions. Such mitochondria-to-nucleus communication named mitochondrial retrograde regulation (MRR) is necessary as the nucleus encodes most organelle proteins. Upon disturbance of the mitochondrial function caused by abiotic stress, mitochondria signal the nucleus to trigger the expression of responsive genes. In this process, organellar redox state and Ca^{2+} and ROS metabolism have been proposed as sources for retrograde signals, which provide the metabolic flexibility that, during stress conditions, plays an important role in the acclimation of plants (Rhoads and Subbaiah 2007; Woodson and Chory 2008). A common effect of all abiotic stresses is increased ROS and RNS production, which are particularly important in mitochondria, acting as a part of the signal transduction induced by stress conditions (Cvetkovska and Vanlerberghe 2012).

The mitochondrial electron transport chain is composed of four large protein complexes, some of them organized into supracomplexes: a proton-pumping NADH dehydrogenase (complex I), a succinate dehydrogenase (complex II), a Cyt bc1 complex (complex III), and a Cyt c oxidase (complex IV). Electron flow through complexes I, III, and IV results in the formation of an electrochemical proton gradient across the inner mitochondrial membrane. This drives the ATP synthase (complex V) to convert ADP to ATP. In addition to this classic ETC, plant mitochondria contain two additional non-phosphorylating pathways including

alternative NADPH dehydrogenases (type II NDH) and alternative oxidase (AOX) (Maxwell et al. 1999). Type II NDH bypass complex I and supply electrons to the ubiquinone pool and thus do not contribute to the generation of the proton motive force needed for ATP synthesis The cyanide-insensitive AOX oxidizes ubiquinol and reduces oxygen to water without translocating protons across the inner membrane. This alternative pathway bypasses the main proton-translocating complexes III and IV and oxidizes NADPH with varying levels of energy conservation and electron transport to help balance the energy status of the cell (Ribas-Carbó et al. 1997; Millar et al. 2011; Moore et al. 2013).

3 Mitochondrial ROS and RNS Production

3.1 ROS Production

An inevitable feature of mitochondrial biochemistry is the generation of ROS, such as $O_2^{\bullet -}$ and H_2O_2, associated with the electron transport chain. In plant cells, the first report that the respiratory chain produced ROS came in 1978 (Rich and Bonner 1978), 12 years after the pioneering work of Jensen (1966), who demonstrated $O_2^{\bullet -}$ generation in mammalian mitochondria. Within mitochondria, $O_2^{\bullet -}$ is produced by the one-electron reduction of O_2, and although it is a reaction thermodynamically favored, only a small proportion of mitochondrial electron carriers do so (Murphy 2009). These carriers include the redox-active prosthetic groups within proteins and complex I and complex III are probably the primary sites of $O_2^{\bullet -}$ generation (Noctor et al. 2007). Under specific conditions, complex II may also be involved in ROS generation, in the course of reverse electron transport (Turrens 2003). This superoxide can, in turn, act as substrate for the generation of H_2O_2 and hydroxyl radicals ($^{\bullet}OH$). An important generalization is that ROS formation increases as the ETC becomes more highly reduced (Moller 2001). Mitochondrial ROS generation is therefore higher in ADP-limiting conditions that increase the mitochondrial transmembrane potential and decrease when ADP is being actively phosphorylated by artificial uncoupler proteins (UCPs) that dissipate the transmembrane potential (Collins et al. 2012). Accepting electrons from ubiquinone, AOX may prevent the over-reduction of the ETC at complex I and/or III. Hence, this route of electron transport could be important for dampening ROS formation under conditions in which Cyt pathway (CP) components have suffered stress-induced damage or, since AOX respiration is less tightly coupled to ATP production, under conditions in which ADP availability is limiting (Maxwell et al. 1999; Millar et al. 2011). Thus, dissipation of the membrane potential directly by uncoupler components may be important in tissues with low AOX expression and/or activities (Trono et al. 2004). ROS accumulation in mitochondria can also be influenced by type II NDH, which could function as "safety valves," to limit ROS production by keeping the ETC relatively oxidized (Rasmusson and Wallström 2010; Millar et al. 2011), by the

activity and modification of antioxidant systems and oxygen concentration (Jiménez et al. 1998b), and by posttranslational modifications of respiratory complexes (Beer et al. 2004).

3.2 NO Production

In recent years, nitric oxide (NO) has emerged as an intracellular and intercellular signal molecule and an important metabolite in plants, where two major enzymatic pathways have been proposed as participating in its formation: the oxidation of L-arginine to L-citrulline by a nitric oxide synthase (NOS)-like enzyme and the reduction of nitrite to NO by a nitrate reductase (NR) (Fröhlich and Durner 2011; Gupta et al. 2011).

In the past decade, the presence of NOS-like activity has been demonstrated in plant peroxisomes. However, the characterization of such an enzyme is still unresolved (del Río et al. 2002). The reduction of nitrite to NO by mitochondrial ETC has been reported to produce small amounts of ATP under hypoxic conditions (Stoimenova et al. 2007). NO production by a mitochondrial nitrite-reducing activity (NR) has been detected in different photosynthetic sources and mitochondria isolated from roots of diverse plant species. Nitrite reduction occurs at the site of cytochrome c oxidase (COX), but it can also occur at the sites of complex III and AOX although a clear mechanism has only been established for COX under hypoxia. However, this may become increasingly important as partial pressures of oxygen are reduced from the ambient level (Igamberdiev et al. 2014; Yu et al. 2014). In mitochondria, the formation of RNS is due to NO's short half-life and reactivity. NO can gain or lose an electron, to form the nitrosyl anion (NO^-) or the nitrosonium cation (NO^+) and to form higher oxides including NO_2 and N_2O_3, or can react immediately with $O_2^{\bullet -}$ originated from ETC, to form peroxynitrite ($ONOO^-$). Through this reaction, superoxide probably plays a role in regulating free NO levels (Leitner et al. 2009). Moreover, NO can react with mitochondrial glutathione (GSH) to form GSNO or with thiols or the catalytic metal centers of proteins. This can result in covalent modification of cysteine residues in a process called S-nitrosylation and, in the NO binding to transition metals, through metal nitrosylation (Yu et al. 2014). The subcellular localization of GSNO in pea mitochondria has been reported (Camejo et al. 2013; Corpas et al. 2013) and it is considered as the most abundant low-molecular-mass *S*-nitrosothiol (SNO) and also a vehicle of NO in the cell, which enables NO biological activity to expand.

Prime targets of NO and its derivates in mitochondria are the electron transport components and enzymes, producing an inhibition of CP, whereas the alternative pathway is only partially inhibited (Day et al. 1996; Martí et al. 2012). These inhibitions may potentially be involved in the regulation of energy metabolism, generation of ROS, cell death, and response to stress. Recent evidences showed that pea Mn-SOD was not inactivated by NO upon mitochondrial treatment with the NO donor DETA-NONOate (Martí et al. 2012). The insensitivity of AOX to NO and the

potential capacity of Mn-SOD to bind and stimulate NO decay under anaerobic and aerobic conditions (Filipovic et al. 2007) represent mechanisms to prevent its deleterious effects on the respiratory activity.

4 Antioxidant and Redox Systems in Plant Mitochondria

As previously noted, mitochondria are important cellular organelles for orchestrating the plant stress response. The mitochondrial antioxidant system, through ROS and RNS detoxification and/or regulation, has a pivotal role affecting redox signaling.

4.1 AOX

As described above, this enzyme is a quinol oxidase that catalyzes the reduction of oxygen to water by directly accepting electrons from the ubiquinone pool, dissipating the energy as heat, and lowering the ADP/O ratio. The AOX is a nuclear-encoded protein linked to the mitochondrial inner membrane present in all kingdoms of life except in archaebacteria (McDonald and Vanlerberghe 2006). In angiosperms, a multigene family has been shown to encode the AOX protein (McDonald et al. 2009), and the genes are both tissue and development specific. In higher plants, AOX exists as a dimer and can be redox regulated by the formation of a disulfide bridge between conserved cysteine residues of each monomer (Vanlerberghe 2013). The reduction level and amount of UQ pool and, in some situations, the concentration of AOX protein are thought to be important in determining the extent of AOX activity (Ribas-Carbó et al. 1995, 1997). Nevertheless, the AOX protein can be activated by posttranslational modifications. Biochemical characterizations have reported that AOX can be both reduced and activated by mitochondrial thioredoxin (PsTrx*o*1) by using its effector pyruvate (Gelhaye et al. 2004). Similarly, pea mitochondrial AOX homodimers are specifically reduced by PsTrx*o*1. This thioredoxin was also able to produce the activation of oxygen consumption by the AOX pathway, using NADPH/NTR system (Martí et al. 2009). While the redox regulation of AOX has been well documented in vitro, its impact on the in vivo activity of alternative oxidase pathway remains controversial (Millenaar and Lambers 2003). A better understanding of the regulation of the AOX protein has been provided by recent advances made in its molecular characterization (McDonald et al. 2009; Kido et al. 2010) that opens new exciting opportunities for the AOX research on the relationship between its structure, regulation, and function.

4.2 Mn-SOD

Manganese superoxide dismutase (Mn-SOD) is a tetrameric isoenzyme initially purified and characterized in pea leaves, as a result of a pioneering work led by Prof. LA del Río (Sevilla et al. 1982). The enzyme is important in providing protection against oxidative stress controlling H_2O_2 production, thus avoiding the formation of $^{\bullet}OH$ radicals (Marques et al. 2014). Mn-SOD suppression in *Arabidopsis* causes decreased growth, although the respiration rate was not affected, whereas the mitochondrial redox balance and some of the tricarboxylic acid cycle enzymes were altered. Unexpectedly, Mn-SOD mutant plants displayed an increased antioxidant capacity, suggesting the existence of a retrograde pathway trying to compensate the lack of this antioxidant enzyme (Morgan et al. 2008). As a result of Mn-SOD, the newly formed H_2O_2 can be decomposed by the mitochondrial peroxidase activities that depend on the ascorbate and glutathione through the ASC–GSH cycle or/on the Trx–Prx–Srx system.

4.3 ASC–GSH Cycle

The first publications describing the presence of the some components of the so-called ascorbate–glutathione cycle or Foyer–Halliwell–Asada pathway in mitochondria (Fig. 1) appeared in 1981 and 1990 with MDHAR and GR of potato and pea mitochondria, respectively (Arrigoni et al. 1981; Edwards et al. 1990). This pathway is considered to be a crucial mechanism for H_2O_2 metabolism in which the heme-containing ascorbate peroxidase (APX) scavenges H_2O_2 using reduced ascorbate that is oxidized to monodehydroascorbate (MDA) or dehydroascorbate (DHA). These oxidized forms are reduced by FAD-containing monodehydroascorbate reductase (MDHAR), using NADPH as reductant, and dehydroascorbate reductase (DHAR), using GSH as electron donor, respectively. The flavoprotein glutathione reductase (GR) is the enzyme in charge of the reduction of oxidized GSSG in a NADPH-dependent manner (Foyer and Halliwell 1976; Asada 1999). In mitochondria, the final proof of the existence of a complete cycle, similar to that in chloroplasts, was described in pea leaves (Jiménez et al. 1997) and further in *Arabidopsis* (Chew et al. 2003). Biochemical and enzymatic latency assays indicated that the mitochondrial APX activity resulted in at least two isozymes in pea mitochondria with different degrees of substrate specificity and sensitivity to inhibitors, when compared to that found in peroxisomes and chloroplasts (Jiménez et al. 1998a). These APX isozymes were linked to both the inner and the outer face of the external membrane in concert with membrane-associated MDHAR, while DHAR and GR were located in the matrix, together with the antioxidants ascorbate and glutathione (Fig. 1). The possible presence of the APX isozymes linked to the inner face of the external membrane was reported in *Arabidopsis* mitochondria by Chew et al. (2003). It was reported that the differential sub-compartmental

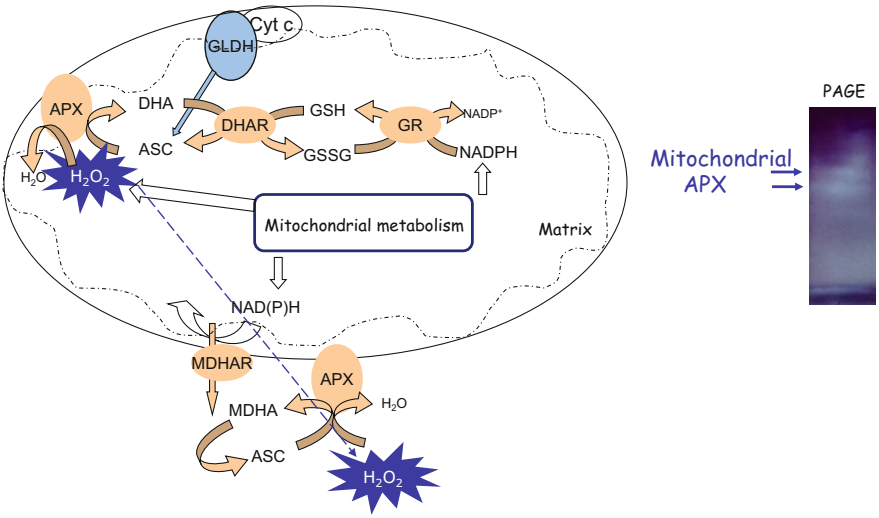

Fig. 1 Mitochondrial ascorbate–glutathione cycle. The hydrogen peroxide in the mitochondria produced by the organelle metabolism is reduced by APX at the expense of ASC to produce DHA that is reduced to ASC by DHAR using GSH as electron donor. The resulting GSSG is regenerated by GR and NADPH. MDHA is reduced back to ASC by MDHAR using NADPH from the mitochondrial metabolism. In the gel, APX isoenzymes detected in isolated pea mitochondria are revealed after PAGE and NBT staining (Jiménez et al. 1998a)

localization of the two APX isoenzymes may indicate slightly divergent roles in ASC recycling: (1) protection against H_2O_2 as it leaks out of mitochondria, thereby preventing oxidative damage to other cell compartments, acting together with MDHAR, and (2) clearance of intramitochondrial H_2O_2 in concert with DHAR and GR (Jiménez et al. 1998a; del Río et al. 1998). The presence of one isozyme of APX on the intermembrane space side of the inner membrane may be also useful for using the ASC generated at this site. Reports including those from Christine Foyer's group described that ascorbate is produced in mitochondria by the terminal enzyme L-galactono-1,4-lactone dehydrogenase (GalLDH) and showed its coupling to the CP has been demonstrated (Bartoli et al. 2000). This enzyme is also attached to the inner mitochondrial membrane, localized on a subset of respiratory chain complex I, and its activity is highly dependent on the availability of oxidized Cyt c (Millar et al. 2003). In addition to the reductive GSH-dependent DHA reduction, ASC regeneration may also be attributed to the respiratory electron transport chain (Szarka et al. 2007) or linked to other redox compounds such as the glutaredoxin (Grx) and Trx systems (Meyer et al. 2012).

The antioxidant role of ascorbate in plants is evident from the relatively high concentrations found in many tissues and in subcellular organelles. On average, the ASC concentration in *Arabidopsis* cells is 5.6 mM (Zechmann et al. 2011). The cellular ASC abundance and homeostasis has profound effects on gene transcription, mainly those regulating plant growth and defense. This is mediated, at least in part, through changes in the plant hormone, abscisic acid (ABA) (Kerchev

et al. 2011). Together with the mitochondria, ASC is found in many subcellular organelles, including peroxisomes, vacuoles, cytosol, the cell wall, and chloroplasts, where concentrations can reach up to 50 mM (Rautenkranz et al. 1994; Jiménez et al. 1997; Takahama 2004). The mitochondrial metabolism has been described as being involved in the modulation of photosynthesis through the suggested complementation of AOX and ASC metabolism in this way protecting against photoinhibition. Changes in mitochondrial ASC synthesis dependent on respiration could regulate retrograde signaling as a common signal from both mitochondria and chloroplasts (Talla et al. 2011). A good example of such an inter-organelle communication is the ASC produced in the mitochondria and then transported into the apoplast. Interestingly, ASC appears to exert its greatest influence by setting thresholds for apoplastic and cytoplasmic signaling (Munné-Bosch et al. 2013). When the ASC and GSH contents are depleted, balanced mechanisms exist to maintain the processes dependent on these antioxidants as well as related signaling responses in specific compartments (Foyer and Noctor 2011).

Glutathione is a key component in the regulation of cellular thiol redox homeostasis. It also plays key roles in the ROS detoxification, redox signaling, modulation of defense gene expression, and the regulation of enzymatic activities (Schnaubelt et al. 2013). In this way glutathione may influence plant development not only under optimal conditions but also in stress situations (extensively reviewed by Noctor et al. 2012; Kocsy et al. 2013; Schmitt et al. 2014). Additionally, glutathione through the posttranslational modification known as glutathionylation is involved in protecting proteins from oxidation (Zaffagnini et al. 2012). Although cellular compartments and changing growth conditions may influence its levels, the biosynthesis of GSH is mainly localized in cytosol and plastids: glutamate cysteine ligase is stromatic, whereas GSH synthetase is targeted to the stroma and cytosol (Preuss et al. 2014). Once synthesized, GSH is transported to mitochondria, although the nature and regulation of these transporters are still unclear (Bachhawat et al. 2013). The presence of glutathione was reported in pea and *Arabidopsis* mitochondria, and in fact, immunolabeling studies have localized this antioxidant in *Arabidopsis* in both mitochondria and chloroplast, containing about 15–25 % and 62–75 %, respectively, of the total pool of GSH (Jiménez et al. 1997; Fernández-García et al. 2009). The role of glutathione in mitochondria is quite an important issue. It seems that the depletion and oxidation of glutathione in mitochondria favors the accumulation of ROS found in senescent leaves (Jiménez et al. 1998b) and could be one reason for the induction of the programmed cell death events after pathogen infection in *Nicotiana tabacum* plants (Zechmann et al. 2014). The involvement of mitochondrial GSH in cell development has been reported in studies using both *rml1* and *pad2-1* mutant (see Zechmann and Müller 2010) pointing to the mitochondrial glutathione level as a key component for the proper plant development in situations when this antioxidant is depleted. Another example of the importance of glutathione in mitochondria is its role in NO scavenging

forming GSNO, which can be further metabolized to oxidized glutathione (GSSG) and NH_3 (Wilson et al. 2008).

As in the case of ASC, a link between complex I (CI) activity and GSH has also been shown in CI *Arabidopsis* mutants. These mutants were insensitive to a GSH biosynthesis inhibitor and showed higher levels of GSH, implying an as yet unexplained effect of mitochondrial respiration on GSH homeostasis (Koprivova et al. 2010).

4.4 Peroxiredoxin System

Peroxiredoxin, thioredoxin, thioredoxin reductase, sulfiredoxin, and NADPH all together constitute a system that ensures not only redox homeostasis in the mitochondria but also signaling. Prxs are thiol peroxidases involved in peroxide detoxification and signaling. They are ubiquitous, are located in different cellular compartments, and are expressed as different isoforms. Plant mitochondria, unlike its mammalian counterpart, have only one type of Prx, PrxIIF, an atypical Prx highly conserved between different species that contains two residues of cysteine (Barranco-Medina et al. 2007; Dietz 2011). Both residues are essential for efficient catalysis, and after hydroperoxide reduction, they form an intramolecular disulfide bridge that is reduced by mitochondrial Trx*o*. Mitochondrial PrxIIF crystallizes as hexamers (Barranco-Medina et al. 2006) which are favored in oxidant conditions but dissociate to dimers upon reduction. The presence of peroxidatic cysteine is critical for hexamer formation, whereas the resolving cysteine is not necessary (Barranco-Medina et al. 2007, 2008b). Posttranslational modification by S-nitrosylation of mitochondrial PrxIIF induces a conformational change in the protein and provokes a reduction in its peroxidase activity while acquiring a novel function as transnitrosylase that is fundamental for the signal transduction role of NO in plants (Camejo et al. 2015).

The involvement of Trx*o* in the detoxification of ROS via PrxIIF has been demonstrated in vivo by interaction between both mitochondrial proteins and in vitro with recombinant proteins (Barranco-Medina et al. 2008a; Martí et al. 2009). Trx harbors a dithiol active site and regulates target enzymes by thiol/disulfide exchange. The presence of Trx in plant mitochondria was shown by Laloi et al. (2001) in *Arabidopsis* and was classified as Trx*o* type. More recently, a pea Trxo was described in both mitochondria and nucleus under normal conditions, and similarly to PrxIIF, pea Trx*o* reduced mitochondrial alternative oxidase (AOX) homodimers (Martí et al. 2009). As we have described previously, Trx*o* through activation of PrxIIF and AOX could also play a role in linking ROS and redox signaling in mitochondria. Mitochondrial Trx*o* is reduced by a homodimeric TR firstly reported by Laloi et al. (2001), which in turn is reduced by NADPH

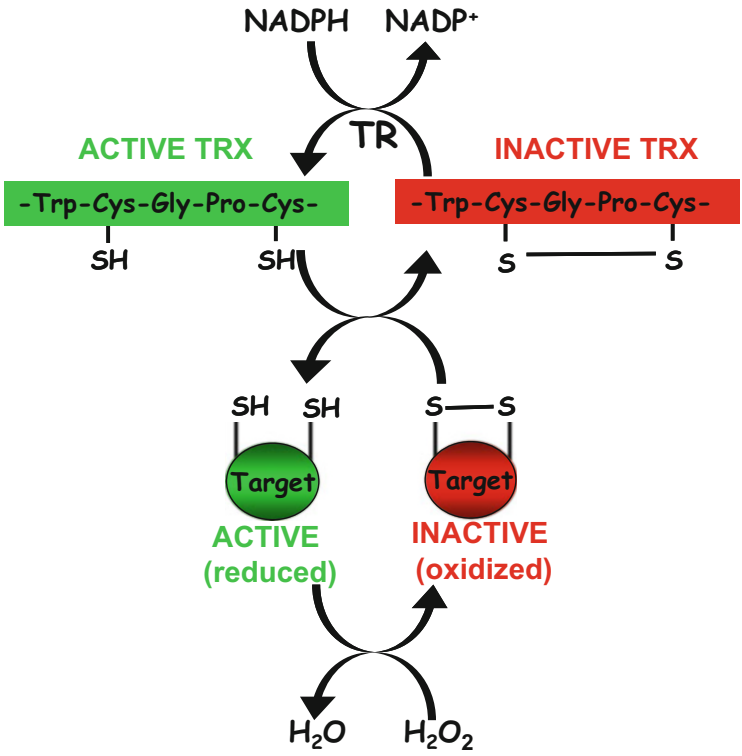

Fig. 2 Thioredoxin (Trx) system. Trx is reduced by NADPH-dependent Trx reductase (TR). Reduced Trx can reduce in turn target proteins from their oxidized forms

(Gelhaye et al. 2005) (Fig. 2). In *Arabidopsis*, there are three TRs, namely, TRA, TRB, and TRC, localized to different cellular compartments, cytosol, mitochondria, and chloroplast, respectively (Serrato et al. 2004).

Under severe oxidative stress, PrxIIF overoxidizes to the inactive sulfinic form, which could serve as a sensor of the hyperoxidative conditions inside the mitochondria. An enzyme called Srx, a special type of ATP-dependent reductase, located in chloroplasts and mitochondria is able to retroreduce the overoxidized form of chloroplastic 2-Cys Prx and mitochondrial PrxIIF (Biteau et al. 2003; Iglesias-Baena et al. 2010, 2011). The interaction between Srx and its mitochondrial targets PrxIIF and Trxo has been proved, and the mechanism of reduction of the sulfinic PrxIIF is similar to the sulfinic 2-Cys Prx, via formation of a phosphoryl intermediate on the sulfinyl moiety, a thiosulfinate between Srx and PrxIIF, and finally, a heterocomplex Srx–Trx. Both complexes strengthen the mechanism for sulfinic PrxIIF reduction by Srx into the plant mitochondria (Iglesias-Baena et al. 2011) (Fig. 3).

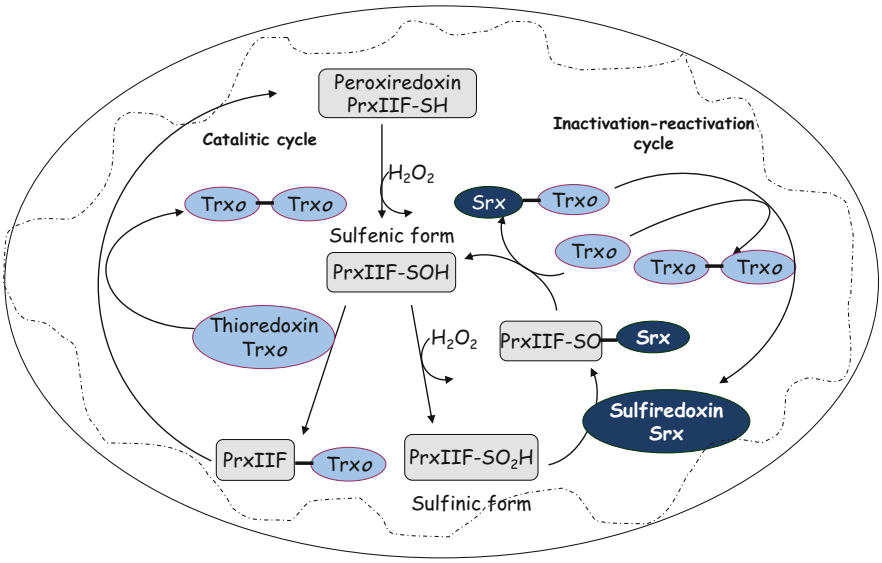

Fig. 3 Catalytic cycle of mitochondrial PrxIIF overoxidation. Under physiological conditions, mitochondrial PrxIIF is oxidized to its sulfenic form acting as peroxidase. At high concentration of H_2O_2, PrxIIF may be overoxidized to the inactive sulfinic form (PrxIIF-SO_2H) that is converted into sulfinate (PrxIIF-SO-S-Srx) with Srx. Mitochondrial Trx*o* reduces the heterocomplex to release PrxIIF-SOH and Srx-Trx*o* which is reduced to Srx-SH by Trx*o*. The sulfenic form of PrxIIF is reduced by Trx*o* that forms PrxIIF-Trx*o* and the active PrxIIF-SH is released by another Trx*o* that forms the dimer Trx*o*–Trx*o*

5 Mitochondrial Antioxidant and Redox System Are Involved in Abiotic Stress Response

Environmental factors have important consequences for plant growth and survival and seriously limit crop production. FAO reports have informed that almost 70 % of the global land area is affected by some environmental constraint (see review by Cramer et al. 2011). Thus, abiotic stresses including salinity, temperature extremes, and drought are a subject of increasing interest due to the significant impact on food yields in a scenario of continued reduction of arable land and water resources and climate change (Lobell et al. 2011).

5.1 Mitochondrial Response Under Salinity

Soil salinization is regarded as a problem for agriculture in the world. In these conditions, the ability of plants to take up enough water decreased, while the Na^+ and Cl^- taken up by roots impair growth by causing an osmotic imbalance, ion

toxicity, the alteration of membranes, redox imbalance, cellular energy depletion, and/or the disruption of photosynthesis (Abogadallah 2010; Maathuis et al. 2014). The response of plants to salinity is complicated and involves metabolic and genetic adjustments. This response is dependent on the salt tolerance of the plant and the severity and duration of the stress. Among the different mechanisms involved in the response, ROS are considered as essential components of salt tolerance, and several examples are known of enhanced capacities to scavenge ROS in mutants or transgenic plants showing higher salt tolerances (Hasegawa et al. 2000). ROS generation generally increases in mitochondria under salinity due to a dysfunction in the respiratory electron transport chain, increasing the rate, with consequent electron leakage to O_2 (Hernández et al. 1993, 2001). In pea plants grown under salinity, the generation of $O_2^{\bullet -}$ and H_2O_2 has been demonstrated in mitochondria of two pea cultivars differing in NaCl sensitivity (Hernández et al. 1993). In tobacco, it was shown that short-term salt stress resulted in a strong increase in leaf $O_2^{\bullet -}$, with only a moderate increase of H_2O_2 (Andronis and Roubelakis-Angelakis 2010). Most reports on mitochondria isolated from plants growing in saline conditions demonstrate an inhibition of the respiratory rate with a decreased enzymatic capacity (see review by Jacoby et al. 2010), although this does not mean a decrease in in vivo respiratory activity. As an example, in *Arabidopsis* after 6 h of saline stress, cytochrome c oxidase decreased transcript and protein abundance, while NDH increased its transcript level but decreased the protein level (Jacoby et al. 2010). The presence of NDH and AOX confers a higher flexibility and complexity to plant ETC than its mammalian counterpart. AOX could represent a key part of a larger stress response at mitochondrial level (Clifton et al. 2006; Elhafez et al. 2005). Oxygen isotope fractionation is presented as the most reliable technique for studying the regulation of the electron partitioning between the two respiratory pathways (Lambers et al. 2005). When the effects of salinity on respiration and partitioning were investigated, the alternative pathway (AP) was found to increase its contribution to respiration during long-term (14 days) NaCl stress conditions in pea plants (Martí et al. 2011) similar to that found in severe drought conditions (Ribas-Carbó et al. 2005b). However, an observed decrease in total respiration was mainly due to a decrease in the CP activity, whereas AP activity remained constant (Florez-Sarasa et al. 2007). This decrease in CP was thought to be due to a reduced energy demand for growth and to the control exerted by an increase in the ATP/ADP ratio (Munns 2002), as was observed from the significant reduction in leaf growth under saline conditions. The maintained AP activity may diminish ROS generation in the mitochondria under saline conditions and reflect the presence of the sustainable active form of AOX, in which the increased PsTrxo1 activity observed could play a role in the response of AOX to salinity which is also reflected at transcript and protein content levels (Martí et al. 2009). Moreover, the analysis of co-expression of AOX genes and other components of the ETC, as external and internal NDH, has revealed that these respiratory components could work together to oxidize external NADPH under a number of conditions including salinity. Another interesting point is that the lack of AOX in antisense *AOX1a Arabidopsis* plants under salinity and drought altered transcript abundance of other

mitochondrial proteins such as glutaredoxin (Grx) and APX6 as well as several antioxidative chloroplast and cytosol proteins (Umbach et al. 2005; Giraud et al. 2008). These changes pointed AOX as a key component in adaptation through the coordination to other antioxidative defenses in the different cell compartments (Clifton et al. 2006). However, the correlation between AOX expression and activity is not always positive and both were induced by salt in shoots, roots, and cell cultures of different plants (see reviews by Jacoby et al. 2010; Lázaro et al. 2013) while discrepancies have been reported and discussed (Guy and Vanlerberghe 2005; Ribas-Carbó et al. 2005b; Vidal et al. 2007; Rasmusson et al. 2009; Martí et al. 2011), revealing the importance of the posttranscriptional and posttranslational regulation of AOX under salinity stress conditions. Another interesting aspect of the response of mitochondria to salinity is the increased abundance of proteins involved in mitochondrial ROS defense, pointing to a huge impact of salinity on the organelle function in vivo. Under salt stress, this enhancement of antioxidants is necessary to defend the mitochondrial components, including respiration function, against increased ROS generation, thus preventing higher damages (Hernández et al. 1993; Sweetlove et al. 2002; Tan et al. 2010). Again, the response is variable. Thus, different antioxidative enzyme isoforms in the cell compartments can present specific profile activity in cultivars differing in salt tolerance (Hernández et al. 1993, 2000; Olmos et al. 1994; Gueta-Dahan et al 1997; Ashraf 2009). Among them, Mn-SOD is a key stress-responsive enzyme in mitochondria. Under salt stress, Mn-SOD activity is increased in various plants like chickpea pea and liquorice plant (Gómez et al. 1999; Eyidogan and Oz 2005; Pan et al. 2006), while its protein content was maintained during the salt treatment in pea plants (Martí et al. 2011; Camejo et al. 2013) or increased in *Arabidopsis* (Jiang et al. 2007). The fact that increased antioxidant activity can prevent the oxidative damage accompanying salt stress was evident in overexpressing Mn-SOD plants in different transgenic plants which showed increased salt tolerance (Wang et al. 2004, 2005). Together with Mn-SOD activity, the activities of Cu/Zn-SOD, Fe-SOD, CAT, and POD were significantly higher in transgenic *Arabidopsis* than in wild-type plants (Wang et al. 2004). Another example of collaboration among antioxidative proteins is the observed coordinated increase in wheat AOX and Mn-SOD proteins which could prevent the over-reduction of the mitochondrial ubiquinone pool, decreasing the content of $O_2^{\bullet -}$ in this organelle (Jacoby et al. 2010). Similarly, in pea mitochondria, an increased expression and protein components of the ascorbate–glutathione cycle, mainly APX and MDHAR, were enhanced under salinity conditions (Gómez et al. 1999). The importance of the subcellular compartments in the response of these enzymes to salt stress was evidenced by these authors showing that the pattern of changes was found different in pea chloroplast isoenzymes. In this organelle, APX behaved differently in thylakoids and stroma in response to NaCl, with a significant increase of stromal APX, whereas the thylakoidal activity was significantly and progressively lost (Gómez et al. 2004). Moreover, GR and DHAR but not MDHAR activities were significantly enhanced. All these inductions in the different cell compartments are thought to contribute to preventing ROS formation and oxidative damage, giving

rise to the tolerance to salt stress in the studied pea cultivar. In sweet potato, the expression of mitochondrial APX together with cytosolic and chloroplast isoforms was tissue specific and dependent on salt stress duration (Lin and Pu 2010). Other authors indicated a higher expression and activities of mitochondrial APX, MHAR, DHAR, and GPX in a salt-tolerant variety of *Lycopersicon pennellii* plants (Mittova et al. 2003). Likewise, the overexpression of MDHAR in transgenic tobacco increased the tolerance against salt and osmotic stresses (Eltayeb et al. 2006) and the overexpression of DHAR increased salt tolerance in *Arabidopsis* (Ushimaru et al. 2006), revealing the importance of these antioxidant components in the tolerance to salinity conditions.

Less information exists related to the involvement of mitochondrial antioxidants ASC and GSH in the salt stress response. Mitochondria are the organelles responsible for ascorbate biosynthesis and although glutathione synthesis is not mitochondrial, glutathione also shows highly compartment-specific changes in plants during stress situations, indicating important subcellular roles for this antioxidant in plant defenses (Zechmann 2014). To sense the oxidative stress, the reduced/oxidized balance of ASC and GSH is crucial for the cell response (Mullineaux and Rausch 2005; Foyer and Noctor 2009, 2011), taking into account their known influence on gene expression (Munné-Bosch et al. 2013). Abiotic stress usually decreases the content of these antioxidants, and balance mechanisms exist to maintain ASC- and GSH-dependent processes in specific compartments (Foyer and Noctor 2011). Therefore, during stress situations, subcellular changes in the glutathione contents may reflect the occurrence of compartment-specific oxidative stress. Moreover, it seems that high and stable levels of this antioxidant in the mitochondria are essential for cell development and survival especially in stress situations in which glutathione is depleted. However, in mitochondria, information about ASC and GSH contents and redox state is scarce and their accurate role in these organelles under abiotic stress is not well established (Lázaro et al. 2013).

The role of Prx system in plant mitochondria in response to abiotic stress is not widely reported in the literature. Nevertheless, there are data that allow the attribution to this system of a redox sensing and signal transduction function (Finkemeier et al. 2005; Dietz 2011; Martí et al. 2011; Lázaro et al. 2013). There are results that suggest a role for mitochondrial PrxIIF in antioxidant response and as a sensitive marker for stress. Transcript and protein levels of this PrxIIF were upregulated in leaves of pea, but not in roots, under salinity and cadmium stress (Barranco-Medina et al. 2007) both causing oxidative stress in sensitive species (Sandalio et al. 2001). However, this contrasts with the results obtained by Horling et al. (2002, 2003) where no changes were observed in PrxIIF mRNA levels in *Arabidopsis* leaves under salt, light, ozone, and hydrogen peroxide treatment. These partially discrepant results in *Arabidopsis* and pea could be attributed to the higher sensitivity of pea (Metwally et al. 2005). On the other hand, in *Arabidopsis* PrxIIF knockout mutants, PrxIIF seems not to be essential for plant survival, and lack of this enzyme in *Arabidopsis* PrxIIF knockout mutants might be compensated by increased mitochondrial ascorbate peroxidase activity or by the presence of the ascorbate–glutathione cycle (Finkemeier et al. 2005; Lázaro et al. 2013). In the response to salt stress, pea mitochondrial PrxIIF was S-nitrosylated over a long time

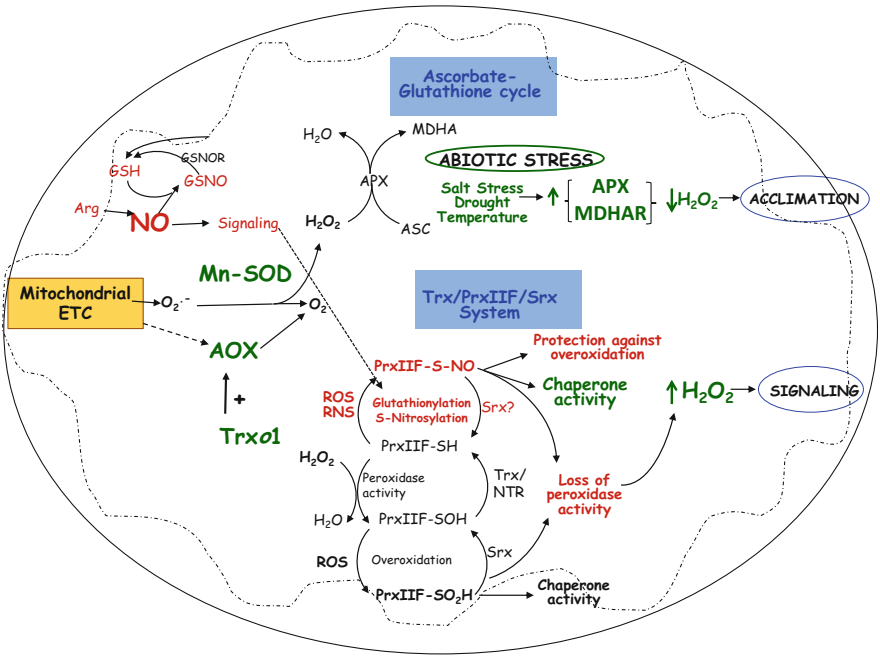

Fig. 4 Interaction between antioxidant and redox systems in ROS and RNS signaling in plant mitochondria under salinity. Superoxide radicals ($O_2^{\bullet -}$) produced by the mitochondrial ETC are dismutated to H_2O_2 by Mn-SOD. H_2O_2 is reduced by the ascorbate–glutathione cycle and the Trx/PrxIIF/Srx system (*in blue*). APX and PrxIIF scavenge H_2O_2 throughout the cycles indicated in Figs. 1 and 3. Under oxidative stress during salinity, AOX activity is induced, potentially decreasing ROS generation by ETC. In parallel, APX and MDHAR activities increased, which produces a decrease in the concentration of H_2O_2 allowing acclimation (*in green*). Also PrxIIF-SOH can be overoxidized to the sulfinic form (PrxIIF-SO_2H) gaining a chaperone activity and losing its peroxidase activity, increasing H_2O_2 concentration in the mitochondria allowing signaling. PrxIIF-SO_2H can be regenerated to the reduced form by the action of Srx and Trx. NO generated in the mitochondria during stress allows signaling in addition of forming GSNO by reduction with GSH from ascorbate–glutathione cycle (*in red*). NO also reacts with $O_2^{\bullet -}$ from the ETC, to form $ONOO^-$ that bursts nitrosative stress. Under nitrosative stress, PrxIIF-SH can be glutathionylated or S-nitrosylated in order to protect the enzyme against overoxidation and to gain chaperone activity, losing the peroxidase activity and allowing the signaling by H_2O_2 (*in red*). These posttranslational modifications could be reverted to PrxIIF-SH by the Srx activity. A similar pattern of induction of the AOX, Mn-SOD, ASC–GSH components, and Prx system has been reported under drought and extreme temperatures (see text)

period, possibly responding to increased NO under long-term salt stress (Camejo et al. 2013). It has been shown that salt stress modifies the S-nitrosylation level of some proteins in *Arabidopsis* (Fares et al. 2011). Subsequently, we have demonstrated that recombinant PrxIIF is S-nitrosylated by in vitro GSNO treatments, resulting in a conformational change and functional switch. Thus, PrxIIF activity was reduced acquiring a new transnitrosylase activity, which could probably function as a protective mechanism under conditions inducing oxidative and nitrosative stress (Camejo et al. 2015) (Fig. 4).

The involvement of mitochondrial TR in oxidative stress has been shown in yeast and mammalian (Lopert et al. 2012; Greetham et al. 2013). Serrato et al. (2004) have observed that *Arabidopsis* chloroplast TR knockout mutant lines showed growth inhibition and hypersensitivity to salt stress, drought, and methyl viologen. To our knowledge, there is no information about the response of mitochondrial TR to salinity as well as of Srx, probably due to its more recent discovery in plants (Biteau et al. 2003). *Arabidopsis* mutant lines lacking Srx are highly sensitive to methyl viologen, a drug that produce severe oxidative stress. In contrast to this observation, Rey et al. (2007) have observed that in vivo grown *Arabidopsis* Srx knockout mutant lines exhibited less oxidative damage than Wt when subjected to photoxidative treatment. This discrepancy could be related to the nature and intensity of the stress conditions. High levels of ROS in the mitochondria overoxidize PrxIIF to the sulfinic inactive form, and Srx, by controlling the reversion of this form, could regulate the signaling process. The absence of Srx in knockout lines of *Arabidopsis* produced an accumulation of inactive sulfinic forms of chloroplast 2Cys Prx and mitochondrial PrxIIF. Besides, the deletion of Srx yielded into more sensitive plants against high concentration of hydrogen peroxide when compared with wild-type plants (Iglesias-Baena et al. 2010, 2011). Therefore, Srx protects chloroplast and mitochondria from the oxidative stress regenerating the inactive sulfinic Prxs. As a summary, a model of response of the antioxidant and redox systems in mitochondria allowing acclimation and signaling under salinity is presented in Fig. 4.

5.2 Mitochondrial Response Under Drought

Drought stress in plants has severe effects, including changes inside the cell, with the result of reduced vegetative growth and cell division accompanied by decreased productivity. Changes in gene expression levels and activation of the enzymes involved in the production and scavenging of ROS as a resulting of drought stress situation have also been widely described (Mahajan and Tuteja 2005; Flexas et al. 2006). Moreover, plants subjected to drought stress suffer a disruption of cellular homeostasis, with inevitable consequences for the functioning of mitochondria (Flexas et al. 2005). These effects included their ability to adjust energy status to cope with the adverse conditions, although chloroplasts are more affected than mitochondria (Taylor et al. 2009). The response of leaf respiration to water stress varied depending on the duration and severity of the drought. In most cases studied (2/3) at whole-plant level, the respiration rate decreased with water stress (see review in Atkin and Macherel 2009). In other studies (1/3), respiration was unaffected but this did not mean that drought had no effect on mitochondrial metabolism. Ribas-Carbó et al. (2005a) found that the partitioning between CP and AP pathways was affected by severe stress in favor to AOX, which increased its activity but not its protein abundance. This could be the result of a greater electron flow through complex I and non-phosphorylating alternative dehydrogenases.

Drought stress also enhanced AOX activity as a percentage of total electron flow in tobacco (Galle et al. 2010). Changes in gene expression have been shown in *Arabidopsis* under drought, with an increase in *AOX1* accompanied by decreases in the transcription of the antioxidants PrxIIF, Trx*o*1, Grx, and Trx x (Mittler et al. 2004) (Fig. 4). In contrast, drought decreased leaf AOX transcript in *Medicago* (Filippou et al. 2011). An increase in AOX protein or capacity in response to drought has also been shown in wheat leaves (Bartoli et al. 2004; Vassileva et al. 2009), while no increase in AOX protein was reported for soybean leaves (Ribas-Carbó et al. 2005a) and drought. Also, the loss of function of mitochondrial AOX1a in *Arabidopsis* mutant plants led to a higher sensitivity to a combination of drought and moderate light stress (Giraud et al. 2008). These mutants, as we described above, showed changes in transcript abundance of other mitochondrial and chloroplast antioxidant enzymes, when growing under drought and salinity stress conditions (Giraud et al. 2008). Another mitochondrial protein involved in the response to drought is Mn-SOD. Its gene expression responded positively but decreased after rehydration in wheat seedlings (Wu et al. 1999), and the increase was greater in tolerant than in susceptible maize plants (Shiriga et al. 2014), suggesting that this enzyme may protect tolerant genotypes upon drought stress (Fig. 4). Moreover, Mn-SOD expression can improve drought tolerance in rice: transgenic plants overexpressing a pea Mn-SOD in rice chloroplasts exhibited less injury, measured by net photosynthetic rate, when treated with PEG, and showed reduced electrolyte leakage compared with wild-type plants (Wang et al. 2005). However, no change in its activity has been reported in other species such as pea plants under drought or after recovery (Moran et al. 1994). Other enzymes involved in the response to drought are the components of the ASC–GSH cycle. GR has been co-localized in chloroplasts and pea mitochondria and it is involved in the response of different species to drought. In pea plants, *GOR1* transcript levels remained constant during the drought phase although an increase was observed upon rewatering (Stevens et al. 1997). However, Sharma and Dubey (2005) described a significant increase in GR activity in drought-stressed *O. sativa* seedlings and also the enzymes involved in regeneration of ASC such as MDHAR and DHAR were higher in the stressed seedlings. Moreover, DHAR overexpression increased drought and ozone stress tolerance in tobacco (Eltayeb et al. 2006) (Fig. 4). Other examples of the involvement of the antioxidant system in the response to water deficit are evidenced in maize in which a mild water stress induced by a PEG treatment led to an increase in the activity of several antioxidant enzymes in mitochondria, including Mn-SOD, GR, APX, ASC, and GSH, during the first 12 h, after which they decreased (Tan et al. 2011). Similarly, in white clover, mitochondrial GR and Mn-SOD activities were found as the main ROS scavengers in mild water stress conditions (Chang-Quan and Rui-Chang 2008). All these imply the cooperation of these defense mechanisms against mild water stress detoxifying ROS at the site of production. In agreement, a decrease in APX protein content in tolerant *Amaranth* plants is probably involved in the observed increase in H_2O_2 which may act as signaling molecule for the stress response (Huerta-Ocampo et al. 2009). Similarly, in tolerant wheat, direct exposure to severe drought without

previous acclimation produced high oxidative stress mediated by a poor response of the mitochondrial antioxidant system (Selote and Khanna-Chopra 2010), although a period of drought acclimation prior to the stress induced the upregulation of SOD, APX, and ASC–GSH cycle components, limiting the accumulation of H_2O_2 and lipid damages (Selote and Khanna-Chopra 2010). Also changes in ascorbate and glutathione contents are a commonly observed stress response of plants during drought (Hernández et al. 2013; Chan et al. 2013). A recent study of drought in vitamin C (*vitc2-1*)- and glutathione (*pad2-1*)-deficient *Arabidopsis* mutants revealed a decrease of glutathione in the nuclei and mitochondria of both mutants, respectively, early in the drought stress, while ascorbate remained unchanged. This suggests that GSH acts as a signal for drought stress from roots to leaves (Koffler et al. 2014). As regards ASC, using two maize varieties differing in their constitutive content Bartoli et al. (2005) showed that the enzyme responsible for the last step in ASC synthesis, GalLDH, was induced by drought in a cultivar-specific manner while not affecting the ASC content in wheat or the regenerating ASC–GSH cycle enzymes. These authors pointed to DHAR and GR, which were higher in the variety containing higher levels of ASC, together with the ETC, as the key components controlling the ascorbate content in the cell and, in this way, possibly influencing the drought stress response.

Changes in the mitochondrial Prx system under drought are not widely described. In *Vitis vinifera*, peroxiredoxin *vvprxIIF* targeted to mitochondria has been shown to be the most responsive gene among the peroxiredoxins to water stress, and it might be involved in drought tolerance through H_2O_2 signaling (Vidigal et al. 2013). Also PrxIIF protein increased under water deficit in *Arabidopsis* in mature but not in young leaves (Gama et al. 2007). However, to our knowledge, the response of mitochondrial Trx*o*, Srx, or TR under this condition is not reported.

All the changes described allow to propose mitochondria as target organelle for improving plant performance under water stress (Tan et al. 2011) and point mitochondria as a key organelle involved in the response and tolerance to salt and drought stresses through the coordinated function of its antioxidative defense metabolism.

5.3 Mitochondrial Response Under Extreme Temperatures

Plants are exposed to changing temperature in natural conditions. Both cold and heat stress induce significant changes in photosynthesis, membrane stability, and respiration and, as those occurring under salinity and drought conditions, constrain agricultural productivity (Camejo et al. 2006; Hasanuzzaman et al. 2013). Low temperature is probably the most studied abiotic stress in relation to AOX respiration, and, as in the case of salinity and drought, it seems that the response of AOX to temperature is tissue and species specific and depends on the severity and length of the stress. In many species, a sharp increase in AOX transcript and/or protein has

been widely reported under this condition (Armstrong et al. 2008), although in other species, such as soybean, no change was observed (González-Meler et al. 1999). Using isotope discrimination, instantaneous (minutes to hours) changes in temperature had little impact on the relative partitioning of electrons between the CP and AOX (MacFarlane et al. 2009). However, longer treatments in *Arabidopsis* produced an increase in AOX activity with no accumulation of protein (Armstrong et al. 2008), while other studies have shown an accumulation of protein at low temperatures (Fiorani et al. 2005; Campbell et al. 2007; Umbach et al. 2009). Also in chilling-sensitive maize, a short-term cold treatment increased the AOX activity with a decreased CP (Ribas-Carbó et al. 2000). The effect of temperature has also been described in field-grown alpine grasses (Searle and Turnbull 2011), which presented higher AOX protein levels in the cold months. When transferred to cold, plants usually exhibited a strong increase in *AOX1a* transcript and protein but also an increase in transcript level of ROS-scavenging enzymes. Also AOX amount is important for the acclimation: plants with strong suppression of *AOX* transferred to cold presented a higher accumulation of these ROS-scavenging enzymes' transcripts than Wt plants, and, as a consequence, mutant plants suffered less oxidative damage (Fig. 4). In this way, cold stress affected *AOX* expression, although this did not always involve an increase in AOX respiration but probably gathered signaling pathways in mitochondria to respond at least at antioxidant level. The role of Mn-SOD in cold stress is observed in experiments targeting this enzyme from *Nicotiana* into maize chloroplasts, with resulting foliar tolerance to the oxidative stress induced by the unfavorable situation (Van Breusegem et al. 1999). Also the Mn-SOD gene was induced in both winter and spring plants when grown in cold-acclimating conditions in controlled environments and in the field (Wu et al. 1999). This similar behavior to drought conditions has been thought to be related to the cellular dehydration occurring in the cold acclimation step (Malone 1993). During freezing stress, overexpression of Mn-SOD in alfalfa plants protected them by minimizing ROS radical production with a rapid regrowth of these plants compared to mutant plants lacking a functional Mn-SOD gene (McKersie et al. 1996). In sacred lotus germinating embryos, chilling stress strongly increased the expression of Mn-SOD gene but was strongly inhibited under heat shock (Li et al. 2009). High temperature causes cell damage through increased ROS levels due to a decline in antioxidant activity in the stressed tissues (Mittler et al. 2004), and tolerance is based in part on an increase in this protective system (Jha et al. 2014).Under a heat shock treatment of two tomato varieties with different thermotolerance, the sensitive variety cv. Amalia presented a higher decrease in Mn-SOD activity than the tolerant one Nagcarlan, pointing to this isoenzyme as implicated in the tolerance of tomato to heat treatments (Camejo et al. 2006) (Fig. 4). It was also shown that an acclimation period to moderate temperatures in tomato cv. Amalia plants induced the Mn-SOD activity when plants were subjected to the heat shock treatment (Camejo et al. 2007).

The role of ASC–GSH cycle enzymes in cold or heat stress is evident from increases in MDHAR activity which contributed to chilling tolerance in tomato

fruits (Stevens et al. 2008). Changes in the GR isoform population as a result of chilling stress have been reported in *Arabidopsis* and pea plants as well as in cucumbers, in which the enhancement of total activity was due to the preferential induction of some of the isoforms (Kubo et al. 1993; Edwards et al. 1994; Lee and Lee 2000). In cucumber plants under heat stress, the activities of mitochondrial, cytosolic, and chloroplastic APX isoenzymes increased after an initial decline (Song et al. 2005) (Fig. 4). Also, studies on heat-acclimated turfgrass have revealed reduced ROS production probably due to enhanced ascorbate and glutathione synthesis (Xu et al. 2006).

Related to redox proteins, little information is reported about their response to changes in temperature. As an example, mitochondrial PrxIIF transcript and protein levels have been shown to increase in leaves of pea and *Arabidopsis* plants under cold stress (Barranco-Medina et al. 2007; Gama et al. 2007). In rice, a Trx*h* OsTrx23 which negatively regulates the activity of kinases OsMPK3 and OsMPK6 is known to be induced after chilling stress (Xie et al. 2009). This induction is coincident with the decline in *OsMPK3* expression and activity. H_2O_2 produced under the stress conditions has been implicated in the negative regulation of the activity and function of this Trx*h*, in this way decreasing its inhibitory action on the kinases. The overexpression of chloroplastic AtTDX in *Arabidopsis* plants conferred more resistance to heat shock stress (Sanz-Barrio et al. 2012) but the role of Trx*o*1 under extreme temperature stress situations is not reported. Related to Srx, an induction of the chloroplast transcript level has been reported in *Arabidopsis* plants responding to cold stress treatment (Liu et al. 2006).

However, although the signaling molecules involved in this response are under investigation, research on this line is imperative to try to enhance antioxidant production to better cope with unfavorable temperature conditions (Dat et al. 1998; Wahid et al. 2007).

6 Conclusions and Prospectives

A major challenge in studying stress responses in plants is to understand how different signaling pathways are activated by similar stimuli at subcellular levels. It has been suggested that, under abiotic and biotic stress conditions, cellular fate depends on several factors, among which ROS and NO play a pivotal role. In this context, timing, kinds, and amounts of ROS production in mitochondria as well as the interactions of ROS/NO are all important factors in determining cellular response. The involvement of the cross talk between ROS/NO in the regulation of protein activity and function and the effect on signaling affecting gene transcription in response to different stress begin to be elucidated. However, we know little about the actual flux of $O_2^{\bullet -}$ and NO within mitochondria in vivo, about how their changes under different physiological and stress conditions, or about their quantitative importance relative to other sources of ROS/NO under these circumstances. More work is required to develop techniques to measure mitochondrial ROS and

NO production in vivo. Such methods are essential to understand the role of mitochondrial $O_2^{\bullet -}$, H_2O_2, and NO production in redox signaling and in processes driving oxidative damage under normal and stress conditions.

An understanding of the subcellular GSH and ASC changes can be critical to our knowledge of redox homeostasis in plant cells under physiological and stress conditions. Such information can be also useful to clarify the importance of both antioxidants as protection mechanisms during abiotic stress and as redox signals.

In this context, there is a need to progress on the molecular identification of the GSH transporters. Despite their importance in glutathione homeostasis, the identification and characterization of mitochondrial glutathione transporters in plants is still not clear and at times controversial. In order to achieve further progress in this field, research on the combination and progression of current methods available for the detection of subcellular glutathione will be also necessary to obtain a combined measurement of the actual GSH concentration and the redox state in each cell compartment. Future research on ASC should be similarly focused on.

Moreover, an induced expression of mitochondrial antioxidant defense genes is usually crucial in determining the ability of a plant to cope with abiotic stress although the molecular mechanisms involved in the regulation of this induction remain unrevealed. In mitochondria, AOX is an important factor in maintaining redox balance and in alleviating ROS formation particularly during stress. APX in the ascorbate–glutathione cycle and PrxII F in the Trx/Prx/Srx system contribute to the redox control by means of the reduction of hydrogen peroxide produced by the Mn-SOD activity. However, changes at a transcript level did not usually correlate well with changes in protein responsive to stress, and posttranscriptional mechanisms are believed to play an important role in defining the mitochondrial stress response. While there has been important progress on the signaling role of redox state of thiol in Trxs and Prxs through posttranslational modifications like protein oxidation, S-glutathionylation, and S-nitrosylation/denitrosylation, many challenges remain about the thiol specificity, when and how they are coordinated to allow specific proteins to respond, and their repercussion in the regulation of target proteins and/or specific genes (Fig. 4). Likewise, as far as we know, the specific function, protein–protein interaction, and redox-network implication for the mitochondrial Trxs are far from being elucidated under abiotic stress. Further studies seeking to identify functional targets for Trx*o*1 in the mitochondria are needed to learn more about new physiological roles of this Trx*o*1 in plant cell. Thus, the interaction between both systems, the ASC–GSH cycle and the Trx–Prx–Srx, needs to be investigated in depth as well as that relative to the functional links between AOX and Trx*o*1. All these studies may help to unravel the role of ROS, RNS, and mitochondrial antioxidant proteins as redox sensors and in the signaling process under normal and stress conditions.

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ROS as Key Players of Abiotic Stress Responses in Plants

Nobuhiro Suzuki

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Abstract Despite their toxic potential, reactive oxygen species (ROS) play an integral role as signaling molecules in the regulation of a broad range of biological processes such as growth, development, and responses to biotic and/or abiotic stimuli in plants. To some extent, various functions of ROS signaling are attributed to differences in the regulatory mechanisms of respiratory burst oxidase homologs (RBOHs) that are involved in a multitude of different signal transduction pathways activated in assorted tissue and cell types under fluctuating environmental conditions. To acclimate or survive under abiotic stress conditions, plants possess powerful strategies involving systemic signaling, retrograde signaling, and programmed cell death (PCD), in which ROS signals are integrated with other pathways to generate highly coordinated signaling networks. In this chapter, beneficial roles of ROS as signaling molecules in the regulation of abiotic stress responses in plants will be addressed.

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Abbreviations

ABA	Abscisic acid
ABI4	ABA-insensitive 4
ACC	1-Aminocyclopropane-1-carboxylic acid
ANAC017	A membrane-bound NAC 017
AOX	Alternative oxidase
APX	Ascorbate peroxidase
BCL2	B-cell lymphoma 2
BR	Brassinosteroid
CDPKs	Calcium-dependent protein kinases
CTR1	Constitutive triple response 1
DPI	Diphenyleneiodonium
EDS1	Enhanced disease susceptibility 1
EIN2	Ethylene-insensitive 2
EEE	Excess excitation energy
GUN1	Genomes uncoupled 1
IAA	Indole-3-acetic acid
JA	Jasmonic acid
LSD1	Lesion simulating disease 1
MAPK	Mitogen-activated protein kinase
NPQ	Non-photochemical quenching
MeJA	Methyl jasmonate
OST1	Open stomata 1
PA	Phosphatidic acid
PCD	Programmed cell death
PAD4	Phytoalexin-deficient 4
PRL1	Pleiotropic response locus 1
PQ	Plastoquinone
RBOH	Respiratory burst oxidase homolog
ROS	Reactive oxygen species
SA	Salicylic acid
SAA	Systemic acquired acclimation
SAR	Systemic acquired resistance
SID2	Salicylic acid induction deficient 2
TPC1	Two-pore channel 1
VPE	Vacuolar processing enzymes

1 Introduction

The reactive oxygen species (ROS) signaling network is highly conserved among aerobic organisms and controls a broad range of biological processes such as growth, development, and responses to biotic and/or abiotic stimuli (Mittler et al. 2011). Although early researches related to ROS metabolism focused on their potential toxicity and the different mechanisms to scavenge them, more recent studies have focused on the roles of ROS as signaling molecules. Why did plants acquire the ability to utilize ROS as signaling molecules during the evolutionary process? The existence of different types of ROS might be an advantage for the fine-tuning of complex signaling networks in cells, because coordinated production of ROS with different properties could at least partially contribute to generation of various signals. Indeed, specificity of ROS signaling was previously indicated by the finding that different sets of genes were upregulated in response to different types of ROS (Gadjev et al. 2006; Suzuki et al. 2011). In addition, almost any changes in cellular homeostasis could lead to alteration of redox state in particular organelles, followed by changes in cellular level of ROS. ROS can be therefore integrated with the activities of many other signaling components such as hormones, Ca^{2+}, and kinases and involved in a number of pathways underlying different biological outcomes (Petrov and Van Breusegem 2012). Mobility of ROS, especially H_2O_2, is also an advantage to act as a signaling molecule. H_2O_2, a relatively long-lived and small ROS, readily penetrates cell membranes and is freely diffusible between cells (Bienert et al. 2006). Diffusion of H_2O_2 that is facilitated by plasma membrane aquaporins could also influence the efficiency and direction of H_2O_2 signaling between cells (Bienert et al. 2007; Dynowski et al. 2008).

To utilize ROS as signaling molecules, nontoxic levels of ROS must be maintained in a delicate balance between production involving enzymatic reactions and the unavoidable production during basic cellular processes and the metabolic counter-process involving ROS scavenging pathways (Mittler et al. 2004). Another possible advantage for using ROS as signaling molecules is the specific localization of their production/scavenging mechanisms in different organelles, cell types, and tissues (Mittler et al. 2004; Miller et al. 2009b; Suzuki et al. 2011). Local increase in ROS production can be limited to particular locations in the cell or plant. Therefore, level of ROS throughout the cell or plant as well as spatial coordination of ROS signals can be tightly regulated. In plants, many antioxidant mechanisms that function in different organelles modulate cellular redox state by detoxifying excess ROS produced during basic biological processes such as photosynthesis and respiration (Miller et al. 2009b). In addition, NADPH oxidases, respiratory burst oxidase homologs (RBOHs), deliberately produce ROS in different cell types, tissues, or stages (Suzuki et al. 2011). The tight regulation of RBOH protein activity makes these enzymes good candidates for the fine-tuning of ROS production in terms of amplitude, duration, and localization in plants (Marino et al. 2012; Gilroy et al. 2014) (Fig. 1).

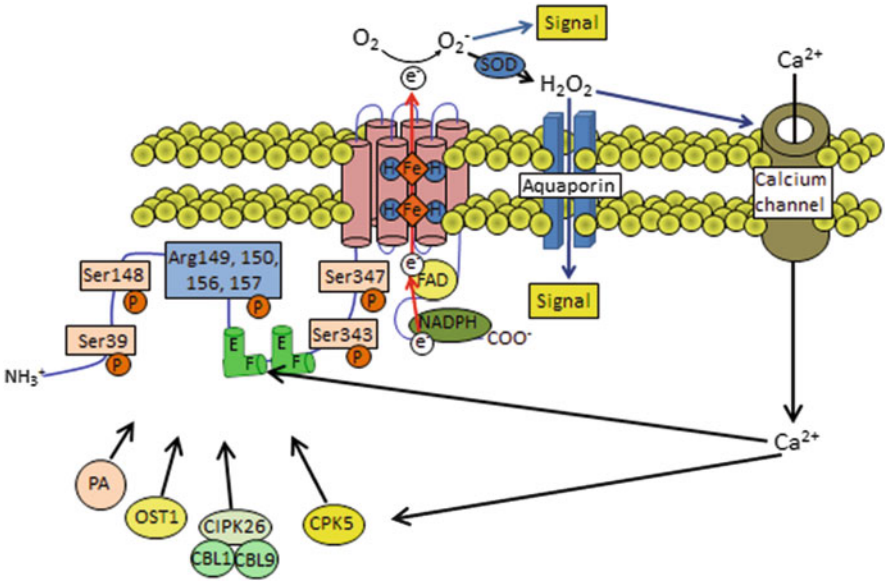

Fig. 1 Regulatory mechanisms of RBOH proteins in *Arabidopsis*. ROS can activate or suppress calcium channels to control the release of Ca^{2+} into cytosol. Ca^{2+} can directly or indirectly regulate the production of ROS by respiratory burst homolog (RBOH) proteins resulting in the generation of superoxide radicals that are dismutated to H_2O_2 spontaneously or via superoxide dismutase (SOD). H_2O_2 diffused into cytosol through aquaporin then functions as a signaling molecule. RBOHs have cytosolic FAD and NADPH-binding domains in the C-terminal region and six conserved transmembrane-spanning domains (*pink cylinders*). The N-terminal domain contains two EF-hand motifs and phosphorylation target sites that are important for activity of RBOHs. Binding of Ca^{2+} to EF-hand motifs is required for activation of RBOHs (Ogasawara et al. 2008; Drerup et al. 2013). Several kinases including OST1 (Sirichandra et al. 2009), CPK5 (Dubiella et al. 2013), and CBL1/9–CIPK26 complexes (Drerup et al. 2013) activate RBOH proteins by phosphorylation of the target sites (Ser or Arg residues). In addition, phosphatidic acid (PA) produced via function of phospholipase Dα1 (PLDα1) was also shown to activate RBOHD (Zhang et al. 2009b). *Abbreviations*: *CBL1* calcineurin B-like protein 1, *CBL9* calcineurin B-like protein 9, *CIPK26* calcineurin B-like interacting protein 26, *CPK5* calmodulin domain protein kinase 5, *OST1* open stomata 1, *FAD* flavin adenine dinucleotide, *NAD* nicotinamide adenine dinucleotide, *PA* phosphatidic acid

Being sessile organisms, plants evolved sophisticated strategies to acclimate to or survive under fluctuating environmental conditions. These strategies involve systemic signaling, retrograde signaling, and programmed cell death (PCD), in which ROS play beneficial roles as signaling molecules (Woodson and Chory 2008; Baxter et al. 2014; Szechynska-Hebda and Karpinski 2013; Petrov et al. 2015). Systemic signaling mechanisms evolutionally improved the ability of plants to alert all remote and unstressed tissues of the plant to the existence of a biotic or abiotic threat and to trigger the activation of resistance or acclimation pathways in these tissues (Suzuki et al. 2013; Szechynska-Hebda et al. 2010). Recent studies have highlighted the importance of rapid systemic responses for

acclimation of plants to abiotic stimuli, focusing on temporal–spatial coordination between several players of complex network of cell-to-cell communication, active propagation of ROS wave and calcium wave, hormones, and electric signals (Miller et al. 2009b; Mittler et al. 2011; Baxter et al. 2014; Gilroy et al. 2014). Signaling networks between the organelles employ ROS as second messenger. Alterations in redox state and ROS metabolism in the chloroplast and mitochondria are sources for retrograde signals to regulate nuclear gene expression, which play an important role in the acclimation of plants to environmental stimuli (Rhoads and Subbaiah 2007; Pogson et al. 2008; Woodson and Chory 2008; Shapiguzov et al. 2012; Szechynska-Hebda and Karpinski 2013). In addition, chloroplastic ROS production and photosynthetic functions were recently shown to be regulated by cues perceived by cell wall or apoplastic spaces (Padmanabhan and Dinesh-Kumar 2010), suggesting that chloroplastic retrograde signaling might be a part of a large signaling network involving various cues generated from different organelles, and ROS might be an important mediator that integrates these different signals. PCD is one of the essential strategies for survival of plants that are subjected to severe biotic or abiotic stresses, because only cells that are destined to die can be eliminated to prevent the spreading of damage to the neighboring cells and maintain appropriate metabolic status in the rest of cells and tissues (Petrov et al. 2015). It is an active and genetically controlled process in which cells are selectively eliminated in multistep fashion through the involvement of high concentrations of ROS and specific proteases and nuclease (Petrov et al. 2015).

In this chapter, beneficial roles of ROS as signaling molecules in the regulation of abiotic stress responses of plants will be addressed, especially in the context of ROS-producing mechanisms, integration of ROS signals with other pathways, and plant-specific mechanisms that underlie response of plants to abiotic stimuli.

2 ROS-Generating Pathways and Their Regulatory Mechanisms in Plants

In plants, NADPH oxidases, respiratory burst oxidase homologs (RBOHs), are responsible for the production of ROS that act as important signaling molecules (Torres and Dangl 2005; Suzuki et al. 2011). In *Arabidopsis*, RBOHs constitute a multigenic family comprised of ten genes (i.e., AtRBOHA–AtRBOHJ). Very specific function of each RBOH isoform was implicated by the finding that expression of genes encoding each RBOH protein, except for RBOHD, is restricted to one or two organs (Suzuki et al. 2011; Marino et al. 2012). Indeed, several studies have revealed that different plant RBOHs are involved in a multitude of different signaling pathways underlying growth, development, and response to biotic and abiotic stress (Torres et al. 2005; McInnis et al. 2006; Monshausen et al. 2007; Jammes et al. 2009; Nishimura and Dangl 2010; Miller et al. 2009b; Suzuki et al. 2011). In addition, a previous study revealed distinctive expression profiles

of nine RBOHs in rice in response to various abiotic stimuli, which demonstrated their linked but diverse functions (Wang et al. 2013).

Plant RBOHs consist of C-terminal region containing cytosolic FAD and NADPH-binding domains, six conserved transmembrane-spanning domains, and N-terminal extension containing two Ca^{2+}-binding EF-hand motifs and phosphorylation target sites that are important for their activity (Kobayashi et al. 2007; Oda et al. 2010; Kimura et al. 2012; Drerup et al. 2013). Previous studies in *Arabidopsis* have revealed several regulatory mechanisms of RBOH proteins, which involve protein phosphorylation, Ca^{2+}, calcium-dependent protein kinases (CDPKs), and phospholipase Dα1 (PLDα1) (Lin et al. 2009; Monshausen et al. 2009; Zhang et al. 2009b; Jakubowicz et al. 2010; Dubiella et al. 2013; Drerup et al. 2013). Mechanical stimulation of plant tissue can induce an increase in cytosolic Ca^{2+} via an influx from the apoplast across the plasma membrane (Monshausen et al. 2009). The increased Ca^{2+} then enhances RBOHC-dependent ROS production followed by the activation of positive feedback loop between Ca^{2+} and RBOHC to regulate root hair development (Monshausen et al. 2007, 2009; Takeda et al. 2008). Ca^{2+} binding and phosphorylation synergistically activate RBOHD and RBOHF in *Arabidopsis* (Ogasawara et al. 2008; Kimura et al. 2012). A Ca^{2+} increase in the cytosol and conformational changes in EF-hand motifs by Ca^{2+} binding were found to be necessary for the activation of RBOHD (Ogasawara et al. 2008). PLDα1 and its lipid product phosphatidic acid (PA) play an essential role in abscisic acid (ABA)-induced production of ROS in guard cells via the function of RBOHD and RBOHF (Zhang et al. 2009b). ABA-dependent stomatal movement involves binding of PA to Arg residues 149, 150, 156, and 157 in RBOHD and phosphorylation of Ser13 and Ser174 in RBOHF by OPEN STOMATA 1 (OST1) kinase (Sirichandra et al. 2009). These findings indicate integration between RBOHD and RBOHF in the regulation of ABA-dependent stomatal closure. The coordination between PA and OST1 however, still needs to be addressed in future studies. In a recent study, systemic acquired resistance to pathogen was shown to involve phosphorylation of RBOHD by calcium-dependent protein kinase 5 (CPK5) and H_2O_2 production (Dubiella et al. 2013), supporting the hypothesis that Ca^{2+}-dependent ROS production is required for the propagation of the ROS wave over long distances (Miller et al. 2009b). In addition, a recent finding demonstrated that the activity of RBOHF is regulated by direct Ca^{2+} binding to its EF-hands and Ca^{2+}-dependent phosphorylation by CBL1/9–CIPK26 complexes (Drerup et al. 2013). Taken together, these findings indicate that the diverse functions of RBOH proteins in plants might be, at least partially, attributed to differences in regulatory mechanisms.

RBOH proteins are not the only source of ROS in plant cells. Numerous pathways for ROS production exist in plants and include photosynthesis, respiration, glycolate oxidase, oxalate oxidase, xanthine oxidase, amine oxidase, excited chlorophyll, fatty acid oxidation, and peroxidases (Mittler 2002). These pathways were also found to play important roles in the response of plants to abiotic stresses. For example, oxalate oxidase was shown to be involved in ROS production in root

cells during drought stress (Voothuluru and Sharp 2013). In addition, recent studies uncovered a role for peroxidase-dependent ROS in the regulation of root growth and response to potassium deficiency (Kim et al. 2010; Jia 2011; Kwasniewski et al. 2013). Interestingly, ROS production by peroxidases might not be functionally equivalent to ROS generated by RBOH proteins (Daudi et al. 2012; Wrzaczek et al. 2013). This hypothesis can be supported by the finding that stomatal closure and ROS burst induced by a yeast elicitor were not inhibited in *rbohD* and *rbohF* mutants in *Arabidopsis* (Khokon et al. 2010). Functional differences between RBOH proteins and peroxidases may be at least partially attributed to differences in the types of ROS generated via functions of these enzymes. Superoxide ($O_2^{\bullet -}$), generated by RBOH proteins, can activate specific signaling pathways distinct from those activated by H_2O_2 (Suzuki et al. 2011). Another possibility is that diverse functions between these different types of enzymes might be due to differences in their respective reductants. RBOH proteins utilize NADPH as a reductant for the generation of $O_2^{\bullet -}$. In contrast, different chemicals or compounds including phenols, organic acids, and auxin have been suggested as candidate reductants employed in the peroxidase-dependent generation of H_2O_2 (O'Brien et al. 2012). Pathways involving these different reductants could be integrated with ROS signals activated via the different functions of these enzymes.

3 Involvement of ROS in the Regulation of Systemic Acquired Acclimation to Abiotic Stress

Recent findings highlight the significance of cell-to-cell communication mediating long-distance systemic signaling in plants. Plants evolved sophisticated acclimation and defense mechanisms that can be activated in the tissue(s) locally exposed to biotic or abiotic stimuli, as well as in distal portions not directly exposed to these stimuli. These mechanisms play an important role in preventing further damage to the entire plant when part of tissues is exposed to biotic or abiotic stimuli. The activation of defense or acclimation mechanisms in systemic or non-challenged tissues is termed systemic acquired resistance (SAR) or systemic acquired acclimation (SAA), respectively (Karpinski et al. 1999; Rossel et al. 2007; Carr et al. 2010; Szechynska-Hebda et al. 2010; Dempsey and Klessig 2012; Spoel and Dong 2012; Shah and Zeier 2013; Baxter et al. 2014). A recent study revealed the existence of an H_2O_2-dependent long-distance signal induced by various abiotic stimuli (Miller et al. 2009b). RBOHD was shown to be required for the initiation and self-propagation of a rapid cell-to-cell signal transduction that is dependent upon H_2O_2 accumulation in the apoplast to generate a "ROS wave" (yellow arrows in Fig. 2) (Mittler et al. 2011). In addition, more recent study demonstrated the significance of the ROS wave in the SAA of plants to heat or high-light stresses

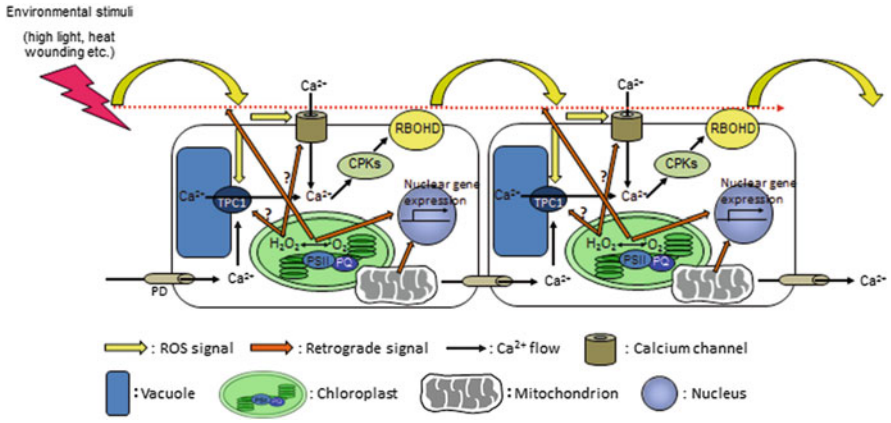

Fig. 2 Cellular pathways that regulate ROS-dependent systemic signaling and retrograde signaling. H_2O_2 generated via function of RBOHD is required for rapid signal propagation from cell to cell in response to different environmental stimuli, such as high light, heat, or wounding (Miller et al. 2009). The ROS and Ca^{2+} waves in cells are integrated via the function of respiratory burst homolog (RBOH) proteins, Ca^{2+}-dependent protein kinases, and calcium channels such as two-pore channel (TPC) 1. Changes in the redox state of PSII and the PQ pools in the chloroplast might activate electric signaling (red dotted arrow) at the plasma membrane involved in systemic responses (Szechynska-Hebda et al. 2010). The electric signal that is dependent upon the chloroplast redox state is also accompanied by ROS generation, implicating a cross talk between the RBOHD-dependent signal, electric signals on the plasma membrane, and ROS/redox signaling from chloroplast. Mitochondrial retrograde signaling (Rhoads and Subbaiah 2007; Pogson et al. 2008; Woodson and Chory 2008) could also play a key role in these responses as part of a cross talk network that senses different metabolic/environmental states and activates rapid systemic signaling. In addition, ROS produced via function of RBOH proteins or basic biological processes in different cellular components might induce different PCD pathways depending on abiotic stimuli. *Abbreviations*: *PD* plasmodesmata, *CPK* Ca^{2+}-dependent protein kinase

(Suzuki et al. 2013). The SAA of plants to abiotic stress is mediated by temporal–spatial interactions of the ROS wave, which function as a general priming signal, with stress-specific hormone or amino acid signals activated in systemic tissues. Calcium wave was recently shown to function as pivotal element of the systemic communication machinery (black arrows in Fig. 2) (Choi et al. 2014; Gilroy et al. 2014). In response to local stimulation with salt stress in the root tip, a wave of increased cytosolic Ca^{2+} level moves systemically through the plant paralleling with the ROS wave. The Ca^{2+} wave that travels at ~400 μm/s can spread through the root system and be transmitted to the aerial part of the plant. Application of the Ca^{2+} channel blocker lanthanum (La^{3+}) inhibited systemic induction of marker gene expression associated with ROS as well as Ca^{2+} wave, implying the link between ROS and Ca^{2+} wave. In addition, plant deficient in two-pore channel 1 (TPC1), a vacuolar ion channel, exhibited disruption of the propagation of the systemic Ca^{2+} wave.

4 Temporal Coordination Between ROS and Other Signals in the Regulation of Systemic Signaling in Plants

In response to changes in environmental conditions, early signaling events including increased Ca^{2+} levels in the cytosol, activation of MAPKs, accumulation of hormones, and production of ROS can all occur within seconds or minutes following application of abiotic stimuli (Benschop et al. 2007; Finka et al. 2012; Miller et al. 2009b). Early systemic responses of plants to abiotic stimuli have been previously described (Karpinski et al. 1999; Rossel et al. 2007; Muhlenbock et al. 2008; Szechynska-Hebda et al. 2010; Gordon et al. 2012). Studies employing transgenic plants expressing a luciferase reporter gene under the control of an APX1, APX2, or ZAT10 promoter demonstrated the activation of acclamatory responses within 5–20 min following application of high light both in leaves locally exposed to the stimuli and in distal tissues that were not directly exposed to the stimuli (Karpinski et al. 1999; Rossel et al. 2007; Szechynska-Hebda et al. 2010). Systemic responses to high light were shown to be associated with redox changes in the plastoquinone (PQ) pool, increased production of ROS and ethylene, reduction of maximal photochemical efficiency and non-photochemical quenching (NPQ), and changes in extracellular electric potential (Karpinski et al. 1999; Rossel et al. 2007; Szechynska-Hebda et al. 2010). In addition, amino acids involved in photorespiratory machinery, such as glycine, serine, and glycerate, rapidly accumulate in leaves directly exposed to high light within 60 s as well as in systemic tissues of plants at 15 and 45 min (Suzuki et al. 2013). Rapid local responses of these metabolites to high light were altered in the mutant lacking cytosolic APX1, demonstrating the involvement of H_2O_2 scavenging in this process.

In *Arabidopsis*, elevated levels of jasmonic acid (JA) accumulate in damaged tissues as well as undamaged systemic leaves within 30 s to 5 min in response to mechanical wounding (Glauser et al. 2009; Koo et al. 2009). The velocity of this long-distance signal leading to synthesis of JA in systemic tissues was 3.4–4.5 cm/min (Koo et al. 2009; Glauser et al. 2009). RBOHD-dependent long-distance signal is also a rapid auto-propagating systemic signal that travels at the rate of approximately 8.4 cm/min and is induced by various abiotic stimuli including mechanical wounding (Miller et al. 2009b). In addition, the potential involvement of electric signals that propagate with similar rates was also implicated in RBOHD-triggered rapid systemic signaling during wounding (Zimmermann et al. 2009; Mittler et al. 2011; Suzuki and Mittler 2012). These findings implicate the integration of JA and mobile signals such as ROS and electric signals. Peroxisomes that are responsible for the production of both H_2O_2 and JA might be a candidate of a key player to integrate JA and mobile ROS signals (Leon 2013). H_2O_2 is produced during the oxidation of glycolate to glyoxylic acid in photorespiratory processes (Mittler et al. 2004). JA is synthesized through the octadecanoid pathway involving the translocation of lipid intermediates from the chloroplast to the cytosol and later on into peroxisomes (Leon 2013). JA synthesized in the peroxisomes is transported to the cytosol, and JA-isoleucine conjugate, the bioactive form of the hormone, is

then produced. Long-term responses to fluctuating environmental conditions regulate phenotypic changes such as growth, development, and survival of cells. Exposure of mature leaves to changes in light conditions and atmospheric CO_2 induces alterations in photosynthetic rate and tolerance to high light in new developing leaves not directly exposed to these environmental changes (Coupe et al. 2006; Araya et al. 2008; Jiang et al. 2012). Although alterations in photosynthetic rate and response to high light implicate ROS and redox signaling in the systemic regulation of long-term responses in new developing leaves (Muhlenbock et al. 2008; Li et al. 2009; Mittler et al. 2011), links between ROS signaling and these responses are still not uncovered.

Previous studies demonstrated that the biphasic production of ROS consists of a primary phase that occurs within minutes and a secondary phase that occurs within hours/days (Nishimura and Dangl 2010; Soares et al. 2009; Kunihiro et al. 2011; Mittler et al. 2011). For example, mechanical wounding induced an initial burst of $O_2^{\bullet -}$ within 3 min followed by later production of $O_2^{\bullet -}$ and H_2O_2 after 6 h (Soares et al. 2009). Inhibition of early phase of ROS production by an NADPH oxidase inhibitor suppresses later production of $O_2^{\bullet -}$ and accumulation of wound response proteins, indicating that an initial burst of ROS is required for the later phase of ROS production which regulates downstream acclamatory responses of plants to stress stimuli. In addition, a recent study suggests that these two phases of the ROS burst are linked via the ROS wave that communicates the initial ROS burst in the local tissue to the systemic tissue via a cell-to-cell relay mechanism (Miller et al. 2009b; Suzuki et al. 2013).

5 Spatial Coordination Between ROS and Other Signals in the Regulation of Systemic Signaling in Plants

To some extent, signals generated in plants during SAA are similar in local and systemic tissues. Rossel et al. (2007) compared the transcriptomes of local leaves, directly exposed to high light, and systemic leaves, not directly challenged by the stimulus. More than 70 % of the transcripts upregulated in local leaves in response to high light were also altered in their expression in systemic leaves, suggesting that similar signals exist between local and systemic tissues during SAA to high light. Similarities between local and systemic responses to high light is also supported by findings that alterations in ROS and redox signals and accumulation of amino acids associated with the photorespiratory pathway occurred both in local and systemic tissues (Muhlenbock et al. 2008; Szechynska-Hebda et al. 2010; Miller et al. 2009b). In addition, local application of heat or cold stimuli also can induce similar stress response proteins or transcripts in both local and systemic tissues (Gorsuch et al. 2010; Suzuki et al. 2013). In particular, induction of heat-responsive proteins in systemic tissue was shown to be RBOHD dependent (Suzuki et al. 2013).

Although signals generated in local and systemic tissues showed considerable overlap, previous studies have also demonstrated differences in alterations of transcripts or metabolites between these types of tissues. For example, ethylene accumulated both in local and systemic tissue in response to local application of high light; nevertheless, the signal regulated by EIN2 was shown to be required for induction of APX2 only in systemic tissues that are not directly subjected to high light (Muhlenbock et al. 2008). In addition, SID2 delays induction of APX2 only in leaves directly exposed to high light. These findings suggest that specific patterns of APX2 expression in local and systemic tissue might be regulated by the coordination between ethylene and salicylic acid (SA) signaling during SAA to high light. Moreover, spatial diversity in high-light responses between different leaves during SAA was also demonstrated by the findings that local high-light treatment resulted in the different expression levels of transcripts associated with regulation of ROS and redox signals depending on leaf position (Gordon et al. 2012).

How are signals generated in local and systemic tissues linked? The ROS wave may play a key role in propagating signals from local tissues to systemic tissues. The initial burst of ROS in a local group of plant cells triggers a cascade of cell-to-cell communication events that carries a systemic signal over long distances throughout different tissues of the plant (Miller et al. 2009b). Szechynska-Hebda et al. (2010) uncovered the pattern of systemic changes in NPQ, H_2O_2 concentration, and APX1 expression during SAA response of plants to high light. Wavelike patterns of APX1 expression in systemic tissue of plants correlate positively with H_2O_2 accumulation but negatively with NPQ (Szechynska-Hebda et al. 2010; Karpinski et al. 2013). The activation of systemic signals by local application of high light was recently shown to be accompanied by plasma membrane electrical signals in a light wavelength-specific manner (Szechynska-Hebda et al. 2010). In addition, the RBOHD-dependent ROS wave is associated with the generation and/or propagation of systemic potential variations (Suzuki et al. 2013). These finding suggest a link between electric signals in plants and ROS production.

6 Integration of ROS Signals with Other Signals

ROS signaling is integrated with various other signals including Ca^{2+} signaling, protein kinases, redox responses, and hormone signals. One good example of signaling networks associated with ROS signaling is MAPK cascade (Mittler et al. 2011; Petrov and Van Breusegem 2012). MPK3, MPK4, and MPK6 can all be activated by ROS and abiotic stresses, but different MKKs might transmit the signal depending on different stimuli (Jaspers and Kangasjarvi 2010). *Arabidopsis* overexpressing MKK2 exhibited constitutive MPK4 and MPK6 activity and resulted in increased tolerance of transgenic plans to salt and cold stress (Teige et al. 2004; Taj et al. 2010; Ismail et al. 2014). In contrast, overexpression of MKK9 that activates MPK3/MPK6 resulted in enhanced sensitivity of the transgenic plants to salt stress (Xu et al. 2008). In addition, activation of MPK6 by MKK3 was shown

to be not required for salt stress response in plants (Takahashi et al. 2007). MEKK1 was suggested to be specifically required for the activation of MPK4 by H_2O_2 (Nakagami et al. 2006), and the signal between MEKK1 and MPK4 is mediated by MKK1 and MKK2 (Qiu et al. 2008). The MEKK1–MKK1/2–MPK4 pathway might play integral roles to regulate transcription factors that are highly responsive to ROS-generating conditions (Pitzschke et al. 2009). MPK8 could be a negative regulator of ROS wave (Marino et al. 2012). MPK8 which is activated by phosphorylation and direct binding of CaM in a Ca^{2+}-dependent manner has been shown to negatively regulate ROS production via control of RBOHD (Takahashi et al. 2011). Various forms of abiotic stress result in increased production of ROS which can be liked to signals caused by changes in the regulation of plant hormones (Fujita et al. 2006). Ethylene biosynthesis was found to be modulated by positive regulation via RBOH proteins and negative regulation via CTR1 (constitutive triple response 1) (Jakubowicz et al. 2010). In *Arabidopsis*, CTR1 can be inhibited by phosphatidic acid (PA) that positively enhances activation of RBOHD and RBOHF (Jakubowicz et al. 2010). Previous studies revealed the involvement of ethylene in the regulation of SAA to high light induced by local high-light application (Muhlenbock et al. 2008; Karpinski et al. 2013). In response to high light, alterations in the redox state of the PQ pool can initiate a signal that induces production of 1-aminocyclopropane-1-carboxylate (ACC, the immediate precursor of ethylene), ROS, and the expression of ethylene-regulated genes (Muhlenbock et al. 2008). Increased ROS production results in bleaching of leaves and programmed cell death that relies on regulation of ethylene-insensitive 2 (EIN2) by lesion stimulating disease 1 (LSD1) (Muhlenbock et al. 2008; Karpinski et al. 2013).

Involvement of brassinosteroid (BR) signaling in ROS-dependent stress responses was also supported by previous findings. For example, exogenous BR treatments resulted in enhanced tolerance to oxidative stress accompanied by induction of H_2O_2 production in apoplast and expression of RBOH, MPK1, and MPK3 (Xia et al. 2009). More recent studies demonstrated the involvement of BR in SAA to high light in cucumber (Xia et al. 2009, 2011; Li et al. 2013a). Although BRs are not directly involved in long-distance signaling, they affect other signals such as auxins and polyamines (Li et al. 2013a). A recent study indicated the involvement of auxin in SAA response of plants to high light. Large portions of the transcripts that are altered in their expression in the distal leaves overlap with auxin-responsive transcripts (Gordon et al. 2012), indicating a connection between SAA and developmental processes mediated by auxin. Integration between ethylene and BRs during SAA response to HL needs to be elucidated in future studies.

ROS can affect auxin biosynthesis, transport, metabolism, and signaling underlying regulation of stress responses in plants (Krishnamurthy and Rathinasabapathi 2013b). The integration of RBOH functions with auxin has been evidenced by the analyses of the various RBOH expressions following the exogenous auxin (IAA: indole-3-acetic acid) treatment in *Arabidopsis*. It was observed that RBOHD was highly activated in response to auxin (Peer et al. 2013). Auxin transport mutant *aux1* exhibited higher sensitivity to arsenite compared to WT plants, and H_2O_2

production was inhibited in *aux1* under this stress condition (Krishnamurthy and Rathinasabapathi 2013a). These results indicate that auxin transport plays positive role in induction of ROS production and protection of plants against arsenite. In addition, the auxin signaling mutant *tir afb2* showed reduced production of H_2O_2 and $O_2^{\bullet -}$ and increased activity of catalase and ascorbate peroxidase accompanied by enhanced tolerance to salt stress (Iglesias et al. 2010).

ABA is involved in a broad range of biological functions, and its integration with ROS has been evidenced in many reports (Ma et al. 2012; Sagi et al. 2004; Drerup et al. 2013; Kwak et al. 2003). For example, RBOHD and RBOHF function together to regulate stomatal closure, seed germination, root elongation, and Na^+/K^+ homeostasis under salt stress (Ma et al. 2012; Kwak et al. 2003). Overexpression of 9-*cis*-epoxycarotenoid in tobacco, an enzyme of ABA synthesis, enhanced tolerance of transgenic plants to drought and salt stress accompanied by increased ABA-induced production of ROS via the function of RBOHs (Zhang et al. 2009a). In addition, mild salt stress triggers biphasic changes in ROS production in *Arabidopsis* and maize, and RBOHD in *Arabidopsis* is required for ROS production following mild salt stress (Lin et al. 2009; Xie et al. 2011). Moreover, SAA of plants to heat stress was shown to be correlated with activation of the ROS wave and transient accumulation of ABA in systemic tissues, and these responses were suppressed in a mutant lacking RBOHD (Suzuki et al. 2013). The SAA response to heat stress was also attenuated in mutants deficient in ABA signaling. These results indicate that temporal–spatial interactions between RBOHD-dependent ROS and ABA mediate SAA to heat stress (Suzuki et al. 2013). Moreover, ABA and SA treatment have been shown to result in transient increases in H_2O_2 production which induces tolerance to heat, salt, high-light, and oxidative stress (Xia et al. 2009).

Integration of SA or JA signaling with ABA underlying stomatal closure has been addressed in a recent review (Song et al. 2014). ABA-deficient mutant *aba2-1* failed to close stomata in response to exogenous SA treatment, whereas guard cells of SA-deficient mutants *sid2* and *NahG* responded to ABA (Zeng and He 2010; Montillet and Hirt 2013; Song et al. 2014), indicating that SA signaling functions upstream of ABA signaling. SA-induced stomatal closure was inhibited by DPI, an NADPH oxidase inhibitor, and plants deficient in RBOHD exhibited defect in stomatal response to exogenous SA treatment (Kalachova et al. 2013). In contrast, several lines of other evidences suggested that SA mediates ROS production, not via NADPH oxidases, but rather via a peroxidase-catalyzed reaction (Mori et al. 2001; Song et al. 2014). The reduced stomatal apertures in SA-accumulating *siz1* mutant were rescued by the application of peroxidase inhibitors, but not by DPI (Miura et al. 2013). In addition, SA induces stomatal closure accompanied by extracellular ROS production mediated by peroxidase (Khokon et al. 2011). Mechanisms that regulate integration of SA signals to ROS-generating pathways need to be elucidated in future works. It was suggested that there is an overlap in the signals associated with stomatal closure between JA and ABA and that many common components including ROS production and Ca^{2+} oscillation are shared between these hormone signalings (Santino et al. 2013). *Arabidopsis* RBOHD and RBOHF were shown to be involved in the expression of methyl jasmonate (MeJA) response genes regulated by MYC2 transcription factor (Maruta et al. 2012).

7 Involvement of ROS in the Regulation of Retrograde Signaling

Under stress conditions, changes in the redox state of the chloroplast and mitochondria, ROS-producing organelles are the source of retrograde signaling that play crucial roles in stress acclimation of plants (Orange arrows in Fig. 2) (Suzuki et al. 2012; Choudhury et al. 2013). Three different processes in *Arabidopsis* were shown to induce chloroplast to nucleus signaling that alter the expression of nuclear genes, depending on the presence of GUN1 in the chloroplast and ABI4 in the nucleus: (1) accumulation of the chlorophyll biosynthesis intermediate Mg-protoporphyrin IX (Mg-Proto IX) and its methylester (Mg-Proto IX-ME), (2) inhibition of plastid gene expression (PGE) at the protein translation stage, and (3) changes in the redox state of the photosynthetic electron transfer (PET) chain (Koussevitzky et al. 2007; Woodson and Chory 2008).

Signals from the chloroplast to nuclei, modulated by changes in cellular redox state depending on the light intensity, might be an important process to respond to fluctuating light conditions (Szechynska-Hebda and Karpinski 2013). Under low light intensity, the transfer of signal from chloroplast to nucleus followed by changes in nuclear gene expression contributes to the adjustment of morphology of the leaves and cells, and number and structure of chloroplasts (Oelze et al. 2012). Light use efficiency is optimized at least partially by modulating stoichiometry of photosystems, light-harvesting antenna size, composition of the stromal enzymes, and activity of antioxidant systems (Muhlenbock et al. 2008; Pfannschmidt 2010; Foyer and Noctor 2011; Ruckle et al. 2012; Szechynska-Hebda and Karpinski 2013). Low light intensity can promote oxidation of PQ and reduction of thioredoxin, whereas high light intensity oppositely affects redox state in PQ pool and thioredoxin (Muhlenbock et al. 2008). Under high light intensity, PQ redox state and non-photochemical quenching (NPQ), the process to dissipate excess energy by heat, were shown to be potential regulators of retrograde signals that are required for at least regulation of APX1 and APX2 expression (Szechynska-Hebda et al. 2010). Imbalance between ATP and NADPH synthesis might be also an initiator of retrograde signaling (Szechynska-Hebda and Karpinski 2013). High proportion of NAD(P)H/NAD(P), ATP/ADP, and ATP/NADPH ratio could lead to inactivation of the photosynthetic electron transport chain components, resulting in production of $^1O_2$ and $O_2^{\bullet -}$ followed by deregulation of chloroplast metabolism and altered expression of stress response genes (Szechynska-Hebda and Karpinski 2013). In addition, excess excitation energy (EEE) also induces the changes in cellular ROS homeostasis and affects the expression of nuclear genes that control SAR and SAA (Rossel et al. 2007; Szechynska-Hebda et al. 2010). Progression of excess energy-induced PCD initiated by redox changes in PQ pool is regulated by the coordination between LSD1, EDS1, PAD4, and EIN2 that are associated with ethylene signaling and ROS production.

Upon illumination, chloroplast precursor, protochlorophyllide (PChlide), Mg-Proto IX, and Mg-Proto IX-ME produce $^1O_2$ that can act as a signaling

molecule from chloroplast to nuclei (Tripathy and Oelmuller 2012). The $^1O_2$-dependent retrograde signaling has been extensively studied using the *Arabidopsis flu* mutant that massively accumulates PChlide and $^1O_2$ and exhibits growth retardation and cell death under constant dark/light cycle (op den Camp et al. 2003; Apel and Hirt 2004; Wagner et al. 2004; Laloi et al. 2007; Lee et al. 2007). Transcriptome analysis revealed a set of genes that can be specifically activated by $^1O_2$, not by H_2O_2 or $O_2^{\bullet -}$ (op den Camp et al. 2003; Gadjev et al. 2006; Suzuki et al. 2011), suggesting that chloroplast to nucleus retrograde signaling is at least partially regulated by $^1O_2$-specific signaling. Two plastid-localized proteins EXECUTER1 and EXECUTER2 are required for $^1O_2$-dependent gene regulation and cell death (Lee et al. 2007). Moderate light exposure induces acclimation mechanisms that protect plants against more severe high-light stress, and $^1O_2$ production and EXECUTER-dependent signal can be activated during this acclamatory process (Zhang et al. 2014), indicating the significance of $^1O_2$-dependent retrograde signaling in the regulation of light acclimation of plants. In addition, nuclear topoisomerase VI and oxygenation derivatives of linoleic acid, the prominent polyunsaturated fatty acid of chloroplast membrane lipid, might also play an important role to integrate $^1O_2$-dependent signal with regulation of nuclear gene expression (op den Camp et al. 2003; Tripathy and Oelmuller 2012). The oxidation of linoleic acid was shown to be not directly caused by $^1O_2$, but enzymatic reactions regulated by $^1O_2$ signals (op den Camp et al. 2003; Tripathy and Oelmuller 2012). Furthermore, $^1O_2$-linked cell death activator (soldat8) that encodes SIGMA6 factor of the plastid RNA polymerase was identified as specific suppressor of $^1O_2$-dependent stress responses in *flu* mutant (Coll et al. 2009). The other protein pleiotropic response locus 1 (PRL1) also affects the expression of $^1O_2$ response genes in *Arabidopsis* (Baruah et al. 2009).

In a recent study, integration between ABI4 and redox metabolism was investigated using the ascorbic acid-deficient mutants, *vtc1* and *vtc2* (Kerchev et al. 2011). The transcriptome signatures of *abi4*, *vtc1*, and *vtc2* mutants extensively overlap with large number of transcription factors and other regulatory genes (Kerchev et al. 2011). In addition, ABA and JA signaling synergistically function to regulate the growth of plants through ABI4 in ascorbic acid-dependent manner (Kerchev et al. 2011). Although it is still not clearly understood, H_2O_2 produced in chloroplasts was also implicated in retrograde signaling (Shapiguzov et al. 2012). H_2O_2-dependent retrograde signaling might be a combination of passive diffusion of H_2O_2 with indirect pathways involving ABA signaling (Mullineaux and Karpinski 2002; Galvez-Valdivieso and Mullineaux 2010). H_2O_2 might not be, however, the signaling molecule that directly affects nuclear gene expression; rather, redox-sensitive components such as oxidized proteins or peptides might mediate the signal transfer from the chloroplast to nucleus (Moller and Sweetlove 2010; Sierla et al. 2013).

Although mitochondrial retrograde signaling is poorly understood compared with the chloroplast to nucleus retrograde signaling, key regulators of mitochondrial retrograde signaling have been identified in recent studies. A membrane-bound NAC transcription factor, ANAC017, was found to be a regulator of

AOX1a, a marker gene of mitochondrial retrograde signaling (Ng et al. 2013). ANAC017 mediates H_2O_2-induced alterations in transcript abundance. The *abi4* mutants are insensitive to transcriptional derepression of AOX1a by the mitochondrial complex I inhibitor rotenone, indicating a role for ABI4 in redox regulation and mitochondria to nucleus retrograde signaling (Giraud et al. 2009). This work demonstrated the integral role of ABI4 to mediate mitochondrial and chloroplast retrograde signaling pathways, and perhaps it is the convergence point for mitochondria–plastid–nucleus coordination.

8 Programmed Cell Death Regulated by ROS Under Abiotic Stress

A low dose of ROS acts as signaling molecules that mediate at least part of stress responses, but they induce programmed cell death (PCD) at higher concentrations. PCD is a genetically controlled process in which only cells that are destined to die are selectively destroyed in a multistep fashion via the functions of specific proteases and nuclease (Petrov et al. 2015). Thus, no damage to the neighboring cells is inflicted. In addition, metabolism in the rest of cells and tissues can be appropriately adjusted to acclimate to or survive under stressed conditions by this process. Here, stress-specific pathways that positively regulate PCD will be mainly discussed. More detail of PCD under abiotic stress has been addressed in more extensive review (Petrov et al. 2015).

Severe drought enhances ROS production mainly due to decreased CO_2 fixation accompanied by increased leakage of electron to O_2, which may result in induction of PCD (Gechev et al. 2012). ROS production is inhibited by suppression of chlorophyll synthesis and photosynthetic activity, and modification of sucrose metabolisms under drought (Petrov et al. 2015; Liu et al. 2013). Although plants possess mechanisms to prevent unnecessary PCD, leaf senescence executed by ROS-triggered PCD acts as an important adaptation process of plants to drought (Petrov et al. 2015). ROS-triggered PCD under drought was shown to be regulated by coordination between ABA and cytokinin (Munn-Bosch and Alegre 2004). PCD characterized by degradation of organelles, increased size of the vacuole, and plasmalemma collapse can be induced in root tip meristems under drought (Duan et al. 2010). This adaptive mechanism in the root might be a strategy of plants to enhance lateral root growth. Reorientation of polyamine metabolism, as well as stomatal closure, is a drought response mechanism in which ABA plays pivotal roles. In grapevine, ABA induces accumulation of polyamine that is metabolized by amine oxidases, and H_2O_2 can be produced as a by-product of this reaction (Toumi et al. 2010). H_2O_2 produced in this reaction might be a regulator of further stress response or induction of PCD. PCD acts as an important process to protect plants against flooding stress. Under this stress condition, aerenchyma is formed by PCD in selected cells to produce air channels in roots (Gunawardena et al. 2001). Significance of ROS in the formation of aerenchyma has been indicated by the

finding that exogenous application of H_2O_2 stimulated formation of aerenchyma in rice (Steffens et al. 2011). In addition, expression of RBOHD was highly enhanced during water logging conditions in maize, suggesting that RBOHD-dependent production of H_2O_2 play a key role in the formation of aerenchyma (Rajhi et al. 2011). Furthermore, mitochondrial dysfunction and accumulation of metal ion was also shown to increase ROS production under flooding stress (Shabala et al. 2014).

Although involvement of $O_2^{\bullet -}$ produced via the function of NADPH oxidase is implicated in PCD pathway under salt and osmotic stress, signals activated by ionic salt and nonionic osmotic stress inducer (i.e., sorbitol) did not show extensive overlap (Monetti et al. 2014; Petrov et al. 2015), indicating the specificity of the mechanism regulating PCD under these stresses. Specific feature of salt-induced PCD is characterized by ion disequilibrium that may be due to invasion of Na^+ into the cytosol accompanied by a decrease in K^+ (Kim et al. 2014). Hydroxyl radicals in cells might affect K^+/Na^+ ratio and regulate enzymes involved in PCD (Demidchik et al. 2010). In addition, an anti-apoptotic protein BCL2 can regulate vacuolar processing enzymes (VPE) by modulating ion fluxes in rice (Kim et al. 2014). Overexpression of BCL2 exhibited enhanced PCD symptoms accompanied by significantly reduced K^+ efflux and represses the expression of VPEs. These findings suggest that maintenance of proper Na^+/K^+ ratios could be a key process to enhance salt stress tolerance in plants (Huh et al. 2002; Teakle and Tyerman 2010).

Mitochondria are responsible for the regulation of PCD induced during heat stress (Vacca et al. 2004). Functionally active cytochrome c can be released from the mitochondria in a ROS-dependent manner, and caspase-like protease that induces PCD is then activated (Vacca et al. 2004). Proline that was implicated in mitochondrial ROS metabolism (Miller et al. 2009a) might be a specific component to cause cell death during heat stress. Although it acts as an osmolyte and ROS detoxifier under salt and osmotic stresses, it can negatively impact on cells under heat stress (Lv et al. 2011). In addition, exogenous application of ascorbate or glutathione (i.e., unspecific antioxidants) induced PCD in *Arabidopsis* cells under heat stress. On the other hand, catalase that specifically detoxifies H_2O_2 suppressed PCD. These finding suggest that H_2O_2 functions as PCD-inducing signal, but other types of ROS might act as negative regulator of PCD during heat stress (Doyle and McCabe 2010). Furthermore, MPK6 was found to be a component of PCD under heat stress. Enhanced accumulation of ROS and cytosolic Ca^{2+} can activate NPK6 that functions upstream to hydrolases and proteases associated with PCD (Li et al. 2012).

Low temperature is also able to induce PCD in plants when combined with elevated light intensity. LSD1, a negative regulator of PCD, interacts with catalase, and its deficiency can enhance low-temperature-induced PCD in plants (Huang et al. 2010; Li et al. 2013b). LSD1 and EDS1 proteins antagonistically regulate the acclimation of plants to UV-C stress. Plants deficient in LSD1 exhibited enhanced PCD following UV-C treatment. On the other hand, knocking out of EDS1 repressed UV-C-induced PCD (Wituszynska et al. 2015). The UV-C response associated with LSD1 and EDS1 is regulated by the modulation of ROS

homeostasis. In response to high dose of UV-B, a defense program involving SA, JA, ethylene, and senescence-associated processes is activated to prevent oxidative damage in plants (Bandurska et al. 2013). These findings indicate the cross talks in the pathways associated with PCD between low-temperature, high-light, UV-B and UV-C, and pathogen responses.

9 Conclusions

ROS play an integral role in the regulation of numerous responses to abiotic stresses in plants. The complexity in ROS responses to various environmental stimuli might be, at least partially, attributed to different regulatory mechanisms of ROS production via basic biological processes and NADPH oxidases (RBOHs) that function in an array of organelles, tissue types, and developmental stages under various environmental conditions. A key mechanism in coordinating the complex spatial and temporal responses in plants is the cascade of cell-to-cell communication events that result in the formation of a wave of ROS production and increase in cytosolic Ca^{2+} that rapidly propagates throughout the different tissues of the plant. Networks of ROS/redox signaling in the chloroplast and mitochondria contribute to a delicate balance of homeostasis within each organelle, as well as to cross talk between different cellular components by regulating important biological pathways such as nuclear gene expression and energy metabolism under stress conditions. Functions of chloroplasts and mitochondria might be the key mediators for signal transductions in the cell because they are involved in the regulation of important pathways including systemic signaling, retrograde signaling, and PCD under stress conditions. In addition, there is a great overlap in the regulatory mechanisms between RBOHD-dependent systemic response, chloroplast/mitochondrial retrograde signaling and PCD, suggesting a cross talk between these pathways. Detailed mechanisms to coordinate or integrate these different ROS-dependent pathways need to be elucidated in future works. In addition, we also need to address how different pathways switched depending on the different environmental stimuli. This question could be answered by studying integration between ROS signaling and sensors of different stimuli.

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Redox Regulation and Antioxidant Defence During Abiotic Stress: What Have We Learned from *Arabidopsis* and Its Relatives?

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Abstract Abiotic stress conditions are a global constraint that affects plant growth and crop yield worldwide, and this phenomenon is expected to be increased in the forthcoming future due to global climate change. *Arabidopsis thaliana* is the model organism for plant science since the early 1990s, and its genome has been known for more than a decade. Studies conducted with *Arabidopsis* created a foundation that could be transferred and used in its close relatives to similarity of genetic sequences. Up to now, studies on *A. thaliana* gave deep insight into different abiotic stress tolerance mechanisms. However, *A. thaliana* is not a stress-tolerant plant species. Therefore some of the stress tolerance mechanisms that are used by its

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stress-tolerant relatives might not even be observed in *Arabidopsis*. This chapter focused on reactive oxygen species (ROS) production during environmental stress and antioxidant defence systems activated against it in *A. thaliana* and its close relatives such as *Thellungiella* sp., *A. halleri, Thlaspi* sp., *Lepidium sativum* and *Arabis paniculata.*

Keywords *Arabidopsis*-related model species (ARMS) • *Arabidopsis* • Antioxidant defence • Drought stress • Heavy metal stress • Redox regulation • Salinity • Oxidative stress

1 Introduction

Arabidopsis thaliana is an invaluable tool for plant biologists since it has been used as a model organism. Its short life cycle, small size, low number of chromosomes, small genome and prolific seed production through self-pollination made *Arabidopsis* a useful tool for genetic studies. Since the production of the first induced mutant with X-ray in 1947 and the development of different protocols for transformation in 1980s and publishing of its full genome in 2000, *A. thaliana* played a pivotal role in the development of plant biology (Koornneef and Meinke 2010). Most of our detailed knowledge about key processes such as plant growth, development, reproduction and response to environment was gained through *A. thaliana*, its mutants, and transgenic lines. Global climate change and rapidly increasing human population brings a new challenge to the field of plant biology: producing enough food to feed everyone even under adverse environmental conditions. For this aim, a better understanding of stress tolerance mechanisms is needed to cultivate marginal lands such as arid or saline areas and to sustain plant production during the climate change. Even though it is an indispensable genetics tool, *A. thaliana* is not a stress-tolerant plant species and some of the stress tolerance mechanisms that are used by its stress-tolerant relatives might not even be observed in *Arabidopsis*. Considering this, some researchers started searching for alternative hosts that evolved to tolerate stress factors at hand (Amtmann et al. 2005). While doing so, selection of plant species that are close relatives of *A. thaliana* provided researchers with the chance to exploit gene sequences and genetics tools available from *Arabidopsis* due to high genetic sequence similarity. In these days, efforts in this area have started to bear its fruits. Model species such as *Thellungiella* as a salt-tolerant model and *A. halleri*, *Arabis* sp. and *Thlaspi* sp. as heavy metal-tolerant models have been very useful tools to investigate tolerance mechanisms to these stresses. In this chapter, we have summarised and discussed studies that are related to redox regulation and antioxidant defence in *Arabidopsis* and its close relative *Arabidopsis*-related model species (ARMS) under abiotic

stress such as salinity, drought and heavy metals. For this, first, we will introduce mechanisms of ROS production, and then we will summarise enzymatic and nonenzymatic antioxidant defence mechanisms. Following this, studies in *Arabidopsis* and its relatives in stressed conditions will be discussed.

2 What is ROS and How it is Produced in Plant Cell?

Reactive oxygen species (ROS), which can also be named as active oxygen species (AOS) or reactive oxygen intermediates (ROI), are formed in all aerobic organisms from bacteria to mammalian cells (Bose et al. 2014). They are inevitable by-products of the aerobic metabolism. There are basically four forms of ROS within the cell: singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$). The main sites of ROS production in a plant cell are mitochondria, chloroplast, peroxisomes, plasma membrane and apoplast. ROS play a dual role: they are involved in cellular signalling on one hand and on the other hand as toxic products that accumulate under different environmental stress conditions such as salinity, drought, etc. To utilise ROS as signalling molecules, nontoxic levels of ROS must be maintained by the control of ROS production and the metabolic counterprocess involving ROS-scavenging pathways and the expression of ROS-regulated genes (Mittler et al. 2004; Lai et al. 2012). On the other hand, uncontrolled accumulation of ROS causes protein denaturation, lipid peroxidation in cellular membrane, oxidation of DNA and RNA and carbohydrate oxidation and also negatively affects enzymatic activity (Scandalios 1993; Noctor and Foyer 1998).

Under normal conditions, plants continuously produce ROS that are localised in different cellular compartments as by-products of various metabolic pathways. While the main sources of ROS production are chloroplast and peroxisomes in the light (Foyer and Noctor 2003), in darkness, it is mitochondria (Moller 2001). Chloroplasts and peroxisomes produce 20-fold more ROS than mitochondria in the light (Rhoads et al. 2006). However, in the dark and in nongreen tissues, mitochondria are the major sources of ROS. In addition, ROS also can be produced at the apoplastic space by plant NADPH oxidases, cell wall-associated peroxidases (POXs) and oxalate oxidases (Kawano 2003), but reliable quantification of apoplastic ROS accumulation is still unavailable (Wrzaczek et al. 2013).

2.1 Chloroplasts

In chloroplasts, the main sites of ROS production are PSI and PSII reaction centres. Under abiotic stresses such as drought and salt stress, decreased availability of CO_2 as a result of limiting gas exchange enhances the production of ROS in chloroplasts. Under normal conditions, the electron flow from excited photosystem centres is

directed to $NADP^+$ to produce NADPH; however, when electron transfer chain (ETC) is overloaded, a part of this flow can be diverted from ferredoxin to O_2, resulting in the production of superoxide anion radical ($O_2^{\bullet -}$) through the Mehler reaction. On the other hand, $^1O_2$ is formed in PSII by energy transfer from triplet excited state chlorophyll, which is produced by excess excitation by light, to $^3O_2$, mainly under high light intensities (Laloi et al. 2004). $^1O_2$ can diffuse distances of only hundred nanometers and react with the first available biomolecule. Moreover, it can cause light-induced activity loss in PSII, triggering cell death (Wagner et al. 2004; Krieger-Liszkay et al. 2008). The production of $^1O_2$ can be prevented by the Mehler reaction due to its ability to relax ETC (Asada and Takahashi 1987). Hence, the production of $O_2^{\bullet -}$ might act as an alternative sink for the consumption of electrons in the ETC, and this is called the 'water–water cycle'. The major site of $O_2^{\bullet -}$ production is thylakoid membrane-bound primary antenna pigments. H_2O_2 is the most stable ROS as compared to other free radicals. While the half-lives of $^1O_2$, $O_2^{\bullet -}$ and $OH^{\bullet}$ are 2–4 μs, the half-life of H_2O_2 is close to a minute (Bhattachrjee 2005; Pitzschke et al. 2006). Among ROS, only H_2O_2 can diffuse some distance from its site of production. Excess accumulation of H_2O_2 in plant cell leads to the occurrence of oxidative stress, leading to necrosis or even programmed cell death (PCD) (Quan et al. 2008). Moreover, it may inactivate enzymes by oxidising thiol groups (Gill and Tuteja 2010). Interestingly, at low concentrations, H_2O_2 can act as a signal molecule, which triggers tolerance to abiotic and biotic stress and is a key regulator in some physiological processes such as growth and development (Foreman et al. 2003), stomatal movement (Bright et al. 2006) and senescence (Peng et al. 2005).

2.2 *Mitochondria*

In mitochondria, under abiotic stress, electrons can be transferred from complex I and complex III to O_2 due to overreduction of electron acceptors, which results in the production of $O_2^{\bullet -}$. $O_2^{\bullet -}$ is reduced by SOD activity to H_2O_2. This H_2O_2 can react with reduced Fe^{2+} and Cu^+ to produce highly toxic $OH^{\bullet}$, which are lethal (Rhoads et al. 2006; Sweetlove and Foyer 2004). Therefore, in mitochondria, the main sites of ROS production are complex I and complex III. ETC system in mitochondria may prevent this overreduction of electron carriers by means of energy-dissipating systems. For example, plants utilise an alternative oxidase pathway (AOX), which is an alternative to the cytochrome pathway in mitochondria. Electrons are taken from the ubiquinone pool and transferred to oxygen to form H_2O in alternative pathway, and energy is dissipated as heat (Millar et al. 2011). In this way, this system prevents ROS generation. Therefore, mitochondria may play a central role in cell adaptation to abiotic stresses, which are known to induce oxidative stress at cellular level.

2.3 Peroxisomes

$O_2^{\bullet -}$ radicals generated as a consequence of their normal metabolism are produced in peroxisomes. In peroxisomes, one of the main sites of ROS production is organelle matrix where xanthine oxidase (XOD) catalyses the oxidation of xanthine and hypoxanthine to uric acid (Corpas et al. 2001). Apart from mitochondria and plastids, peroxisome membranes have sites for ETC composed of a flavoprotein NADH and cytochrome b. In peroxisome membranes, $O_2^{\bullet -}$ is produced by peroxisome ETC (del Río et al. 2002). H_2O_2 is produced by the main metabolic processes such as β-oxidation of the fatty acids, photorespiratory glycolate oxidase reaction, enzymatic reactions of flavin oxidases and the disproportionation of $O_2^{\bullet -}$ radicals. Similar to chloroplast and mitochondria, peroxisome-generated ROS especially H_2O_2 at low levels also act as mediators in intracellular signalling (Masters 2001). In addition, in peroxisomes, transition metal ions like Fe^{2+} and Cu^{2+} that can catalyse the formation of hydroxyl radical ($OH^{\bullet}$) in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$) are also abundant. Excess formation of this ROS can lead to lipid peroxidation, followed by damage to peroxisomal membrane and ultimately resulting in loss of peroxisomal functions (Yokota and Oda 2001). Therefore, peroxisomes are a source of oxidative stress, but it is a compartment where controlled production and scavenging of ROS (mostly due to photorespiration) simultaneously occur.

2.4 Other Sources of ROS Production

ROS are also produced at the apoplastic space and plasma membrane. This production is stimulated by process like ozone fumigation and during pathogen attack. H_2O_2 can be produced in apoplast by pH-dependent cell wall peroxidases, germin-like oxalate oxidases, amine oxidases and glycolate oxidases (Kawano 2003). Moreover, in plasma membranes, other sources of this radical are NADPH-dependent oxidases (NADPH oxidases, known in plants as respiratory burst oxidase homologues [RBOH]) (Baxter et al. 2014). Also, oxalate oxidase was shown to be involved in ROS production in root cells during drought stress (Voothuluru and Sharp 2013), whereas glycolate oxidase (GOX) has been shown to play a role in nonhost pathogen defence in *Arabidopsis* and tobacco (Rojas and Mysore 2012; Rojas et al. 2012).

3 Antioxidant Defence Mechanism

Plants possess an array of antioxidant defence enzymes to cope with detrimental effects of ROS. These enzymes work in coordination to scavenge $O_2^{\bullet -}$, H_2O_2 and other free radicals. Among different ROS mentioned above, $OH^{\bullet}$ and $^1O_2$ cannot be scavenged by enzymatic antioxidants, and their detoxification is purely dependent on nonenzymatic low-molecular-weight antioxidants. Although mechanisms underlying the production of ROS in plants under stress are universal, based on plant species, different antioxidant defence strategies are involved to scavenge ROS. The mechanism of different types of enzymatic and nonenzymatic antioxidants will be briefly discussed with special emphasis on *Arabidopsis*. Our aim of focusing discussion in *Arabidopsis* is that although most of the enzymes were first identified in other species such as spinach, maize or barley, molecular mechanisms underlying their activities, their regulation and their interactions with other cellular components were first studied and elucidated in *Arabidopsis* due to genetic and molecular tools available.

3.1 Superoxide Dismutase

Among antioxidant enzymes, SOD has a unique role: it is the only enzyme that can scavenge $O_2^{\bullet -}$ and convert it to H_2O_2. Therefore, cellular defence against $O_2^{\bullet -}$, which is the main ROS produced during photosynthesis and respiration, is dependent on SOD activity. As it can be seen, SOD activity is the first step in the detoxification of the $O_2^{\bullet -}$, and hence, it has been named as the 'first line of defence' against oxidative stress. Delicate balance between SOD and other antioxidant enzymes that scavenge H_2O_2 such as APX, CAT, peroxidases and peroxiredoxins needs to be maintained in the cell for full detoxification of ROS. If SOD activity exceeds the capacity of H_2O_2-scavenging enzymes, H_2O_2 can accumulate and exert its toxic effects. On the other hand, insufficient SOD activity leads to the accumulation of $O_2^{\bullet -}$, which can also cause oxidative damage.

It is known that phospholipid membranes are not permeable to $O_2^{\bullet -}$ due to its negative charge (Takahashi and Asada 1983). Therefore $O_2^{\bullet -}$ that is produced in a compartment needs to be scavenged in the same organelle. For this reason, plants have a wide arsenal of SOD isoforms that work in different cellular compartments. Three types of SOD have been identified in plants that differ by their cofactor in the active site (Cu and Zn, Fe and Mn). These enzymes are located in different compartments of the cell and scavenge $O_2^{\bullet -}$ in their specific locations. FeSOD is believed to be the most ancient isoform due to the abundance of iron in soluble Fe (II) form at that time (Bannister et al. 1991). As the levels of O_2 in the environment increased and minerals were oxidised, Fe (II) become less available and Mn (III) was utilised in the active site of SOD (MnSOD). Similarly, increasing O_2 concentration in the atmosphere caused the conversion of insoluble Cu (I) to soluble

Cu (II). At this phase, Cu (II) began to be used at the active site of SOD (Cu/ZnSOD) (Alscher et al. 2002).

FeSOD is localised in chloroplasts, while MnSOD is localised in mitochondria. Cu/ZnSOD can be found in different compartments such as chloroplasts, cytosol and peroxisomes. In *Arabidopsis*, there are three genes encoding FeSOD (*FSD1*, *FSD2*, *FSD3*), three genes encoding Cu/ZnSOD (*CSD1*, *CSD2*, *CSD3*) and one gene encoding MnSOD (*MSD1*). Among these *CSD* genes, *CSD1* and *CSD2* encode cytoplasmic and chloroplastic isoforms, respectively, whereas *CSD3* encodes the peroxisomal Cu/ZnSOD isoform. It has been shown that *FSD1* expression is under circadian regulation in response to a diurnal rhythm, while other isoforms of SOD respond to oxidative stress (Kliebenstein et al. 1998).

3.2 Catalase

Catalase was first described in 1900 by Oscar Loew, and it is the first antioxidant enzyme to be discovered. This enzyme was named as 'catalase' by the author because of 'its catalytic action on hydrogen peroxide' (Loew 1900). Catalase dismutates two molecules of H_2O_2 to water and O_2. All known eukaryotic catalase are haem containing tetramer proteins, and each polypeptide in a tetramer contains a haem prosthetic group (Regelsberger et al. 2002). Investigations showed that this enzyme is encoded by three genes in all angiosperms species studied till date. Products of all three genes encode 492 amino acid polypeptides, which show a high similarity between each other. In *Arabidopsis*, *CAT1* gene is mainly expressed in pollen and seeds, while CAT2 is expressed in photosynthetic tissues. *CAT3* is expressed in vascular tissues and leaves. Among these genes, *CAT2* follows a circadian rhythm similar to photosynthetic enzymes, while *CAT3* follows an opposite one. Although there are three genes encoding CAT in *Arabidopsis*, seven different bands can be detected in native activity gels (Hu et al. 2010). This is due to heterotetrameric nature of the enzyme. According to this, if three CAT genes encode a heterotetrameric protein, there can be 15 possible distinct isoforms (McClung 1997). However, fewer than the possible number of catalase heterotetramers might indicate a specific interaction between different subunits, eventually limiting the number of isoforms (McClung 1997).

In plants, CAT is mainly found in peroxisomes and is responsible for scavenging of photorespiratory H_2O_2 and H_2O_2 produced by β-oxidation of fatty acids. In *Arabidopsis*, leaf CAT activity is reduced by 80 and 20 % in *cat2* and *cat3* knockout plants, respectively, while there is no evident decrease in *cat3* knockouts. Moreover, double *cat2 cat1* and *cat2 cat3* knockout plants have similar CAT activities to that of *cat2* knockout plants (Bueso et al. 2007). When grown at irradiances between 50 and 200 μmol m^{-2} s^{-1}, *cat2* plants showed a dwarf phenotype, which became more severe with the increasing light intensity. This phenotype under increasing light intensity was caused by redox perturbation due to insufficient scavenging of H_2O_2 (Queval et al. 2007). Contrary to *cat2*, *cat1* and *cat3* plants

did not show any obvious leaf phenotype, which was also consistent with the reduction in leaf CAT activity (Hu et al. 2010). In addition, Hu et al. (2010) showed that *cat2* phenotype could be complemented with CAT2 or CAT3 protein expressed under *CAT2* promoter, indicating that the function of CAT2 is rather regulated at transcriptional level. Previously it was reported that calmodulin (CaM) binds to CAT3 in Ca^{2+}-dependent manner and regulates its activity, indicating an interaction between Ca^{2+} signalling and the regulation of CAT activity (Yang and Poovaiah 2002).

3.3 Ascorbate Peroxidase and Other Ascorbate–Glutathione Cycle Enzymes

Ascorbate peroxidase (APX) is a Class I peroxidase that exclusively uses ascorbate (AsA) as its electron donor to scavenge H_2O_2. During this reaction, two AsA molecules are oxidised to yield two monodehydroascorbate (MDHA) molecules and H_2O_2 is converted to water. MDHA formed is spontaneously disproportionated to AsA and dehydroascorbate (DHA). MDHA can also be converted directly to AsA via the action of NADPH-dependent enzyme monodehydroascorbate reductase (MDHAR). On the other hand, oxidised DHA can be regenerated back to AsA by dehydroascorbate reductase (DHAR), which utilises GSH as a source of reducing power. Oxidised GSH (GSSG) is then regenerated back to GSH by glutathione reductase (GR) with NADPH. In conclusion, APX supported by an efficient AsA–GSH cycle for the regeneration of electron donor AsA prevents the accumulation of toxic levels of H_2O_2 in the plant cell. This regeneration mechanism is also important for sustainable activity of APX enzyme. When the concentration of AsA is lower than 20 μM in the medium, APX activity was rapidly and irreversibly lost. This inactivation occurred in 30 s for chloroplastic APX isoforms, while it was approximately an hour for cytosolic APX isoforms (De Leonardis et al. 2000).

In *Arabidopsis*, there are at least nine different isoforms of APX (Mittler et al. 2004). The chloroplast contains at least three isoforms, which are located in different parts of the chloroplasts such as lumen (*APX4*), stroma (*sAPX*) and thylakoids (*tAPX*). Stromal APX can also be targeted to mitochondria (Chew et al. 2003). In some plant species, stromal and thylakoid isoforms of APX are encoded by a single gene, and different isoforms are a product of alternative splicing. However, in *Arabidopsis*, there are two separate genes for each isoform (Shigeoka et al. 2002). It is known that many photosynthetic enzymes can be regulated via oxidation and reduction of their thiol groups (Dietz and Pfannschmidt 2011); therefore fine-tuning of H_2O_2 concentrations in chloroplasts is vital and APX plays a major role in this regulation. In contrast to a variety of chloroplastic APX isoforms, cytoplasm of *Arabidopsis* only contains one APX isoform (*APX1*) under normal conditions, and an additional isoform (*APX2*) was induced under high light stress (Karpinski et al. 1999). In addition, one isoform of APX (*APX3*) was

reported in peroxisomes, site that is known to be a major source of H_2O_2 in plant cells. Similar to APX, isoforms of AsA-GSH cycle enzymes are also diverse. There are five *MDHAR*, five *DHAR* and two *GR* genes in *Arabidopsis*, which are dispersed to cytosol, chloroplasts and mitochondrial compartments. Like stromal APX, products of *MDHAR1* and *DHAR1* and *GR2* genes are also dual targeted to chloroplasts and mitochondria.

3.4 Glutathione Peroxidase

Glutathione peroxidise (GPX) is the general name for the family of enzymes that catalyse the reduction of H_2O_2, lipid peroxides and organic peroxides by utilising GSH as electron donor (Ursini et al. 1997). The global phylogenetic analysis of GPX between vertebrates, invertebrates, bacteria, fungi and plants showed that plant GPXs form an independent cluster (Margis et al. 2008). An ancestral gene led to the origin of all plant GPX genes, and major duplication events of this gene to generate known GPXs occurred before the divergence of monocots and dicots. There are 8 genes encoding GPX in *Arabidopsis* (Mittler et al. 2004). Among these *GPX1*, *GPX6* and *GPX7* are predicted to be localised in chloroplasts, while *GPX3* is in mitochondria and *GPX5* is in endoplasmic reticulum. Besides these organellar isoforms, there are also two additional cytoplasmic isoforms *GPX4* and *GPX8* (Mittler et al. 2004). These isoforms show spatiotemporal expression pattern. Among these, *GPX1* is strongly expressed in leaves, stems and flowers; however, it is not expressed in roots indicating a role for defence against photosynthetic ROS (Milla et al. 2003). Milla et al. (2003) also showed that these genes were differentially induced under abiotic stresses such as salinity, osmotic stress, cold and high temperature. Under salinity, *GPX1*, *GPX2*, *GPX 5* and *GPX 6* were induced, while under osmotic stress, all of the *GPX* genes were induced. However, *GPX1* was the only gene to respond to high temperature stress. Recently, Passaia et al. (2014) showed that *GPX*s can regulate root phenotype of *A. thaliana* plants. By using different *gpx* mutants, they demonstrated that *gpx1*, *gpx4*, *gpx5*, *gpx7* and *gpx8* mutants had a greater lateral root density, when compared to wild type, while, *gpx2* and *gpx3* mutants had lower lateral root densities.

3.5 Peroxiredoxins

Peroxiredoxins (Prxs) are a family of thiol-specific reductases or peroxidases that catalyse the reduction of a range of different peroxides such as H_2O_2, peroxynitrites and alkyl hydroperoxides to water, nitrite or corresponding alcohol (Hofmann et al. 2002). They are nonheme-containing peroxidases that require an external electron donor (Rouhier and Jacquot 2002). When Prxs reduce a substrate and become oxidised, they lose their enzymatic activity, and for the next catalytic cycle,

they need to be regenerated (Dietz et al. 2002). It was shown that for many Prxs, thioredoxins (TRXs) serve as electron donor to regenerate the enzyme as well as other dithiols such as glutathione (Dietz et al. 2002; Finkemeier et al. 2005). Four out of six subclasses of Prx have been reported in plants, and these are 1-Cys Prx, 2-Cys Prx, type II Prx and PrxQ (Dietz et al. 2002). 1-Cys Prx contains a single catalytic cysteine, and it gets its name from this single cysteine. 2-Cys Prx is a dimeric enzyme with two cysteine residues in each polypeptide, and in plants, they are localised exclusively in chloroplasts. Similarly, type II Prx contains two conserved cysteines, but these Prxs are localised in other subcellular compartments such as chloroplasts, mitochondria and cytosol (Finkemeier et al. 2005; Bréhélin et al. 2003). The fourth type of Prxs is PrxQ, which are monomeric enzymes that contain two cysteines separated by four amino acids (Rouhier and Jacquot 2002). In *Arabidopsis,* there are ten genes that encode Prx genes (Dietz et al. 2002). These encode one 1-Cys Prx, two 2-Cys Prxs (2-Cys Prxs A and B), one PrxQ and six type II Prxs (type II Prxs A–F). Baier et al. (2000) demonstrated that antisense suppression of *Arabidopsis* 2-Cys Prx leads to increased oxidation of Asc pool. In response to this, stromal and thylakoid APX and MDHAR expressions were increased. Induction of H_2O_2-scavenging mechanism by APX under decreased 2-Cys Prx expression and oxidation of Asc pool under these circumstances suggested that 2-Cys Prx plays a major role in the detoxification of H_2O_2 in chloroplasts. Although 2-Cys Prx is not efficient as APX in terms of H_2O_2 reduction, it can detoxify other hydroperoxides such as tert-butyl hydroperoxide and cumene hydroperoxide. By this way, Prx can combat with lipid peroxidation, which is in agreement with thylakoid localisation of this enzyme where most of the lipid peroxides are produced in chloroplasts (König et al. 2002).

3.6 Nonenzymatic Antioxidants

There are many nonenzymatic antioxidants such as ascorbate, glutathione, tocopherols, carotenoids, polyphenols like flavonoids, and some compatible solutes such as proline. As compared with enzymatic antioxidants, nonenzymatic antioxidants have higher potential to avoid the toxic effects of ROS. Among them, AsA and GSH are the most important and well-studied compounds that mediate the redox status of the cell under strict control.

Ascorbate is one of the most important molecules that directly scavenge H_2O_2 and is the electron donor of APX (Foyer and Noctor 2003). GSH is a part of AsA–GSH cycle and plays crucial role in diverse physiological processes like plant growth, sulphate transport, signal transduction, cell cycle, cell death and senescence (Kranner et al. 2006). Moreover, it is responsible for the removal of excess H_2O_2 and a strong partner of redox regulatory system (Mullineaux and Rausch 2005). Detailed information of AsA–GSH cycle compounds and enzymes of relatives of *Arabidopsis* will be discussed under environmental stress sections in this chapter.

α-Tocopherol prevents the membranes from lipid peroxidation by scavenging lipid peroxyl radicals and also $^1O_2$ in chloroplasts (Munne-Bosch 2005). In the presence of AsA, tocopherols can be regenerated and continue to scavenge the lipid peroxyls. For instance, ascorbate-deficient *vtc1* mutant of *Arabidopsis* showed α-tocopherol deficit under stressed conditions due to the absence of ascorbate in chloroplasts (Munné-Bosch and Alegre 2002). Overexpression of tocopherol biosynthesis genes (*HPPD*, *VTE1*, *VTE2*, *VTE3*, *VTE4*) in *A. thaliana* enhanced the expressions of genes that encode APX, DHAR and MDHAR enzymes and altered AsA and GSH pool in the cell (Li et al. 2010). Recently, an excellent review was published on the relation between stress tolerance and tocopherols, which can be used for further information (Miret and Munné-Bosch 2015).

Carotenoids are another group of low-molecular-weight antioxidants playing a central role in scavenging $^1O_2$ in chloroplasts and preventing damage of $^1O_2$ and peroxyl radicals in the cell. There are many side products of the oxidation of β-carotene such as β-cyclocitral, which are accumulated as a consequence of environmental stress. β-Cyclocitral was recently identified to enhance the expressions of $^1O_2$-responsive genes but not H_2O_2-responsive genes in *Arabidopsis*. These findings suggest that oxidation products of nonenzymatic antioxidants might act as signalling molecules in the transduction of $^1O_2$ signalling in plants (Ramel et al. 2012). This phenomenon was only documented in *Arabidopsis*, and it was not investigated in any other related model plants which are more tolerant to abiotic stress. It might be interesting to test if this signalling type also exists in other stress-tolerant relatives of *Arabidopsis*.

Besides their effects on water balance in the cell, there are many studies suggesting that compatible solutes such as proline can act as nonenzymatic antioxidants, which can scavenge $HO^{\bullet}$ (Szabados and Savouré 2010). *Arabidopsis* overexpressing Δ(1)-pyrroline-5-carboxylate synthetase under hsp 17.6II promoter enhanced proline biosynthesis after heat induction, which also resulted in increase in the activity of SOD, GPX and CAT (Lv et al. 2011). Kant et al. (2006) showed that salt tolerance of *Thellungiella halophila* is highly related to proline metabolism. Exogenous proline application to cultured cells of *Thellungiella* before H_2O_2 application alleviated the damage of oxidative stress, demonstrating the protective role of proline in *Thellungiella* (Soshinkova et al. 2013). Also, drought stress increased the proline content and activities of antioxidant enzymes in *B. juncea* (Fariduddin et al. 2009). Lee et al. (2012) examined the proline contents of 16 *Thellungiella* accessions and compared them with the data obtained from 54 *A. thaliana* accessions under low temperature (4 °C, 14 days) and found that proline content was higher in *Thellungiella* species.

4 ROS Formation and Antioxidant Defence Under Abiotic Stress

4.1 Salt Stress

In the last 20 years, understanding of the mechanism underlying plant tolerance and adaptation to salt stress has been based on comparative analyses between halophytic and glycophytic (salt-sensitive) species, rather than genetic studies that were only conducted in halophytes. Moreover, multigenes involved in salt tolerance have been revealed by mutational approaches derived from salt-sensitive *A. thaliana*. However, *A. thaliana* may not be suitable to study salinity tolerance due to its sensitivity to this stress. Therefore, although it is a genetic model, *A. thaliana* may not provide sufficient information about the mechanism of salt tolerance. This situation has raised the idea of investigating halophytic species that are closely related to *A. thaliana* for this kind of research. In this part, responses of several species, which are close relatives of *A. thaliana*, to salt stress will be discussed.

Thellungiella parvula, a close relative of *Arabidopsis*, can withstand salt concentrations up to 600 mM, while *A. thaliana* (genetic model, glycophyte) shows significant damage and even plant death at 50 mM (Orsini et al. 2010; Uzilday et al. 2015). While 50 mM NaCl treatment did not negatively influence growth of *T. parvula*, 300 mM (Ghars et al. 2008; Uzilday et al. 2015) and 500 mM NaCl (Kant et al. 2006) significantly decreased it. These results showed that 50 mM NaCl could be counted as a 'nonstress' condition for *T. parvula*, while higher salt concentrations become stressful as reported by Uzilday et al. (2015) (Table 1). Recently, *Thellungiella parvula* is renamed as *Eutrema parvulum*, but for the sake of recognition and wide use of *Thellungiella* name in the literature, we preferred to use the genus name *Thellungiella* in this chapter.

Salt stress can significantly decrease maximum quantum yield of PSII (Fv/Fm), which is an indicator of damage to the photosynthetic machinery (Lim et al. 2007; Kim et al. 2010). However, Fv/Fm of *T. parvula* was not altered even at 300 mM NaCl. This result suggested that there is no any apparent damage in photosynthetic machinery of *T. parvula* (Uzilday et al. 2015). On the other hand, M'rah et al. (2007) found that 50 mM NaCl did not affect the photosynthetic activity of *Thellungiella halophila* due to decreased stomatal conductance and unchanged Rubisco pool. But 100 and 200 mM NaCl decreased photosynthetic activities in both *T. halophila* (halophyte) and *A. thaliana* (glycophyte). However, it was observed that the detrimental effect of 200 mM NaCl in *T. halophila* was less than *A. thaliana* as evidenced by lower contents of lipid peroxidation (M'rah et al. 2007).

M'rah et al. (2007) determined also the expression level of CDSP32, which is a plastidic thioredoxin-like protein, in the abovementioned study. CDSP32 plays a role in limiting the accumulation of alkyl hydroperoxides in chloroplast and is accepted as one of the components of the defence system against oxidative damage. It was found that its expression level increased in both *T. halophila* and *A. thaliana*

Table 1 Antioxidant enzyme activities in close relatives of *Arabidopsis* under stress conditions

Species	Reference	Treatment	SOD	CAT	POX	APX	GR	MDHAR	DHAR
Thellungiella halophila	M'rah et al. (2006)	NaCl 100 mM	−	+					
Thlaspi caerulescens	Wójcik et al. (2006)	Cd 25 μM	0	−	−	−			
Thlaspi caerulescens	Wójcik et al. (2006)	Cd 500 μM	−	−	0	0			
Thlaspi caerulescens	Wójcik et al. (2006)	Zn 500 μM	+	+	−	+			
Thlaspi caerulescens	Wójcik et al. (2006)	Zn 2000 μM	+	−	+	−			
Arabis paniculata	Qiu et al. (2008)	Cd 178 μM	+	+		+	+		
Thellungiella parvula	Uzilday et al. (2015)	NaCl 300 μM	+		+	+	+	+	+
Lepidium sativum	Gill et al. (2012)	Cd 100 μM	+	+		+	+		

SOD Superoxide dismutase, *CAT* Catalase, *APX* Ascorbate peroxidase, *GR* Glutathione reductase, *MDHAR* Malondialdehyde reductase, *DHAR* Dehydroascorbate reductases. +: increase, −: decrease, 0: no change

under salt stress. However, the highest expression level was observed in *T. halophila*. These results suggest that salt tolerance of *T. halophila* might be associated with the upregulated protection systems such as CDSP32. Lipid peroxidation is widely used as a marker of oxidative stress (Mittler 2002). Similar to those of other halophytes such as *Crithmum maritimum* (Ben Amor et al. 2005) and *Cakile maritima* (Ellouzi et al. 2011), lipid peroxidation levels of *T. parvula* and *T. halophila* were not altered by salinity (Uzilday et al. 2015; M'rah et al. 2006). Uzilday et al. (2015) and M'rah et al. (2007) reported that salinity tolerance of *T. parvula* and *T. halophila* might be closely related with the increased capacity of antioxidant system to scavenge ROS. As compared with lipid peroxidation level in whole cell of *T. parvula*, peroxidation level in chloroplast isolated from 300 mM NaCl-treated plants increased with salinity (Uzilday et al. 2015), but, even so, this situation did not affect the rate of linear electron flow in chloroplasts (Stepien and Johnson 2009) due to the upregulation of redox regulatory enzymes such as TRX–Prx (Uzilday et al. 2015).

Several studies have demonstrated that salt-tolerant species increase their antioxidant enzyme activities and antioxidant contents in response to salt stress, whereas salt-sensitive species failed (Olmos et al. 1994; Jbir et al. 2001; Shalata et al. 2001). M'rah et al. (2006) also investigated the effects of NaCl on the activities of antioxidant enzymes of *T. halophila*. It was found that SOD activity in leaves of salt-treated *T. halophila* decreased, which was probably associated with the decrease of Cu/Zn SOD isoform. Moreover, the activities of CAT increased only in 50 and 100 mM NaCl-treated plants, but it did not change with 200 mM NaCl. Similarly, guaiacol peroxidase activity was also not changed under salt stress (M'rah et al. 2006). These results showed that antioxidant system of *T. halophila* was not induced with salinity as expected. On the other hand, Uzilday et al. (2015) determined changes in antioxidant defence in both chloroplast and whole cell of *T. parvula* under salinity. In contrast to *T. halophila*, the activities of water–water cycle enzymes (SOD, APX, MDHAR, DHAR and GR) and expressions of genes encoding these enzymes in chloroplast of *T. parvula* were significantly induced by salt stress, associated with increase in $O_2^{\bullet -}$ production caused by overload of electron transport system. This increase in the activities of water–water cycle enzymes might cause the relaxation of electron transport chain in the chloroplast of *T. parvula*. All the above results suggest that *T. parvula* was able to protect the photosynthetic machinery with the efficient use of water–water cycle as reported by Uzilday et al. (2015). Similar to chloroplast, the activities of APX, GR, MDHAR and DHAR were increased in whole cell of *T. parvula* indicating the importance Asada–Halliwell–Foyer enzymes in preventing salt stress-induced oxidative stress (Uzilday et al. 2015).

Besides the activities of water–water cycle enzymes, Uzilday et al. (2015) also determined the expression levels of some of key chloroplastic redox components such as ferredoxin (*FED*), ferredoxin thioredoxin reductase (*FTR*), NADPH thioredoxin reductase (*NTRC*), some thioredoxins (*TRX*) and four types of peroxiredoxins (*Prxs*) in *T. parvula*. The relative expression of *TRX-X*, *Prxs*, *FTR*, *NTRC*, *2CPA* and *2CPB* reached their highest level in 200 mM NaCl-treated

T. parvula. TRXs can reduce Prxs, which scavenge H_2O_2 and detoxify alkyl hydroperoxides and peroxynitrite. They are also involved in regulating the activity of enzymes involved in carbon metabolism. Therefore, induction of TRX–Prx system under salt stress prevented any change in the rate of linear electron transport in chloroplast of *T. parvula*. As opposed to *T. parvula* and other halophytes (such as *Spartina alterniflora* (Baisakh et al. 2008) and *Nitraria sphaerocarpa* (Chen et al. 2012)), in *Arabidopsis*, the capacity of TRX–Prx system was decreased with salinity implying decrease in linear electron flow in chloroplasts.

Perez-Ruiz et al. (2006) reported that NTRC is an alternative system for chloroplast protection against oxidative damage. Moreover, Perez-Ruiz et al. (2006) observed damage to photosynthetic apparatus of *Arabidopsis ntrc*-knockout mutants due to overload of ETC. On the other hand, Uzilday et al. (2015) found that in *T. parvula*, similar to FTR expression, NTRC expression was also induced by salinity implying protection of photosynthetic apparatus. These results revealed that *T. parvula* was able to protect its chloroplast from salinity-induced oxidative stress by the induction of redox regulatory components (Uzilday et al. 2015).

Under salt stress, proline (Pro) accumulation leads to the reduction of osmotic potential of the cell, which results in the attraction of water into the cell and maintenance of turgor (Cechin et al. 2006). In addition to this, Pro might act as a compatible solute that can protect membrane structure and conformation of proteins as well as scavenge ROS to regulate cellular redox status (Verslues and Bray 2006). Hence, proline may act as a regulatory or signalling molecule to activate multiple stress response mechanisms that are part of the adaptation process (Claussen 2005). Pro accumulation was studied also in some halophytes, which are closely related to *Arabidopsis* such as *T. parvula* and *T. halophila* under salt stress. Pro accumulation increased in *T. parvula* and *T. halophila* under salt stress as determined by Uzilday et al. (2015) and M'rah et al. (2006), respectively. To understand this increase in proline accumulation with salinity, Uzilday et al. (2015) determined expressions of genes related to proline biosynthesis (*P5CS1* and *P5CS2*) and its degradation (*PRODH1* and *PRODH2*) in *T. parvula*. It was found that in *T. parvula* under salt stress, the expression of *P5CS1* was increased, while expressions of *PRODH1* and *PRODH2* were decreased, which results in the accumulation of Pro. However, expression of *P5CDH*, which converts P5C to glutamate, was increased to degrade P5C which is a toxic compound and can cause tissue damage. Uzilday et al. (2015) reported that in *T. parvula*, Pro takes the role of both being an osmotic regulator to maintain turgor of the cell and an antioxidant to prevent ROS induced damage.

Pro accumulation in response of *T. halophila* to salt stress was determined by M'rah et al. (2007). It was found that while Pro accumulation increased in both *A. thaliana* and *T. halophila* under moderate salinity (50 and 75 mM NaCl), at NaCl concentrations exceeding 300 mM, additional increase in Pro content was not observed. These results suggested that Pro is not effective in imparting tolerance to salt stress of *T. halophila* as indicated in *T. parvula*. On the other hand, Ghars et al. (2012) also investigated the relationship between lipid signalling enzymes and Pro level in *T. halophila* and found that Pro accumulation was prevented in phospholipase C (PLC) inhibitor-treated *T. halophila* under salt stress. In contrast,

Table 2 The proteomic studies that identified changed protein profile in antioxidant defence of close relatives of *Arabidopsis* under abiotic and oxidative stress conditions

Species	Stress	ROS-related proteins	References
Thellungiella halophila	50–150 mM NaCl	APX, DHAR, Prx, GST, APX1	Pang et al. (2010)
Thellungiella halophila	300 mM NaCl	GST, TPX, Cu/ZnSOD	Zhou et al. (2010)
Thellungiella halophila	0–200–400–600 mM NaCl	NADPH dehydrogenase, MnSOD	Wang et al. (2013)
Thellungiella halophila	4 °C Low temperature	Prx, dehydrin, 2-Cys Prx, aldo-keto reductase	Gao et al. (2009)
Arabidopsis halleri	1 mM Cd, 10 mM Zn	GST, FeSOD, MDHAR	Farinati et al. (2009)
Thlaspi caerulescens	2 μm Zn	APX, Prx, Cu/ZnSOD, DHAR	Tuomainen et al. (2006)
Brassica juncea	H_2O_2	GST, DHAR, TRX	Alvarez et al. (2011)

SOD Superoxide dismutase, *CAT* Catalase, *APX* Ascorbate peroxidase, *MDHAR* Malondialdehyde reductase, *Prx* peroxiredoxin, *TRX* thioredoxin, *GST* glutathione-S-transferases

under control conditions, this inhibitor induced Pro accumulation. These results suggested that in the absence of stress, PLC negatively regulates proline accumulation. In contrast to PLC, the phospholipase D (PLD) inhibitor inhibited Pro biosynthesis under control conditions. These results indicated that PLD positively controls Pro accumulation in *T. halophila* via Ca^{2+} signalling under severe stress.

In addition, 100 and 150 mM NaCl treatments increased the carotenoids and proline levels of *B. juncea*. Antioxidant defence system was also induced by salinity such as activities of SOD, POX, CAT, GR and APX (Ahmad 2010). The salinity tolerance of *B. juncea* showed differences between varieties. Salinity increased proline accumulation in *B. juncea* var. Bio902, and SOD, APX and CAT activities of this cultivar were higher than *B. juncea* var. Urvaslu (Mittal et al. 2012).

In a comparative proteomic study between *T. halophila* and *A. thaliana*, it was found that 5 of 32 and 1 of 79 proteins were identified as related with antioxidant defence system under salt stress (Pang et al. 2010) (Table 2). In *Thellungiella* roots, differential expression of phosphoproteome was reported under less than 300 mM NaCl (Zhou et al. 2010). Some of the identified proteins also include GST, TRX and Cu/ZnSOD. Wang et al. (2013) also compared the proteomic profiles of *T. halophila* which were subjected to different salt concentrations (0, 200, 400, 600 mM NaCl) and found that salinity changed the expressions of antioxidant defence system elements such as NADPH dehydrogenase and MnSOD.

4.2 Drought

Drought is a global constraint that affects plant growth. According to IPCC forecasts, frequency and duration of drought will increase in the forthcoming future due to global warming. These future scenarios urged plant scientist and agronomists to develop drought-resistant crops and to better understand the mechanisms underlying drought tolerance.

There are several reports on the involvement of antioxidant defence as an important component of drought stress response. As also mentioned above, limitation of gas exchange due to stomatal closure impairs the balance between light reactions and carbon reactions of photosynthesis and causes a disturbance in the metabolism. Especially under high light conditions, load on chloroplastic electron transfer chain is increased, and this causes excess production of ROS. Particularly in C_3 plants, photorespiration is one of the major sources of ROS (H_2O_2) due to decreased availability of CO_2. If plant cannot cope with this increased ROS production, damage to biological molecules is an inevitable phenomenon.

Considering this, some antioxidant genes were overexpressed (OE) in plants to confer drought stress tolerance. For example, overexpression of different *APX* isoforms (Murgia et al. 2004; Yan et al. 2003; Badawi et al. 2004a, b; Lu et al. 2007), *MDHAR* (Eltayeb et al. 2007), *DHAR* (Ushimaru et al. 2006), *MnSOD* (Wang et al. 2004) and *Cu/ZnSOD* (Badawi et al. 2004a, b) conferred tolerance to drought as compared to wild types. In addition, Xi et al. (2010) overexpressed *MnSOD* and *CAT* (single OE and double OE plants) under control of seed-specific promoter in *A. thaliana* and observed drought tolerance during seedling stage. Increased expression of both enzymes conferred stress tolerance by decreasing ROS levels and preventing oxidative damage.

As expected, loss of the function of antioxidant genes resulted in plants to be more sensitive to drought. *gpx3* knockout *Arabidopsis* exhibited a higher rate of water loss under drought stress, higher sensitivity to H_2O_2 treatment during seed germination and seedling development (Miao et al. 2006). Also, AsA-deficient *Arabidopsis* plants (*vtc1*) exhibited stress sensitivity due to insufficient ROS scavenging (Huang et al. 2005). Davletova et al. (2005) showed that the absence of cytosolic *APX1* resulted in altered H_2O_2-scavenging system, accumulation of ROS and oxidation of proteins during photooxidative stress. However, interestingly, *apx1* plants grow better than WT plants under osmotic stress, although it was more sensitive to photooxidative stress (Miller et al. 2007). When *apx1* mutants were compared to double *cytosolic apx1*/*thylakoid apx* mutants, again it showed that double mutant plants developed a different cellular response that results in late flowering, low protein oxidation under high light and enhanced accumulation of anthocyanin (Miller et al. 2007). These findings suggest that plant antioxidant defence system is a dynamic process and there is some degree of redundancy and overlapping signals can induce different responses.

Most of the drought-related molecular studies are conducted with *A. thaliana*. Interestingly, there is no such model to investigate drought tolerance mechanisms

such as *Thellungiella* or *A. halleri* that is used for salinity or heavy metal tolerance. However, researchers focused on comparatively investigating drought tolerance mechanisms between different *A. thaliana* ecotypes. Unfortunately none of these studies tried to elucidate ROS defence and redox regulation mechanisms under drought in detail. In this part, some reports on *A. thaliana* drought responses with a special focus on antioxidant defence and redox regulation will be discussed.

While some of the studies investigated plant responses to drought at whole plant level, Jung (2004) investigated drought responses of young and mature leaves of *A. thaliana*. In this study, it was shown that both young and mature leaves exhibited an increase in nonphotochemical quenching (NPQ), which aids in dissipating excess light as heat and reduced electron load on chloroplastic electron transport chain. Increase in NPQ is a mechanism that is used by both young and mature leaves to avoid ROS production in chloroplasts. In addition, although nonenzymatic antioxidants increased in both young and mature leaves, increase in enzymatic antioxidant including SOD, CAT, total POX and GR were only observed in mature leaves but not young leaves. These findings suggest that tolerance mechanisms are diverse even in young and mature leaves of the same plant.

In another study, Koffler et al. (2014) investigated compartment-specific response of antioxidants to drought stress in *Arabidopsis*. They showed that in early stages of drought (4–7 days of water withholding), Asc content does not decrease in mitochondria, chloroplasts, cytosol or nuclei and it increased in peroxisomes and vacuole. Asc content reduced only after severe drought (7–10 days after stress) in these compartments, and it is still at levels as the start of the experiment in vacuoles. Contrastingly, a significant decrease in GSH was observed starting at the 4th day of drought treatment. These results indicate that GSH can serve as an early signal for drought stress. Especially decrease of glutathione in the nuclei can regulate gene expression through redox regulation of transcription factors.

Ozfidan et al. (2012) investigated regulation of antioxidant defence in *A. thaliana* under osmotic stress and ABA treatment. As it is widely known, ABA is a regulator of stress responses in plants and takes part in development, germination and stomatal closure. In this study, the authors showed that osmotic stress increased activities of SOD, CAT and APX, but these inductions were more prominent in the groups that were treated with osmotic stress with the addition of ABA. H_2O_2 levels were lower in plants treated with osmotic stress + ABA, when compared with only osmotic stress-treated plants, demonstrating the effect of ABA on antioxidant defence system.

For a full picture of transcriptomic response of antioxidant response of *Arabidopsis*, Noctor et al. (2014) defined 406 *Arabidopsis* genes that encode core antioxidant and redox homeostasis genes. Of these, probes for 302 genes were available in ATH1 microarray chips. Using data from two recent drought experiments that was available through Genevestigator, the authors investigated the changes of these 302 genes under drought and observed that only 15 genes respond to the same trend (increase or decrease) in both experiments with a twofold cutoff. Among all, only ROS-producing enzyme was increased, i.e., annexin-1, which has a peroxidase activity, while none of the NADPH oxidases were increased.

Similarly, CAT2 expression and expression of cytosolic and chloroplastic SODs did not change. A clear response was observed in a gene that encodes ascorbate oxidase, *Senescence-Related Gene 1*. The expression of two TRX-linked genes and subunit of a chloroplastic 2-Cys Prx was decreased. In addition, two glutaredoxin genes (GRX) were observed to be responsive to drought in both experiments, which are known to interact with transcription factors (Zander et al. 2012). Also inductions in several GSTs were observed. Among these GSTs, GSTU4 was observed to be induced in response to drought, salt, ABA and osmotic stress. As it can be seen, the accumulation of available data makes it possible to dissect and extract more detailed information related to events under stressed conditions.

4.3 Temperature Stress

Another important stress factor is low and high temperatures such as cold, freezing and heat shock. These stress factors can also cause the induction of ROS production. Heat stress causes lipid peroxidation which is a marker of oxidative stress (Larkindale and Knight 2002). Griffith et al. (2007) investigated the freezing temperature effects on Yukon ecotype of *Thellungiella halophila*, which is native to subarctic Canada. It has genetic potential as model species whose genome had been published, and it naturally resides in extreme cold temperatures such as −18 °C. In their study, Griffith et al. (2007) found that seed germination and viability of Yukon ecotype of *T. halophila* were resistant to cold temperature and it was ascribed as freezing plant, which continues to live in extreme cold under the formation of ice in its tissues.

Under chilling stress (4 °C), GSH content of *T. halophila* was measured as two times higher than that of *A. thaliana*, but GSSG content of *A. thaliana* was slightly higher than *T. halophila*. In the same study, low-temperature marker genes were induced by chilling conditions in both species which are *C0R15A*, *COR47*, *RD29A*, *RD29B*, *LEA*, *ELIP2* and *ADH1* (Benina et al. 2013).

Proteomic analysis of *T. halophila* under low temperature showed that 16 % of total protein profile was related to plant defence. Among them, four proteins were involved in chloroplastic redox balance, and also two nonchloroplastic ROS-scavenging proteins were elevated by cold stress (Gao et al. 2009) (Table 2).

As discussed below, heavy metal accumulation alters membrane integrity and increases ROS. Taulavuori et al. (2005) suggested that together with freezing, this membrane damage by heavy metals led to severe membrane damage as a result of ROS accumulation (Taulavuori et al. 2005).

4.4 Heavy Metal Stress

Heavy metals are a group of elements in the periodic table that especially refer to cadmium (Cd), zinc (Zn), nickel (Ni), mercury (Hg) and others. These metals are the main environmental pollution factors around old and current industrial areas. Some of the heavy metals such as Cu are essential for plants, but excess levels of these essential elements and nonessential elements such as Cd can be toxic after a certain threshold. It is important to determine the toxicity levels of these elements in plants and to evaluate their effects on plant growth and development. Heavy metal stress transforms the metabolism of plants by affecting enzyme activities and obstructing membrane integrity. Heavy metal stress also disturbs ROS balance in the cell and induces plant-detoxifying mechanisms such as antioxidant machinery. For example, excess accumulation of Cd leads to the production of ROS and inevitably oxidative stress (Gill and Tuteja 2010; Foyer and Noctor 2003).

Cho and Seo (2005) demonstrated that Cd-resistant *A. thaliana* had lower H_2O_2 and TBARS content as compared to wild type. Resistant type had greater activities of SOD, GPX, APX and GR as compared to wild type. The authors suggest that decreased H_2O_2 accumulation of resistant type enhanced Cd tolerance. Similarly, Maksymiec and Krupa (2006) investigated the effects of 100 μM Cu and Cd on antioxidant defence system of *A. thaliana*. This study revealed that Cd treatment increased the ROS and MDA levels and the activities of SOD, CAT and APX, while Cu treatment enhanced the ROS and MDA levels and APX activity, but it decreased the activities of SOD and CAT. In another study, in *A. thaliana*, 10 μM Cd treatment increased ROS and MDA levels and also enhanced the activities of SOD, CAT, APX, GR and GSH content. Moreover, 5 μM Cu elevated ROS, MDA and GSH contents, while it decreased the activities of CAT, APX and GR in contrast to Cd treatments (Cuypers et al. 2011).

There are many *Arabidopsis*-related heavy metal hyperaccumulators such as *Arabis paniculata*, *Thlaspi* sp. and *A. halleri*. Many researchers exploited the use of these plant species for the elucidation of heavy metal stress tolerance due to their close relation to *A. thaliana*. Similarity between genome sequences of these plants and *A. thaliana* makes it possible to use available sequence data and molecular tools for this research.

Qiu et al. (2008) investigated the effects of Cd treatments in the range from 0 μM to 178 μM (0, 22, 44, 89, 178 μM Cd) on antioxidant system of Cd hyperaccumulator *A. paniculata*. They found that 22–89 μM Cd decreased the lipid peroxidation and activities of SOD, GPX and APX in roots, whereas these concentrations did not have any effects in leaves. While in roots, 22–89 μM Cd inhibited the oxidative stress, 178 μM Cd enhanced the accumulation of ROS in *A. paniculata* (Qiu et al. 2008) (Table 1).

T. geosingense is a hyperaccumulator of Ni and it can tolerate Zn, Co and Cd. It is a well-known metal hyperaccumulator which is related to model plant *A. thaliana*. The accumulation of glutathione (GSH) in *T. geosingense* increased the oxidative stress tolerance capacity of the cell and enhanced the tolerance to Ni,

Co and Zn by the activity of mitochondrial serine acetyltransferases (SATm) (Freeman and Salt 2007).

A. halleri can tolerate Zn, Cd, Pb and can hyperaccumulate Cd and Zn (Farinati et al. 2009). According to proteomic studies, 1 mM Cd and 10 mM Zn treatments enhanced the levels of several stress response proteins including oxidative stress-related GST and FeSOD in *A. halleri* (Farinati et al. 2009) (Table 2). In their transcriptomic study, Chiang et al. (2006) revealed that expressions of Asc–GSH cycle genes such as APX, MDHAR4 and POX were higher in *A. halleri* than that of *A. thaliana*. They also found that the activities of APX and POX were induced in *A. halleri*, when compared to *A. thaliana*. Under paraquat and Cd treatments, *A. halleri* was able to efficiently scavenge the ROS, but this was not the case for *A. thaliana*. The higher antioxidant activities of *A. halleri* resulted in heavy metal tolerance by decreased levels of ROS (Chiang et al. 2006).

Phytochelatins (PCs), which bind metals in the cytosol and then segregate them to vacuole, are formed by the polymerisation of GSH. γ-EC synthetase and GSH synthetase are two enzymes that are responsible for the production of GSH from glutamate. Expressions of these GSH synthesis genes were highly induced by Cd or Cu application to *A. thaliana* (Xiang and Oliver 1998).

The content of Ni, an essential micronutrient, can reach up to 10 μg g^{-1} dry weight in plants grown in normal soil, while generally it is toxic over this concentration. On the other hand, in Ni-rich soils, there are many plant species that can accumulate Ni up to 1000 μg g^{-1} dry weight. Till date, approximately 400 species were characterised as heavy metal hyperaccumulators (Assunção et al. 2003). In the late 1800s, *Thlaspi* was identified as growing in Zn-rich soils, and even now, it is widely used as a model species in heavy metal tolerance studies. *T. caerulescens* is a well-known species, which is a hyperaccumulator of Ni (up to 4700 μg g^{-1} dry weight), Zn (30,000 μg g^{-1} dry weight) and Cd (14,000 μg g^{-1} dry weight).

A comparative study between Ni hyperaccumulators (*T. geoesingense*, *T. oxyceras*, *T. rosulare*) and nonaccumulators (*T. perfoliatum*, *T. arvense*, *A. thaliana*) exhibited that GSH concentration was highly related with hyperaccumulation capability (Freeman et al. 2004). Serine acetyltransferase (SAT) is another key regulator enzyme in Cys and glutathione synthesis in plants. Enhanced activity of SAT showed a significant correlation with tolerance to oxidative stress in hyperaccumulator *T. geosingense*, which also caused elevated tolerance to Ni. The same study also revealed that when *TgSAT* was overexpressed in *A. thaliana*, it led to Ni tolerance in transgenic lines as compared to wild type (Freeman et al. 2004). These results prove the importance of genetic diversity of *Arabidopsis*-related model plants and give us the opportunity to engineer these characters to *A. thaliana* or other crop plants.

In a comparative study between hyperaccumulator *T. caerulescens* and nonaccumulator *Nicotiana tabacum*, *T. caerulescens* showed higher antioxidant defence capacity than that of *N. tabacum* under Cd. For instance, *T. caerulescens* had higher basal levels of CAT and SOD activities, and after Cd treatment, their activities were increased. Lipid peroxidation was elevated by Cd treatments in both species; however, it was 83 % higher in *N. tabacum* as compared to *T. caerulescens*.

T. caerulescens controlled H_2O_2 levels by CAT activity, which indicated the pivotal role of this antioxidant enzyme in heavy metal stress response (Boominathan and Doran 2003).

Craciun et al. (2006) crossed *A. halleri* and *A. lyrata* to produce genotypes that have different degrees of heavy metal tolerance. They ascribed the transcriptomic differences between Cd-tolerant and Cd-sensitive genotypes and found that genes involved in cellular detoxification and DNA repair were upregulated in Cd-tolerant genotypes.

Lepidium sativum is another Cd-tolerant plant which is related to *Arabidopsis* and belongs to *Brassicaceae*. While high concentrations of Cd (100 mg Cd kg^{-1} soil) inhibited the photosynthetic rate, growth, and decreased N content and nitrate reductase activity, it increased the activities of SOD, CAT, GR, APX and GSH levels. Moreover, antioxidant defence mechanism of *L. sativum* was efficiently working at low Cd concentrations, and as a result of this, no effects of low Cd concentrations on *L. sativum* had been reported by Gill et al. (2012) (Table 1).

Although it is an essential element, Cu as well as Cd are also considered as a heavy metal stressor for plants and have impacts on antioxidant defence system. For example, excess Cu (5, 25, 50, 100 μM) application to *A. thaliana* for 1, 3 and 7 days influenced the antioxidant defence system (Drążkiewicz et al. 2003). This study revealed that responses of antioxidant defence system of *Arabidopsis* against Cu depend on both the duration of treatment and Cu concentration used. Cu affected the activities of DHAR, MDHAR, GR, APX and levels of DHA, AsA, GSH and GSSG (Drążkiewicz et al. 2003). Similar to this, it was shown that the activity of another GSH-related enzyme GST was induced by Cu (Skórzyńska-Polit et al. 2010). In the same study, it was also shown that in *Arabidopsis*, membrane damage by Cu is higher than that of Cd application, which was evidenced by electrolyte leakage.

Proteomic approaches are widely used to understand the metal tolerance of hyperaccumulator *T. caerulescens*. *T. caerulescens* is a widely used plant for heavy metal stress studies because its genome is 87 % identical to *Arabidopsis* (Peer et al. 2003) (Table 2). In a study where three accessions of *T. caerulescens* were used to compare proteomic profiles under Zn and Cd treatments, ROS-scavenging proteins such as Cu/Zn SOD, APX, DHAR and Prx were identified under Zn treatment (Tuomainen et al. 2006). In an experiment, 500, 1000, 1250 and 2000 μM Zn and 25, 200 and 500 μM Cd were applied to *T. caerulescens* for 14 days by Wójcik et al. (2006) (Table 1). Elevated Zn increased the SOD and POX activities but decreased the APX and CAT activities, while Cd application increased the APX and POX activities and decreased the SOD activity.

B. juncea is a member of Brassica family related to *A. thaliana* and is an important oil plant worldwide. Under toxic levels of Zn, activities of CAT, POX, APX, MDHAR, DHAR and GR enzymes were increased in *B. juncea* and AsA and GSH contents were enhanced (Prasad et al. 1999). *B. juncea* is also an accumulator of Cd, and for this attribute, it was proposed for bioremediation for contaminated lands. It was found that under high levels of Cd, it had high antioxidant capacity due to increased levels of GSH (Seth et al. 2008). Markovska et al. (2009) reported that

100 μM Cd induced the H_2O_2 levels but not MDA and enhanced the activities of APX, MDHAR and GR in *B. juncea*. Moreover, Mobin and Khan (2007) compared the antioxidant capacity of two different cultivars of *B. juncea* under excess Cd. In this study, it was shown that two cultivars had different responses to Cd and increased activities of CAT, APX and GR played roles in the alleviation of Cd stress. Mohamed et al. (2012) demonstrated difference between antioxidant responses of roots and shoots of *B. juncea* to Cd stress and showed that roots could tolerate more Cd than shoots, which can be associated with GSH levels. The effects of Cu stress on antioxidant system was also investigated in *B. juncea*, and it was found that Cu application decreased the content of GSH and excess Cu (200 μm) increased the activities of CAT and APX (Devi and Prasad 2005). A comparative study on antioxidant defence systems of *B. juncea* and *B. napus* under Cd stress showed that MDA levels of *B. napus* were increased by stress and the activities of SOD, CAT, APX and GR were decreased upon Cd stress, whereas antioxidant defence system was not changed in *B. juncea* that showed higher tolerance to Cd than *B. napus* (Nouairi et al. 2009).

All the studies mentioned above indicated that there is a strong correlation between antioxidant capacity and heavy metal stress tolerance. Hyperaccumulator plants usually display high amounts of antioxidants within the cell either enzymatically or nonenzymatically, and they are capable of conserving the membrane integrity by reducing reactive oxygen species.

5 Conclusions

As it can be seen from the studies above, relatives of *A. thaliana* can give us insights to improve performance of crop plants when cultivated in the marginal areas such as arid, saline and heavy metal-contaminated soils. In this stage, researchers are trying to understand how basic mechanisms that are well known in *A. thaliana* work in its stress-tolerant relatives. The next step will be to analyse the differences between sensitive and tolerant mechanisms and then transfer this knowledge to application via crop plants.

Due to the availability of comprehensive microarray chips, most of our knowledge at transcriptomic level depends on experiments that use *A. thaliana*. However, recent developments in non-Sanger next-generation DNA sequencing technologies such as 454 and Illumina sequencing make it possible to investigate transcriptomic of any species that is interested. Combined with sequence and gene annotation data of *Arabidopsis*, these developments can make it possible to unravel yet to be discovered tolerance mechanisms in its relatives.

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ROS Signaling: Relevance with Site of Production and Metabolism of ROS

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Abstract One of the inevitable consequences of aerobic metabolism is the production of ROS in biological organisms including plants. Accordingly, plants have evolved antioxidant system (antioxidant enzymes and antioxidant molecules) to protect the cell components from oxidative threat. Usually cells accumulate ROS to a fatal level due to imbalance between generation and scavenging under stress or pathogenic attack. Recent observations led to an idea of involvement of ROS in signaling for plant growth and development. Although common sites of ROS generation are chloroplasts, mitochondria, and other organelles, ROS produced by plasma membrane localized NADPH oxidase (Rboh) in extracellular space has been implicated to participate in signaling process. Calcium, being most important signal molecule, has a cross talk with Rboh through a positive feedback loop that forms the basis of ROS-driven signaling network. Such signaling most often works behind plant growth and developmental processes like seed germination, root growth, stomatal regulation, and stress tolerance. Recent researches establish a ROS wave with an integration of Ca^{2+} signal that may operate for long distance signaling in plants. This may help to explain the event of systemic signaling in case of systemic acquired resistance (SAR) during pathogen attack and systemic acquired acclimation (SAA), which is achieved by gradual exposure to stress.

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Establishment of the possibilities of intracellular ROS signaling through vesicular trafficking and involvement in regulation of nuclear activities are on the way.

Keywords Antioxidant • Extracellular space • NADPH oxidase • Oxidative metabolism • Reactive oxygen species • ROS signaling • ROS wave

1 Introduction

Since the origin of life on the earth, it has been a challenge to perpetuate with the constantly changing environment. Accordingly, the life forms have undertaken great deal of modifications to cope up with newer milieu. Therefore, a long history of evolution includes diversity not only in shape, size, and morphology but also metabolism and behavior. Divergence of plants from animals has been a critical point during such evolution since a radical difference in respect of metabolism makes them two discrete opposite groups—autotrophic (producers) and heterotrophic (consumers), respectively. At the same time, plants, being sessile organisms, were more exposed to unavoidable adverse conditions that exerted an enormous selection pressure for suitable adaptations.

Another breakthrough in the history of metabolic evolution is the introduction of oxygen in the earth's outer layer, atmosphere, with the advent of oxygenic photosynthesis in cyanobacteria. Besides the advantages of living with O_2 (like energy efficient aerobic respiration, formation of ozone layer for UV filtration), generation of reactive oxygen species (ROS) as inevitable byproducts of aerobic metabolism (Halliwell 2006) has been presumed to be a curse to life, as ROS can attack major biomolecules viz. lipids, proteins, nucleic acids, and many others. But from the recent knowledge developed by extensive research for last one or two decades, it appears to be boon in a disguise. In recent years, role of ROS as signaling molecule for growth and development and also for defense has been mostly unraveled.

2 ROS: Types and Chemistry

Molecular oxygen as such mostly unreactive in ground state, by univalent reduction produces a series of free radicals and other chemical species through electron transfer reactions. Most important products are superoxide ($O_2^{\bullet -}$), that is further dismutated to H_2O_2 either spontaneously or enzymatically, and hydroxyl radical ($OH^{\bullet}$) (Gechev et al. 2006). Also energy transfer reactions lead to the formation of singlet oxygen ($^1O_2$) (Kim et al. 2008). Transition metals (Fe or Cu) having unpaired electrons react with O_2 to form $O_2^{\bullet -}$ frequently. These are also responsible for generation of $OH^{\bullet}$ from H_2O_2 by Fenton reaction or Haber–Weiss reaction

(Halliwell and Gutteridge 2000; Lee et al. 2007; Quan et al. 2008). Such different forms of reactive oxygen species (ROS) may be connected by a reaction cascade where $^1O_2$ or $O_2^{\bullet -}$ are sequentially reduced to H_2O_2 and $OH^{\bullet}$ (Halliwell and Gutteridge 2000). Besides, $O_2^{\bullet -}$ can react with $NO^{\bullet}$ to produce peroxynitrite ($OONO^-$) or may give rise to $HO_2^{\bullet}$ through protonation. Different ROS species are mostly short-lived and attack locally major cellular ingredients. Besides, other radicals like alkoxy radical ($RO^{\bullet}$), peroxy radical ($ROO^{\bullet}$), excited carbonyl (RO*) that may be generated under different situations are all cytotoxic to plant cells (Vellosillo et al. 2010). $O_2^{\bullet -}$ and $OH^{\bullet}$ are highly unstable (half-life at the level of microseconds and nanoseconds, respectively) and cannot cross membrane, while H_2O_2, though not a free radical but a ROS, is relatively stable (half-life around 1 ms) (Møller et al. 2007) and can cross membranes through aquaporins. Therefore, under cellular conditions $O_2^{\bullet -}$ and $OH^{\bullet}$ cause damage locally, but H_2O_2 may move across cellular distances and thus act as signal molecules apart from damaging biomolecules.

3 ROS: Sites of Production

Reactive oxygen species are synthesized in plant cells through various reactions as a part of normal metabolism (Suzuki et al. 2011). But their rate of production aggravates under stress and pathogenic conditions, and such production is confined to particular cell compartments (Mittler 2002; Asada 2006; Navrot et al. 2007).

In green plants, chloroplast is the most important among the organelles in respect of ROS generation as O_2 is continuously synthesized and readily available inside the chloroplast because of photosynthetic electron transport activity (Tripathy and Oelmüller 2012). Thus, due to over-excitation of chlorophylls under stress excitation energy from triplet chlorophyll may be transferred to O_2 producing $^1O_2$ near the reactions centers of photosystem PSII. Besides, $O_2^{\bullet -}$ may also be formed at PSI via Mehler reaction (Karuppanapandian et al. 2011) or at PSII during electron transfer to O_2 through Q_A and Q_B (Das and Roychoudhury 2014). On the other hand, mitochondria, the major source of ROS in mammals, may generate $O_2^{\bullet -}$ and H_2O_2 by univalent reduction of O_2 near electron transport chain (ETC) in plants. Apart from these organelles, single membrane bound peroxisomes are the major sites of H_2O_2 production due to activities of flavin oxidases (del Río et al. 2006; Palma et al. 2009). Plant peroxisomes also produce NO during different metabolic reactions (Corpas et al. 2001). Cell wall and apoplastic space are active sites of ROS production and possibly play pivotal roles in defense and signaling. Cell wall localized peroxidase(s), diamine/polyamine oxidases, and oxalate oxidase produce H_2O_2 (Spiteller 2003; Higuchi 2006) that may, in turn, be metabolized to $OH^{\bullet}$ by the activity of class III peroxidases (Kärkönen and Kuchitsu 2015). Moreover, plasma membrane localized NADPH oxidase transfers electrons from NADPH on cytoplasmic side to O_2 producing $O_2^{\bullet -}$ in cell wall or apoplastic space. This superoxide is further dismutated to H_2O_2 which plays important roles in cell wall

Table 1 Different intracellular ROS production sites and associated ROS-action(s)

Site of production	Source(s) of ROS	ROS action(s)	Reference(s)
Chloroplasts	Photosynthetic electron transport chain, lipoxygenase	Induce chloroplast avoidance movement	Wen et al. (2008); Foyer and Noctor (2009)
Mitochondria	Complexes I and III of mitochondrial electron transport chain	Interorganellar signal transduction	Møller (2001); Sweetlove and Foyer (2004); Rhoads et al. (2006)
Peroxisome	Flavin oxidases (e.g., xanthine oxidase), glycolate oxidase, peroxisomal membrane polypeptides	Involved in leaf senescence, responses to some abiotic stresses	López-Huertas et al. (1999); Mittler et al. (2004); del Río et al. (2006)
Endoplasmic reticulum	NAD(P)H-dependent electron transport	Ca^{+2} transmission	Mittler (2002)
Plasma membrane	NADPH oxidase (NOX)	Defense in biotic–abiotic stresses	Kwak et al. (2003); Apel and Hirt (2004); Sagi and Fluhr (2006)
Apoplast	Class III Peroxidase, germin-like oxalate oxidase, polyamine oxidase	ABA-induced stomatal closure, modulation of wall extensibility	Mittler (2002); Müller et al. (2009)

modifications and pathogenic defense (Kärkönen and Kuchitsu 2015). A summarized chart showing the cellular sites for ROS production and possible action is given in Table 1.

4 Oxidative Metabolism and Antioxidant System

Plants growing under natural condition are always prone to be subjected to abiotic and biotic stresses. As a response to such stresses, plant cells invariably generate significant level of ROS leading to oxidative stress (Dangl and Jones 2001; Mittler et al. 2004; Gunes et al. 2008; Xiao et al. 2008). Plants thus developed the ability to control the oxidative load that finally results in stress tolerance (Cheeseman 2007). Stress-induced increase in ROS level can cause different degree of oxidation of cell components and a gross change in redox status. Thus, an oxidative outburst as a consequence of stress is reflected in the levels of ROS molecules like $O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$, which are biochemically connected through metabolic reactions (Halliwell and Gutteridge 2000; Quan et al. 2008). However, plants have evolved defense system against such oxidative attack that may even occur under normal condition. Antioxidant system in plant comprises of enzymatic as well as nonenzymatic components. Superoxide dismutases (SODs) are considered as first

line of defense that may become active in different cellular compartments (Alscher et al. 2002). Depending on the metal requirement, SODs are grouped under Fe SOD (chloroplasts), Mn SOD (mitochondria and peroxisomes), and Cu-Zn SOD (chloroplasts, cytosol, peroxisomes, and extracellular space), and all these are involved in dismutation of $O_2^{\bullet -}$ to H_2O_2 and O_2 (Mittler 2002; Alscher et al. 2002; Chen et al. 2010). Once H_2O_2 is formed, it may further be metabolized by the activities of several enzymes like catalase, ascorbate peroxidase, and glutathione peroxidase, the latter two enzymes requiring electron donor (ascorbate and glutathione, respectively) (Dat et al. 2000). Nonenzymatic antioxidants includes ascorbate and glutathione (GSH) as important members while other members like carotenoids, tocopherol, flavonoids, etc. also participate in ROS scavenging process (Apel and Hirt 2004). As major redox buffers ascorbate and GSH help in bringing down the oxidative load involving ascorbate–glutathione cycle (Noctor and Foyer 1998; Apel and Hirt 2004; Halliwell 2006). Therefore, a balance between ROS generation and ROS scavenging maintain the redox homeostasis, and a complex network of prooxidant and antioxidant system coordinates such balance (Quan et al. 2008). Often this balance is perturbed under stress, senescence, and pathogen attack leading to oxidative burst and plants having stress tolerance show upregulation of antioxidant system to reestablish the redox homeostasis (Kar 2011).

5 Role of ROS in Signaling

Although ROS have been initially envisaged as offenders causing damage to cell components under adverse conditions like stress and pathogen attack (Halliwell 2006), role of ROS as signaling system has emerged quite recently. Some properties of ROS that makes them likely to be signaling molecules are their compartment-specific rapid generation and scavenging, instability (very short half-life), and their oxidative reactions with other signal molecules and phytohormones that may integrate with the signaling cascade (Mittler et al. 2011; Kar 2011). But playing with potentially toxic molecules is dangerous unless there is some safeguard mechanism prevailing in the respective compartments. In fact, evolution of ROS scavenging mechanism occurred even before ROS has been used for signaling (Mittler et al. 2011). Therefore, it seems that the compartment chosen for ROS signaling must have tight regulation for combating oxidative effects. Moreover, effective signaling requires some sensor and related components in specific compartments that form a cascade ending with the final response that may involve gene expression (Apel and Hirt 2004). Reports are accumulating on ROS sensing by histidine kinases, MAPK cascade, inhibition of protein phosphatases, and activation of transcription factors (Apel and Hirt 2004). Besides, other redox molecules like thioredoxins, peroxiredoxins, glutaredoxins, and NADPH that constitute cellular redox networks also connect ROS for signaling (Dietz et al. 2010; Rouhier 2011). As a signaling component ROS may act downstream of hormones like ethylene and ABA, although there may be feedback loop where ROS may induce

hormonal increase (e.g., ethylene synthesis may be induced by ROS). An interaction of hormones like GA, ABA, and ethylene with ROS has been demonstrated to control seed germination in *Vigna radiata* (Chaudhuri et al. 2013).

For last decade, ROS have been found to play key role in different plant growth and developmental processes like seed germination, root growth and root hair elongation, pollen tube growth, stomatal regulation, and gravitropic movement apart from stress and defense responses (Kar 2011; Müller et al. 2012; Baxter et al. 2013).

6 Signaling in Growth and Development

Plant growth depends on the cell division and cell elongation, the latter being the central event in case of seed germination, pollen tube formation, and root hair growth. Cell elongation growth is mostly driven by turgor and cell wall relaxation. ROS-mediated cell wall relaxation has been demonstrated in such process where ROS production is initiated by the activity of NADPH oxidase that generates $O_2^{\bullet -}$ in the apoplast (Singh et al. 2014). In fact, plant NADPH oxidases, known as respiratory burst oxidase homologues (rbohs), are homologues of catalytic domain of mammalian $gp91^{phox}$ (Keller et al. 1998) playing a key role in ROS production for signaling (Torres and Dangl 2005; Suzuki et al. 2011). That is why Rbohs have been aptly designated as engines of ROS signaling (Suzuki et al. 2011). Superoxide-producing plant NADPH oxidases or Rbohs are plasma membrane bound enzymes that contain EF-hand for Ca^{2+} binding and phosphorylation sites potentially controlled by cytosolic Ca^{2+} level (Ogasawara et al. 2008). On the other hand, ROS can induce increase in cytosolic Ca^{2+} level by activating hyperpolarization-activated Ca^{2+}-permeable cation channels (Pei et al. 2000), thus forming a positive feedback loop that help in amplifying the signal. Such ROS-Ca^{2+} interaction may form the basis of several ROS-mediated plant processes (Mori and Schroeder 2004). Further, extracellular $O_2^{\bullet -}$ thus formed by NADPH oxidase is immediately dismutated to H_2O_2 spontaneously or by the activity of SOD. Being relatively a stable form of ROS, H_2O_2 may enter into cytoplasm through aquaporins and become instrumental in causing elongation growth possibly through modulation of cytoskeleton including actin. Ca^{2+} ions may also have some role in this process, but less is known about this mechanism. However, a direct involvement of ROS ($OH^{\bullet}$) resulting from further metabolism of $O_2^{\bullet -}$ and H_2O_2 in the cell wall by cleaving polysaccharides (Müller et al. 2009) has also been implicated for seed germination and secondary root growth (Kranner et al. 2010; Roach and Kranner 2011; Singh et al. 2014). Apparently, apoplastic space is an ideal site for ROS signaling since this compartment has low redox buffering capacity (Noctor et al. 2002), thus less affecting signal strength.

7 Systemic Signaling and Acclimation

Recently, ROS are also proposed for systemic signaling thus constituting a cell-to-cell communication system that is involved in case of systemic acquired resistance (SAR) when pathogens invade the plant tissues and systemic acquired acclimation in case of exposure to different kinds of stress (Mittler et al. 2011; Baxter et al. 2013). During such systemic communication, ROS and Ca^{2+} interact with each other constituting a positive feedback loop, and this forms a wave of signal that can propagate from exposed tissues to systemic or non-challenged tissues (Gilroy et al. 2014). It is reported that such systemic signal, which is self-propagating, can move faster (8.4 cm min^{-1}), and this wave is essential for long distance signaling (Mittler et al. 2011). In this signaling process, NADPH oxidase initiates the ROS cascade being activated by stimuli followed by accumulation of H_2O_2 that can move to neighboring cells and activate Ca^{2+} influx channel on plasma membrane. Subsequently, cytosolic Ca^{2+} again activates Rboh by binding at EF-hands and phosphorylation to produce extracellular H_2O_2 that forms the ROS wave (Steinhorst and Kudla 2013).

Although the extracellular space has been emphasized for ROS signaling in most of research works based on the idea that this compartment has least interference with other components including antioxidants, arguments may be placed regarding any signaling role of intracellularly generated ROS like that are produced in chloroplasts, mitochondria, peroxisomes, and other organelles or compartments. Rhee (2006) first highlighted the possibility of intracellular signaling through H_2O_2. There are reports describing endosome-associated ROS generation by NADPH oxidase or ROS generation from endoplasmic reticulum and its intracellular compartments that suggest for intracellular communication system in response to stress (Park et al. 2003; Fedoroff 2006). ROS molecules have been demonstrated to be carried in vesicle and delivered to the destination by tightly regulated vesicular trafficking process in response to salt stress (Leshem et al. 2006; Leshem and Levine 2007). Therefore, a possibility of intracellular signaling by ROS through vesicular trafficking cannot be ruled out. Another important cell compartment is the nucleus where major signaling cascades end with modulation of nuclear functions like transcription and also epigenetic chromatin modifications. Extension of activities of ROS along with its cross talk with Ca^{2+} to the nucleus for signaling purpose has already been attempted (Mazars et al. 2010). Further research in near future will establish a complete array of ROS signaling in plant growth and development and interaction with environment. A working model including different subcellular sites of ROS production and possible downstream action towards signaling for plant growth and development and stress responses has been shown in the Fig. 1.

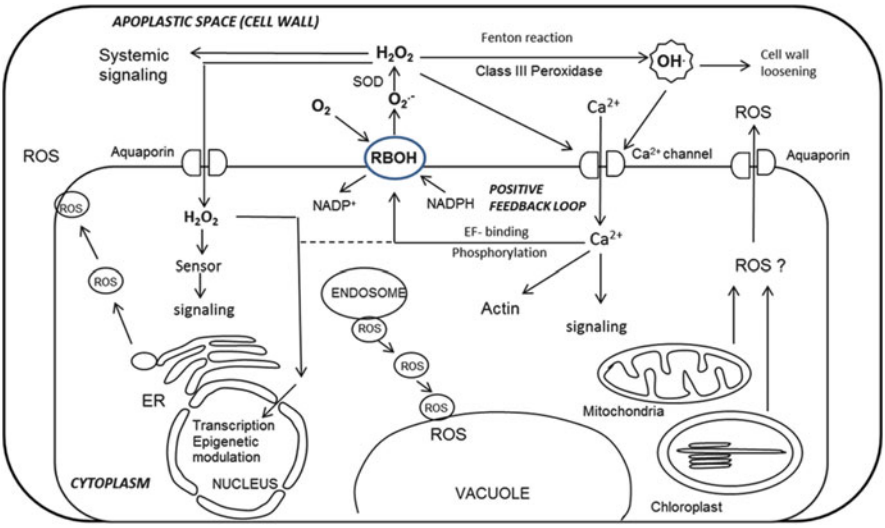

Fig. 1 An integrated model encrypting ROS production and metabolism at different subcellular sites and possible action towards signaling for plant responses. In the extracellular space (apoplast), the major site of ROS signaling, plasma membrane-located NADPH oxidase (RBOH) generates superoxide ($O_2^{\bullet-}$) that sequentially produces hydrogen peroxide (H_2O_2) and then hydroxyl radical ($OH^{\bullet}$), which directly causes cell wall loosening, required for cell elongation growth and/or activate hyperpolarization-activated calcium channels (HACC). Ca^{2+} influx into cytosol, in turn, activates RBOH though EF-binding and phosphorylation forming a positive feedback loop. Intracellular Ca^{2+} is also involved in interfering with actin and other signaling processes. Extracellular ROS (H_2O_2) may also enter into cytosol through aquaporins and lead to intracellular signaling through binding with redox censor in cytosol or move to nucleus along with Ca^{2+} to modulate nuclear activities. Besides, extracellular ROS (H_2O_2) may migrate to the neighboring cells and trigger the positive feedback loop with Ca^{2+} and sequentially propagate as systemic signal to far away cells. Mitochondria and chloroplasts, as intracellular sites, produce ROS that may participate in signaling. ROS may also be produced intracellularly in endoplasmic reticulum (ER) and endosomes and move for signaling either to apoplastic space or vacuole through vesicular trafficking

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Heavy Metal-Induced Oxidative Stress in Plants: Response of the Antioxidative System

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Abstract Heavy metals (HMs) are among the most important environmental pollutants, particularly in areas with strong anthropogenic pressure. For plants, high levels of HMs are extremely toxic since they may act in several different modes: by the direct inhibition of plant growth and biosynthetic pathways or through the production of reactive oxygen species (ROS). Certain metals generate ROS due to their involvement in redox reactions like Fenton and/or Haber–Weiss reactions, while metals without redox capacity enhance ROS production by reducing the antioxidant glutathione pool, activating calcium-dependent systems and

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influencing iron-mediated processes. ROS production affects lipids, proteins, and DNA and consequently leads to cell death. In response, plants are equipped with complex enzymatic and nonenzymatic mechanisms involved in antioxidative defense to neutralize HM toxicity, and the main components of these mechanisms will be reviewed in this chapter.

Keywords Heavy metals • ROS • Antioxidants

1 Introduction

From a biological point of view, the term "heavy metal" refers to a series of metals that can be toxic for plants and animals even at very low concentrations. Some metals, such as copper (Cu), cobalt (Co), chromium (Cr), iron (Fe), nickel (Ni), zinc (Zn), manganese (Mn), and molybdenum (Mo), are essential elements for plant growth and metabolism, while others, such as cadmium (Cd), mercury (Hg), lead (Pb), selenium (Se), as well as metalloids like arsenium (As), have no known biological function (Rascio and Navari-Izzo 2011). However, it has been shown that carbonic anhydrase may bind Cd as a cofactor in the marine diatom *Thalassiosira weissflogii* (Lane et al. 2005).

Heavy metals (HMs) occur naturally in the Earth's crust and are found in rocks, soils, sediments, and marine and freshwater ecosystems. High levels of HMs enter in the environment from different anthropogenic activities such as mining and smelting, usage of phosphate fertilizers and pesticides, energy production from coal, metal-contaminated wastes, and sludge disposal and from various industrial processes such as plating, alloying, and production of NiCd batteries, pigments, and plastic (Alloway and Steinnes 1999). Generally, soil and water pollution by HMs is a serious environmental problem and in recent years, it has been reported from across the world (Ikenaka et al. 2010; Su et al. 2014; Naser 2013).

Both, essential and nonessential HMs may become extremely toxic for plants, if present in excess.

Higher plants take up metals from water and air by the shoots and leaves or from the soil and sediment by their roots, while algae use their thallus to accumulate metals from water (Greger 2004). Absorption by roots can be passive or active process. Passive uptake includes diffusion of ions from the soil into the root endodermis, while active uptake takes place against concentration gradient and requires metabolic energy. Although absorption differs between the metal ions, ions which are absorbed by the same mechanisms are likely to compete with each other (Alloway 2013). In addition to root absorption, plants can take up significant concentrations of different metal ions through foliar absorption. Absorption of atmospheric pollutants mainly depends on plant species, as well as epidermal and cuticle characteristics (Tomašević and Aničić 2010; Schreck et al. 2012). Once they

have been absorbed through the roots or leaves, metal ions will be translocated into the xylem and then through the whole plant. In the xylem, metals could be chelated to different compounds such as histidine (His) (Krämer et al. 1996), nicotianamine (NA), citrate, malate, or oxalate (Senden et al. 1995). There is a considerable variation in the mobility of HM ions (Alrawiq et al. 2014). Mn, Zn, Cd, B, Mo, and Se have a high capability to be readily translocated. Ni, Co, and Cu are less mobile, while lowest mobility is typical for Cr, Pb, and Hg. Plants have highly specific mechanisms to translocate and store micronutrients as well as toxic elements. In some plant species, Ni and Cr were accumulated in their roots (Poniedziałek et al. 2005; Nematshahi et al. 2012), while in other plants, the highest concentration of HMs was found in stems and leaves (Rafati et al. 2011). Zn and Cu accumulation occurs mainly in the roots with poor translocation to the aboveground parts of the plants (Marques et al. 2007; Rivelli et al. 2012). Cd is accumulated mainly in the plant roots and to a smaller extent in other parts of the plant such as leaves, fruits, and grains (Grant et al. 1998). For different plant species, the most of the accumulated Pb is retained in the roots and only a small part can be translocated (Gichner et al. 2008; Gupta et al. 2009; Shahid et al. 2011). It has been shown that some hyperaccumulator plant species with specific detoxification mechanisms are capable of translocating higher concentrations of Pb to aboveground plant parts (Xiong 1998).

Manifested HM toxicity includes chlorosis, growth inhibition, and yield depression (Chaudri et al. 2000; Oancea et al. 2005; Sharma and Dubey 2005; Ebbs and Uchil 2008). For example, high levels of Cu may cause disruption of root cuticle, deformation of the root structure, and reduction of root hair proliferation (Sheldon and Menzies 2005). Excessive amount of Cr can not only inhibit seed germination, limit seedling growth (Akinci and Akinci 2010), and reduce root and shoot elongation but also can reduce chlorophyll (Chl) concentration (Amin et al. 2013). Exposure of algae to Cr (III) and Cr (VI) resulted in a significant inhibition of cell development after mitosis (Volland et al. 2012). Pb accumulation inhibits seed germination (Yang et al. 2010) and plant growth (Malar et al. 2014) and affects root system (Obroucheva et al. 1998; Fahr et al. 2013). Furthermore, high levels of Pb may cause reduction in: chlorophyll content (Zengin and Munzuroglu 2005), net photosynthetic and transpiration rate, stomatal conductance, and water use efficiency (Bharwana et al. 2013).

Algal growth inhibition is highly related to the amount of HM ions bound to the algal cell surface or present in intracellular spaces (Franklin et al. 2000, 2001; Ma et al. 2003) as well as to the chemical nature of the HMs (Tripathi and Gaur 2006).

According to their chemical and physical properties, HMs affect normal plant functioning in a variety of ways, including displacement of essential metal ions from biomolecules and blocking of essential functional groups in proteins and enzymes (Schützendübel and Polle 2002; Ali et al. 2013) and also distorting the integrity of cytoplasmic membrane (Janicka-Russak et al. 2008), adversely

affecting processes such as respiration, photosynthesis, and enzymatic activities (Farid et al. 2013). In the case of algae, toxicity results from metal binding to sulphydryl groups of proteins and from the disruption of protein structure and displacement of essential metal ions (Arunakumara and Zhang 2008). Rise in HM levels is associated with the increased formation of reactive oxygen species (ROS). Highly reactive singlet oxygen $^1O_2$ is produced after spin conversion which usually occurs when triplet oxygen (O_2) absorbs sufficient energy. Transferring of one, two, or three electrons to O_2 results in the formation of superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), or a hydroxyl radical ($^{\bullet}OH$), respectively (Mittler 2002; Tripathy and Oelmüller 2012). Superoxide radical is a relatively stable and unreactive molecule. It is a reductant of the transition metal ions in the Haber–Weiss reaction to produce $^{\bullet}OH$ from H_2O_2. Furthermore, $^{\bullet}O_2^-$ oxidizes the quinols produced via disproportionation of $^{\bullet}OH$ and thiols to produce thiyl radicals which may initiate radical chain reactions. H_2O_2 in plant cells is mainly generated during the process of photosynthesis, photorespiration, and, to a lesser extent, cellular respiration (Bhattacharjee 2005). It can easily diffuse across the membranes, where it exerts specific functions at the subcellular level (Corpas et al. 2001; Neill et al. 2002; Mittler et al. 2004). It oxidizes thiols to the sulfenic acids, slowly reacts with cysteine in proteins, and also, inhibits CO_2 fixation in chloroplasts. H_2O_2 may also act as a signaling molecule that mediates resistance of plants to biotic and abiotic stresses but also in the process of growth and development (Ślesak et al. 2007). Hydroxyl radical reacts nonselectively with organic and inorganic molecules nearby its production site and does not diffuse across a long distance (Mano 2002). Certain metals such as Cu and Fe generate oxidative stress through their involvement in redox cycles like Fenton and/or Haber–Weiss reactions which result in an increased production of ROS (Moura et al. 2012). On the other hand, metals without redox capacity (Cd, Pb) enhance oxidative stress by reducing the antioxidant glutathione (GSH) pool, activating calcium-dependent systems, and influencing iron-mediated processes (Pinto et al. 2003). The majority of ROS are generated in the plasma membrane, apoplast, chloroplasts, mitochondria, peroxisomes, and glyoxysomes (Ahmad et al. 2008). Injury of the plasma membrane is one of the primary events of HM toxicity in plants. In the cell walls, high level of $^{\bullet}O_2^-$ and H_2O_2 occurs as a consequence of impaired function of oxidoreductive enzymes and electron transport chain leakage caused by HM uptake. Plasma membrane-bound NADPH oxidase is involved in HM-induced ROS generation (Hao et al. 2006). Radicals generated close to cell membranes may attack the unsaturated fatty acid side chains of membrane lipids which results with the formation of lipid hydroperoxides (Bestwick et al. 2001). From the apoplast, HM enters the symplast and bind to different molecules—proteins, nonenzymatic macromolecules, and other metabolites and may compete with essential cations. This occurs simultaneously in both, cytoplasm and cell organelles (Fodor 2002). Peroxisomes and glyoxysomes, single membrane organelles, contain enzymes involved in β-oxidation of fatty acids and C2 photorespiratory cycle. In these organelles,

H_2O_2 is produced via glycolate oxidase, while xanthine oxidase, urate oxidase, and NAD(P)H oxidases generate $^{\bullet}O_2^-$ (Perl-Treves and Perl 2002). The disruption of electron transfer by HM within the electron transport chain in mitochondria may also lead to an increase of ROS production (Keunen et al. 2011). Furthermore, HM accumulation may disrupt chloroplast ultrastructure, inhibit chlorophyll biosynthesis (Shukla et al. 2008), and interrupt O_2-evolving reactions of PSII and electron flow around PSI and PSII (Mallick and Mohn 2003). Thus, chloroplasts are very important source of ROS in plants under the metal-induced oxidative stress (Parmar et al. 2013; Wang et al. 2013).

In plants, resistance to toxic metal ions includes different mechanisms such as: exudation of complexing agents into the rhizosphere, binding in the cell wall, efflux of metal ions from the symplast, prevention of upward transport of metal ions into aboveground plant parts, complexation with various ligands in the symplast, transport of metal-ligand complexes into the vacuole, storage of metal ions in the vacuole by complexation with vacuolar ligands, and formation of metal-resistant enzymes (Gratão et al. 2006). Algal tolerance to HM is mainly dependent on the defense responses against the possible oxidative damages (Pinto et al. 2003), exudation capacity of chelating compounds, active efflux of metal ions by primary ATPase pumps, and reduced uptake (Gaur and Rai 2001). When exposed to HM-induced oxidative stress, plants will evolve enzymatic and nonenzymatic defense mechanisms (Sharma and Dietz 2009). These ROS-scavenging pathways are often species and tissue specific, as well as dependent on the metal used for the treatment and the stress intensity (Table 1). These comprise firstly the enzymatic pathways (Fig. 1):

(a) Ascorbate–glutathione cycle in chloroplasts, cytosol, mitochondria, apoplast, and peroxisomes which includes ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR);
(b) Water–water cycle in chloroplasts that includes superoxide dismutase (SOD);
(c) Catalase (CAT) in the peroxisomes.

Nonenzymatic antioxidant endpoints include substances such as ascorbate (AsA), phenolics (PHE), tocopherols and tocotrienols, sulfur-containing antioxidants including GSH, metallothioneins (MTs) and phytochelatins (PCs), soluble sugars, organic acids and amino acids, polyamines (PA), nicotianamine, phytates, carotenoids (Car), and phycobilines (Fig. 1). Many studies have indicated a positive relationship between the accumulation of these compounds during HM stress and plant stress tolerance but also a dual action of some antioxidants which also autoxidize, especially in the presence of metal ions, generating reactive substances and acting as prooxidants. Among the well-established and optimized antioxidant networks in plant cells induced by HMs, the most common and potent scavenging mechanisms are addressed in this chapter.

Table 1 Summary of the responses of antioxidative system to various HMs in different plant species

Species	Metal	Concentration	Duration	Organ	Enzyme	Nonenzymatic antioxidants	References
Ulva fasciata	Cd	0, 5, 10, 20, 50 μM	4 days	Thallus	MnSOD=, FeSOD↑, CAT↑, APX↑, GR↑	tAsA↑, tGSH↑,	Wu et al. (2009)
Scenedesmus sp.	Cu	2.5 μM	6 h	Whole algal cells	SOD↑, CAT↑, APX↑, GR↓	Pro↑, Car=	Tripathi et al. (2006)
		10 μM			SOD↑, CAT=, APX↓, GR↓	Pro↑, Car=	
	Zn	5 μM	6 h		SOD=, CAT=, APX=, GR=	Pro↑, Car=	
		25 μM			SOD↑, CAT=, APX=, GR↓	Pro↑, Car=	
Scenedesmus quadricauda	Cr (III)	1 μM	24 h	Whole algal cells	CAT=, APX↑, GR↑	PHE=, Thiols↑	Kováčik et al. (2015)
		10 μM			CAT↑, APX↑, GR=	PHE=, Thiols=	
	Cr (IV)	1 μM			CAT↑, APX=, GR=	PHE=, Thiols=	
		10 μM			CAT↑, APX↑, GR↓	PHE↓, Thiols↑	
Gracilaria manilaensis	Cu	2 mg/L	24 h	Whole algal cells	CAT↑, APX↑		Ahamad and Shuhanija (2013)
	Pb				CAT↑, APX↑		
Lemna minor	Cu	0, 0.05, 0.5, 5, 10 and 20 mg/L	4 days	Fronds	SOD↑, CAT↑↓, GPX↑↓	Car↓	Hou et al. (2007)
	Cd				SOD↑, CAT↑↓, GPX↑↓	Car↓	
Groenlandia densa	Ni	0, 0.05, 0.5, 5, 10, and 20 mg/L	7 days	Leaf	SOD↑, CAT↑↓, APX↑↓, GR↑↓	Pro↑	Yilmaz and Parlak (2011)
Maize	Cd	0, 0.3, 0.6 and 0.9 mM	8 days	Leaf	SOD↑, GPX↑↓, APX↑↓, GR↑↓	Car↓	Ekmekçi et al. (2008)

Arachis hypogaea L. seedlings	Cd	25, 50, and 100 μmol/L	10, 15, 20 and 25 days	Leaf Root	SOD↑, CAT↑, GPX↑, APX↑, GR↑	Pro↑	Dinakar et al. (2008)
Lepidium sativum L.	Cd	0, 25, 50 or 100 mg/kg soil	30 days	Leaf	SOD↑, CAT↑, APX↑, GR↑	tGSH↑	Gill et al. (2012)
Triticum aestivum	Cd	0.010, 0.101, 0.504, 0.971, 5.10, 9.68, 46.1, 92.3 μM	72 h	Root	SOD↓, CAT↓,		Dandan et al. (2011)
Cultured tobacco cells	Cd	100 μM	7 days	Whole cells	SOD↓, CAT↓, GPX↑	Pro↑, Betaine=	Islam et al. (2009)
Jatropha curcas L.	Pb	0.5, 1, 2, 3, 4 mM/kg soil	6 days	Leaves of seedlings	SOD=↑, CAT↑↓, GPX↑	Car↓	Shu et al. (2011)
Alyssum bertolonii	Ni	426 μM	21–28 days	Hairy roots	SOD↓, CAT↓, APX↓		Boominathan and Doran (2002)
Nicotiana tabacum					SOD=, CAT↓, APX↓		
Jatropha curcas L.	Ni	100, 200, 400, 800 μmol	7 days	Cotyledons	SOD↑↓, CAT↑↓, GPX↑↓		Yan et al. (2008)
Nasturtium officinale R. Br.	Ni	0, 1, 5, 10, 25 mg/L	1, 3, 5,7 days	Leaves	SOD↑↓, CAT↑↓, APX↑↓		Duman and Ozturk (2010)
				Roots	SOD↑↓, CAT↑↓, APX↑↓		
Triticum aestivum L. cv. 'Zyta'	Ni	100 μM	3, 6, 9 days	Leaves	SOD↓, CAT↓, APX↑, GPX↑		Gajewska and Skłodowska (2007)
Raphanus sativus L.	Cu	0.5 mM	2 days	Leaves	CAT↑, GPX↑	Pro=	Teklić et al. (2008b)
	Pb				CAT↑, GPX↑	Pro↑	
Saccharum spp.	Zn	65.0, 130 mg/L	30 days	Leaves	SOD↑, CAT↑, GPX↑	Car↑	Jain et al. (2010)

(continued)

Table 1 (continued)

Species	Metal	Concentration	Duration	Organ	Enzyme	Nonenzymatic antioxidants	References
Triticum aestivum	Zn	0.1, 1, 10, 100 mM	2, 4, 6, 6, 8 days	Leaves	SOD↓, CAT↓, GPX↓	Pro↑, Car↓, AsA↑, GSH↑↓	Panda et al. (2003)
	Cr				SOD↓, CAT↓, GPX↓	Pro↑, Car↓, AsA↑, GSH↑↓	
Lablab purpureus	Zn	100, 300, 600 µM	72 h	Leaves	APX↑, GR↑, GPX↑,	GSH↑↓, AsA↑, Pro↑, Put↑, Spd↑, Spm↓	D'Souza and Devaraj (2012)
				Roots	APX↓, GR↓, GPX=	GSH↑↓, AsA↑, Pro=, Put↑, Spd↑, Spm↓	
Hordeum vulgare L. cv. "Obzor"	Cu	15, 150 and 1500 µM	5 days	Leaves	SOD↑↓, CAT↑, GPX↑, APX↓↑,	AsA=↑	Demirevska-Kepova et al. (2004)

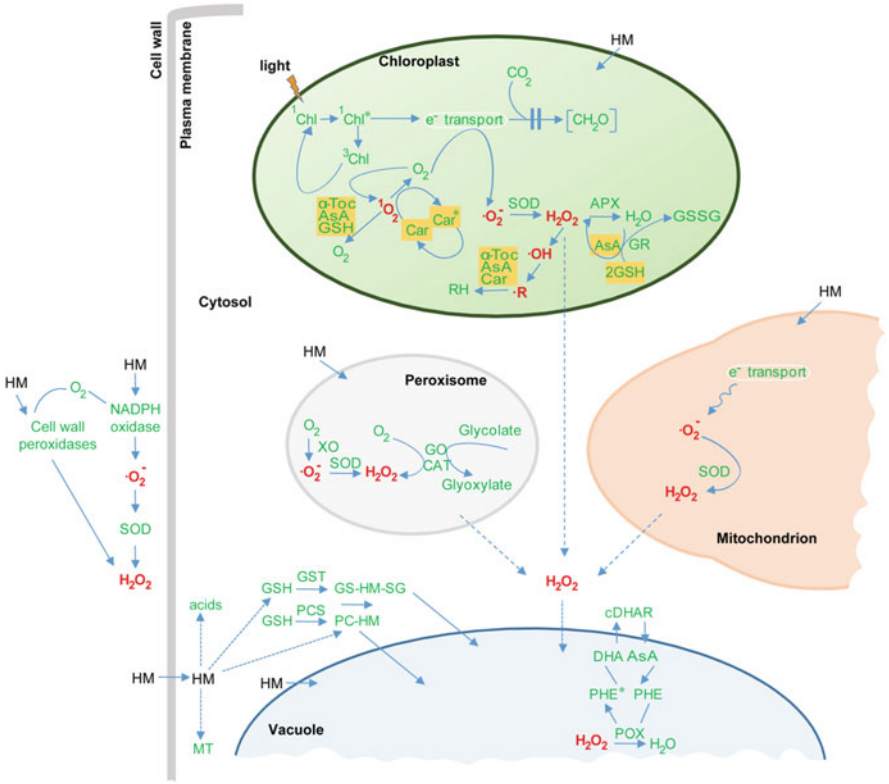

Fig. 1 General scheme of HM antioxidative mechanisms in plant cells

2 Antioxidative Enzymes

2.1 Superoxide Dismutase

Superoxide dismutase (SOD) is an essential component of plant antioxidative defense system as it dismutates two $^{\bullet}O_2^-$ radicals to H_2O_2 and O_2 and represents the first line of defense (Alscher et al. 2002). In this way, SOD maintains $^{\bullet}O_2^-$ at a steady state level, and it may contribute to a shift in the balance of free-radical metabolism towards H_2O_2 accumulation. In response to HM treatment, SOD activity can show induction of activity correlated with increased concentration of HMs, biphasic response—induction at low concentrations of HMs, and inhibition of activity at high concentrations of HMs (Table 1).

In algae, HM treatment (Cd, Cu, and Zn) caused the significant increase in SOD activity (Table 1). Data concerning the influence of HM treatment on SOD activity in plants are contradictory since both enzyme activation and inhibition can be observed (Table 1). Likely, SOD activity showed no contribution to the difference

in Cd tolerance between the two groups of lines of the same species (Shah et al. 2001; Guo et al. 2007; Ci et al. 2009). The Cr, Pb, and Ni treatment causes biphasic SOD activity response in plants. HMs at low concentrations caused the significant increase in SOD activity, while prolonged exposure to high concentrations caused the significant reduction in the activity of SOD (Zou et al. 2009; Yan et al. 2008; Duman and Ozturk 2010). Bah et al. (2011) showed that Cd, Cr, and Pb treatments increase the SOD activity in the order Cd > Cr > Pb in seedlings of *Typha angustifolia*.

Noteworthy, the same HM treatment can cause differential response in different plant organs. An excess of Zn exhibits different response in leaves and roots of wheat plant, while it causes dose-dependent induction of SOD activity in roots and no changes in leaves (Li et al. 2013). Dixit et al. (2001), however, showed that SOD activity in Cd-exposed pea plants increased significantly in leaves, while it mostly remained lower than the control in the roots. Kumar et al. (2012) found that treatment of the barley with lower concentration of Ni caused a significant induction of SOD activity in both roots and leaves, while the SOD activity decreased with increasing Ni treatments only in leaves. The Pb treatment generally enhances SOD activity, which is more pronounced in roots than in shoots of rice seedlings (Verma and Dubey 2003).

In general, increase in SOD activity due to HM toxicity may be linked to an increase in $^{\bullet}O_2^-$ formation (Chongpraditnun et al. 1992) as well as to the de novo synthesis of enzyme protein (Cakmak and Horst 1991; Verma and Dubey 2003), which in turn may be associated with an induction of genes of SOD by superoxide-mediated signal transduction (Fatima and Ahmad 2005). Decrease in SOD activity caused by excess of HM can be a result of ROS overproduction and unspecific enzyme degradation (Filek et al. 2008) or the consequence of the binding of HM ions to the active center of the enzyme and replacement of elements such as Zn, Fe, and Mn, which are essential cofactors of SOD isozymes (Stroiński and Kozłowska 1997; Lopez-Millan et al. 2009).

Based on the metal cofactor used by the enzyme, SODs are classified into three groups: iron SOD (FeSOD) located in the chloroplast, manganese SOD (MnSOD) located in the mitochondrion and peroxisome, and copper–zinc SOD (CuZn SOD) located in chloroplast, cytosol, and extracellular space (Alscher et al. 2002). A completely distinct SOD class that contains Ni (NiSOD) was discovered in *Streptomyces* and cyanobacteria (Barondeau et al. 2004). The number of isoenzymes of each type of SOD varies greatly from plant to plant (Gratão et al. 2005). CuZnSOD proteins appear in both charophycean algae and land plants but other green algal groups lack them (De Jesus et al. 1989).

Different HMs can alter the SOD isoforms pattern in a different way in different plant species.

In green macroalga *Ulva fasciata*, MnSOD was not affected by Cd, but FeSOD was increased by Cd at higher concentrations, reflecting also SOD activity induction (Wu et al. 2009). In the marine dinoflagellate *Gonyaulax polyedra*, the activities of FeSOD and MnSOD, but not CuZnSOD, were induced by exposure to acute Cd (Okamoto and Colepicolo 1998). The Ni treatment caused increase in MnSOD

activity and no changes in FeSOD activity in coffee cells, while no Cu/ZnSOD isoenzymes were detected (Gomes-Júnior et al. 2006a). On the contrary, Ni application can also result with a decrease in the total SOD activity in both leaves and roots of pea plants due to the inhibition of the activity of CuZn-containing isoenzyme (Gajewska and Skłodowska 2005). Similar results were found in pea plants treated with Cd, where CuZnSODs were the most sensitive forms to HM toxicity (Sandalio et al. 2001). This may be explained by the fact that Ni and Cd disturb Cu and Zn uptake by plants (Parida et al. 2003). In *Arabidopsis thaliana*, differences between the expressions of SOD isoenzymes were found between the roots and leaves. Smeets et al. (2008) found a significant increase in FSD1 (plastidic FeSOD) transcript level and small increase in MSD1 (mitochondrial MnSOD) transcript level, whereas reduction in CSD2 (plastidic CuZnSOD) transcript level in roots of *A. thaliana* due to Cd stress. On the other hand, transcript levels of CSD2, FSD1, and MSD1, as well as general SOD activity at the enzymatic level, are not increased in the leaves of *A. thaliana*.

In roots of *A. thaliana,* Cd and Cu treatment caused different response in gene expression level of SOD isoenzymes (Cuypers et al. 2011). In roots exposed to Cd stress, a slight increase in MSD1, strong induction in FSD1, and reductions in CSDs and FSD2-3 gene expression were noticed after exposure to Cd, while application of Cu resulted in an overall reduction in the gene expression of all superoxide scavenging enzymes except for CSD2. SOD-transcript levels are known to be posttranscriptionally regulated by miRNAs (Cuypers et al. 2011). MicroRNAs (miRNAs) are noncoding small RNAs, and they regulate gene transcription by targeting epigenetic modifications at the DNA level or act posttranscriptionally via RNA cleavage or translational repression (Mallory and Bouché 2008; Khraiwesh et al. 2010).

2.2 Antioxidative Enzymes That Remove H_2O_2

APX and CAT belong to two different classes of H_2O_2-scavenging enzymes because of their different affinities, with APX having a Km in the μM range and CAT in the mM range. Thus, while APX might be responsible for the fine modulation of ROS for signaling, CAT might be responsible for the removal of excess ROS during stress. Plants also contain plastidic and cytosolic isoenzymes of APX, which participate in the scavenging of H_2O_2 in the different compartments (Davletova et al. 2004).

2.2.1 Catalase

The main role of catalase (CAT), a heme-containing enzyme, is scavenging of hydrogen peroxide (H_2O_2) generated during different pathways under standard and stress condition (Mittler 2002; Foyer and Noctor 2005). CAT has a very low affinity

for substrate (H_2O_2) compared to other peroxidases as for the actual reaction, the simultaneous entry of two molecules of H_2O_2 in the active site is required (Willkenes et al. 1997). Consequently, CAT is responsible for the gross removal and control of high H_2O_2 levels but is less suited for a fine tuning of sensitive redox balances with low H_2O_2 concentrations that may be important for regulatory mechanisms. In plant cells, CAT is primarily present in peroxisomes and plays a central role in maintaining the cellular redox balance (Corpas et al. 2001).

The contradiction between CAT activation and suppression in the presence of HM may depend on the element, its concentration, and the plant species (Cardoso et al. 2005).

In algae cells, CAT activity is generally induced or unchanged due to HM treatment (Table 1). HM treatment may cause biphasic response of CAT activity, it increases at moderate toxic level of HM and then decreases at high toxic level in different plant species (Table 1). It also may cause activation or reduction in CAT activity (Table 1).

Romero-Puertas et al. (2007) showed that Cd treatment causes reduction of CAT activity and its protein expression in pea leaves, while it causes induction of CAT transcripts. So they assumed that Cd promotes posttranslational modifications of this enzyme. On the other hand, the increase of CAT mRNA by the metal treatment could be induced by the higher H_2O_2 production that takes place in pea leaves in these conditions (Romero-Puertas et al. 2004). H_2O_2 is proven to be a signal molecule in cat1 induction in response to ABA and wounding in maize plants (Guan and Scandalios 2000, 2002). In *Brassica juncea,* four distinct CAT sequences have been cloned, and it has been shown that Cd exposure causes an increase of CAT3 transcript (Lang et al. 2005). Smeets et al. (2008) observed an increase in CAT transcription but no significant change in catalase enzymatic activity in the leaves. These discrepancies can be due to the presence of multiple allo- or isozymes. Thus, CAT activity was confirmed to closely correlate to Cd tolerance and can be an alternative biological indicator of Cd toxicity in wheat due to its high sensitivity to Cd, while it increases only in Cd-tolerant lines or species and decreases in sensitive ones (Ci et al. 2009; Li et al. 2011). In Ni hyperaccumulator *Thlaspi goesingense,* CAT activity also increased after Ni treatment (Freeman et al. 2004). Results in the study of Heidari and Saran (2011) also showed that at the highest concentration of heavy metals, the activity of CAT was higher in Cd treatment than Pb treatment.

HM treatment also showed different effect on CAT activity in plants due to the different plant organs. As an example, pea plants subjected to Ni stress generally exhibited no changes in CAT activity in leaves and roots (Gajewska and Skłodowska 2005). However, in barley, a significant increase in CAT activity was observed in Ni-treated leaves, while roots showed no significant changes (Kumar et al. 2012). Enhancement in the activity of CAT was recorded at low Cr(VI) stress in roots as well as in leaves of *Zea mays* seedlings, while after prolonged treatment with high concentration of Cr(VI), only CAT activity in roots was decreased (Zou et al. 2009). CAT activity was increased in all bioparts of heavy

metal (Pb, Cr, and Cd)-stressed plant *Jatropha curcas*, while leaves showed the greatest increase in CAT activity (Devi Chinmayee et al. 2014).

In general, according to Pandey and Sharma (2002), decline in the activity of CAT might be attributed to the inhibition of synthesis of this iron-porphyrin enzyme and other oxidase proteins (Das et al. 1978), while some HMs have some similar characteristics to Fe and may interfere with its absorption and reduced the availability of Fe for heme biosynthesis (Siedlecka and Krupa 1999).

2.2.2 Peroxidases

Another enzyme class responsible for degrading H_2O_2 is the peroxidases (POX), which are capable of reducing H_2O_2 to H_2O with the concomitant oxidation of a series of substrates. POX isolated from plants are distinguishable from APX in both amino acid sequence and in physiological function. Peroxidases (POX) with large number of isoenzymatic forms participate in a variety of cellular functions such as growth, development, differentiation, senescence, auxin catabolism, and lignification having broad specificity for phenolic substrates (Passardi et al. 2004). Guaiacol peroxidase (GPOX) is present in vacuoles, the cell wall, cytosol, and extracellular spaces and consumes H_2O_2 to generate phenoxy compounds that are polymerized to produce cell wall components such as lignin (Mishra et al. 2006). Overall results indicate an enhancement in the activity of guaiacol peroxidase, suggesting that this enzyme serves as an intrinsic defense tool to resist HM-induced oxidative damage and may serve as a biochemical stress indicator for HM pollution (Van Assche and Clijsters 1990; Demirevska-Kepova et al. 2004; Radotic et al. 2000).

In the marine alga *Nannochloropsis oculata,* Cd treatment causes induction of GPX activity (Lee and Shin 2003). HM treatment can cause increase and decrease in GPX activity due to different HM and different plant species (Table 1). The GPX activity can also display biphasic responses due to HM treatment (Table 1) when low-level metal stress increased the activity, while the excess of HMs decreases the activity of GPX. Decline in the activity of GPOD might be due to the formation of protein complex with metals, to change the structure or integrity of proteins (Florence and Stauber 1986; Mohan and Hosetti 1997).

Van Assche et al. (1986) demonstrated that increase in GPX activity might be a result of de novo protein synthesis or the activation of enzymes already present in plant cells to diminish ROS deleterious effects. Enhanced POX activity reflects the modified mechanical properties of the cell wall and cell membrane integrity through participating in lignin biosynthesis which can be used in building up physical barrier against toxic HM (Hegedüs et al. 2001). Degenhardt and Gimmler (2000) revealed that cell walls isolated from the endodermal layer of *Z. mays* cultivated on metal-rich slag, including Cu and Zn, contain higher amounts of lignin than cell walls of control roots.

The toxicity of HM might not be the same at all stages of plant development (Hegedüs et al. 2001). The POD activity in roots of green or greening barley seedlings was not influenced by Cd concentration, but it increases in leaves treated

with toxic concentrations of Cd (Hegedüs et al. 2001). Baccouch et al. (1998) showed that Ni treatment did not cause any changes in GPX isozyme profiles; the increased activity of the three GPX isoforms is the most likely explanation for the increase in GPX activity. Changes in isozyme profiles of peroxidases have been observed by others under HM stress conditions. In fact, Van Assche et al. (1986) showed the quantitative changes of GPX isozyme profile throughout Ni exposure.

2.2.3 Ascorbate–Glutathione Cycle

Ascorbate–glutathione cycle (AsA–GSH) operates in cytoplasm, mitochondria, peroxisomes, as well as chloroplasts, where it is the primary mechanism of H_2O_2 detoxification, and it appears to be an important defense mechanism against oxidative stress caused by HM (Cuypers et al. 2000, 2001). The reduction of H_2O_2 by AsA can occur directly or it can be catalyzed by ascorbate peroxidase (APX) (del Río et al. 2006). During this process, AsA is oxidized to monodehydroascorbate (MDH). The monodehydroascorbate formed can be directly reduced back to AsA by monodehydroascorbate reductase (MDHAR) or may first be converted to dehydroascorbate (DHA) and then reduced by dehydroascorbate reductase (DHAR). Efficiency of APX depends on rapid regeneration of AsA from DHA, which in turn is dependent upon the availability of GSH. To maintain homeostasis, GSH must be generated from oxidized glutathione (GSSG) by glutathione reductase (GR) at the expense of NADPH (Noctor and Foyer 1998). The enzymes of AsA–GSH cycle also play a significant role in scavenging H_2O_2 mainly in chloroplasts and in maintaining the redox status of the cell.

Cuypers et al. (2011) pointed out a central role for GSH in the regulation of the antioxidative gene expression under Cd stress. Also, a higher GSH/GSSG ratio is a marker of HM tolerance in hyperaccumulating ecotype of *Sedum alfredii,* which shows less ROS production than the non-hyperaccumulating ecotype under excess Cd (Tian et al. 2011). Cd treatment causes the enhancement of the transcript levels of genes responsible for GSH synthesis, which implies possible de novo synthesis of both enzymes, resulting in higher GSH production, although levels of GSH were lower in Cd-treated leaves (Semane et al. 2007). The decrease of GSH found in the study of Semane et al. (2007) coincides with the strongly increased phytochelatin (PC) level, which assumes an active complexation of free Cd in the leaves by PC. Although the Cd treatment causes the increase in GR activity, it still reduces the GSH/GSSG ratio in *A. thaliana* leaves. The same authors also showed reduction of AsA content, another important antioxidant in AsA–GSH cycle that correlates with the reduction of APX activity (Semane et al. 2007). One hypothesis to explain the reduction in reduced AsA is through a diminished biosynthesis as a consequence of the inhibition of the mitochondrial electron transport chain and increased levels of ROS after Cd exposure (Wang et al. 2004). The AsA biosynthetic pathway is linked to the mitochondrial enzyme, L-galactono-1,4-lactone dehydrogenase, a reaction coupled with cytochrome c oxidoreductase (complex I) from mitochondria (Millar et al. 2003; Smirnoff and Wheeler 2000). On the other hand, it is possible that

regeneration of DHA to AsA by DHAR using GSH as an electron donor could not be maintained at a sufficient level because of the decreased GSH level under Cd stress (Semane et al. 2007). The activation of AsA–GSH cycle also depends upon duration of HM exposure. Schützendübel et al. (2001) gave the timetable of Cd-induced reactions of AsA–GSH cycle in roots. Initially, Cd causes a depletion of GSH, and it binds to thiol groups of GR (Creissen and Mullineaux 1995). The same inhibition mechanism may be possible for APX being sensitive to thiol reagents (Chen and Asada 1992). This causes inhibition of AsA–GSH cycle and an accumulation of H_2O_2 that acts as a signaling molecule in the induction of APX genes (Prasad et al. 1994; Karpinski et al. 1999). In contrast to APX gene expression, which is activated by H_2O_2, GR was not stimulated by H_2O_2 but by jasmonic acid (Xiang and Oliver 1998). The different time courses for the induction of APX and GR activities observed here also support the finding that these enzymes are regulated by different stimuli.

In algae, different HM treatment can cause different response of APX and GR activities (Table 1). The activities of APX and GR can also be enhanced, decreased, or unchanged by HM treatment or even showed biphasic response (Table 1).

Chou et al. (2012) found that the activities of APX and GR increased with increased Cd treatment duration in rice seedlings with no differences in expression of OsAPX and OsGR.

In contrast, APX2 expression is only induced during stress conditions and was clearly induced in roots of Cu and Cd (Cuypers et al. 2011). Smeets et al. (2008) showed the increase in APX activity and also enhanced level of APX1 transcript and DHAR in the Cd-treated leaves of *A. thaliana*. Interestingly, the GR1 gene was transcriptionally upregulated in both plant parts, while a significant decrease of the GR enzyme activity was observed. So, it is possible that Cd interacts with the translation mechanisms, disturbs the activity of GR, or even more likely influences the turnover of this enzyme.

Cd differentially altered GR activity and isoforms in leaves and roots of wheat plants. Wheat leaves did not show any change in their GR activity over time, whereas roots presented a remarkable increase after treatment (Yannarelli et al. 2007). Dixit et al. (2001) have showed in pea that GR was more activated in roots than in leaves when plants were treated with Cd. Moreover, GR activity did not show significant changes in pea leaves exposed to Cd stress (Sandalio et al. 2001). On the other hand, Cd treatment has decreased the activity of this enzyme in *Helianthus annuus* and *Ceratophyllum demersum* (Gallego et al. 1996; Aravind and Prasad 2005). Ni treatment strongly inhibited APX activity in maize root (Gajewska and Skłodowska 2005), which can disturb ROS homeostasis causing oxidative stress. In contrast to roots, APX activity in leaves of *Z. mays* plants increased in response to Ni treatment in Baccouch et al. (1998).

Cr treatment decreases the GSH and increases GSSG content in green gram leaves and induces the APX activities (Karuppanapandian et al. 2006). APX and GR activities were also upregulated upon exposure to Cr exceeding levels in cotton (Daud et al. 2014) and *Amaranthus viridis* (Liu et al. 2008).

D'Souza and Devaraj (2012) indicated that APX and GR activities in leaves of Hyacinth bean increase in the presence of Zn, and the early enhancement of APX activities suggests that the Zn-triggered antioxidative capacity mainly uses APX for the removal of H_2O_2. At the same time, GR activity declined with time in roots suggesting operation of AsA–GSH cycle only in leaves of Hyacinth bean (D'Souza and Devaraj 2012). Zn affects the AsA–GSH pathway in leaves and roots of *Phaseolus vulgaris*; nevertheless, the mechanism of action is different (Cuypers et al. 2001). In roots, an overall oxidation of metabolites and inhibition of APX and GR capacities were observed together with increased biosynthesis of AsA, which imposes the signal transport from roots to shoots. On the contrary, in primary leaves, the AsA–GSH pathway plays a prominent role in the defense against Zn stress. Nevertheless, the capacities of the four participating enzymes increased at various moments. Cu treatment, on the other hand, caused an increased capacity of the four enzymes (APX, MDHAR, DHAR, and GR) involved in the AsA–GSH cycle in the roots of *P. vulgaris* (Gupta et al. 1999). These results, therefore, clearly indicate that enhancement of the AsA–GSH cycle is part of the defense against oxidative stress that is imposed by Cu, shown also by Weckx and Clijsters (1996) in the primary leaves.

3 Nonenzymatic Antioxidants

3.1 Phenolics

Phenolics constitute very numerous groups of secondary plant metabolites. Through two most important ways of biosynthesis (shikimic and malic acid pathways), their production is indirectly stimulated by proline (Pro) which induces the synthesis of their precursors through pentose phosphate pathway (Hare and Cress 1997). These molecules, especially the complex flavonoid structures are among the most important plant antioxidants. Their functional diversity lies in their structural diversity with many biological functions, including multiple antioxidative mechanisms (Tahara 2007). Due to their structural properties, especially dihydroxy B-ring substitution, flavonoids perform antioxidant activities by directly scavenging $^{\bullet}OH$, HOCl, and $^1O_2$ and lipid peroxyl radicals, by inhibiting lipoxygenase and by metal chelation. For example, anthocyanins chelate metal ions enable their transportation to vacuole and even form complexes with other antioxidants, such as AsA, to prevent their oxidation (Sarma et al. 1997). In some cases, the resulting metal-flavonoid complexes are found to be more efficient ROS scavengers than the initial flavonoids (Malešev and Kuntić 2007). Flavonoids usually concentrate in plant vacuoles and are abundant in leaves, seeds, bark, flowers, and fruits. Efficient mechanisms have been recently identified for the transport of flavonoids from the endoplasmic reticulum, the site of their biosynthesis, to different cellular compartments. As summarized by Brunetti et al. (2013), the content of antioxidant

flavonoids is high in leaf mesophyll cells, where they act in quenching of H_2O_2 and H_2O_2-generated $^{\bullet}OH$ in the nucleus, trapping of singlet oxygen in vivo in chloroplasts and their outer envelope membrane limiting the diffusion of ROS, and enhancing membrane rigidity. Also, flavonoids accumulate in vacuoles where they are capable of removing H_2O_2 freely diffusing out of other cellular compartments, when the activity of APX (or CAT) is strongly depressed, being of primary significance in plants suffering from severe stress conditions. Flavonoids can facilitate HM tolerance, which is seen in *A. thaliana* (Keilig and Ludwig-Müller 2009). Absorption profile of flavonoids showed a decrease in flavonoid contents in pea plants influenced by Cd under field conditions (Agrawal and Mishra 2009). Several classes of flavonoids can be found in microalgae and cyanobacteria, especially a large amount of phenolic acids, mostly salicylic, trans cinnamic, synaptic, chlorogenic, chimic, and caffeic acids (Miranda et al. 2001). Due to the high content of phenolic substances and their contribution to overall antioxidant activity in higher plants and algae, as well as a variety of beneficial biological properties of these compounds, they represent a good source of natural antioxidants (Hajimahmoodi et al. 2009).

3.2 Ascorbic Acid

The very important role of AsA in the plant antioxidative defense system was addressed earlier in this chapter in the context of AsA–GSH pathway which enables successive oxidations and reductions of AsA, GSH, and NADPH by the complex set of enzymes which operate together in the removal of ROS (Anjum et al. 2014). Hence, AsA is the most important reducing substrate for H_2O_2 detoxification in plants and exists in relatively high concentrations in leaves representing over 10 % of the total soluble carbohydrates (Noctor and Foyer 1998). Except for the high millimolar concentrations in chloroplasts and cytosol, AsA was found in mitochondria, peroxisomes, and vacuoles and also as one of the rare antioxidants in the apoplast (Gratão et al. 2005). Besides being a substrate for APX, AsA reacts directly with $^{\bullet}OH$, $^1O_2$, and $^{\bullet}O_2^-$ and also regenerates Car and tocopherols in thylakoid membranes (Mallick and Mohn 2000). Besides the total level of AsA in the cell, the ratio between its reduced and oxidized form maintained by associated enzymes plays an important role in the defense mechanisms from ROS and their products (Anjum et al. 2014). AsA content in plants and algae shows distinct trend under HM treatments (Table 1). Its content is found to decrease in different plant species (Rodríguez-Serrano et al. 2006; Romero-Puertas et al. 2007; Agrawal and Mishra 2009; Chen et al. 2010). In case of algal cells treated with HMs, slight increase in AsA pool of *Chlorella vulgaris* was observed in lower Cu concentrations, while a drastic reduction was evident at higher concentrations (Mallick 2004).

3.3 Tocopherols and Tocotrienols

Tocopherols and tocotrienols (group of vitamin E compounds) are denoted as lipid soluble membrane-associated antioxidants (Singh 2005). Tocotrienols are only found in certain plant families and almost exclusively in seeds and fruits, while tocopherols are more ubiquitous and accumulate in chloroplasts, vacuoles, and nuclei in plant leaves or in seeds within plastids and associated with cytoplasmic lipid bodies, while in green algae, they are present inside chloroplasts and mitochondria (Falk and Munné-Bosch 2010). As antioxidants tocopherols act as lipid protectors, membrane-stabilizing agents, inhibitors of lipid peroxidation, and ROS scavengers (Matringe et al. 2008), and their interplay with carotenoids in scavenging $^1O_2$-induced damage to photosystem is seen as the essential cell response in limiting photo-oxidative stress (Trebst et al. 2002). Out of four isomers found in plants, the greatest number of studies are focused on antioxidative properties of α-tocopherol (α-toc), which is the most potent scavenger of $^1O_2$, which is, like AsA, a relatively poor electron donor in physiological conditions that acts primarily by transfer of single hydrogen atoms (Kruk et al. 2005). Different methods were applied in studying tocopherol functions in plants, such as exogenous applications at the whole-plant or cellular level, studying variations in their content prior to and during the various physiological processes, and the use of mutants and transgenic lines with altered biosynthesis and levels of these compounds (Falk and Munné-Bosch 2010). In the case of heavy metal-induced stress, among other antioxidants, a significant increase of α-toc in *P. vulgaris* leaves was observed after 10-day exposure to HMs, decreasing in the following order Hg > Cd > Cu > Pb (Zengin and Munzuroglu 2005). Also, the increase of α-toc was observed after the exposure of *Solanum lycopersicum* (Hédiji et al. 2010) and *Vitis vinifera* cv. cell suspension cultures (Cetin et al. 2014) to Cd as well as Eridano *Populus* sp. clones to elevated Zn concentrations (Fernàndez-Martínez et al. 2014). It has been shown that oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in higher plants (Tang et al. 2011). For example, overexpression of the γ-tocopherol methyl transferase gene from *A. thaliana* in transgenic *Brassica juncea* plants resulted in an over sixfold increase in the level of α-toc, induced by a variety of abiotic stresses (Yusuf and Sarin 2007). Moreover, α-toc-enriched transgenic *B. juncea* was found to increase the tocopherol levels which enhanced its tolerance against Cd-induced stress (Kumar et al. 2013). Also, some other transgenic plants, such as potato, with the reduced expression of some other proteins, e.g., manganese stabilizing protein, an important component of the PSII, were found to accumulate α-toc and other antioxidants as a consequence of Zn treatment (Gururani et al. 2013).

3.4 Amino Acids and Peptide Derivates

The importance of amino acids and peptide derivates accumulation in plant cells is especially pronounced under metal stress (reviewed by Gill et al. 2014). The main

focus is attributed to Pro, but certain other amino acids and oligopeptides, i.e., glycinebetaine, polyamines (PAs), and nicotianamine, are also addressed (Sharma and Dietz 2006). Most of these compounds act as osmolytes which accumulate in response to water deficit conditions regulating the osmobalance which is often disturbed by HMs. Glycinebetaine and Pro are two major organic osmolytes with positive effects on enzyme and membrane integrity and important roles in osmotic adjustment in plants grown under stress conditions (Ashraf and Foolad 2007). While many studies have indicated a positive relationship between accumulation of these osmolytes and plant stress tolerance, some have proposed that the increase in their concentrations under stress is a product of and not an adaptive response to stress. Also, it is unclear if the Pro accumulation under HM stress is a sign of its role in metal detoxification or only an indirect response to metal-induced disturbances in plant water balance (Schat et al. 1997). However, besides osmoregulation, amino acids, particularly Pro, were shown to have many beneficial functions under metal stress, such as metal chelation and reducing metal uptake, $^1O_2$ and $^{\bullet}OH$ quenching, signaling, serving as a source of carbon and nitrogen, maintaining cytosolic pH and NAD(P)+/NAD(P)H ratio, and preventing denaturation of enzymes (Mehta and Gaur 1999; Sharma and Dietz 2006). Its accumulation can be achieved by increased synthesis or reduction of degradation which may be regulated by enzymes (Hong et al. 2000). *Lablab purpureus* plants exposed to Zn stress exhibited elevation in Pro levels in leaves and roots as a function of time (D'Souza and Devaraj 2012), which is also assumed to be connected with the role of Pro in osmotic stress during long-term metal exposure (Sharma et al. 1998). Accumulation of Pro was observed due to Cd treatment in different plant parts of *P. sativum* (Agrawal and Mishra 2009), as well as in carrot and radish roots (Chen et al. 2003). The same effect is seen in lettuce leaves of two cultivars after the administration of Cu in nutrient solution, as well as in soil (Teklić et al. 2008a). Also, the Pro concentration rises in the leaves of *V. faba* plants treated with Cd, Cu, Ni, Pb, or Zn, as well as in the stems of plants grown on Zn and Ni-contaminated soil. The concentration of Pro was higher in the leaves than in the stems of all treated plants, whereas the control plants had comparable amounts of this amino acid in both organs (Nadgórska-Socha et al. 2013). The content of Pro in radish plantlets treated with Cu or Pb differed depending on plant part (leaf or hypocotyl) and stress intensity (heavy metal content in growth medium and exposure duration) (Teklić et al. 2008b). This has been mainly linked to Ni hyperaccumulator plants (Sharma and Dietz 2006), and its content can be increased even 36-fold in the xylem of the hyperaccumulator *Alyssum lesbiacum* compared to nonaccumulator *Alyssum montanum* (Krämer et al. 1996). Another amino acid, NA, is a potent plant HM chelator involved in HM transport and HM tolerance in hyperaccumulating plants (Sharma and Dietz 2006).

Due to their effects on plant resistance against HMs or other abiotic stresses, exogenous applications of some amino acids, such as glycinebetaine and Pro to plants could lead to the significant increases in growth and final crop yield under the stress conditions (Ashraf and Foolad 2007). However, in some cases of metal toxicity, such as Cd application in barley roots, Pro accumulation is disturbed

leading to water and oxidative stress responsible for the root growth inhibition (Tamás et al. 2008).

Some algae also respond to heavy metal toxicity by producing Pro (Table 1). Due to small size and specific uptake of metals in algal cells, Pro accumulation in unicellular microalgae was observed within a few hours, which differs from higher plants that require a longer period of exposure to metals (Mehta and Gaur 1999). *Chlorella vulgaris* cells accumulated high levels of Pro, with the highest content observed in maximum concentrations of Cu and Cr, followed by Ni and Zn treatment. Also, Pro pretreatments inhibited metal-induced lipid peroxidation in *C. vulgaris*. There is also a very important role for Pro in algal recovery from stress. Pro hyperaccumulation also seems to be an important strategy for overcoming oxidative stress induced by long-term exposure of *Scenedesmus* sp. to elevated levels of Cu and Zn (Tripathi et al. 2006).

PAs are low molecular-weight amines important in plant growth and development and are also involved in protection against environmental stresses (Gill and Tuteja 2010). Their hydrophilicity enables them to buffer intracellular pH and work as effective ROS-scavenging compounds, being mediators in protective reactions against different stresses (Grzesiak et al. 2013). Like with amino acids, the accumulation of these compounds in plants is one of the responses to water stress, as a consequence of HM influence or some other type of abiotic stresses. Their exact role in heavy metal defense is not clear, but a participation in the stabilization and protection of the membrane systems has been proposed (Sharma and Dietz 2006). It can be also related to their antioxidative properties in reducing the $^{\bullet}O_2^-$ accumulation when applied exogenously, like in tobacco protoplasts, which can be related to the inhibition of microsomal membrane NADPH oxidase, mostly by spermine (Spm) (Papadakis and Roubelakis-Angelakis 2005). The changes in PA levels vary among plant species and the stress duration. Individual PAs have different roles during stress response, and spermidine (Spd) could be implicated in enhancing HM tolerance, possibly by exerting an antioxidant activity and/or by metal chelation (D'Souza and Devaraj 2012). The most abundant of the three PAs, Spd, was found in twofold and onefold higher concentrations in leaves and roots of *L. purpureus* under Zn stress after 72 h at maximum concentration. It has been suggested that stress-tolerant plants increased exogenous PAs level to a greater extent than sensitive ones and, those PAs with a higher number of amino groups, i.e., Spm and Spd, are more effective in scavenging ROS, than diamine putrescine (Put) suggesting the involvement of amino groups in ROS scavenging (Kubiś 2008). In addition to common types of PAs, certain algae also have homospermidine, 1,3-diaminopropane, norspermidine, and norspermine, whose content varies due to the HM treatment (Agrawal et al. 1992).

3.5 Soluble Sugars

Soluble sugars, besides amino acids, present another important group of plant osmoregulators with antioxidant properties. An elevated level of total soluble sugars is important for energy production, stabilization of cellular membranes, maintenance of turgor, vitrification of the cytoplasm, and signaling, which may account for tolerance to heavy metal stress. Soluble sugars have different roles in events associated to metabolism of HM-stressed plants. Although soluble sugars have been linked to metabolic pathways that produce ROS, they can also have an important role in ROS-scavenging mechanisms. Sucrose and glucose either act as substrates for cellular respiration or as osmolytes to maintain cell homeostasis, while fructose is not related to osmoprotection and seems related to secondary metabolites synthesis (Rosa et al. 2009). Increased glucose levels can increase the production of NADPH (via the pentose phosphate pathway), that is, an important intermediate in the AsA–GSH cycle (Couée et al. 2006) as NADPH is the primary electron donor that assures an intracellular reduction status. Both glucose and sucrose levels have been shown to increase in some plant species treated with HMs. Both of these sugars also participate in signaling mechanisms. Van den Ende and Valluru (2009) suggest that sucrose might have a protective role against stress due to its capacity to scavenge ROS. Other sugars like raffinose and fructans are also reported to have a protective role as membranes against several stresses, namely freezing and drought stress (Van den Ende and Valluru 2009). Besides the benefits of endogenously derived sugars, exogenous application of trehalose in *Lemna gibba* also reduces the deleterious effects of Cd and Pb increasing the metal accumulation and is identified as a potential phytoremediation-enhancing agent (Duman et al. 2011; Duman 2011). Plant carbohydrate levels are often directly correlated to Pro synthesis, which was previously described in *L. purpureus* leaves and roots after Zn treatment (Sharma et al. 1998).

3.6 Thiols/Glutathione

These sulfur-containing antioxidants in plants are usually measured as total thiols, non-protein thiols, and GSH, as well as protein thiols i.e., PCs and MTs. As seen in algae (*Scenedesmus* sp.), the binding of metals to thiols can protect cell metabolism from metal toxicity only temporarily; however, during severe stress, depletion of thiol pool is known to make cells vulnerable to oxidative stress (Rijstenbil et al. 1994). Depletion of thiol content in *Scenedesmus* sp. after Cu or Zn treatments was significantly higher in longer treatments which can be related to high GSH demand needed for PC synthesis and was also followed by the inhibition of GR. Cd uptake is strongly connected to alterations in plant thiol content, having dual action. Elevated thiol content connected with lipid peroxidation and root growth inhibition

was detected in all individual segments of barley root after Cd treatment (Tamás et al. 2008), as well as in *P. sativum* (Agrawal and Mishra 2009). On the other hand, 10 μM Cd decreased thiol content and induced oxidation of AsA and GSH indicating oxidative stress. Zn supplementation to Cd-treated plants can restore thiols, showing the protective role of Zn in modulating the redox status of the plant system through the antioxidant pathway for Cd-induced stress tolerance (Aravind and Prasad 2005). Thiols can play an important antioxidative role, protecting membrane lipids. Lipoic acid, both in its reduced and oxidized form, is reported to have antioxidative properties due to its direct scavenging of ROS. It is also able to chelate several metal ions that induces oxidative stress (Navari-Izzo et al. 2002) and thus can have an important role in cell protection. Glutathione is a low molecular-weight thiol tripeptide (Glu-Cys-Gly) that exists in plant cells in the reduced (GSH) and oxidized form (GSSG) with the typical GSH/GSSG ratio of 100:1 in glutathione homeostasis (Viehweger 2014). Being one of the major antioxidants, it is ubiquitous in plant tissues and active in many intracellular compartments (Noctor and Foyer 1998). Some of its most important functions include antioxidant activity in scavenging $^1O_2$ and H_2O_2, overall defense reactions against oxidative stress, and involvement in sulfur metabolism. Besides being a potent HM-ligand, it is a precursor of PCs. Upon HM exposure, GSH concentrations can decrease as a consequence of initiated PC biosynthesis. As it was mentioned in the previous chapter, GSH plays another key role in the plant antioxidative defense system by regenerating AsA via Asa–GSH pathway (Foyer and Halliwell 1976). In addition, GSH is a substrate for GPX and GST (glutathione peroxidase and glutathione-S-transferase) involved in the removal of ROS (Noctor et al. 2002). Exposure to heavy metals results in two different ways. In some cases of metal toxicity, the severe depletion of GSH is observed which is often strongly positively correlated with the inhibited activity of antioxidative enzymes, while in some cases, GSH content is found to increase (Table 1). A Cd-dependent reduction of glutathione (GSH) has been described in different tissues and plant species (Rodríguez-Serrano et al. 2006; Gomes-Júnior et al. 2006b). This reduction is often seen in total glutathione content, although the reduced form (GSH) is the most affected (Romero-Puertas et al. 2007). Moreover, exogenous GSH can modulate the antioxidant defense system against Cd stress in the two barley genotypes differing in Cd tolerance (Chen et al. 2010). Very large difference in antioxidant response related to GSH was also seen between the two species of green microalgae exposed to Cu. Among other measured endpoints, *Scenedesmus vacuolatus* exposed to Cu showed significantly higher GSH levels compared to *Chlorella kessleri* (Sabatini et al. 2009).

PCs are thiol-rich compounds formed in metal-exposed plants which are the main plant cellular mechanisms for HM detoxification and tolerance (Gupta et al. 2013). PCs are synthesized non-translationally in a transpeptidation reaction catalyzed by the enzyme phytochelatin synthase, a reaction needing GSH as a precursor. These peptides have chelating properties but also play other important roles in plant cells, including essential heavy-metal homeostasis, sulfur metabolism, and antioxidant action (Dietz et al. 1999; Cobbett 2000). Some in vitro

analyses show that compared to GSH, PCs show stronger scavenger properties for H_2O_2 and O_2^- radicals (Tsuji et al. 2002). PCs have been most widely studied in plants, particularly in relation to Cd tolerance (Cobbett 2000). Some heavy metal hyperaccumulator plants show that their hypertolerance mechanisms to Cd accumulation are not dependant exclusively on PC-based sequestration (Schat et al. 2002), while in some hyperaccumulators like *Sedum alfredii,* it is probably related to GSH chelation (Sun et al. 2007; Gupta et al. 2010). The synthesis of PCs has also been reported in algal species exposed to high concentrations of metals such as Cu in *Scenedesmus bijugatus* (Nagalakshmi and Prasad 2001) and Cd in *Vaucheria* spp. (Skowroński et al. 1998). Scheidegger et al. (2011) suggested that PCs play a minor role in Pb detoxification in *Chlamydomonas reinhardtii* at environmentally relevant concentrations but are highly involved in Cu and Zn homeostasis. In several species of marine algae, PCs are synthesized even in untreated cultures, while elevated PC concentrations were induced also by very low but environmentally relevant concentrations of Cd (as low as 10^{-12} M free ion concn). Also, the treatment of *Stigeoclonium* spp. with individual metals (Cd, Pb, and Zn) induced higher content of PCs, opposed to metal mixture contained in the mining water (Pawlik-Skowrońska 2001). In certain algal species, such as *Dunaliella tertiolecta,* the Zn pretreatment induces the PCs synthesis-related tolerance toward other heavy metals (Cd, Hg, Cu, and Pb) and As and also increases tolerance toward oxidative stress caused by H_2O_2 or herbicide paraquat (Tsuji et al. 2002).

MTs are the second type of cysteine-rich, metal ion-binding proteins. They are special for their highly conserved cysteine arrangement, although in plants, MTs make a uniquely diverse family with many isoforms which make, based on the cysteine pattern, four different topologies (types 1–4 plant MTs) (Leszczyszyn et al. 2013). The biosynthesis of MTs is regulated at the transcriptional level, and MT genes can be differentially regulated under various HM stresses (Ahn et al. 2012). MTs are suggested to be involved in metal tolerance or homeostasis, by complexing ions (most commonly Cu(I), Zn(II), and Cd(II)) through the thiol groups of their cysteine residues in characteristic metal-thiolate clusters (Leszczyszyn et al. 2013). MTs are reported to be much more important in HM tolerance of certain plants than PCs (Mijovilovich et al. 2009). Except for their main function as metal chelators, novel investigations show that MTs are also involved in ROS removal, plasma membrane repair, and signaling, while the interplay between these functions needs to be fully characterized (Hassinen et al. 2011). Transgenic tobacco plants overexpressing GhMT3a-type protein showed increased tolerance against abiotic stresses, including HM treatments, compared with wild-type plants (Xue et al. 2009). Tsuji et al. (2002) suggested that MT2 in algae could play a role not only in detoxification of HMs but also in mitigation of oxidative stress. Morelli and Scarano (2004) found that metal-free MT3 was present in cell extracts of *Phaeodactylum tricornutum* treated with Cu, hypothesizing that this oxidized MT3 form participates in ROS scavenging.

3.7 Carotenoids and Phycobilins

Carotenoids (Car) function as accessory pigments in light absorption and as structural units of photosystem complexes serving also as photoprotective agents, quenchers of the excited states of Chlorophyll, and scavengers of $^{\bullet}O_2$ during photosynthesis, protecting the reaction center from photoinhibition (Yamamoto and Bassi 1996). The Car molecules become themselves excited but lack energy to form other ROS species what makes them very efficient antioxidants (Taiz and Zeiger 2002). Car contributes significantly to the total antioxidant capacity of both microalgae and plants. However, in most cases, HMs are known to target the pigment apparatus leading to Chl degradation followed by reduced Car content (Table 1), causing a marked effect on the entire metabolism of a plant. In some plants, like Cd-treated *Pisum sativum,* a decrease in total Chl and Car contents was observed in all treatments at all time points (Agrawal and Mishra 2009). Certain plants, like *P. vulgaris*, react adversely by increasing the levels of Car, with the strongest effect observed in plants exposed to Ni, followed by the sequence Co > Cr > Zn (Zengin 2013). In algae, such as *C. vulgaris*, a rising trend in Car content was observed after Cu treatment (Mallick 2004). Also, in some heterotrophically grown ROS-treated algal cultures, the employment of oxidative stress can be used to promote the formation of secondary carotenoids including astaxanthin (Ip and Chen 2005). Moreover, the biosynthesis of both primary pigments (chlorophylls and primary Car such as β-carotene and lutein) and secondary Car (astaxanthin, canthaxanthin, and adonixanthin) depends on the nature of applied ROS. In some cases, like in Cu and Zn-treated *Scenedesmus* sp. cells, Car content was unchanged during the treatments showing that some algal species use other mechanisms in protection from metal-induced oxidative stress (Tripathi et al. 2006), while in some red algae, Pb and Cu treatments significantly decreased total carotenoid content (Gouveia et al. 2013).

Phycobilins are open-chain tetrapyrroles that function as chromophores of light-harvesting chromoproteins called phycobiliproteins. Phycobiliproteins, such as phycoerythrin, phycoerythrocyanin, phycocyanin, and allophycocyanin, are highly absorbent, and fluorescent components of the photosynthetic light-harvesting complex (phycobilisome) found in cyanobacteria, rhodophytes, cryptomonads, prochlorophytes, and glaucocytophytes and their ratio in cells vary due to the chromatic adaptation (Larkum 2003). Their antioxidation activity was shown in cell-free extracts isolated from several species of cyanobacteria, which was mostly attributed to phycoerythrin as the main phycobiliprotein (Pumas et al. 2011). Moreover, phycocyanobilin, isolated from *Spirulina platensis*, prevented the peroxidation of linoleic acid (Hirata et al. 1999). However, it is indicated that some compounds such as phycocyanin exhibit pro-oxidant activity, generating $^{\bullet}OH$ in the light, while having radical scavenging activity in darkness attributed to phycobilin part of the compound (Zhou et al. 2005). Because of their linear tetrapyrrole structure, which resembles that of biliverdin and bilirubin, phycobilins are expected to have strong antioxidant properties (Wada et al. 2013).

The phycocyanobilin found in *S. platensis* cell extracts inhibited the initiation of lipid peroxidation and was proven to be responsible for majority of the antioxidative activity of phycocyanin with the potential as effective antioxidant agent (Hirata et al. 2000). Studies on *Anabaena flos-aquae* show that cellular proteins, including phycocyanin, represent the main targets of metal toxicity, which was more pronounced in the protein profile after Cu treatment under different temperature levels, compared to Cd treatment in the same temperature range (Surosz and Palinska 2005). Pb and Cu exposure significantly decreased the total phycobiliprotein content in rhodophyte *Gracilaria domingensis* (Gouveia et al. 2013), which was also observed for several cyanobacterial species, such as in *S. platensis* when exposed to Pb (Arunakumara and Zhang 2007) and after Ni or Pb exposure in *Oscillatoria* sp. and *Westiellopsis* sp., particularly in longer treatments. Phycocyanin and phycoerythrin were more susceptible to HM toxicity than allophycocyanin (Balakrishnan and Narayanan 2007).

4 Conclusion

The presented results reveal a high diversity in antioxidative responses not only among different plant or algal species but also among cultivars and different plant parts or algal strains. Therefore, the HM-induced oxidative stress is probably a general symptom in several species, but the antioxidative response is specific and dependent on genetic potential of individual cultivars or strains of given species and on HM concentration and/or a given period of exposure. Also, the key challenge in the current research of HM-induced oxidative stress in plants is to delineate the individual contribution of enzymatic and nonenzymatic biomolecules in the overall antioxidant capacity of plant organisms subjected to stress. In the last several decades, antioxidant systems including AsA, GSH, and their related enzymes which exist in relatively high content in cellular compartments shared a spotlight in the research field of HM-induced oxidative stress, and their roles have been examined in detail in the existing literature. However, other antioxidants which share a (potentially) minor role in the overall antioxidant capacity of plant cells are less studied, thus having great research potential.

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Arsenic and Chromium-Induced Oxidative Stress in Metal Accumulator and Non-accumulator Plants and Detoxification Mechanisms

Sarita Tiwari and Bijaya Ketan Sarangi

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Abstract Mitigation of arsenic (As) and chromium (Cr) pollution is a topical environmental issue of high R&D priority due to its toxicity on living organisms and deleterious effects on the environment. Following uptake by plants, As and Cr generate reactive oxygen species (ROS) and induce oxidative stress, which exerts negative effects on biochemical, molecular, and cellular levels that hinder plant growth and development. When the stressor level reaches the threshold level of plant tolerance, the stress response is manifested physiologically and beyond that level, the plant succumbs. However, some plants termed as hyperaccumulators, i.e., those accumulating metal ions inside their cellular milieu with $BF > 1$, have evolved detoxification mechanisms due to their physiological and genetic makeup which facilitates scavenging of indigenously generated ROS. Various enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), ascorbate, glutathione, and phenolic compounds have been reported to be involved in neutralising ROS. It seems that the antioxidant defence system plays a significant

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role in combating metal stress and confers metal tolerance to these plants. Understanding the biochemistry of plants exposed to As and Cr stress would be beneficial for selecting As and Cr tolerant plants that are better equipped with such defence mechanisms. This chapter reviews different aspects related to antioxidant defence mechanisms in As and Cr hyperaccumulator and non-hyperaccumulator plants. This chapter also highlights usefulness of these biomarkers for screening plants with competent biochemical mechanisms for metal stress tolerance. This information, in turn will help to design efficient phytoextraction treatment systems through deployment of such competent plants.

Keywords Arsenic • Chromium • Phytoremediation • Oxidative stress • Antioxidant enzyme

1 Introduction

Amongst all biological organisms, terrestrial plants are most vulnerable to environmental stress in their habitat. Being non-motile, they are exposed to the ecosystem dynamics at different stages of growth and times out of which abiotic stress is more acute. These plant species try to maintain homeostatic interrelationship between ecosystems dynamics for their existence and survival. However, sustained stresses for long duration become stressors which are more profound with abiotic stress. Industrialisation and urbanisation have become inevitable to sustain human needs resulting in a footprint in the ecosystem which is ever being magnified due to improper environmental management practices. Uncontrolled disposal of waste, natural catastrophes, reckless mining and smelting of metallic ores, agricultural use of waste sludge and water, accidental and process spillage, etc. have led to serious pollution resulting in conversion of natural resources and ecosystems to contaminated wastelands or waterbodies (Bartolomeo et al. 2004; Landajo et al. 2004; Lokeshwari and Chandrappa 2006; Ogbonna et al. 2009). Wastes emanating from different industrial processes are both inorganic and organic compounds comprising heavy metals, combustible and putrescible substances, hazardous wastes, explosives, and petroleum products which become recalcitrant for treatment due to their complex nature (Xia et al. 2011). Heavy metals and inorganic constituents are indestructible and persist in most environmental matrices (Alloway 1990; Ghosh and Singh 2005). Heavy metal toxicity such as arsenic (As) and chromium (Cr) is one of the major environmental concerns that warrant urgent attention for mitigation and sustainable management.

Several end-of-the-pipe treatment methods are available for mitigation of As and Cr pollutions, but they are invasive, *ex situ*, have secondary impacts on the environment, and are non-sustainable. Moreover, application of these methods in a large stretch of land and water ecosystem is techno-economically not feasible.

Phytoremediation is an in situ cleanup technology which entails use of green plants for environmental restoration (Cunningham and David 1996; USEPA 2000, 2001; Mwegoha 2008; Das and Maiti 2008; Purakayastha and Chhonkar 2010; Raymond and Felix 2011; Witters et al. 2012). This remediation process follows the principles of green technologies and is sustainable (Li et al. 2003). It is a solar-driven biological process and in average, tenfold cheaper than engineering-based remediation methods, such as soil excavation, soil washing or burning, or pump-and-treat systems (Glass 1999; USEPA 2001; Sheoran et al. 2011; Conesa et al. 2012; Pokhrel and Dubey 2013).

Metal accumulation in plant species growing in metal-polluted soils is due to uptake and eventual accumulation along with other inorganic nutrients (Baker 1981; Ross 1994; Verbruggen et al. 2009; Sarangi et al. 2009; Sheoran et al. 2011) in their biomass, although, heavy metals also exert toxic effects on their metabolism. Different plant species significantly vary in their ability to tolerate metal stress and metal accumulation in the biomass depending on the genetic and biochemical organisation of the plant system. The ideal plants for phytoremediation application should possess multiple traits, such as high contaminant uptake capacity, relatively fast absorption kinetics, high level of biomass production, and adaptability to different climates and soil environments (Pichai et al. 2001; Padmavathiamma and Li 2007; Sarangi et al. 2009; Verbruggen et al. 2009). Effective remediation of contaminated soil and water systems using a specific plant species is an immensely complex task whose success depends on a multitude of factors: first and foremost, the ability of the client plant to uptake, translocate, detoxify, and accumulate the target contaminant in its biomass. Heavy metals are toxic to plants, but some plants known as metallophytes have evolved mechanisms to sustain and cope with this stress, and hyperaccumulator plants accumulate high levels of heavy metals in their biomass with a higher bioaccumulation factor (Nedelkoska and Doran 2000; Sheoran et al. 2011; Sarma 2011; Raymond and Felix 2011). This chapter presents recent progress in the research on mechanisms of metal tolerance in non-hyperaccumulating and hyperaccumulating plants.

1.1 Prevalence of Arsenic and Chromium Stress

Arsenic (As) Arsenic exists in the earth's crust, averaging about 3 mg kg^{-1} (Mandal and Suzuki 2002; Zhao et al. 2010), but it locally gets concentrated from various anthropogenic activities (Buchet and Lison 1998; Mandal and Suzuki 2002; Benner 2010). In nature, As is distributed ubiquitously throughout earth crusts, soil, sediments, water, air, and living organisms in over 200 different mineral forms, of which approximately 60 % are arsenates, 20 % sulphides and sulphosalts, and the remaining 20 % includes arsenides, arsenites, oxides, silicates, and elemental As (Mukherjee et al. 2008; Kim et al. 2009). Arsenic exists in a variety of inorganic and

organic compounds in the environment, and inorganic species are highly mobile and toxic (Meharg and Hartley-Whitaker 2002; Giacomino et al. 2010). So far, four As species: As(V), As(III), As(0) and As (-III) have been studied extensively in the water–soil–plant system (Onken and Hossner 1995; Smith et al. 1998; Kertulis et al. 2005). Under aerobic soil conditions, arsenate (AsO_4^{3-}) is the thermodynamically stable form, whereas arsenite (AsO_3^{3-}) is the predominant inorganic species under reducing conditions (Smith et al. 1998; Chen et al. 2009).

Chromium (Cr) Chromium is listed as the seventh most abundant metal in the earth's crust (Panda and Choudhury 2005) and exists in bound form in the range of 100–300 mg kg^{-1} soil (Sarangi et al. 2009). The prime sources of Cr contamination in the environment are utilisation of Cr-containing compounds, pesticides and insecticides, indiscriminate disposal of industrial, urban, and mine wastes, denudation of soil cover, over exploitation of aquifers resulting in geogenic formations, etc. (Han et al. 2002; Da Costa et al. 2012). Chromium-based compounds are widely used in different industries such as tannery, metal plating industries, metallurgical and processing, wood preservation, and alloy preparation (Dhal et al. 2013; Fan et al. 2012). The discharge limit of Cr from industries is less than 1 mg L^{-1}. In the environment, Cr occurs in different oxidation states but primarily exists as a soluble, highly toxic Cr(VI) anion and the less soluble, less toxic Cr(III) form (Mohanty et al. 2006) Usually it occurs in association with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions. Although Cr is required as a trace element in humans and animals, it exerts toxicity above 0.5 mg L^{-1}, which is its permissible discharge limit. Cr(VI) is both toxic and mutagenic to biological organisms and induces cancer and teratogenic effects in animals and plants (Shanker et al. 2005).

2 Phytotoxic Effects of As and Cr Stress in Hyperaccumulator and Non-hyperaccumulator Plants

Arsenic and Cr are not essential nutrients for plants, and their entry inside plants has deleterious effects at multiple levels. As a chemical analogue of phosphate, As (V) interferes with oxidative phosphorylation, while As(III) exerts inhibitory effects on enzyme activity by binding to thiol groups (Dixon 1996; Zhao et al. 2010; Hughes 2002). The primary targets of As toxicity are unknown, but As(V) can substitute for phosphate in phosphorylation reactions, including ATP synthesis. However, in plant cells, As(V) is rapidly reduced to As(III), catalysed by ACR2 arsenate reductases (Bleeker et al. 2006; Dhankher et al. 2006). Toxicity of As(III) is probably due to its high sulphydryl reactivity. Arsenic, either supplied as As(V) or As(III), causes oxidative stress and can deplete reduced glutathione, an important cellular antioxidant, through the formation of As(III)–glutathione (As(III)–GSH) and As(III)-induced phytochelatin (PC) synthesis. As in animals, the mitochondrial electron transfer chain of plant cells is one of the major targets of

As toxicity and is the site of rapid As-induced ROS production (Heyno et al. 2008). Increased ROS production induces lipid peroxidation. Furthermore, As, either supplied as As(V) or As (III), is both mutagenic and an inactivator of DNA mismatch repair in yeast and human cells (Jin et al. 2003), and the same mechanisms may also be effective in plants.

2.1 Alterations in Physiological and Biochemical Mechanisms of Stressed Plants

A putative chromatin remodelling factor, named *OXS3,* was recently identified in a screen for metal tolerance of a *Brassica juncea* cDNA library in *Schizosaccharomyces pombe* (Blanvillain et al. 2008). An *OXS3* mutant was hypersensitive to metal stress and overexpression improved tolerance. It was postulated that *OXS3* might protect DNA or alter its transcriptional selectivity. Interestingly, *OXS3* overexpression also enhanced tolerance to As, Cu, and Zn or oxidising chemicals like diamide. As-induced increase in ROS (H_2O_2) production is likely mediated by glycolate oxidase and may act as a cellular signal triggering the stress response (Gupta et al. 2013). Stress-responsive mitogen-activated protein kinases (MAPK) seem to be involved in transcriptional responses possibly activated by ROS under excess As (Jonak et al. 2004). It is thought that MAPK activation is actually mediated by ROS production, a secondary effect of oxidative injury by heavy metals. Similar to other abiotic stresses, different plant hormones and growth regulators may also participate in the plant response to metal(loid) stress (Dalcorso et al. 2008). Exposure to As (Norton et al. 2008) or other metal ions has been reported (Weber et al. 2006; Craciun et al. 2006; Van de Mortel et al. 2008) to induce many different transcription factors. Some of them are constitutively upregulated in *Arabidopsis halleri* or *Thlaspi caerulescens*. Large arrays of genes are constitutively highly expressed in metal hyperaccumulators compared to a related non-hyperaccumulator species (Verbruggen et al. 2009). Gene duplication and modification of *cis*-regulatory elements are demonstrated mechanisms of enhanced expression of these stress-related genes (Hanikenne et al. 2008). The molecular study of metal hyperaccumulators has unravelled the role of genes involved in metal homeostasis and detoxification previously identified in *Arabidopsis thaliana*. The major metal(loid) detoxification mechanisms in plants are (1) transport to the major storage organs or tissues, (2) chelation, (3) subcellular compartmentalization, or (4) efflux from the plant body. The specific mechanisms of metal-induced stress response and mitigation measures evolved by plants are discussed in the following sections.

2.2 *Non-hyperaccumulators*

Induction of abiotic stress triggers production of several metabolites and proteins, some of which may be responsible for conferring a certain degree of protection to these stresses (Jaleel et al. 2009). Plant species found in metal-polluted/contaminated soils are prone to take up metals (Baker 1981; Ross 1994) in their biomass, which also includes edible parts. As(V) is easily incorporated into plant cells through the high-affinity Pi transport system. Non-hyperaccumulators generally rely on decreased As(V) uptake, because of suppression of the high-affinity Pi uptake system (Meharg and Hartley-Whitaker 2002). It has been shown in *Holcus lanatus* that As(V) hypertolerance is not associated with any hypertolerance to As (III) because of the different uptake mechanisms of As(V) and As(III) (Bleeker et al. 2006). Arsenite has a molecular size and structure similar to that of silicic acid, and the silicon transporter OsNIP2;1/Lsi1 was recently demonstrated to be responsible for most of the As(III) influx into rice roots (Ma et al. 2008). However, As(III) efflux from the roots into the rhizosphere has also been demonstrated in a number of plant species. In non-hyperaccumulators, very considerable fractions (50–85 %) of the arsenic taken up may be removed from the plant body via root As (III) efflux, unlike in the As hyperaccumulator, *Pteris vittata*, where hardly any As (III) efflux was observed (Zhao et al. 2009).

Arsenic and Cr-mediated disorders in plant physiology have been observed by many researchers (Panda and Choudhury 2005; Shanker et al. 2005; Dhankher et al. 2006; Kramer 2010). Chromium and As-induced phytotoxicity results in plant growth inhibition, decrease in protein content, nutrient imbalance, and inhibition on enzymatic activities and mutagenesis (Panda and Choudhury 2005; Shanker et al. 2005; Yadav et al. 2010; Sharma 2012). Requejo and Tena (2005) reported up or downregulation of about 10 and 15 %, respectively, of total detected maize root and shoot proteins under As stress. Various investigators have proved that As toxicity in non-resistant plants results in considerable stress upon exposure, ranging from inhibition of root growth to death (Meharg 2003; Barrachina et al. 1995). Length and biomass of root and shoot were significantly affected due to As stress. Reduced root length growth in response to As exposure has been observed by a number of investigators in different plants (Shri et al. 2009; Liu et al. 2005; Hartley-Whitaker et al. 2001).

Uptake and concentration of Cr in plants bring many disorders in physiological and biochemical processes that has been studied in different plants like mosses, rice, pea, wheat, etc. in relation to oxidative stress (Choudhury and Panda 2005; Panda 2007; Hayat et al. 2012). Both forms of Cr [Cr (III) and Cr (VI)] are found to have adverse impacts on plant growth, root wilting, and chlorophyll content of plants (Panda and Choudhury 2005). Chen et al. (2001) reported a decrease in root weight and root length in maize seedlings grown in soil containing 20 mg Cr (VI) kg^{-1}. The inhibition in root growth is due to shutdown of root cell division/elongation or extension of the cell cycle in roots. In the presence of both forms of Cr, the root gets affected due to direct contact between roots and metals which in

turn inhibits the roots ability to absorb water from the medium (Barcelo et al. 1985). Effect on inhibition of seedling germination under different Cr concentration was reported by many investigators. Plants such as *Echinochloa colona*, barley, *Phaseolus vulgaris*, sugarcane bud, etc. were reported to display a reduction in seedling growth in response to Cr stress (Skeffington et al. 1976; Parr and Taylor 1982; Rout et al. 2000; Shanker et al. 2005). The reduction in seed germination under Cr stress has predicated the suppressive effect of Cr on the activity of amylases and on the subsequent transport of sugars to the embryo axes. Inhibition of chlorophyll biosynthesis by Cr has also been reported in terrestrial plants (Vajpayee et al. 2000). In fact, Cr also causes deleterious effects on plant's physiological processes such as photosynthesis, water relations, and mineral nutrition. Barcelo et al. (1985) described the inhibition of P, K, Zn, Cu, and Fe translocation within the plant parts when bean plants were exposed to Cr in nutrient solutions. Sujatha et al. (1996) reported that tannery effluent irrigation caused micronutrient deficiencies in several agricultural crops. Metabolic alterations by Cr exposure have also been described in plants either by a direct effect on enzymes and metabolites or by the ability of Cr to generate ROS (Shanker et al. 2005).

2.3 Reactive Oxygen Species Generation Under Metal Stress

Under standard growth conditions, uneven harmony of a plant scavenging system with ROS buildup leads to uncontrolled oxidation and free radical chain reactions, which result in oxidative stress to the plant (Srivastava et al. 2005). During stress, electrons that have a high-energy state are transferred to molecular oxygen (O_2) to form ROS. ROS that most often occur under metal toxicity are singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), peroxides, hydroxyl radicals ($OH^{\bullet}$), and the widely distributed hydrogen peroxide (H_2O_2) (Ahmad et al. 2010). Heavy metals including As and Cr have been reported to stimulate the formation of ROS, mostly free radicals, leading to oxidative stress (Singh et al. 2007). Excess ROS formed within cells can provoke oxidation and modification of cellular amino acids, proteins, membrane lipids, and DNA. The functionality of protein can be affected by ROS either by oxidation of amino acid side chains or by secondary reactions with aldehydic products of lipid peroxidation (Reinheckel et al. 1998). Plant cell membranes are generally considered to be primary sites of metal injury. Membrane destabilisation is frequently attributed to lipid peroxidation due to an enhanced production of toxic amounts of oxygen free radicals after exposure to metal. The polyunsaturated fatty acids (PUFAs) are the major fatty acids in the plant membrane, and ROS attacks unsaturated fatty acids inducing peroxidation which gives rise to complex mixtures of lipid hydroperoxide products like malondialdehyde (MDA) (Mueller 2004; Singh et al. 2007; Mishra et al. 2008). Malondialdehyde (MDA) is one of the final decomposition products of lipid peroxidation and has been used as an index for lipid peroxidation status (Zhou 2001; Yamauchi et al. 2008; Singh et al. 2010). In rice seedlings, the levels of MDA were used as

an indicator of lipid peroxidation under As stress (Shri et al. 2009). Extensive PUFA peroxidation decreases the fluidity of the membrane, increases leakiness, and causes secondary damage to membrane proteins (Ahmad et al. 2010). In high concentrations, ROS are predominantly employed in causing cell damage but in lower concentrations, they play a major physiological role in several aspects of intracellular signalling and regulation (Palmer and Paulson 1997). It has been clearly demonstrated that ROS interfere with the expression of a number of genes and signal transduction pathways (Ahmad et al. 2008; Jaleel et al. 2009) playing a significant role as secondary messengers.

In case of As, although As is a non-redox active element, there are significant evidences that both inorganic forms of As i.e., As(III) and As(V) generate ROS which cause toxicity and damage to cell systems (Meharg and Hartley-Whitaker 2002). There is significant evidence that exposure to inorganic As species results in the generation of ROS. Using a proteomics approach, it has been reported that As exposure induces proteins related to oxidative stress in plants (Requejo and Tena 2005). *P. vittata* is a well-known established hyperaccumulator which has a capacity of accumulating high concentrations of As inside plants without any toxicity symptoms (Ma et al. 2001). Whereas in most of the plants, As accumulation causes adverse effects by enhancement of ROS (Kertulis et al. 2005). After entry into the plant's cellular milieu, arsenic causes injury to the major biomolecules such as proteins, DNA, and lipids of the cell system (Miller et al. 2008; Mittler et al. 2004). However, the complete mechanism of As toxicity in plants is not clear so far, but inactivation of essential enzymes by SH-As binding, displacement of essential ions from enzymes, and interference of As with phosphate metabolism are some predictable mechanisms of As toxicity in plants (Sharma 2012). A comparative study by Srivastava et al. (2005) between *P. vittata* (As hyperaccumulator) and *P. ensiformis* and *N. exaltata* (As sensitive) observed elevated levels of TBARS (thiobarbituric acid reactive substances), which are lipid peroxidation products, in the fronds of *P. ensiformis* and *N. exaltata* in comparison to *P. vittata* after As exposure. The increase in levels of TBARS in the fronds with increasing concentrations of arsenate indicates that As induces oxidative stress in fern plants. Lower production of TBARS in the fronds of *P. vittata* corresponds to its higher As accumulation.

Chromium(III), on the other hand, apart from generating ROS, if presents in high concentrations, coordinates various organic compounds resulting in inhibition of some metalloenzyme systems. Significant increase in lipid peroxidation and H_2O_2 generation was seen in Cr-treated green gram (*Vigna radiata*) plants (Shanker et al. 2004). H_2O_2 levels increased in both roots and leaves of sorghum treated with either 50 AM Cr(VI) or 100 AM Cr(III) (Shanker et al. 2005). Under Cr stress, the plants induce certain metabolic modifications such as alterations in pigment production, e.g., chlorophyll, anthocyanin (Boonyapookana et al. 2002), increased production of antioxidant metabolites (e.g., glutathione, ascorbic acid) as a direct response to Cr stress which may cause damage to the plants (Shanker 2003), and production of biochemically related metabolites which confer resistance or tolerance to Cr stress (Schmfger 2001).

3 Mechanism of As and Cr Detoxification in Hyperaccumulator Plants

3.1 ROS Scavenging Mechanisms

Plants under metal stress induce different detoxification mechanisms, of which compartmentalization of metals inside the plant biomass is a primary action. However, failure to concentrate toxic metals inside plants turns on different biochemical processes through maximising the metal thiol binding, resulting in lower levels of free ionic species involved in stress-related detoxification reactions (Srivastava et al. 2005; Singh et al. 2006).To neutralise effects of generated ROS, endogenous defence mechanisms are activated which lead to the synthesis of adaptive biochemical responses such as (1) synthesis of phytochelatins, (2) increased production and activity of enzymatic and non-enzymatic antioxidants, and (3) prevention of the degradation of biomolecules (Shri et al. 2009; Schmoger et al. 2000). Antioxidant enzyme activity assays have been commonly used as indicators for evaluation of oxidative stress in some plants (Foyer and Noctor 2005; Zhang et al. 2007; Gusman et al. 2013) since the induction of antioxidants is often the only evidence that oxidative stress has occurred in vivo (Halliwell and Gutteridge 1999). Metal-tolerant plants have a well-organised defence system to combat metal stress. Antioxidative systems play a key role in maintaining ROS levels below its toxic threshold limits which get imbalanced due to abiotic stresses. Literature review reveals that heavy metal tolerance by plants is the result of enhanced production of antioxidant enzymes (Gratao et al. 2005). Stress-tolerant plants had evolved intricate biochemical mechanisms to sustain metal toxicity through reduced influx, complexation of ions, and enhanced production of antioxidants that detoxify ROS generated in response to the exposure to toxic metals (Gunes et al. 2009). The stimulation of enzymatic activities of, e.g., superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidases (APX), glutathione reductase (GR) and non-enzymatic antioxidants like glutathione, ascorbate and carotenoids have been considered as basic defence mechanisms against As-induced oxidative stress in ferns and some higher plants (Sharma et al. 2007). Many reports demonstrate that As and Cr hyperaccumulators show an increase in enzymatic antioxidants in response to As and Cr stress (Singh et al. 2010; Srivastava et al. 2005; Diwan et al. 2010). Hyperaccumulator plants have developed efficient mechanisms to combat such adverse environmental stresses like heavy metal toxicity. The Chinese brake fern (*P. vittata*), a versatile and fast growing As hyperaccumulator, showed no toxicity symptoms, low levels of lipid peroxidation, and high activities of SOD, CAT, and APX in response to elevated As concentrations when compared with two other As-sensitive ferns, namely *P. ensiformis* and *N. exaltata*, implicating the antioxidative defence in As tolerance (Srivastava et al. 2005; Singh et al. 2010).

3.1.1 Enzymatic Antioxidants

Enzymatic antioxidants act as shields against metal stress by eliminating ROS and preventing degradation of biomolecules in plants (Schmoger et al. 2000; Shri et al. 2009). The major antioxidant enzymes which are involved in ROS scavenging mechanisms of plants include SOD, CAT, APX, and GPX (Ashraf 2009; Wang et al. 2010). In plant cells, each cellular compartment has been reported to contain more than one type of enzyme to neutralise ROS (Table 1). That is, normally, each cellular compartment contains more than one enzymatic activity that detoxifies a particular ROS. For example, the cytosol contains at least three different enzymatic activities that scavenge H_2O_2: APX, GPX, and PrxR (Suzuki and Mittler 2006). Specific roles for antioxidant enzymes have been explored via transgenic approaches. Reactive oxygen species initially produced during metal stress are eliminated, e.g. SOD, by virtue of converting $O_2^{\bullet -}$ to H_2O_2 which can further be removed by CAT, APX, and GPX enzymes as shown in Fig. 1 (Sharma and Dietz 2008). Enhanced activity of these enzymes had been reported in plants exposed to metal stress (Sun et al. 2007; Shri et al. 2009; Gusman et al. 2013). A comparative study of enzyme activity between As hyperaccumulator and non-accumulator plants showed an increased enzymatic activity in stress-resistant (i.e., As hyperaccumulator) plants (Srivastava et al. 2005).

3.1.1.1 Superoxide Dismutase, EC 1.15.1.1

SOD is one of the early induced enzymes known to scavenge ROS in response to metal stress (Gechev et al. 2006; Bowler et al. 1992). Several isomeric forms of SOD exist in plants which convert superoxide radicals ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2). The upregulation of SODs is implicated in combating oxidative stress caused due to abiotic stress and have a critical role in the survival of plants. SOD activity as a function of treated As concentration was evident in *P. vittata,* and higher activity was observed in aerial parts due to a higher concentration of As in fronds. SOD activity in treated fronds was 46–61 % higher as compared to controls (Srivastava et al. 2005; Singh et al. 2010). An increase in SOD activity in response to As(V) and/or As(III) toxicity was also reported in *H. lanatus* (Hartley Whitaker et al. 2001) and *Zea mays* (Requejo and Tena 2005). Comparative analysis of SOD activity vs. As tolerance between different species of *Pteris* revealed that SOD

Table 1 Localization of enzymatic antioxidants in plant cells

Organelles	Enzyme
Chloroplast	APX/SOD/GR/glutathione
Mitochondria	APX/GR/Mn-SOD/glutathione
Cytosol	APX/GR/Mn-SOD/glutathione
Vacuole	Glutathione/peroxidase
Peroxisomes	CAT/SOD

Cited from Ahmad et al. (2010)

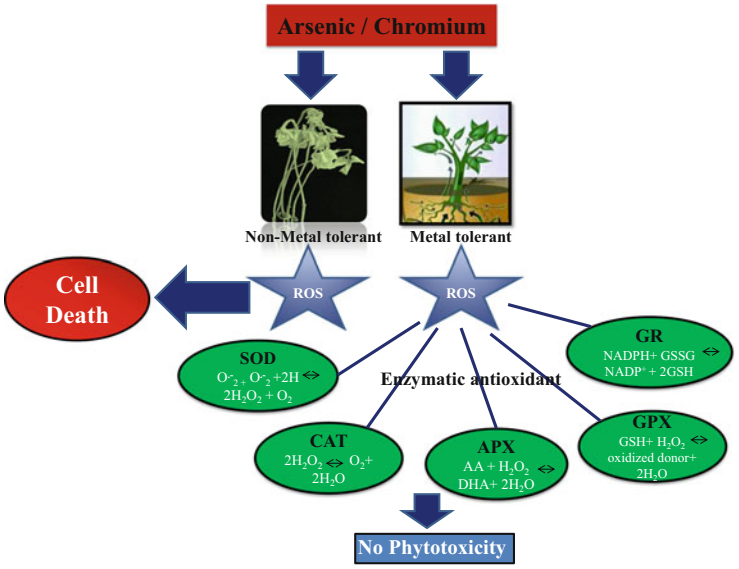

Fig. 1 Enzymatic antioxidant defence mechanism in hyperaccumulator and non-accumulator plants

activity was the highest in *P. vittata* followed by *P. ensiformis* and *N. exaltata* showing highest and lowest As tolerance, respectively (Srivastava et al. 2005). Similarly, decreased SOD activity in pea and other plants is related to their inability to fight against oxidative stress caused by As (Gong et al. 2005; Gunes et al. 2009). The study by Hartley-Whitaker et al. (2001) on *H. lanatus* concluded that SOD levels are depressed at higher As concentrations but enhanced at lower As concentrations. In maize, it has been shown that expression of *sod* genes in the leaves was increased on exposure to low levels of arsenate and arsenite but was decreased at higher levels of As treatment (Mylona et al. 1998). Altered expression of SOD may in turn lead to expression of other enzymes associated with the stress resistance (McKersie et al. 1999). Similarly, the highest level of SOD activity was observed in the fronds of the As hyperaccumulator *P. vittata*. Similar to As, exposure of pea to low Cr concentrations (20 μM) increased SOD activity, and this activity was inhibited at much higher (200 μM) concentrations which was also lethal to the plant. In another study, it has been shown that SOD was active in scavenging the superoxide produced by both Cr species in green gram, whereas catalase did not participate in active H_2O_2 reduction irrespective of Cr speciation (Shanker et al. 2004).

3.1.1.2 Catalase, EC1.11.1.6

A higher SOD activity during detoxification of ROS results in an increased generation of H_2O_2, and its higher concentrations may promote further oxidative damage. Therefore, a rapid removal of H_2O_2 produced as a result of SOD activity is another important requirement. The generated H_2O_2 is more permeable across membranes than other ROS (Mittler et al. 2004) and also toxic. Therefore, scavenging of the generated H_2O_2 is followed by an increase in the activity of other enzymes responsible for scavenging such as CAT, APX, GPX, and POX (Asada 1992; Srivastava et al. 2005; Gill and Tuteja 2010). The function of CAT is the direct degradation of H_2O_2 (i.e., without the involvement of an electron donor), a potential source of highly reactive hydroxyl radical and singlet oxygen and the conversion of H_2O_2 into water and oxygen (Ahmad et al. 2010). This enzyme is mainly localised in the peroxisomes, and the fact that peroxisomes proliferate during stress optimises the scavenging of H_2O_2 by CAT that diffuses from the cytosol. However, it is important to point out that under extreme conditions of stress, a plant may be too weak to produce enough antioxidant enzymes to protect it. Increased CAT activity in response to metal stress is observed in many plants (Fayiga et al. 2004), and an increase in catalase activity is supposed to be an adaptive trait (Sekmen et al. 2007; Vital et al. 2008). CAT has been shown to be upregulated by As at the transcriptional level. Srivastava et al. (2005) found that *P. vittata* (As hyperaccumulator) and *P. ensiformis* (an As-sensitive fern) had similar CAT activities in the fronds in the absence of As. After exposure to As, CAT activity significantly increased in the fronds of both plants (21–28 %). This may imply the role of CAT in As tolerance by both plants. CAT was not only induced in the fronds of *P. vittata*, but it was also activated, up to 300 %, upon As exposure. Although a similar activation pattern was observed in the non-hyperaccumulator *P. ensiformis*, the magnitude was much smaller. The activation of CAT at two different concentrations of arsenate may be the result of different CAT isozymes present in the fern. It was also observed that in the As-treated *P. vittata*, CAT activity was found primarily in fronds rather than in roots. This response is corroborated by the study of Duquesnoy et al. (2010), where high As(V) and As(III) concentrations increased CAT activity in leaves and roots of *Zea maize*. Activity of CAT in response to Cr has also been studied in many crops like rice, wheat, green gram, and mosses (Panda and Patra 2000; Choudhury and panda 2005; Panda and Choudhury 2005). In most of the studies, a gradual decrease in CAT activity was observed in the treated plants with increasing Cr concentrations. Chatterjee and Chatterjee (2000) had reported restricted CAT activity in cauliflower leaves at 0.5 mM Cr.

3.1.1.3 Ascorbate Peroxidase, EC 1.11.1.1

APX, which is produced mainly in the chloroplasts and other cell organelles, is required to scavenge H_2O_2 and to maintain the redox state of the cell (Asada 1992). APX has a higher affinity for H_2O_2 than CAT and POD, and it may have a more crucial role in the management of ROS stress or may be responsible for the fine

modulation of ROS signalling. In contrary to SOD levels, APX was remarkably high in roots of As-treated *P. vittata* and *P. ensiformis* but not in *N. exaltata*. The As hyperaccumulator, *P. vittata*, demonstrated a significant increase in APX activity in its frond, rhizome, and root tissues under As treatment (Srivastava et al. 2005). Similar results were obtained in *P. ensiformis*. However, in the case of the sensitive species, *N. exaltata*, there was no significant increase in the activity of APX in the frond tissues (Singh et al. 2006).

3.1.1.4 Glutathione Peroxidase, EC 1.11.1.9

Glutathione peroxidases are the family of multiple isozymes which catalyse the reduction of H_2O_2 and cytotoxic hydroperoxides to alcohols (Dixon 1996). Thus, besides scavenging of H_2O_2, GPX also serves to detoxify products of lipid peroxidation formed due to the activity of ROS. The activity of GPX under As stress in frond tissues of *N. exaltata* and rhizome tissues of *P. ensiformis* and *N. exaltata* was significantly high. This indicates that an enhancement of GPX activity upon exposure to As serves as an intrinsic defence tool to resist As-induced oxidative damage in these plants. Similar increases in GPX activity in leaves of *Zea Mays* and *Oryza sativa* in response to As stress was also observed (Shri et al. 2009). In certain plants, like lettuce roots, APX activity in treated plants was lower than in controls. It was presumed that this could be due to non-equilibrium between the activation of CAT, SOD, POX, and APX (Gusman et al. 2013).

3.1.1.5 Glutathione Reductase, EC 1.6.4.2

GR is responsible for scavenging H_2O_2, catalysing disulphide bonds of glutathione, and maintaining the levels of glutathione (Yannarelli et al. 2007). GR is mainly localised in the chloroplast stroma but is also found in mitochondria, cytosol, and peroxisomes. GR catalyses the rate limiting last step of the ascorbate–glutathione pathway. A study by Kertulis-Tartar et al. (2009) after observation of combined results of induction, kinetics, and inhibition studies interpreted that GR is not affected by As in *P. vittata* and *P. ensiformis*. This non-induction of GR activity upon As exposure in *P. vittata* and *P. ensiformis* assumes no significant role of GR in *Pteris* in As tolerance (Cao et al. 2004; Srivastava et al. 2005; Kertulis-Tartar et al. 2009).

3.1.2 Non-enzymatic Antioxidants

3.1.2.1 Phenolic Compounds

Phenolics are diverse secondary metabolites (flavonoids, tannins, hydroxycinnamate esters, and lignin) abundant in plant tissues, having at least

one aromatic ring (C_6) and bearing one or more hydroxyl groups. The phenolic compounds are potential non-enzymatic antioxidant acting as ROS scavenging compounds (Blokhina et al. 2003). Polyphenols possess an ideal structural chemistry for free radical scavenging activity, and they have been shown to be more effective antioxidants in vitro than tocopherols and ascorbate (Rice-Evans et al. 1997; Balasundram et al. 2006). Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and the ability to chelate transition metal ions (termination of the ROS-generating Fenton reaction). Phenolic antioxidants (PhOH) interfere with the oxidation of lipids and other molecules by the rapid donation of hydrogen atom to radicals $ROO^{\bullet} + PhOH \rightarrow ROOH + PhO^{\bullet}$. The phenoxy radical intermediates are relatively stable, so they do not initiate further radical reactions. They even act as terminators of the chain reaction by interacting with other free radicals. Induction of polyhydroxy phenolic compounds in response to different heavy metals has been reported (Zhang et al. 2002). During heavy metal stress, phenolic compounds can act as metal chelators, on the other hand, phenolics can also directly scavenge molecular species of reactive oxygen. In an As non-accumulator, *A. filiculoides* Sánchez-Viveros et al. (2011) had observed a decrease in the total amount of phenolic compounds and presumed that this phenomenon is the reason for susceptibility of these plants to As-induced stress.

Flavonoids are one of the important phenolic compounds found abundantly in plant organs that act as ROS scavengers by identifying and neutralising radicals before onset of their toxic action (Lovdal et al. 2010). Their functionality depends on the number and arrangement of their hydroxyl groups attached to ring structures. Their ability to act as antioxidants depends on the reduction potentials of their radicals and accessibility of the radicals. Many flavonoid biosynthetic genes are induced under stress conditions, and there is significant enhancement in flavonoid level towards metal toxicity (Gill and Tuteja 2010). Another mechanism underlying the antioxidative properties of phenolics is the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes. This could delay diffusion of free radicals and restrict peroxidative reactions. In the presence of H_2O_2 and phenolic substrates, peroxidases operate in the peroxidatic cycle and are engaged in the synthesis of lignin and other phenolic polymers. However, if the phenolic substrates are replaced by NADPH or related reduced compounds, a chain reaction starts that provides the basis for the H_2O_2-producing NADH-oxidase activity of peroxidises (Apel and Hirt 2004). Increased activity of peroxidise in root tips of pine were linearly related with elevated level of phenolics against cadmium stress (Schutzendubel and Polle 2002).

3.1.2.2 Ascorbate and Glutathione

These are probably the most extensively studied antioxidants and can be detected in the majority of plant cell types, organelles, and the apoplast (Potters et al. 2002;

Smirnoff 2000). Normally, ascorbate occurs in the reduced form (AsA), and its intracellular concentration ranges from 20 mM in the cytosol to 20–300 mM in the chloroplast stroma (Noctor and Foyer 1998). A fundamental role of AsA in the plant defence system is to protect metabolic processes against H_2O_2 and other potentially toxic derivatives of oxygen. AsA acts essentially as a reductant and scavenges many types of ROS. Two molecules of AsA are utilised by APX in the ascorbate–glutathione cycle to reduce H_2O_2 to water, with the concomitant generation of monodehydroascorbate (MDA) which is converted into dehydroascorbate (DHA) through regeneration of AsA. MDA can also be reduced directly to AsA. AsA can react directly and reduce superoxide, hydrogen peroxide, and hydroxyl radical or quench singlet oxygen. Ascorbate also functions as a co-substrate of plant oxidases, such as the ascorbate peroxidase system, which produces dehydroascorbate (Singh et al. 2006). Dehydroascorbate is reduced to ascorbic acid in a GSH (glutathione)-dependent reaction catalysed by dehydroascorbate reductase.

Glutathione (GSH) is a non-enzymatic low-molecular weight thiol-type antioxidant involved in cellular defence against toxic xenobiotics (Terry and Banuelos 2000; Gill and Tuteja 2010). Glutathione levels in plant tissues are known to increase under metal stress (Sun et al. 2007). Glutathione is involved in a range of metabolic processes and constitutes an important plant defence system against environmental stress, including heavy metals. Glutathione levels in plant tissues are known to increase under metal stress (Sun et al. 2007). Glutathione levels are constitutively higher in plants adapted to metal(loid) stress conditions (Mishra et al. 2008, 2009). Glutathione is the major source of nonprotein thiols and a precursor of the metal-binding phytochelatins (PC). The chemical reactivity of the thiol group of glutathione makes it particularly suitable to serve a broad range of biochemical functions in all organisms. Reaction of GSH by ROS forms oxidised glutathione (GSSG) and ascorbate is oxidised to monodehydroascorbate (MDA) and dehydroascorbate (DHA) through the ascorbate–glutathione cycle (Gill and Tuteja 2010). GSSG, MDA, and DHA can be reduced to reform GSH and ascorbate. A high ratio of reduced to oxidised ascorbate and GSH is essential for ROS scavenging in cells. GSH also prevented the toxic effects of As(V). Plants exposed to As substantially increase the synthesis of GSH and PC, which is a polymer of GSH. Depletion of GSH by oxidants may alter the redox status of the cell and present a stressful and toxic situation (Hughes 2002). However, the heritable metal tolerance in *P. vittata* (Kertulis-Tartar et al. 2009) and *Silene vulgaris* (De Vos et al. 1992) was not found to correlate with constitutively high GSH concentrations, implying the species specificity of GSH functions. GSH and Cys gave partial protection against As stress. Kertulis-Tartar et al. (2009) found a slight increase of ascorbate and glutathione in *P. vittata* and *P. ensiformis* under As exposure. The contents of non-enzymatic antioxidants (GSH, and –SH) were significantly increased at high levels of As exposure (>20 mg kg^{-1}), though no significant change at low As levels was observed (Cao et al. 2004). Mutants with decreased ascorbic acid levels or altered GSH contents are hypersensitive to As stress.

4 Importance of Detoxification Mechanisms for As and Cr Phytoremediation

There are a variety of existing physical and chemical technologies for remediation of As and Cr-contaminated sites (USEPA 1992; Choong et al. 2007). For As remediation treatment, conventional approaches like co-precipitation, adsorption, ion exchange, and membrane processes are effective in removing As from contaminated groundwater (Cheremisinoff 1998; Brenner and Lazarova 2012). On the other hand, in case of Cr, leaching of pollutants, vitrification, electro kinetic treatments, excavation, and off-site treatments are in use (Fu and Wang 2011). However, these remedial technologies are *ex situ*, expensive, and restricted for remediation of small areas due to techno-economic limitations (Paspaliaris et al. 2010). In recent times, R&D are more focused towards development of sustainable processes based on 'green chemistry' approaches taking into consideration human health, resources availability, energy input, and atom efficiency. Phytoremediation is a promising technology for pollution management in comparison to other physico-chemical methods with minimal maintenance requirement and public acceptance (Kramer 2010), and phytoextraction is suitable for metal extraction from contaminated matrixes. This plant-based biological remediation process can function with minimal maintenance after its establishment, as the costs of growing vegetation is minimal compared to those of soil removal, treatment, and replacement. Because biological processes are solar driven, phytoremediation is on average tenfold cheaper than engineering-based remediation methods, such as soil excavation, soil washing or burning, or pump-and-treat systems (Purakayastha and Chhonkar 2010; Macek et al. 2011). However, implementation of this technique for remediation of metal and other contaminants has met with limited success due to lack of enough evidence about competency of a potential plant for application based on phytoremediation principles (Zhao and McGrath 2009).

Plants ideal for phytoremediation should posses multiple traits viz. high contaminant uptake capacity, relatively fast absorption kinetics, high biomass, and adaptability to suit various climates and soil environments (Pichai et al. 2001; Sarangi et al. 2009). Employment of hyperaccumulator plants having higher ability for metal uptake and accumulation in their biomass has a promising scope to enhance efficacy of the phytoextraction process. A large number of hyperaccumulator plants specific for different metals have been experimentally discovered, and their extraordinary ability for phytoremediation has been proven. So far, many fern species within the *Pteridaceae* family have been reported to be As hyperaccumulators, some of these are listed in Table 2 (Zhao et al. 2009). *P. vittata* is an initially discovered and established As hyperaccumulator having an exceptional ability for As uptake and accumulate up to 2.3 % of its biomass (Ma et al. 2001; Zhao et al. 2009). In case of Cr, very few plants such as *Dicoma niccolifera*, *Sutera fodina,* and *Leersia hexandra* passed the criterion of being a Cr hyperaccumulator having >1000 mg kg^{-1} accumulation in their leaves (Reeves and Baker 2000; Zhang et al. 2007).

Table 2 Details of some As and Cr hyperaccumulator plants

Plant species	Metal concentration in frond (mg kg^{-1})	Treatment medium	Reference
Arsenic (As)			
Pteris vittata	4360	Soil	Ma et al. (2001)
P. biaurita	1770	Soil	Srivastava et al. (2005)
P. quadriaurita	2800	Soil	Srivastava et al. (2005)
P. ryukyuensis	3700	Soil	Srivastava et al. (2005)
P. cretica	2046	Soil	Ma et al. (2001)
Pityrogramma calomelanos	5390	Soil	Francesconi et al. (2001)
P. longifolia	2361	Soil	Meharg (2003)
P. umbrosa	5300	Soil	Zhao et al. (2002)
Chromium (Cr)			
Leersia hexandra	2978	Water	Zhang et al. (2007)
Dicoma niccolifera	1500	Water	Wild (1974)
Sutera fodina	2400	Water	Baker and Brooks (1989)

Identification of plant species suitable for phytoremediation is crucial for a successful deployment of phytoremediation technologies. The conventional approach for identification of plant species based on dose-dependent responses could be erroneous, which has been found to be true in some plants such as *H. lanatus* and other fern species from the genus *Pteris* which were considered to be As tolerant/accumulators (Meharg and Hartley-Whitaker 2002). Biochemical mechanisms in plants for scavenging stress-induced reactive molecules are efficient strategies evolved in those species that confer metal accumulation in the plant biomass with a high bioaccumulation factor. It is essential to be sure about such a capability of the plant by using molecular tools. Molecular markers using proteomics and genomics with a state-of-the-art knowledge base are potential tools to generate science-based evidence and overcome the bias in selection. The biochemical mechanisms discussed in this chapter could be used as molecular markers for identification of suitable candidate plants with hyperaccumulation ability for As and Cr phytoextraction.

5 Conclusions and Prospective

Metals are indestructible in the environment, and their increased concentrations act as stressors to plants at multiple levels. Remediation of As and Cr pollution through phytoextraction by hyperaccumulator plants is being increasingly investigated to offer a better alternative over other existing conventional *ex situ* methods. Plants respond differently under As and Cr stress due to differential endogenous mechanisms. Oxidative damages are caused by ROS, and excess amounts of ROS are harmful to many cellular components, including membrane lipids. The difference in antioxidant functions between metal tolerant and non tolerant plants can be further utilised for the selection of appropriate plants for phytoremediation. However, the plants encompassing systemic stress-response mechanisms are able to tolerate the oxidative stress generated by metals. For optimising and strengthening phytoremediation technologies, there should be a better understanding of fundamental biochemical and molecular processes regulating metal uptake and detoxification in the potential plant species. The inherent ability of a plant for metal accumulation is the primary requisite to make phytoextraction a practicable alternative to the existing conventional processes. Effective remediation of contaminated soil and water systems using a specific plant species is an immensely complex task whose success depends on a multitude of factors, first and foremost, the ability of the client plant to uptake, detoxify, translocate, and accumulate the contaminant in its biomass. The effectiveness of metal phytoremediation can be enhanced by two means, i.e., identification of plants with high metal hyperaccumulating potential and knowledge of factors to maximise metal accumulation. Antioxidant systems could be used as a defined set of markers to predict tolerance towards a particular type of stress. Enhancing antioxidant mechanisms of plants by manipulating biochemical mechanisms for more synthesis of antioxidant biomolecules and enzymes is a prospective research area to enhance As accumulation and simultaneously increase biomass generation in order to confer efficient phytoremediation.

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Phytochelatin and Oxidative Stress Under Heavy Metal Stress Tolerance in Plants

Weitao Liu, Xue Zhang, Lichen Liang, Chen Chen, Shuhe Wei, and Qixing Zhou

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Abstract With the rapid developing of industry and agriculture, heavy metal pollution in environment has been both serious and widespread worldwide. To cope with adverse environmental heavy metal toxicity, plants have evolved a variety of adaptive responses, which include immobilization, exclusion, chelation, and compartmentalization of metal ions and often involve metal-binding ligands. Particularly, phytochelatins (PCs), a family of peptides, have been regarded as the

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best-characterized heavy metal chelators especially in detoxication of heavy metals such as cadmium (Cd) in plants and some microorganisms. Generally, PCs have the general structure (γ-Glu-Cys)$_n$-Gly ($n = 2$–11) and are produced by the enzyme phytochelatin synthases, which can bind to various metals including Cd, As Cu, or Zn. In this chapter, we focused on the biosynthesis and function of PCs and the role of PCs in metal detoxification and tolerance. Finally, the molecular biology of PCs has been briefly reviewed.

Keywords Heavy metals • Plants • Oxidative damage • Tolerance • Phytochelatins

1 Introduction

The term heavy metal generally refers to a series of metals and metalloids with atomic mass over 20 and specific gravity above 5, which can be toxic to both plants and animals even at much low concentrations (Liu et al. 2013; Rascio and Navari-Izzo 2011). Heavy metal contamination in soils has dramatically increased during the last century due to mining, smelting, manufacturing, agricultural soils amended with municipal sewage sludge, and waste disposal practices (Liu et al. 2015). Unlike organic pollutants, heavy metals could not be degraded naturally or by microbial communities. Therefore, heavy metals could persist in soils for a long period of time after its introduction (Zhou and Song 2004).

Many heavy metals are essential for plant physiological processes e.g., copper (Cu), zinc (Zn), manganese (Mn), and nickel (Ni), while other metals especially cadmium (Cd), lead (Pb), and mercury (Hg) are nonessential (Pal and Rai 2010). Both excessive essential metals and a small quantity of nonessential metals could be toxic to plant species. Heavy metal toxicity to plant is mainly induced oxidative stress. Reactive oxygen species (ROS), e.g., $^{\bullet}O_2^-$, H_2O_2, and $^{\bullet}OH$, was the primary response of plants exposure to high levels of heavy metals (Mithöfer et al. 2004). Malondialdehyde (MDA), one of the decomposition products of polyunsaturated fatty acids of membrane, is regarded as a reliable indicator of oxidative stress (Demiral and Türkan 2005).

Plants have developed a variety of mechanisms to prevent excessive accumulation of nonessential metals within cells and/or transform these metals into less toxic forms (Cobbett 2000b). Some plants produce metabolites that bind to heavy metals in the cytosol, such as glutathione (GSH), polypeptides, and proteins (e.g., metallothioneins (MTs) and phytochelatins (PCs)) (Zenk 1996). Particularly, the principal mechanism of intracellular detoxification of heavy metals in plants is complexation by phytochelatins (PCs). PCs are a family of metal-binding peptides with the general structure (γ-Glu-Cys)$_n$-Gly ($n = 2$–11), which have been regarded as the best-characterized heavy metal chelators in plants, especially in the context of Cd tolerance (Cobbett and Goldsbrough 2002; Cobbett 2000a). The enzyme catalyzing the biosynthesis of PCs from GSH, phytochelatin synthase, was first

characterized by Grill et al. (Grill et al. 1989). After that, the presence of phytochelatins has been reported in a wide variety of plant species, including algae, mosses, ferns, gymnosperms, monocots, dicots, and some fungi species (Wójcik and Tukiendorf 2004). PCs are synthesized in the cytosol and then transported as complexes to the vacuole. Moreover, the synthesis of PCs is rapidly activated in the presence of heavy metals such as Cd, Cu, Zn, Ag, Au, Hg, and Pb (Manara 2012).

During the last decades, multiple excellent research articles and reviews have been published describing our knowledge on PCs (Pawlik-Skowrońska et al. 2002; Clemens 2006; Clemens and Peršoh 2009; Cobbett 2000b; Hirata et al. 2005; Lindberg et al. 2007; Liu et al. 2011a; Machado-Estrada et al. 2013; Pal and Rai 2010; Rauser 2003; Rea 2012; Stolt et al. 2003; Zhang et al. 2010, 2012; Schulz et al. 2008). This chapter reviews the recent advances in understanding of the biosynthesis and functions of PCs, involving metal detoxification especially in plants to provide updated information about these unique peptides applicable to heavy metal detoxification in plant. Furthermore, the genetic manipulation of plants with various genes involving directly or indirectly in phytochelatins metabolism and their role in heavy metal stress tolerance have also been discussed.

2 Metal Toxicity in Plants

Heavy metals contamination of soils has become a critical environmental issue (Wei et al. 2005, 2008a, b). Metal concentrations in soil typically range from less than one to as high as 100,000 mg kg^{-1} (Pal and Rai 2010). Many heavy metals are essential micronutrients for plants (i.e., Co, Cu, Fe, Mn, Mo, Ni, and Zn) because they are involved in numerous metabolic processes as constituents of enzymes and other proteins (Hirata et al. 2005). However, they can become toxic if their concentration is higher than a specific critical point, as they can lead to a range of interactions at the cellular and molecular level (Baldisserotto et al. 2007; Prasad and de Oliveira Freitas 2003). Soil and plant characteristics, e.g., mineralogical and organic matter properties of the soil and plant metal susceptibility and prediction of heavy metal uptake by plants by the common soil, have influence on bioavailability of heavy metals in soils (Liu et al. 2010a; Wei et al. 2011). Therefore, heavy metal toxicity in plants usually varied, and plant chemical analysis techniques are often unreliable (Zhou and Song 2004).

Soils contain high levels of Cd because of natural source and human activity (Liu et al. 2010a, 2011b). However, Cd is nonessential to plant nutrition, and high levels of Cd can cause great damage to the growth and development of plant (Manara 2012). Growth and seed germination inhibition, chlorosis, leaf roll, dwarfing, phenological phase delay, and biological production reduction that finally even lead to plant death are obvious visible symptoms (Haghiri 1974; Mohanpuria et al. 2007; Wójcik and Tukiendorf 2004). Cd can lead to Fe(III) reductase inhibition of root, reduce the generation of Fe(II), and cause the lack of Fe(II), which

finally affect the photosynthesis of plants (Alcántara et al. 1994). Photosynthesis is one of the most important factors in plant growth and the lower of photosynthesis can lead to reduction of plant production directly. Previous studies have highlighted that, even at low level, soil Cd contamination could pose a significant risk to human health through the soil–crop–human exposure pathway (Liu et al. 2010a, 2011b).

Lead (Pb) is one of the major heavy metals of soils and has gained considerable importance as a potent environmental pollutant (Sharma and Dubey 2005). Pb contamination in soil has been serious and is not likely to decrease in the near future due to mining and smelting activities, Pb-containing paints, gasoline, and the disposal of municipal sewage sludge enriched with Pb (Yang et al. 2000). Moreover, Pb is easily taken up by plants from soils and accumulated in different organs (Liu et al. 2010b). When Pb uptake in plants exceeds a certain value, it will produce certain toxicity to plants, disorder the metabolism of plants, inhibit growth of plants, and even cause plant death (Pourrut et al. 2011; Sharma and Dubey 2005). The visual symptoms of Pb toxicity are rapid inhibition of root growth, stunted growth of plant, and chlorosis (Burton et al. 1984; Pourrut et al. 2011). Pb toxicity also causes inhibition of enzyme activities, water imbalance, alterations in membrane permeability, and disturbs mineral nutrition (Sharma and Dubey 2005). Plants under heavy metal stress can induce the generation of reactive oxygen and show the effect of plant poisoning (Liu et al. 2010b). Lead stress caused significant changes in the activity of antioxidative enzymes. Plants exposed to Pb can increase malondialdehyde (MDA) content coupled with the increase in the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR), and glutathione S-transferase (GST) compared to control (untreated) plants (Reddy et al. 2005).

Copper (Cu) is an essential micronutrient to plants growth and development, which widely participates in various life activities (Thomas et al. 1998). Cu plays an important role in the synthesis of cytochrome oxidase, ascorbic acid oxidase, polyphenol oxidase, and polyamine oxidase. However, excessive Cu causes injury to plants, including plant growth retardation and leaf chlorosis (Lewis et al. 2001). The dynamic balance of copper in cellular is controlled mainly from three aspects: (l) absorption, (2) intracellular storage, and (3) exosmosis of copper (Nelson 1999). Phytotoxic effect of Cu on plant is higher than most of heavy metal contaminants (Xu et al. 2006). It was reported that toxic effect of Cu and other heavy metals on wheat growth was in the following order: $Cd > Cu > Ni > Zn > Pb > Cr$ (Athar and Ahmad 2002). Most of the excess Cu in soil was transferred to and accumulated in plant leaves in which the storage rate in vacuoles and chloroplasts was 48 % and 7 %, respectively (Boojar and Tavakoli 2010). In response to copper exposure, plants showed significant induction of proteins and enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT) and glutathione reductase (GR), however, only up to moderate exposures (Srivastava et al. 2006). Stress due to copper toxicity resulted in an increase in total catalase (CAT) and superoxide dismutase (SOD) activity and a simultaneous induction of SOD and CAT gene expression (Lombardi and Sebastiani 2005). Moreover, Cu toxicity removed the correlation

between sulphur metabolism-related gene expression and the suggested regulatory metabolites (Shahbaz et al. 2014).

Arsenic (As) has been known as a poison for years (Sun et al. 2009). Arsenic is used in several industries like paints, dyes, metals, soaps, insecticides, and semiconductors and is also released into the environment through burning fossil fuels, paper production, cement manufacturing, and mining activities (Singh et al. 2015). Arsenic exists mainly in four oxidation states—arsenate (As^{V}), arsenite (As^{III}), arsenic(As^{0}), and arsine (As^{-III}), but the two biologically important species are As^{V} and As^{III}, which are interconvertible depending on the redox status of the environment (Elangovan and Chalakh 2006; Singh et al. 2015). Plants take up As mainly as As^{V}, which causes considerable stress in plants, including inhibition of growth, physiological disorders, and finally death (Garg and Singla 2011). Plants with As toxicity have obvious symptoms, such as leaf roll, chlorosis, evidently inhibition of root growth, and root and leaf tissue destruction (Rascio and Navari-Izzo 2011). Plants exposed to As substantially increase the synthesis of glutathione (GSH) and phytochelatins (PCs), the polymers of GSH (Tripathi et al. 2007).

Natural source, mining and smelting activities, sewage water irrigation, and fertilizers and pesticides cause mercury (Hg) contamination in soils (Xu et al. 2015). Hg is not an essential nutrition element to plant growth, which can cause plant damage. Hg has various existence forms, such as HgS, Hg^{2+}, Hg^{0}, and methyl-Hg (Yadav 2010). Many studies have shown that Hg is preferentially accumulated in plant roots (Beauford et al. 1977; Chen et al. 2009b; Iglesia-Turino et al. 2006). Cellular integrity and biological activity might be compromised due to its strong affinity for sulfhydryl residues of proteins and other biomolecules (Hall 2002). Mercury has also been found to be a potent inducer of oxidative stress (Rellan-Alvarez et al. 2006).

Manganese (Mn) is an essential trace element for plant tissues, but it can cause phytotoxicity at supraoptimal level (Lei et al. 2007). High concentrations of Mn in soil are a widespread environmental problem in the world (Yang et al. 2013). Natural processes and anthropogenic activities, e.g., acid rain, acidic fertilizer, mines, and industrial wastes, cause serious soil contamination of Mn (Yang et al. 2013). Brown spots on mature leaves, interveinal chlorosis and necrosis, deformation of young leaves, and growth retardation are obvious symptoms of Mn toxicity (Baldisserotto et al. 2007; Khabaz-Saberi et al. 2010).

Nickel (Ni) is an essential micronutrient for higher plants (Brown et al. 1987). However, Ni may be toxic to plants at high levels of contamination (Bingham et al. 1986). Nickel phytotoxicity varies with concentration of Ni in soil solution as well as with the plant species (Kramer et al. 1997; Sreekanth et al. 2013). Excess Ni can affect physiological/biochemical process like decreasing leaf chlorophyll contents and leaf photosynthetic and transpiration activities and impairing membrane permeability associated with enhanced extracellular peroxidase activity (Yang et al. 1996). Toxic levels of Ni may depress yield and disturb the pattern of nutrient uptake, resulting in reduced uptake of some nutrient elements and increased supply of the others (Chen et al. 2009a; Kramer et al. 1997).

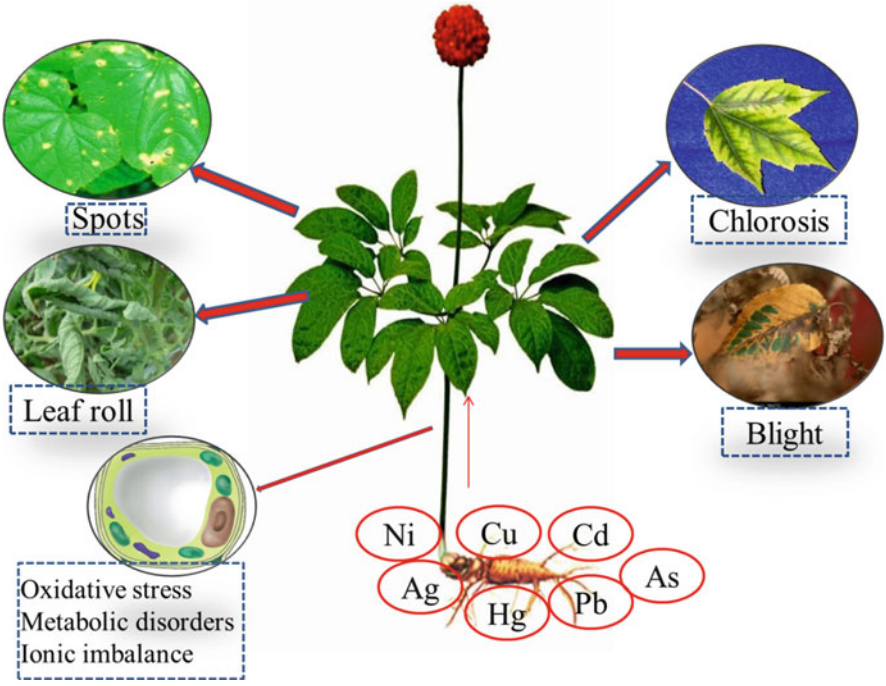

Fig. 1 Metal toxicity to plants

Further, heavy metals also can induce oxidative stress. Plants respond to oxidative stress by increasing the production of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), or peroxidase (POD) (Shri et al. 2009). The excess of Cd causes a decline in the antioxidant protection of the organ in *Triticum aestivum* L., with the consequent generation of considerable amounts of H_2O_2, a direct agent of oxidative stress (Ranieri et al. 2005). Requejo and Tena (2005) studied the effect of arsenic exposure on maize (*Zea mays* L.) root proteome and found that the induction of oxidative stress is the main process underlying arsenic toxicity in plants. Pb and Ni can induce the overproduction of reactive oxygen species (ROS) such as superoxide radicals ($^{\bullet}O_2^-$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) in plant cells (Reddy et al. 2005). Indian mustard (*Brassica juncea* L.) effectively generated an enzymatic antioxidant defense system (especially CAT) to scavenge H_2O_2, resulting in lower H_2O_2 in shoots with higher mercury concentrations and generated adaptation system to mercury-induced oxidative stress (Shiyab et al. 2009).

As mentioned above, high levels of heavy metals can cause great damage to plants, and the main visible symptoms are shown in Fig. 1. To cope with excessive heavy metals uptake by plants, plants have evolved a variety of adaptive responses, which include immobilization, exclusion, chelation, and compartmentalization of metal ions and often involve metal-binding ligands. For example, plant cells developed a mechanism by which the metal ion, entering the cytosol of the cell, is immediately complexed and inactivated, thus preventing the metal from

inactivating catalytically active or structural proteins (Zenk 1996). Shimwell and Laurie (1972) firstly pointed out that plants can excrete excessive heavy metals to ensure their normal growth. Similarly, the toxic metal ions in Lichens were immobilized in biologically non-active forms and then were secreted by plants glands (Tyler 1989). Heavy metals uptake by plants can be compartmentalized and sequestrated in less-active organelles (e.g., cell wall and vacuole) and/or be chelated with metal-binding ligands to improve the resistance to heavy metals (Wierzbicka 1986). It was reported that Cd can deposit in the vacuole in the form of oxalic acid salt (Van Balen et al. 1980). Up to now, it has been acknowledged that heavy metals can induce phytochelatins in plants and that the binding of heavy metals with phytochelatins is one of the main detoxification mechanisms (Chen et al. 2008, 2009b; Clemens 2006, 2009; Cobbett and Goldsbrough 2002).

3 Phytochelatin Biosynthesis

The peptide was first discovered by Hayashi and his groups as the Cd-binding complexes in fission yeast, *Schizosaccharomyces pombe*, exposed to Cd^{2+} and was named as "cadystins" (Murasugi et al. 1981). In 1984, cadystins was second discovered in wine yeast (*S. pombe*), which can chelate with metals (Kondo et al. 1984). A set of novel heavy-metal complexing peptides were isolated from plant cell suspension cultures. These peptides, named as phytochelatins (PCs), appear upon induction of plant cells with heavy metals and represent the principal metal-binding activities in the cells (Grill et al. 1985). PCs are synthesized inductively by exposure to not only Cd but also by other heavy metals such as Hg, Cu, Zn, Pb, and Ni (Yadav 2010).

3.1 Structure of PCs

PCs are a class of heavy-metal-binding peptides previously isolated from cell suspension cultures of several dicotyledonous and monocotyledonous plants. These peptides consist of repetitive γ-glutamylcysteine units with a carboxyl-terminal glycine and range from 5 to 17 amino acids in length and with the general structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2$–11) (Fig. 2) (Grill et al. 1987). They are structurally related to glutathione (GSH; γ-Glu-Cys-Gly) and were presumed to be the products of a biosynthetic pathway. However, in some plants, the C-terminal glycine can be replaced by serine, glutamine, glutamate, or alanine, such as $(\gamma\text{-Glu-Cys})_n\text{-β-Ala}$, $(\gamma\text{-Glu-Cys})_n\text{-Ser}$, and $(\gamma\text{-Glu-Cys})_n\text{-Glu}$ (Rauser 1995, 1999; Zenk 1996) (Table 1). These peptides were unequivocally identified as homologues of the phytochelatins by chemical analysis, and the chain length pattern of these compounds is identical to the PCs series. These compounds were called "homo-phytochelatins" (h-PCs) or iso-phytochelatins (iso-PCs) (Ducruix et al. 2006; Gekeler et al. 1989) (Fig. 3). Synthesis of iso-PCs mostly depends upon the

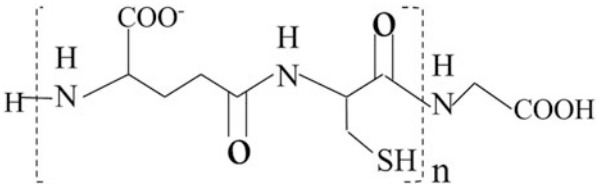

Fig. 2 Structure of PCs (after Pal and Rai 2010)

Table 1 Species of PCs in plants

PCs and h-PCs	Main plant species	References
$(\gamma\text{-Glu-Cys})_n\text{-Gly}$	*Rauvolfia serpentina*, *Lycopersicon esculentum*	Grill et al. (1985); Rauser (1990)
$(\gamma\text{-Glu-Cys})_n\text{-}\beta\text{-Ala}$	*Glycine max*, *Pisum sativum* L.	Klapheck et al. (1995); Rauser (1990)
$(\gamma\text{-Glu-Cys})_n\text{-Ser}$	*Oryza sativa* L. cv Strella	Klapheck et al. (1994)
$(\gamma\text{-Glu-Cys})_n\text{-Glu}$	*Zea mays* L.	Meuwly et al. (1993)
$(\gamma\text{-Glu-Cys})_n$	*Armoracia rusticana*	Kubota et al. (2000)

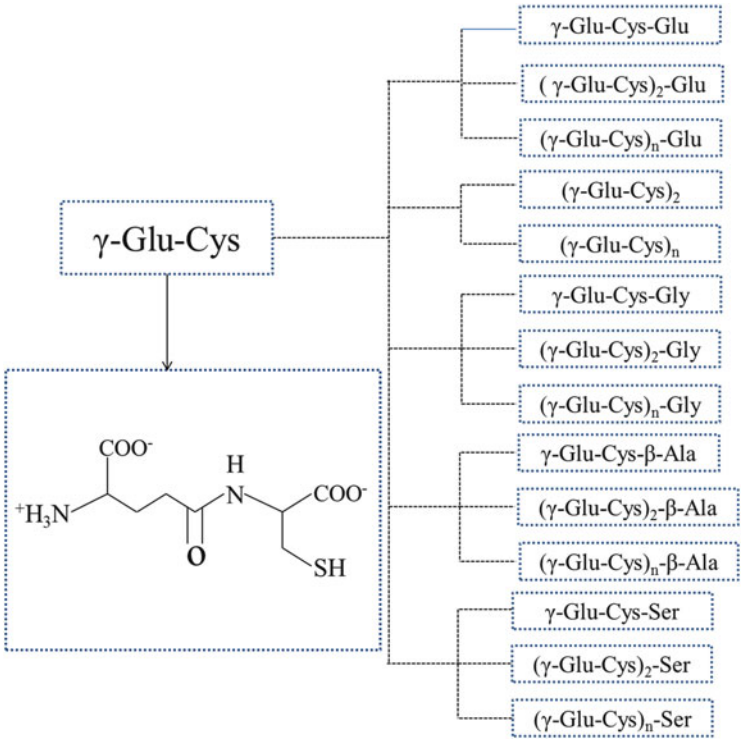

Fig. 3 Structure and classification of γ-Glu-Cys

availability of Gly or glutathione synthetase in the cells and may switch over to synthesize iso-PCs when the plant comes under stress (Rea 2012).

3.2 PCs Biosynthesis

3.2.1 Pathway of PCs Biosynthesis

Plants can develop a very potential mechanism to combat with heavy metal toxicity. Therefore, plants produce low-molecular weight thiols that show high affinity for toxic metals such as GSH—a sulfur containing tri-peptide thiol (Rauser 1995). The formula of GSH is γ-glutamate-cysteine-glycine. Steffens (1990) first suggested GSH is the precursor for PCs, heavy-metal-binding peptides involved in heavy metal tolerance and sequestration. The steady-state glutathione concentration in *Arabidopsis* plants was modified by expressing the cDNA for γ-glutamylcysteine synthetase (*GSH1*) in both the sense and antisense orientation, and the resulting plants had glutathione levels that ranged between 3 and 200 % in wild-type plants. *Arabidopsis* plants with low glutathione levels were hypersensitive to Cd due to the limited capacity of these plants to make phytochelatins (Xiang et al. 2001). Therefore the pathway of PCs biosynthesis is mostly related with that of GSH biosynthesis, which is generally divided into two stages (Fig. 4) (Noctor and Foyer 1998). The first step is that γ-glutamylcysteine synthetase (γ-ECS, GSH1, EC 6.3.2.2) catalyzes the formation of a peptide bond between the carboxyl group of glutamate and the amino group of cysteine, to yield γ-glutamylcysteine (γ-Glu-Gys, γ-EC), and glutathione synthetase (GS, GSH2, EC 6.3.2.3) catalyzes ligation between the carboxyl group of γ-Glu-Gys and glycine (Gly), to form γ-Glu-Gys-Gly (GSH) (Noctor and Foyer 1998). In this step, γ-ECS is one of the most important/critical synthetase to form GSH, and ATP is essential to complete this process. The second step is that PCs were synthesized, which derived from GSH by the function of phytochelatin synthases (PC synthases, EC 2.3.2.15) (Rea 2012). The biosynthesis of PCs is catalyzed by phytochelatin synthase, an enzyme that requires heavy metals as activating factors (Grill et al. 1989). It has been shown that Cd is the most effective activator of this enzyme, whereas other heavy metals activate it to a much lesser extent than Cd (Hirata et al. 2005). The γ-ECS is a rate-limiting

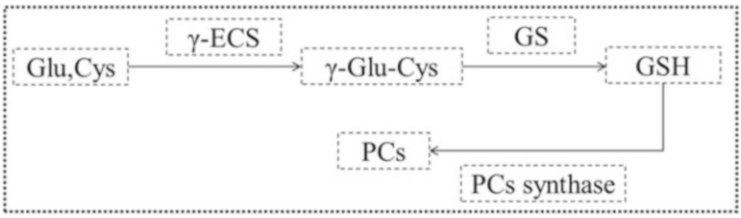

Fig. 4 Pathway of PCs biosynthesis

enzyme for GSH synthesis, and its activity is enhanced by metal ions like Cd^{2+} and suppressed by treatment with buthionine sulfoximine (BSO) (Inouhe 2005). In particular, genetic studies have confirmed that GSH-deficient mutants of *S. pombe* as well as *Arabidopsis* are PC deficient and hypersensitive to Cd (Cobbett and Goldsbrough 2002). Cells treated with BSO fail to produce phytochelatin and become extremely sensitive to the metals used to induce phytochelatin synthesis (Rauser 1990).

PCs synthases play a key role in the process of PCs synthesis, mainly for catalysis. Catalytic process is divided into two periods. The first period is activation. The signal detection zone of the carboxyl group of PC synthases detects signal of Cd^{2+} in the medium and immediately combines Cd^{2+} and Cys to form special space structure. Therefore, the amino group of PC synthases has catalytic activity (Yadav 2010). The second period is catalytic. The transfer of the γ-Glu-Cys moiety of a molecule of GSH onto a second molecule of GSH (or an existing PC molecule) to form a PC product by the function of the amino group of PC synthases activation. The reaction process can be repeated until the synthetic of $(\gamma\text{-Glu-Cys})_n$-Gly ($n = 2$–11) forms (Cobbett 2000a), which was firstly synthesized by the use of GSH as the substrate in *Silene cucubalus* cell suspension cultures (Grill et al. 1989).

3.2.2 Regulation of PCs Biosynthesis

Lots of researchers have dedicated to the studies on the regulation of PCs biosynthesis since the success of PCs synthesis. At present, regulation of GSH biosynthesis and regulation of PC synthase activity are the two key strategies for regulating PCs biosynthesis (Zhou and Song 2004).

The intracellular level of GSH has been found to regulate PC synthesis (Chen et al. 2008; Collin-Hansen et al. 2007), and oxidative stress can regulate GSH synthesis (Liang et al. 2009). PC biosynthesis and Cd tolerance are increased in transgenic *Brassica juncea* in which either γ-glutamylcysteine synthetase (γ-ECS) or glutathione synthetase (GS) was overexpressed, which proved the importance of the regulation of GSH biosynthesis in modulating PC expression (Zhu et al. 1999a, b). The GS enzyme is rate limiting for the biosynthesis of glutathione and phytochelatins in the presence of Cd (Zhu et al. 1999b). Similarly, it was also found that γ-ECS and GS were both increased in *Arabidopsis* plants when exposed to Cd and Cu (Xiang and Oliver 1998). Indian mustard (*Brassica juncea*) was genetically engineered to overexpress the *Escherichia coli gshI* gene encoding γ-ECS, targeted to the plastids, and the results show that the γ-ECS transgenic seedlings showed increased tolerance to Cd and had higher concentrations of PCs, γ-Glu-Cys, glutathione, and total non-protein thiols compared with wild-type seedlings (Zhu et al. 1999a). Similarly, it was indicated that in roots, Cd-induced PC synthesis correlates with a moderate increase of expression of genes involved in GSH synthesis, the change for γ-ECS being most pronounced (Schäfer et al. 1998). Moreover, the activity of γ-ECS was approximately twofold higher in *CdR6-0* cells than in *CdS* cells of *Lycopersicon esculentum*, which thus increases the activity of

the first enzyme of GSH biosynthesis in *CdR6-0* cells and can cause higher cadmium tolerance and capacity to synthesize GSH and PCs (Chen and Goldsbrough 1994). Therefore, regulation of PCs biosynthesis can be achieved by regulating the activity of γ-ECS.

Regulation of PC synthase is the other important way to regulate PCs biosynthesis. PC synthase is isoelectric near pH 4.8 and has a temperature optimum at 35 °C and pH 7.9 (Zenk 1996). It has been demonstrated that PC biosynthesis occurs within minutes and is independent of de novo protein synthesis using plant cell cultures exposed to Cd, consistent with the observation of enzyme activation in vitro (Cobbett 2001). In *Arabidopsis*, the *CAD1* gene, identified by using Cd-sensitive, PC-deficient *cad1* mutants, has been proposed to encode PC synthase (Ha et al. 1999). It was reported the suppression cloning of a cDNA (*AtPCS1*) from *A. thaliana* encoding a 55-kDa soluble protein that enhances heavy-metal tolerance and elicits Cd^{2+}-activated phytochelatin accumulation when expressed in *Saccharomyces cerevisiae* (Vatamaniuk et al. 1999). *Cad1* mutants of *A. thaliana* are sensitive to cadmium to different extents, deficient in their ability to form cadmium–peptide complexes, and deficient in its ability to accumulate phytochelatins (PCs) (Howden et al. 1995b). As mentioned above, PC synthase is primarily regulated by activation of the enzyme in the presence of heavy metals.

3.3 Factors Affecting PCs Biosynthesis

3.3.1 Types of Heavy Metals

The biosynthesis of PCs can be affected by the types of heavy metal (Cobbett 2001). Although a range of heavy metals can induce PCs in vivo, only a few heavy metals can form stable complex compound with PCs (Cobbett 2000a). It was reported that exogenous lead can induce a stronger stimulation of the synthesis of PCs by the *Chlorella vulgaris* cell (Bajguz 2002). Transgenic *Arabidopsis* plants exposed to high levels of Cd^{2+} could form PCs quickly, but the number of PCs reduces and eventually disappears with the decrease of metal concentration (Lee et al. 2003). PCs are rapidly induced in vivo by a wide range of metal ions, which follow the order $Cd^{2+} > Pb^{2+} > Zn^{2+} > Sb^{3+} > Ag^{+} > Hg^{2+} > As^{5-} > Cu^{+} > Sn^{2+} > Au^{3+} > Bi^{3+}$ (Grill et al. 1987). Similarly, it was found that the order of PCs induction by heavy metals in *Stigeoclonium* sp. was Cd > Pb > Zn (Pawlik-Skowronska 2001). PCs were the most strongly induced peptides under Cd and Hg stress, whereas As only tended to synthesize small thiols such as glutathione and γ-glutamylcysteine, which indicates that PCs are induced at different rates depending on the metal stressor used. (Dago et al. 2014).

3.3.2 Concentrations of Heavy Metals

The concentrations of heavy metals can also influence the induction of PCs. PCs induced significantly at 10 μM Cd in roots and decreased in roots at 50 μM Cd, which may be correlated with reduced level of GSH (Mishra et al. 2006). However, PCs binding efficiency in *Nitzschia palea* was enhanced with the increase of Cd exposure concentration (Figueira et al. 2014). It has been highlighted that PC_n ranged from PC_1 to PC_3 in Vetiver (*Vetiveria zizanioides*) roots exposed to 1200 mg Pb L^{-1}. The most abundant PC_n in both root and shoot tissues of Vetiver was PC_1, while PC_3 was observed only in the root tissues owing to the high levels of Pb (Andra et al. 2010). PC formation could be induced in the leaf, stem, and root tissues of *Sedum alfredii* upon exposure to 400 μM Cd and only in the stem and root when exposed to 700 μM Pb. However, no PCs were found in any part of *S. alfredii* when exposed to 1600 μM Zn (Zhang et al. 2008). The maximum amount of As chelated by PCs was found to be about 39 % in *Hydrilla verticillata* (L.f.) Royle exposed to As^{III} (at 10 μM) and 35 % in As^{V} exposed plants (at 50 μM) (Srivastava et al. 2007). Therefore, the induction of PCs depends not only on the concentrations of heavy metals but also on the existing form of heavy metals.

3.3.3 Species and Growing Condition of Plant

In addition, the induction of PCs is correlated with the species and growing condition of plants. The total concentrations of PCs in plants under As stress confirm the order of *Agropyron repens* > *Lolium perenne* > *Leonurus marrubiastrum* > *Zea mays* > *Glecoma hederacea* > *Urtica dioica* (Schulz et al. 2008). During mercury stress, both free (*Hydrilla verticillata*) and rooted (*Vallisneria spiralis*) submerged plants synthesized different species of phytochelatins (PCs), which bind with the accumulated mercury and showed high levels of cysteine and non-protein thiols. However, plants of *V. spiralis* showed more amount of PCs than *H. verticillata* despite relatively more Hg tolerance of *H. verticillata* (Gupta et al. 1998). Application of heavy metals to cell suspension cultures and whole plants of *Silene vulgaris* and tomato induces the formation of heavy metal-phytochelatin complexes with Cu and Cd and the binding of Zn and Pb to lower molecular weight substances. However, *S. vulgaris* cells can tolerate 5–10 times higher concentrations of Cu^{2+}, Cd^{2+}, Zn^{2+}, and Pb^{2+} in comparison to tomato cell cultures (Leopold et al. 1999). Transgenic *A. thaliana* contained almost 2 and 1.6 times more cadmium in the form of Cd-PC_2 than did the corresponding wild-type plants in their roots and shoots, respectively (Sadi et al. 2008).

Moreover, the growing condition of plant (e.g., pH, light and temperature) can also influence the induction to PCs. Higher production of phytochelatins was observed in response to metals at the lower studied pH 6.8 than at the alkaline pH, which might be that zinc availability to algae was improved due to the changed chemical Zn speciation after water acidification than at alkaline pH (Pawlik-

Skowronska 2001). The production of PC in the light is increased as compared to the dark and also at high (16 °C) as compared to low (4 °C) temperatures in *Vaucheria compacta* and *V. debaryana* (Xanthophyceae). Increased production at high temperature was related with the temperature optimum (35 °C) of phytochelatin synthase responsible for phytochelatin biosynthesis. However, decreased production in the dark, as well as at low temperature (4 °C), was related with the decreased rates of other cellular reactions including the reduced transport of cadmium leading to its decreased availability for PC induction (Gaur and Rai 2001).

4 Function of PCs

The function of PCs mainly reflects in two aspects: improve resistance of plants to heavy metals and detoxify the toxicity of heavy metals and maintain intracellular metal ions homeostasis (Rauser 1995). Particularly, the main function of PCs is the ability of complexing metals (plant micronutrient and nonessential metal elements).

4.1 Improve Resistance of Plants to Heavy Metals and Detoxify the Toxicity of Heavy Metals

PCs are peptides that function in heavy-metal chelation and detoxification in plants and fungi (Hirata et al. 2005). Cadmium is a kind of important chelating peptide resultant. PC-metal complexes are one of the ways to improve resistance of plants to heavy metals, which proved that the roles of PCs are to protect plants against toxic metals (Cobbett and Goldsbrough 2002). There are enough and genetic evidences to prove the role of PCs in Cd detoxification. Two different forms of PC-metal complexes in fission yeast cells exposed to Cd were reported, i.e., the first, composed mostly of PCs and Cd, elutes from gel filtration columns with an apparent molecular weight of 3–4 kDa (LMW PC–Cd complex), and the second, more highly charged form has an apparent molecular weight of 6–9 kDa and contains sulfide in addition to PCs and Cd (Ortiz et al. 1992). In extracts of *S. pombe*, two PC–Cd complexes (referred to as HMW and LMW) can be clearly resolved using gel-filtration chromatography. Sulfide ions play an important role in the efficacy of Cd detoxification by PCs. HMW PC–Cd complexes contain both Cd and acid-labile sulfide. The incorporation of sulfide into the HMW complexes increases both the amount of Cd per molecule and the stability of the complex (Fig. 5) (Cobbett 2000b; Cobbett and Goldsbrough 2002). An allelic series of *cad1*, cadmium-sensitive mutants of *A. thaliana*, was isolated. These mutants were sensitive to cadmium to different extents and were deficient in their ability to form cadmium–peptide complexes. Moreover, the amount of PCs accumulated by

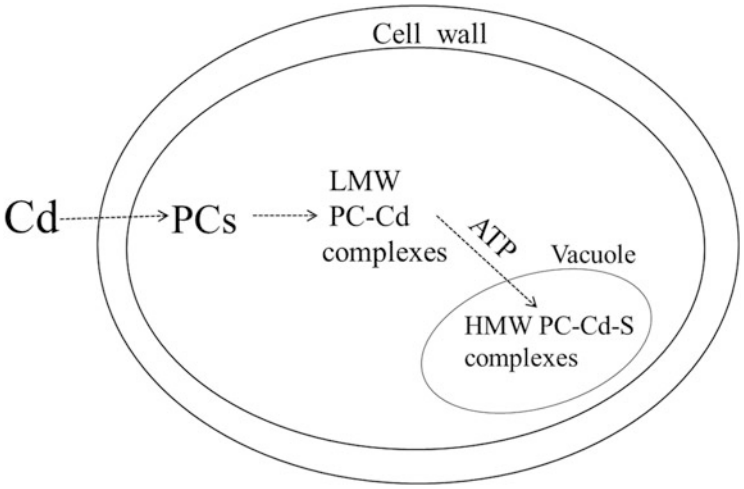

Fig. 5 Mechanisms of PCs detoxification (an example of cadmium)

each mutant correlated with its degree of sensitivity to cadmium (Howden et al. 1995b). In *B. juncea* L., Cd accumulation is accompanied by metabolic adaptation, in particular, the rapid induction of phytochelatin (PC) biosynthesis (Haag-Kerwer et al. 1999). It was highlighted that stringent control of Cd detoxification by PCs protects photosynthesis but does not prevent a decline in transpiration rate (Zhu et al. 1999a, b). Sequestration of Cd by PCs provides an essential cellular mechanism for Cd detoxification (Haag-Kerwer et al. 1999). Similarly, suspension-cultured cells of azuki bean (*Vigna angularis*) as well as the original root tissues were hypersensitive to Cd, and the azuki bean cells challenged to Cd did not contain phytochelatin (PC) peptides, unlike tomato (*L. esculentum*) cells that have a substantial tolerance to Cd (Inouhe et al. 2000). It was reported that enhanced Cd tolerance of Ce-PCS tobacco was accompanied by an increased cytosolic and vacuolar SH of PC/Cd ratio, suggesting the important role of mechanisms other than PC–Cd transport in Cd translocation to the vacuole. The key role of the PCs in Cd tolerance is temporary binding of Cd^{2+} in the cytosol (Sylwia et al. 2010). Exposure to Cd^{2+} leads to activation of phytochelatin synthase (PCs) and the formation of phytochelatins (PCs) in the cytosol. Binding of Cd by PCs and the subsequent transport of PC–Cd complexes to the vacuole are essential for Cd tolerance (Sylwia et al. 2010).

Induction of PCs in vivo and activation of PC synthase in vitro are conferred by a range of metal ions. However, there is little evidence supporting a role for PCs in the detoxification of such a wide range of metal ions (Cobbett 2000b). The ability of PCs detoxification is based on the ability of heavy metal ions complex. PCs were induced to various degrees by exposure to various metals (Ag^{+}, As^{3+}, As^{5+}, Cd^{2+}, Cu^{2+}, Ca^{3+}, Hg^{2+}, In^{3+}, Ni^{2+}, Pb^{2+}, Pd^{2+}, Se^{4+}, and Zn^{2+}) (Maitani et al. 1996). It was found that in a medium with low cation content, PC-deficient mutants show

pronounced Zn^{2+} hypersensitivity. This phenotype is of comparable strength to the well-documented Cd^{2+} hypersensitivity of *cad1* mutants (Tennstedt et al. 2009). Andra et al. (2009) indicated that Vetiver grass (*Vetiveria zizanioides* L.) exposed to Pb induced the synthesis of phytochelatins and the formation of Pb-PC_n complexes to alleviate the phytotoxic effects of free Pb ions. Similarly, Estrella-Gómez et al. (2009) suggested that the accumulation of PC in *Salvinia minima* is a direct response to Pb^{2+} accumulation, and phytochelatins do participate as one of the mechanisms to cope with Pb^{2+} of this Pb-hyperaccumulator aquatic fern.

However, there are also some queries on the detoxifying of PCs. de Knecht et al. (1994) pointed out that in response to a range of Cd concentrations, the root tips of Cd-tolerant plants of *S. vulgaris* exhibit a lower rate of PC production accompanied by a lower rate of longer chain PC synthesis than those of Cd-sensitive plants. Similarly, phytochelatin synthesis and loss of total glutathione were observed in both the roots of copper-sensitive and tolerant *S. cucubalus* (L.) exposure to Cu (De Vos et al. 1992). A study on Cu-sensitive and Cu-tolerant populations of *S. vulgaris* found that both the populations produce PCs when exposed to Cu. However, when exposed to their own highest no-effect concentration or 50 %-effect concentration of Cu for root growth, the two plants exhibit equal phytochelatin contents in the root apex (Schat and Kalff 1992). It seems that Cu tolerance in *S. vulgaris* does not rely on phytochelatin production.

4.2 Maintain Intracellular Metal Ions Homeostasis

PCs are involved in metal ion homeostasis in plants. PCs accumulation is correlated with the initial concentration of zinc ions in the nutrient solution. PCs had almost disappeared from the cells after reaching stationary growth phase (Grill et al. 1988). PCs play a critical role in homeostasis of heavy metals in plants by regulating the availability of metal ions in the plant cell (Zenk 1996). Metal ions homeostasis requires intracellular complexation of metals when there is a cellular surplus and later release of metals to metal-requiring apoproteins and perhaps to final storage sites within cells (Rauser 1995). Kneer and Zenk found that heavy metal ions entering cells at sublethal concentrations are totally complexed by phytochelatins using $^{109}Cd^{2+}$ in *Rauvolfia serpentina* (and to a much lesser extent to some high molecular weight proteins) and a series of metal-sensitive plant enzymes such as alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, nitrate reductase, ribulose-1,5-diphosphate carboxylase, and urease tolerate Cd^{2+} in the form of a phytochelatin complex from 10- to 1000-fold the amount as compared with the free metal ion (Kneer and Zenk 1992). Therefore, the complexation of PCs and Cd protects the activity of the enzyme, so as to guarantee the normal metabolism of cells. It was reported that *AvPCS* (phytochelatin synthase genes isolated from *Avicennia germinans*) was the most active gene involved in the regulation of essential metals (e.g., Cu^{2+}) in *A. germinans* leaves (Gonzalez-Mendoza et al. 2007). PCs play a dual role in plants: the first is that PCs complex to the

metal ion tightly to inactivate and store it in the vacuole, the second is that PCs transfer the essential metal to newly synthesized apoenzymes (Thumann et al. 1991).

Metal ions in multicellular organisms have to be moved to target cells, tissues, and organs distant from the site of uptake without being sequestered by other available sites along the way (Colangelo and Guerinot 2006). Key homeostatic processes include (1) tightly controlled transport of metal ions across membranes, (2) chelation by low-molecular weight molecules, (3) delivery to target proteins, e.g., via metallochaperones (Clemens and Peršoh 2009; Palmer and Guerinot 2009).

4.3 Other Functions

PCs can also function as long-distance carriers for heavy metal ions. Gong et al. (2003) found that transgenic expression of the *TaPCS1* gene increases long-distance root-to-shoot Cd^{2+} transport and reduces Cd^{2+} accumulation in roots. In the fungus *S. pombe*, complexes of heavy metals bound to PCs are transported across the tonoplast and sequestered in vacuoles by means of the ATP-binding cassette (ABC) transporter *HMT1* (Ortiz et al. 1995). The wheat (*Triticum aestivum*) PC synthase (*TaPCS1*) gene was expressed under the control of a shoot-specific promoter (*CAB2*) in an Arabidopsis (*A. thaliana*) PC-deficient mutant, *cad1-3* (CAB2: *TaPCS1*/*cad1-3*). The study shows that *TaPCS1* is expressed only in shoots and that CAB2: T*aPCS1*/*cad1-3* lines complement the cadmium (Cd) and arsenic metal sensitivity of *cad1-3* shoots, and PC_2 is transported over long distances in the shoot-to-root direction (Chen et al. 2006). These results imply that transported PCs play a role in the transport of Cd.

Vetiver grass (*V. zizanioides* L.) exposure to Pb induced the synthesis of phytochelatins (PC_n) and the formation of Pb–PC_n complexes, alleviating the phytotoxic effects of free Pb ions. PCs can transport Pb to vacuoles in vascular tissues of root and shoot (Andra et al. 2009). Li et al. (2006) showed the long-distance movement of thiol peptides from shoots down to roots by expressing a bacterial γ-glutamylcysteine synthetase (ECS) in the shoots of an *Arabidopsis* ECS-deficient mutant using a shoot-specific, light-induced regulatory cassette.

Based on the above evidences, the functions of PCs are summarized: (1) the phytochelatin pathway has an important role in essential metal homeostasis; (2) phytochelatin detoxifies the toxicity of heavy metals; and (3) Cd and As are essential elements, and the phytochelatin pathway is a part of the homeostatic network for these elements (Clemens 2006; Clemens and Peršoh 2009).

5 Role of PCs in Metal Detoxification and Tolerance

Response to heavy metal stress in plants involves immobilization, exclusion, chelation, and compartmentalization of metal ions and often involves metal-binding ligands (Cobbett 2000a, 2001). Since immobilized metals are less toxic than free ions, PCs are considered to be part of the mechanism detoxifying heavy metals in higher plants (Cobbett and Goldsbrough 2002). High concentrations of heavy metals such as Cd, Cu, Zn, Ag, Au, Co, Ni, and Bi can induce the synthesis of phytochelatin in plants, fungi, cyanobacteria, algae, and animals (Gaur and Rai 2001), and trace levels of essential metals can also induce PCs synthesis. Appearance of such metal-binding peptides (PCs, iso-PCs) in plants is one of the most important biochemical indications of heavy metal contamination in various environments (Li et al. 2006). The induction of PCs in *Phaeodactylum tricornutum* cells was tested, which exhibited a decrease of the PC pool and a concomitant increase of glutathione, suggesting a mechanism of degradation and release of metal-phytochelatin complexes (Morelli and Scarano 2001). More sensitivity was found toward Cd and arsenate for the *cad1-3* mutant of *A. thaliana* than wild-type plants. However, no considerable difference was found for metals like Zn, selenite, and Ni ions (Ha et al. 1999). Figueroa et al. (2008) revealed that metal bioavailability has effect on PCs production. Their results indicated that humic substance (HS) in soil restricted the availability of metal for creating stress and thereby resulted in reduced PC production in *Ricinus communis*. *Hydrilla verticillata* and *Vallisneria spiralis* can synthesize different species of phytochelatins (PCs), which bind with the accumulated mercury and showed high levels of cysteine and non-protein thiols during mercury stress and play a role in mercury detoxification (Gupta et al. 1998). Cysteine content and accumulation of acid soluble thiols inside *H. verticillata* tissue and synthesis of phytochelatins enhanced with concentrations of Pb, which indicated that PC synthesis was induced under lead stress utilizing glutathione as a substrate (Gupta et al. 1995). Pb-binding complex indicated involvement of phytochelatins in Pb detoxification. These evidences all demonstrated that PCs play a critical role in the detoxication of heavy metals.

Not all studies have supported the role of PCs. The phytochelatin-inhibitor, BSO, can increase the Zn tolerance of roots, which argues against a key role of phytochelatins in the Zn-tolerance mechanism of roots of *Festuca rubra* L. (Davies et al. 1991). Further, this preliminary evidence suggests that BSO stimulates cell division activity in roots of *F. rubra* L. The role of PCs in metal tolerance in *T. caerulescens* and the related non-accumulator *T. caerulescens* was examined. Results show that total PCs levels in the hyperaccumulator were generally lower, and the lack of a role for PCs in the hyperaccumulator's response to metal stress suggests that other mechanisms are responsible for Cd tolerance, but PCs were generally present at lower levels than in the non-accumulator *T. caerulescens* (Ebbs et al. 2002). PCs may play a secondary role in Cd tolerance of *T. caerulescens*. PCs were most abundant in leaves followed by stems but hardly detected in roots of *S. alfredii*. There is a linear correlation between PC synthesis and Cd accumulation

in leaves, suggesting that PCs do not detoxify Cd in roots of *S. alfredii* (Zhang et al. 2010). When Cd hyperaccumulating ecotype (HE) *S. alfredii* was exposed to Cd, larger proportion (about 70 % in leaves and 47 % in stems) of Cd was bound with malic acid (the major organic acid in the shoots of the plants) (Tian et al. 2011). Their results indicate that a majority of Cd accumulates in the parenchyma cells, especially in stems, and is likely associated with calcium pathways and bound with organic acid (malate). PCs might not act in the major intracellular heavy metals detoxification mechanism in plants.

6 Molecular Biology of PCs

In 1989, Grill had begun to study the purification of PCs in plants (Grill et al. 1989). However, it was not until 1999 that the phytochelatin synthase genes from *A. thaliana* and *S. pombe* wheat were isolated, cloned, and identified (Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999). They are *AtPCS1* (CAD1), *SpAtPCS*, and *TaPCS1*, respectively, encoding a 55-kDa soluble protein. These proteins have 40–50 % sequence similarity between each other.

Later, phytochelatin synthase genes were isolated and purified. Howden et al. (1995a) isolated *Cad1-1* and *Cad2-1* from cadmium-sensitive mutant of *A. thaliana*. Cazale and Clemens (2001) isolated *AtPCS2* from *Arabidopsis*, a highly homologous gene to *AtPCS1*, and they concluded that *AtPCS2* gene encodes a functional phytochelatin synthase as shown by expression in *S. cerevisiae* and the complementation of a *S. pombe* phytochelatin synthase knockout strain. When *AtPCS2* is expressed in yeast, it confers Cd-resistance, indicating that its gene product is active (Cobbett 2000a). And compared with *AtPCS1*, the expression of *AtPCS2* is at a relatively low level. Oven et al. (2002) isolated and functionally expressed a cDNA *GmhPCS1* encoding homo-phytochelatin synthase from *Glycine max* and proved that *GmhPCS1* can accept homo-glutathione as the sole substrate for the synthesis of homo-phytochelatins. Loscos et al. (2006) further isolated a cDNA clone encoding a protein (*LjPCS1*) from legume Lotus (*Lotus japonicus*), a highly homologous to a homo-phytochelatin synthase (hPCS) of *Glycine max* (*GmhPCS1*). Osaki et al. (2008) successfully isolated *CmPCS*, a product of a *PCS*-like gene from the genomic DNA of the red alga *Cyanidioschyzon merolae*. Dong et al. (2005) reported the isolation of a full-length cDNA sequence encoding a phytochelatin synthase (PCS) from *P. vittata* L., which designated *PvPCS1*, predicts a protein of 512 amino acids with a molecular weight of 56.9 kDa. And they also demonstrated that *PvPCS1* expressed in *S. cerevisiae*, and PvPCS1 mediated increased Cd tolerance. Ramos et al. (2007) identified *LjPCS1*, *LjPCS2*, and *LjPCS3* genes in *L. japonicus*, through screening of transformation-competent artificial chromosome libraries, encoding protein products viz. *LjPCS2-7N* and *LjPCS3-7N* which conferred Cd tolerance when expressed in *S. cerevisiae*. It was reported that *CdPCS1* (from *Ceratophyllum demersum*, an aquatic As-accumulator

plant) expressing rice transgenic lines showed marked increase in PCs activity and enhanced synthesis of PCs in comparison to non-transgenic plant (Shri et al. 2014).

The *Escherichia coli gshII* gene encoding glutathione synthetase (GS) was overexpressed in the cytosol of Indian mustard (*B. juncea*). The results indicate that the transgenic GS plants accumulated significantly more Cd than the wild type, and the GS plants showed enhanced tolerance to Cd at both the seedling and mature-plant stages. Cd accumulation and tolerance are correlated with the *gshII* expression level, and transgenic plants have more glutathione, phytochelatin, thiol, S, and Ca than wild-type plants (Zhu et al. 1999b).

7 Conclusive Remarks

PCs play a key role in heavy metal detoxification and accumulation in plants. The most significant recent advances of PC biosynthesis and function have come from molecular genetic studies using a variety of model systems. The biosynthesis of PC should be regulated to make the balance between PCs and GSH (the PCs synthesis substrate). In addition, further study should be made in the function of antioxidant molecular genetic regulation mechanism of the PCs synthesis. Especially, the pathway of biosynthesis and regulation mechanism of PCs in transgenic plants and the detoxification mechanism of transgenic plants to heavy metal stress need deep explorations for the breeding of suitable transgenic plants used for phytoremediation of heavy metal-contaminated soil.

The studies on the processes of different heavy-metal detoxification mechanisms will benefit to select plant species capable of hyperaccumulating heavy metal(s). Since it is an established fact that PCs are involved in metal detoxification and/or accumulation in plants, the above problem can be overcome by engineering PC synthase genes in common plants capable of growing fast and producing large biomass (Pal and Rai 2010). Therefore, understanding well of the function of PCs will potentially improve remediation efficiency of heavy metal pollution in future. Moreover, much more attention should also be paid on the influence of PCs on crops cultivated in contaminated agricultural soil. The surprising evidence for long-distance transport of PC-metal complexes raises the question of a PC involvement in intercellular metal ion transport, which should be intensively studied in future (Gong et al. 2003).

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General Roles of Phytochelatins and Other Peptides in Plant Defense Mechanisms Against Oxidative Stress/Primary and Secondary Damages Induced by Heavy Metals

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Abstract Phytochelatins (PCs) are nonprotein cysteine-rich oligopeptides having the general structure of (γ-glutamyl-cysteinyl)n-glycine ($n = 2–11$). They are synthesized from the precursor glutathione (a reduced form, GSH) by the activity of

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phytochelatin synthase (PCS). The biosynthesis is stimulated by several heavy metals (HMs), especially Cd and metalloid As. PCs can bind to various HMs like Cd, As, Cu, Pb, Zn, and Ag, via their sulfhydryl (–SH) and carboxyl (–COOH) groups. The complexations become more stable and massive in vacuole where acid-labile sulfides (S^{2-}) are incorporated to make the PCs–S–HMs conjugates. Both the thiols and S^{2-} are originated from sulfate through a partially common energy-dependent metabolism (sulfur assimilation), which is again enhanced by Cd, besides essential metals (Co, Mg). To date, fundamental roles of PCs and also related iso-peptides such as hPCs in intracellular detoxification and/or transport of HMs are well demonstrated in various plants, especially in experiments targeting genes and enzymes for PC and GSH biosynthesis. However, how they function as a defense molecule in the oxidative stresses or other biological processes are still unknown or conceiving subtle problems. Some of the possible functions are highlighted in this chapter as tentative examples for further discussion: (1) PCs–S–HMs complex as a potent pool/stock of thiols or reducing powers to be reusable for further robustious responses by the tolerant plants against various abiotic and biotic stresses including oxidative stress and (2) PCs as a possible mediator for metal translocation or redistribution via phloem rather than xylem, regardless of a trait of "hyperaccumulator" for HMs in land plants. Apart from the positive roles of PCs in HM-tolerant plants, arguments still hot arise an issue (3) the roles of PCs, GSH, and other thiols as delicate barometer or indicators in the mineral and redox balance and/or homeostasis, in addition to their well-known functions being substrates and antidotes. In the absence of HMs, the levels of PCs are too minute to account for their sufficient bindings to the essential metals. Although GSH is ubiquitous and abundant, it is a multifunctional peptide that rapidly consumed or oxidized for numerous enzymic or nonenzymic antioxidants/redox systems as well as direct substrate for PCS. Eventually, importance of preservation of thiols and sulfide (S^{2-}) as resource for reducing powers in sensitive sessile plants against various oxidative stresses is again emphasized in return for PCs in the HM-tolerant plants in metalliferous habitats.

Keywords Glutathione • Heavy metal • Phytochelatin • Reactive oxygen species • Thiol-sulfide pool

1 Introduction

Degradation of environmental quality due to metal and metalloid pollution has become a universal problem. Dispersions of untreated industrial and municipal wastes are widespread and create instability of natural equilibrium increasing the risk that the toxic pollutants would inflict their harmful effects upon the ecosystem and the individual organisms in the community. Constituting a diverse group of elements, heavy metals (HMs) having a density equal or greater than 4.0 or 5.0 g/cm^3 vary in their chemical characteristics, biological functions, and toxicity (Chatterjee et al. 2007). Most of them are microelements essential and vital for

Table 1 Some symptoms and remarks related to HM attacks in plant functions and metabolisms

HM	Essential	Redox active	ROS yield	Molecules targeted and damages (compositional molecules or enzymes)	HMs-binding ligands	Transporter: membrane (tonoplast)
Cd	n (no)	n (no)	y (yes)	Damages in Zn/Ca/Cu-enzymes, kinases, and DNA. Chorosis, lignification, PCD	PC, GSH, S^{2-}	ZIP, YSL, NRAMP (ABC, CAX, HMA, CDF)
Ni	n	n	y	Mg/Zn-protein, chlorosis, necrosis	GSH, PC, His	ZIP, YSL (CDF)
Pb	n	n	y	Damages in pigments, membrane permeability, enzyme activities	PC, S^{2-}	HMA, ABC? (HMA)
Hg	n	n	y	Membrane, aquaporin, exo-glucanase, other SH enzymes, phytotoxic, plastid	GSH, (PC), S^{2-}	? (ABC)
As[a]	n	n	y	Disturbance of P and Si, damages in DNA and SH enzymes	PC, GSH, Cys	PiT, NIP (ABC)
Zn	y	n	y	Lipid, protein, DNA damage if excess (SOD, RNA polymerase, ADH, CA, many Zn-enzymes)	PC, GSH, MA, CI, NA	ZIP, HMA, YSL (ZAT, HMA, FDR, CDF, CAX)
Co	y	y	y	Chlorosis, necrosis if excess (VitamineB_{12})	PC GSH	HMA (CDF)
Cr	n	y	y	Cell membrane, plastid	GSH, (PC?)	?
Cu	y	y	y	Chromosome or nucleus changes if excess (Cu-enzymes, SOD, cyt-c, cyanin)	GSH, (PC), S^{2-}, NA,	YSL (HMA)
Fe	y	y	y	Phytotoxic if excess (SOD, Cyt-c, Fd, other redox enzymes, heme, plastids)	MU, NA	ZIP, NRAMP, YSL (CDF)

PC phytochelatin (including hPC and iso-PC), *GSH* glutathione (+hGSH), *NA* nicotianamine, *MU* mugineic acids, *His* histidine, *MA* malate, *CI* citrate, *PiT* phosphate transporter, *CA* carbonic anhydrase, *ADH* alcohol dehydrogenase. See text for other abbreviations. After Shahid et al. (2014) and Socha and Guerinot (2014)

[a]Metalloid

plant growth and nutrition at optimum concentrations but toxic at excessive concentrations (i.e., supra-optimal concentration). The others are nonessential and just toxic even at low concentrations, simply called as "toxic HMs." Among the representatives as shown in Table 1, Cd, Pb, Hg, and Ni are very toxic. As is a metalloid but often put in to a member of HMs for convenience mainly because of the highly poisonous chemical property. Those elements are actually listed up in the high rankings applied for the various toxic and hazardous chemicals, i.e., As, Pb, Hg are no.1–3, and Cd is no.7 of the list (CERCLA 2007).

The toxicity of HMs lead to interference with metabolism and other biological activities through the generation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide molecule (H_2O_2), in addition to their direct disruptive functions to the essential enzymes and other molecules (Prasad and Freitas 2003). HMs are divided into two groups (redox-active or -inactive) for the characteristics whether or not having a redox activity by itself to produce free radicals (Viehweger 2014). According to this category, Fe, Cu, Cr, Co, Mn, and V are redox-active, and most of the rests (including Cd, Zn, Ni, Pb, and As, as noted above) are redox-inactive like Al and Na in light metals (Hossain et al. 2012a; Shahid et al. 2014). The redox-active metals are directly involved in the redox reaction in cells resulting in the formation of $O_2^{\bullet -}$ and $^{\bullet}OH$ radicals, while the redox-inactive metals indirectly increase the levels of $O_2^{\bullet -}$, $^{\bullet}OH$, and H_2O_2 by inhibiting various enzyme activities directing towards ROS sequestrations (Hossain et al. 2012b; Shahid et al. 2014). After all HMs are responsible in activating ROS formation and causing strong oxidative stress in plant cells. In conjunction to this commonality, anti-oxidation systems and processes that resume the HMs-induced stresses have attracted attention of scientists and experts (Shahid et al. 2014). Various types of anti-oxidative systems operate using many types of molecules. These are named as the redox (reduction and oxidation) cycling molecules, quenching antioxidant molecules (low molecular substances with anti-oxidation powers), detoxification enzymes (high molecular proteins involved in sequestration of ROS using low molecular antioxidants or substrates), etc. In these systematic strategies, quantitatively abundant molecules used are small antioxidants such as GSH and ascorbic acid (AsA), which reach or exceed 1 mM order at the intracellular concentrations. These play a central role in the maintenance of redox status and the nutritional homeostasis by buffering or pooling of the reducing powers within the soluble organic matters that are usable for the respective cases and places in plants under HMs and/or oxidative stresses. Concurrently, biological roles of these and other antioxidation systems especially in relation to plant's HM stress have been reviewed by several researchers (Shahid et al. 2014).

As measures to deduce the toxic HM ions within plant cells, vacuolar compartmentalization has been also suggested. However, traffic movements of HMs as an inert binding form from the outside to vacuole are necessary through the cytoplasm, the vital site of cells. Here, plant cells produce quite unique HMs-chelating thiol peptides named phytochelatins (PCs) (Grill et al. 1987; Rauser 1999; Inouhe 2005). These peptides were first recognized to be present in Cd-binding complexes in plants and yeast cells exposed to Cd as peptides having a function homologous to metallothioneins (MTs) in animals and other living organisms (Rauser 1999). It has been demonstrated that PCs are derivatives from glutathione (GSH), after all, not a protein unlike MTs (Rauser 1999; Inouhe 2005). The roles of PCs in HMs binding and detoxification have been demonstrated in various plants. However, their roles in the other biological processes and functions are still unknown in plants and other living organisms and now being very important questions to be addressed.

Further, mechanisms that appear independent from PCs and PC-related peptides have been reported from different aspects, i.e., plant species, HMs, and various molecular species involved in HM sequestration and/or transportation. Especially, regulation at transportation level can be useful as HMs defense mechanism by blocking the entrance via traffic channel or eliminating the toxic matter to outside of cells or plants. These defense mechanisms as well as intracellular PC- and antioxidant-mediated mechanisms are all required for normal life cycles of sessile plants that depend on the mineral uptakes and photosynthetic activities under different environmental conditions.

In this chapter, we summarize some information's of HMs-induced oxidative stress in plants, especially focusing on the functions of PCs and other thiols. Thereafter, some pros and cons for their biological functions of them and later are taken for discussion about a possible benefit for the pooling of PCs, GSH, and S metabolites. All these topics are highlighted on what factors are best involved in the decrease in ROS evolution in cells.

2 Input and Impact of HMs

2.1 Route into Plant Cells from Environment

Natural or anthropogenic routes are the major source of Cd contamination in soil. Natural or edaphic stress factors may influence plants development, growth, or productivity due to alteration of concentrations of different bio-reactive metals (Schützendübel and Polle 2002; Chatterjee et al. 2011). Natural phenomenon like Cd-rich rock weathering can enhance natural mineral outcrops which in turn pollute the environment. While, burning of fossil fuels such as coal or oil and the incineration of municipal wastes, cement factories, and as a by-product of phosphate fertilizers are the major anthropogenic sources of the Cd in environment (Mengel et al. 2001; Chen 2005; Kirkby and Johnson 2008; Lux et al. 2011). The concentrations of Cd may be up to 40–300 nM in natural non-polluted soils; however, the concentration may increase with clay concentration up to 1 μg/g dry soil (Wagner 1993; Mengel et al. 2001; Inaba et al. 2005). Availability of Cd to plants is greater in acid soils and its solubility increases with exudates of roots (Mengel et al. 2001; Lux et al. 2011). Delivery of Cd to plant roots is dominated by a transpiration-driven mass-flow process of the soil solution (Sterckeman et al. 2004; Ingwersen and Streck 2005).

Accumulation of HMs such as Ni, Pb, and Hg is also the result of several anthropogenic activities (Gupta et al. 2013a). Nonessential metals like Cd, As, Pb, and Hg present along with the essential one may also enter into the plant systems. Varied tactics are followed by plants in response to HMs toxicity, which include immobilization, exclusion, chelation, and compartmentalization of the metal ions, and expression of the general stress responses (Cobbett 2000). Several plants have been identified that possess the unique capability to live on toxic

conditions at HMs contaminated sites and also been found to accumulate a considerable amount of such metals within their biomass (hyperaccumulators). Various studies have shown that natural hyperaccumulators like As hyperaccumulating fern species *Pteris vittata* (Gumaelius et al. 2004) and Ni hyperaccumulating species *Thlaspi caerulescens* (Freeman et al. 2004) can withstand higher amount of metal accumulation without having significant damage within cell system. Further studies on the conspicuous properties and functions of the hyperaccumulators will disclose the different and diverse mechanisms for HMs detoxification by plants (Inouhe et al. 2012).

2.2 Toxicity to Plant Cells

Biological impacts of HMs are different by the metal species, as well as plant species, their origins, growth stage, and condition. For example, Cd is very toxic for plants even at low concentrations and often interferes with other essential metals containing enzymes (enzymes of Zn, Fe, Cu, Mn, Mg, and Ca) by displacing these elements (Wagner 1993). Cd primarily damages photosystems and some other enzyme systems in plants. As noted earlier, Cd is a redox-inactive metal, like Zn or Ni, but usually accompanies an oxidative stress by causing a transient depletion of GSH and inhibition of antioxidant enzymes (Romero-Puertas et al. 1999). Thus, the strong and versatile phytotoxicity of Cd in growth, cell death, photosynthesis, and induced lignification, etc. are due to these direct and indirect effects (Hossain et al. 2012b). Ni causes inhibition of growth, chlorosis, necrosis, and flaccidity in plants, and this toxicity is also due to the generation of oxidative stress. Pb affects many processes of plants, causing a decrease in photosynthetic pigments, an increase in membrane permeability, and a disturbance of the mineral nutrition and affecting many enzyme activities. Hg is known to provoke oxidative stress in many plants accompanying overall increases in the antioxidant enzyme systems. Arsenic is not strictly a heavy metal since it is classified as a metalloid. However, it is an important poison, which induces toxicity in plants. Usually, As is present in two toxic inorganic forms, arsenate (AsO_4^{2-}) and arsenite. Arsenate disrupts energy flows in cells and is taken up by plants through high-affinity phosphate transporters. Arsenite provokes toxicity by reacting with sulfhydryl groups of enzymes and tissue proteins and consequently resulted in inhibition of cellular function. Both forms of arsenic induce the formation of ROS leading to oxidative stress.

Apart from these toxic HMs, some others (microelements) are indispensable for living organisms at low doses, but exposure of plants above certain metal threshold concentrations, specific for each one, develops damaging effects linked to disturbances of the oxidative balance. Thus, contrary to other HMs reported above, Cu at an adequate concentration is strictly necessary for plants, since it serves as a cofactor of enzymes required for normal growth and development such as Cu/Zn-superoxide dismutase (Cu/Zn-SOD), cytochrome *c*, or plastocyanin. However, Cu at high concentrations causes multiple toxic effects in plants. Fe is also a key

element in a large number of plant metabolic routes requiring a redox exchange. Although Fe is abundant in soils, its availability is depressed in alkaline soils provoking to plant Fe deficiency, which is a common nutritional disorder for many dicotyledonous species. However, an excess of Fe have also phytotoxic effects. Conclusive remarks with examples for the hazardous effects of HMs in plants are also shown in Table 1.

Almost all HMs are potently to be toxic if present in excess as free ions (e.g., Hg^{2+}) or some organo-metallic forms (e.g., methyl-Hg) that are hydrophilic and hydrophobic, respectively. As mentioned above, free HM ions penetrated via root systems are the most probable xenobiotics for plants. Further considerations about the organic forms of HM contaminants are put aside in this chapter, while this will be a serious problem if the environmental pollution and contamination proceed and become more complex in the ecosystems during artificial activities.

2.3 ROS Production

Since pathways in which different ROS evolve are quite complex even in common plants under the influence of HMs, candidate pathways are shown here briefly (Fig. 1). ROS are produced during normal aerobic metabolisms, especially in

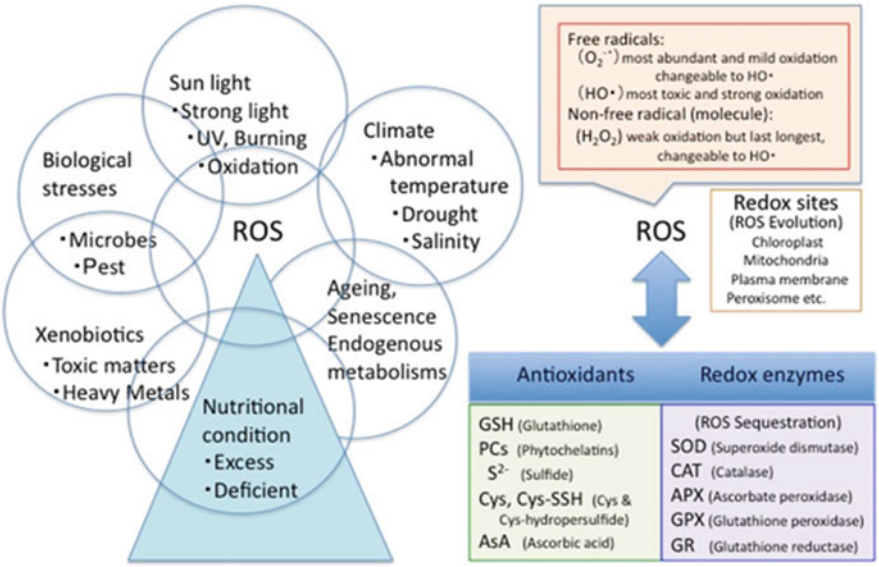


Fig. 1 Reactive oxygen species (ROS) induced by stresses and the possible antioxidants and detoxifications enzymes involved in the ROS sequestration in plants. Different abiotic and biotic stresses including HMs and oxidative stresses induce ROS in different sites in cells of plant tissues. The antioxidants (mainly in water-soluble forms) and enzymes are collaborating to the ROS sequestration in cases and places under stresses. GSH is a multifunctional thiol peptide which is also an important precursor for PCs and other HM-binding substances

chloroplast, mitochondria, peroxisome, apoplast (cell wall), and plasma membrane. HMs enhance the formations of ROS such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and H_2O_2 in those sites by inhibiting the enzymatic or nonenzymatic antioxidant systems (Shahid et al. 2014). The $O_2^{\bullet -}$ is generated when oxygen molecule (O_2) is reduced via electron transfer or energy transfer reactions. This radical is toxic but short-lived and readily converted to (H_2O_2) by the enzyme superoxide dismutase (SOD). The H_2O_2 molecule is weaker in toxicity than $O_2^{\bullet -}$ but stable, long lasting, and permeable across membrane and hence also serves as inter/intracellular second signals for various oxidative stresses. These ROS are able to generate much toxic $^{\bullet}OH$ radical, in the presence of redox-active HMs such as Fe and Cu. This radical is extremely reactive and causes strong oxidation damages in bio-membranes (usually known as lipid peroxidation reaction trigging a self-propagating chain reaction in membranes resulting in serious problems of the cell viability) and in other macromolecules including proteins, DNA, conjugated lipids, and photosynthetic pigments (Gechev et al. 2006; Hossain et al. 2012a, b). After all, ROS interact with HMs resulting in various damages in several cell sites and components (Shahid et al. 2014). ROS production is common in all living cells but the rate of production of ROS in chloroplasts is increased by influence of excessive light energy, and HM contamination is quite unique to plants. Typically, HM stress reduces photosynthesis rate and hence lead to increased production of ROS such as $O_2^{\bullet -}$ and H_2O_2 (Takahashi and Murata 2008). These adverse effects of metal stress can be observed in several places of photosynthesis, including PS I, PS II, and carboxylating enzymes like RuBisCO and phosphoenol pyruvate carboxylase (PEPC) (Siedlecka and Baszynaski 1993; Hossain et al. 2012b). Usually PS II reaction center in the chlorophyll is mostly affected by metals like Cd that replaces Ca and Mn (Atal et al. 1991). Similarly, ROS generation is also evident in mitochondria at complex I and the ubiquinone (Q) zone (Blokhina and Fagerstedt 2006). Furthermore, several reports suggest that NADPH oxidase-dependent ROS induction can take place in response to Cd stress in *Pisum sativum* (Rodrıguez-Serrano et al. 2006), As stress in *Arabidopsis thaliana* (Gupta et al. 2013a), Pb stress in *Vicia faba* (Pourrut et al. 2008), Cd and Cu stress in *A. thaliana* (Remans et al. 2010), and Ni stress in wheat (Hao et al. 2006).

3 Mechanisms Against Heavy Metal Toxicity

3.1 Overview of Phytochelatin-Binding Defense Mechanism

3.1.1 Phytochelatins

Because details for research history and topics of findings for PCs are available in reviews (Grill et al. 1987; Cobbett 2000; Inouhe 2005), the framework is shown briefly. PCs are nonprotein cysteine-rich oligopeptides having the general structure of (γ-glutamyl-cysteinyl)*n*-glycine ($n = 2$–11). They are synthesized from the

precursor GSH by the activity of phytochelatin synthase (PCS), encoded in genes (PCS1, CAD1) isolated in 1999 at three laboratories (Ha et al. 1999; Vatamaniuk et al. 1999; Clemens et al. 1999). The PCs biosynthesis is stimulated by several HMs, especially Cd and metalloid As. PCs can bind to various HMs like Cd, As, Cu, Pb, Zn, and Ag, via their sulfhydryl (–SH) and carboxyl (–COOH) groups, and these complexations become more stable and massive in vacuole where acid-labile S^{2-} are incorporated to make the PCs–S–HMs conjugates. Both the thiols and S^{2-} are originated from sulfate through a partially common energy-dependent metabolism (sulfur assimilation), which is again enhanced by Cd, besides essential metals. Coordinative roles and functional linkages in these sulfur-containing compounds are expectable as discussed in detail later.

PCs and structurally PC-related peptides have been described in various plants and other organisms. Initially, PCs with different degrees of polymerization were reported from more than 300 species of plants and other organisms (Grill et al. 1989; Gekeler et al. 1989). In angiosperms, more than 23 species of monocotyledonous plants and 90 species of dicotyledonous plants were tested, and all of the plants were shown to produce PCs after Cd treatments (Gekeler et al. 1989). The PC synthesis was also confirmed either in suspension cultures or differentiated plant seedlings of various higher plants as well as in the lower plants that belong to groups of mosses or ferns (Gekeler et al. 1989). Such ubiquitous occurrences of PC peptides with the same structures among plant kingdom strongly suggest significant role of PCs as common metabolites in plants, while their physiological roles in the absence of heavy metals are still in open question at present, as described later.

Fundamental roles of PCs in intracellular detoxification are well demonstrated in various plants but especially for Cd (and As). This metal might be nonessential for most living organisms as so far known; nevertheless, why most plants have the most favorite response with PCs to this metal remains as an interesting question. Recent molecular phylogenetics approaches have started disclosing the ubiquitous roles of widespread PCS enzymes and genes in various organisms, which would have been diverged, specified, or converged during more than hundred billions of years of evolutionally time-span. However, evidences still show very low levels of PCs produced in the ancient type of plants like bryophyte as compared with their substantial levels in the some group of fungi (yeast), green algae, and various species of vascular plants (Hayashi et al. 1991; Mehra and Winge 1990; Inouhe et al. 1996; Murasugi 2008). Besides Cd, in response to other HMs stress in plants, PCs may play a significant role in detoxification in higher plants (Cobbett and Goldsbrough 2002) and make a complex, as immobilized metals are less toxic than the free ions. Synthesis and emergence of these metal-binding peptides in plants indicate HMs contamination under various environments (Gupta et al. 2002, 2004). It has been reported that in plants, PCs–HMs complexes form during detoxification process against a wide range metal ions, like Cd, Pb, As, Ag, Hg or Zn, Cu, Ni (Maitani et al. 1996; Mehra et al. 1996; Rauser 1999; Ha et al. 1999; Manara 2012). More direct evidence for the role of PCs against HMs contamination was presented through study on isolated PC-deficient cad1 mutants in heavy metal stress condition. Indeed, such a mutant of *A. thaliana* was more sensitive towards Cd and

arsenate (AsO_4^{2-}) than wild-type plants; however, no considerable difference was found for the others like Zn, selenite (SeO_3^{2-}), and Ni (Ha et al. 1999). A PCS-deficient mutant of *Schizosaccharomyces pombe* showed moderate sensitivity to Cu and Hg and modestly to Ag (Maitani et al. 1996; Ha et al. 1999; Manara 2012). Further evidence for Cu-induced triggering of PCs biosynthesis in Cu tolerance has been shown in Cu-tolerant species *Mimulus guttatus*. In contrast, a differential tolerance was reported in *Silene vulgaris*, on exposing root tips to Cu; both the Cu-tolerant and Cu-sensitive ecotypes produced comparable quantity of PCs. It is also manifested that PC–Cu complexes are comparatively transient and relatively poorly sequestered to the vacuole (Schat and Kalff 1992; De Knecht et al. 1994; Cobbett and Goldsbrough 2002). However, in plant *Rubia tinctorum*, exposure to different heavy metals leads to the formation of PC-metal complexes in the roots. Heavy metals ions like Ag, As, Cd, Cu, Hg, and Pb were appeared most effective in stimulation of PCs, though, PC complexes known in vivo were with Cd, Ag, and Cu ions (Maitani et al. 1996; Cobbett and Goldsbrough 2002). Moreover, the key role of PCs against heavy metal stress in plants and detoxification of different heavy metals has been corroborated in many studies. However, why such a change appears in the contribution of PCs to tolerance and detoxification against different HMs is still not fully understood.

3.1.2 Variation in Phytochelatins: Homo- and Iso-phytochelatins

PCs and structurally PC-related peptides have been described in various plants and other organisms. Such a ubiquitous occurrence of the PC peptides with the similar structures among plant kingdom strongly suggests their significant roles as primary metabolites common in the plants. However, here are some exceptional cases for the ubiquity of PCs in some restricted plants and yeast, i.e., some diversity is known for the molecules. PC peptides have Gly in the C-terminal end in general. The presence of some des-Gly variants of PCs in Cd-binding complexes was reported in *S. pombe* (Hayashi et al. 1991) and *Candida glabrata* (Mehra and Winge 1990). They have a structure of (γ-Glu-Cys)n. Similar peptides were not abundant in many higher plants but its substantial level can be found in *Zea mays* roots treated with Cd ions. Furthermore, four other PC-related peptides were discovered from plant sources. They have different amino acid residues at the C-terminal end of (γ-Glu-Cys)n peptides, Ala, Ser, Glu, or Gln. The (γ-Glu-Cys)n-Ala peptides first isolated from plants belonging to Fabaceae (Phaseoleae) are called homo-phytochelatins (hPCs) because they are synthesized from homo-glutathione (hGSH) with the structure of γ-Glu-Cys-Ala. Some other variants of those peptides have been also detected in maize and other plants and named as iso-phytochelatins (iso-PCs), which have the structures of (γ-Glu-Cys)n–Ser, (γ-Glu-Cys)n–Glu, or (γ-Glu-Cys)n–Gln (Cobbett 2000; Rea 2012). Biological roles of these variant peptides have not been well understood for a long time; however, their biochemical functions as thiol peptides are assumed to be basically equivalent to that of PCs. They also have common pathways in metabolisms, at least, some enzymes and

precursors such as (γ-Glu-Cys) dipeptidyl units, or glutamate (Glu, E) and cysteine (Cys, C), except glycine (Gly, E), for biosynthesis. Amount and distribution of these PC-related peptides may differ in different plant species; as for example, cells of *A. thaliana* are capable of synthesizing different PCs and iso-PCs (Ducruix et al. 2006). Synthesis of iso-PCs typically depends upon the availability of Gly or GSH synthetase in the cells that helps to switch over to synthesize the peptide (as, e.g., synthesis of dipeptide γ-glutamyl cysteine (γ-EC) when plant comes under stress (Ducruix et al. 2006; Rea 2012). The appearance of the mixture of PCs and iso- PCs such as (γ-Glu-Cys)n–Ser, (γ-Glu-Cys)n–Glu, or (γ-Glu-Cys)n–Gln, and (γ-Glu-Cys)n has been conceivably demonstrated as characteristic of Poaceae family (grasses) under Cd stress by several workers (Klapheck et al. 1994; Cobbett and Goldsbrough 2002) and also under As stress (Zhang et al. 2010; Duan et al. 2011; Batista et al. 2014). As induced hPCs and other variants PCs in *Lotus japonicus* (Ramos et al. 2008). However, it is suggested that for the PCS1 and hPCS enzymes, hGSH is a good acceptor, but a poor donor, of γ-EC units. Purified AtPCS1 and LjPCS1 were activated (in decreasing order) by Cd, Zn, Cu, and Fe, but not by Co or Ni, in the presence of 5 mM GSH and 50 mM metal ions. Activation of both enzymes by Fe was proven by the complete inhibition of PC synthesis by the Fe-specific chelator, desferrioxamine. *Arabidopsis* and Lotus plants accumulated hPCs only in response to a large excess of Cu and Zn, but to a much lower extent than did with Cd, indicating that hPC synthesis may not significantly contribute in vivo to Cu, Zn, and Fe detoxification.

3.1.3 Glutathione and Homo-glutathione

GSH is a direct precursor for PC synthesis but itself a very multifunctional metabolite and antioxidant important for metal tolerance and many other biological processes. GSH synthesis consists of two steps of energy-dependent processes that can occur in the cytosol or in the cell organelle like chloroplasts and mitochondria (Zechmann and Müller 2010). First step is an ATP-dependent rate-limiting reaction catalyzed by γ-EC synthetase (EC 6.3.2.2) producing γ-EC from glutamate and cysteine. Second step is an addition of glycine to γ-EC by GSH synthetase (EC 6.3.2.3) activity (Noctor et al. 2012). Both enzymes (named as GSH1 and GSH2, respectively) are encoded by single genes with alternate transcription initiation sites, and GSH1 is exclusively localized in the plastids, whereas GSH2, albeit also present in the chloroplasts, is to a large extent, a cytosolic protein. Thus, the compartmentalization of GSH synthesis functionally links the different cellular compartments and may provide a platform for intracellular redox signaling (Wachter et al. 2005). These and other data lead to the view that the synthesis of γ-EC is restricted to the plastid but that GSH synthesis can also occur in the cytosol using γ-EC, transported from plastids (Wachter et al. 2005; Noctor et al. 2011). In general, GSH synthetase expression and activity increased concurrently with that of γ-EC synthetase, both of which are otherwise indispensable for early developmental stages in plants. GSH-deficit *Arabidopsis* resulted in increased sensitivity to Cd

(Sengupta et al. 2012). The γ-EC synthetase is a rate-limiting enzyme for GSH synthesis (Noctor and Foyer 1998) whose activity is elevated by the presence of metal ions like Cd^{2+} and repressed by the treatment with buthionine sulfoximine (BSO), a specific inhibitor of this enzyme (Grill et al. 1987; Scheller et al. 1987). GSH plays also an important role in Pb detoxification in *Sedum alfredii*, under stress conditions, where PCs are absent, and chelated Pb, in conjunction with PCs synthesis and complexation, reduces stress in Pb-tolerant plants (Gupta et al. 2010, 2013b). Likewise, other reports suggested that *Vigna radiata* under a long-term stress with water deficit condition showed a decrease in both γ-ECS activity and its transcript levels in roots but with higher mRNA levels during the recovery period (Zagorchev et al. 2013). Homo-glutathione (hGSH) has antioxidant activity and serves functions in the transport of reduced sulfur and as direct substrate for hPC synthesis in legumes, as GSH does for PCs in these and other plants (Sobrino-Plata et al. 2009; Zagorchev et al. 2013). If the PCS or hPCS activities are same or samely reduced, these tripeptide levels become important factors that control the major antioxidative reactions for HMs and ROS sequestration. Here, the γ-ECS or similar enzyme has been shown to be involved in hGSH synthesis, while still unknown for the other iso-peptides. Whereas PC synthases (PCSs) are categorized as the γ-EC dipeptidyltranspeptidase (EC 2.3.2.15) that adds a γ-EC-unit of GSH to PCs or another GSH in vitro (Grill et al. 1989; Loeffler et al. 1989), which has been reported to be effective for the formations of hPCs and other iso-PCs (Ramos et al. 2008). Biochemical functions of hGSH and GSH are therefore basically similar if the metabolic or enzymic backgrounds are fulfilled in plants. It was shown that hGSH is an important regulator of root nodule formation, symbiotic interactions and nitrogen fixation in legumes (Zagorchev et al. 2013). Furthermore, their levels, distributions, and redox balances change differently in specific plants in response to different stress or hormone treatments and the developmental stages of the plants (Becana et al. 2010; Clemente et al. 2012; Zagorchev et al. 2013). The biological and evolutionary importance of hGSH and those for substitutions or deletion of the C-terminal amino acid in GSH to form other isotypes in different plants or organ sites await further investigations.

3.2 Other Mechanisms

3.2.1 Transport

Metal ions are vital for life and therefore maintenance of homeostasis of those ions is tremendously important (Fig. 2). Loss of homeostatic balance of elements may create severe metabolic and physiological dysfunction leading to death or severe illness of the plants. The homeostatic maintenance is a highly regulated process integrating uptake, storage, and secretion, where a number of transporters and antiporters proteins are involved. Inhibition in essential nutrient will decrease the plant vitality and its ability to cope with (metal) stress (Huang et al. 2008). Precise

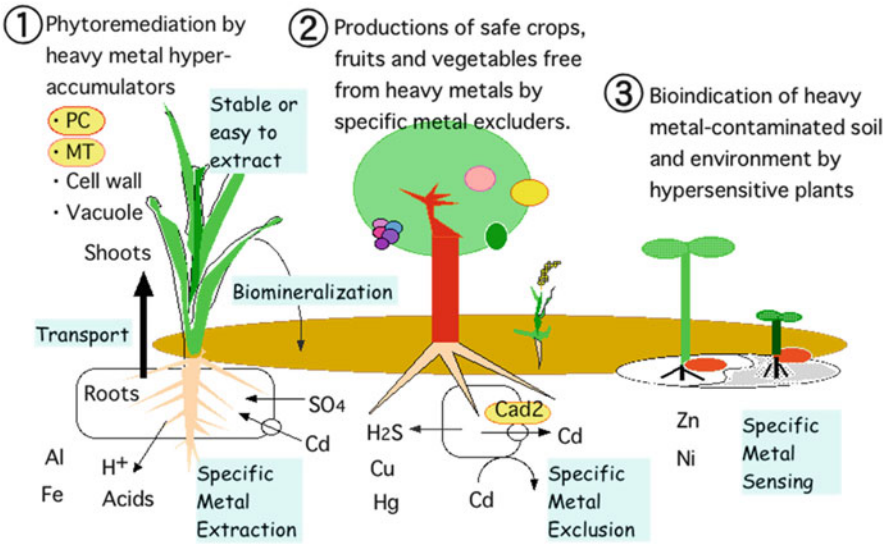


Fig. 2 Impact and route of HM in three ideal types of plants. (1) Hyperaccumulators absorb HMs from roots and transport them to shoots via xylem transport, where various kinds of HM-transports have critical roles. In shoots (and roots), special detoxification/sequestration mechanisms operate. (2) Plants that developed the HM exclusion mechanisms at roots can be useful in agricultural purpose as safety products for other organisms. (3) HM-sensitive plant will be good biological/biochemical index or markers against HMs contaminations

activation of metal-responsive genes to counteract the stress through the synthesis of proteins and signaling molecules related to stress takes place (Maksymiec 2007). Physiological transport of nutrients like Ca, Fe, Mg, Mn, Co, and Zn is unique in plants and some of these are inhibited by HMs. For example, Cd competes with these essential nutrients during transportation through transmembrane energy-dependent nutrient transporters and ion channels (Clemens et al. 1998; Curie et al. 2000; Thomine et al. 2000; Papoyan and Kochian 2004). Cortical tissues of the root help entering metal ions and usually become accumulated in the roots. It gets into the xylem by apoplastic and/or symplastic pathway and further transported to shoots. During the journey, the metal may be complexed by a number of ligands such as organic acids and/or PCs. Here, plant roots have the ability either to exclude and/or chelate or sequester Cd and other HMs from the plant tissues (Cataldo and Wildung 1983; Salt et al. 1995).

HMs-hyperaccumulator plant ecotypes include the Cd-hyperaccumulators such as *Noccaea caerulescens* (J&C Presl.) FK Mey, *Phytolacca americana* L., and *A. halleri* (L.) O'Kane and Al-Shehbazsetc (Lux et al. 2011). These plants also have the defensive mechanism through the production of Cd-chelators (such as organic acids, etc., as described below) other than PCs at the root zone that confining the entry of Cd to the xylem to prevent the metal accumulation in shoot tissues (Liu et al. 2010; Lux et al. 2011). Cd accumulation in shoot of species of the

Caryophyllales and Lamiales was much higher than other species (Broadley et al. 2001). In general, Cd concentrations are mostly (but not always) higher in roots than in shoots, indicating that transportation of Cd to the xylem and phloem is limited in most plants and lowest in seeds, fruits, and tubers (Seregin and Kozhevnikova 2008; Conn and Gilliham 2010). Absorption of HMs in higher plants is a critical issue, where especially rhizosphere region interacts with HMs (Wenzel et al. 2003). They are usually cotransported in the form of cation across the plasma membrane (Manara 2012). Reports suggest that plant roots primarily secrete exudates in its surrounding soil matrix that helps in the chelation of unwanted metals to prevent transportation inside the cell (Marschner 1995). For example, histidine (His) and citrate (CA) are secreted as root exudates to prevent the Ni uptake from the soil (Salt et al. 2000). Pectic sites and a number of extra cellular carbohydrate molecules present on the cell wall play an important role for immobilization of toxic heavy metal ions (Manara 2012). However, HM homeostasis is mainly maintained by transporters present on the plasma membrane. Typical examples of these transporters are the ZIP, the HMA, the YSL, the NRAMP, the CDF, and the CAX families (Williams et al. 2000; Guerinot 2000; Hossain et al. 2012a, b; Sochia and Guerinot 2014), as shown below briefly.

1. The ZIP (zinc-regulated transporter/iron-regulated transporter [ZRT/IRT1]-related protein) family transporters are well characterized for divalent metal uptake, which consists of eight transmembrane domains with similar topology at N- and C-termini exposed to apoplast also containing a histidine-rich domain supposed to involve in specific metal binding (Guerinot 2000; Nishida et al. 2008). ZIP protein gets activated in response to Fe or Zn loading. In *A. thaliana*, IRT1, the founding member of ZIP family, was the first reported transporter in root cells and has an important role in Fe uptake from the soil (Vert et al. 2002). IRT1 can also transport Mn, Zn, and Cd (Korshunova et al. 1999). AtIRT1 in yeast enhanced the Ni-uptake activity (Nishida et al. 2011). Furthermore, AtZIP4 proteins, expressed in roots and shoots, are involved in Zn transport and also helps in Cd uptake from soil into the root cells and Cd transport from root to shoot (Krämer et al. 2007).
2. The HMAs family transporters (P_{1B}-type ATPases that belong to P-type ATPase superfamily) efflux various metal cations across biological membranes (Axelsen and Palmgren 2001). They are basically internal transporters to load Cd and Zn metals into the xylem from the surrounding tissues and act as an efflux pump. The HMAs were categorized as both monovalent and divalent cation transporters (Baxter et al. 2003; Krämer et al. 2007). In *A. thaliana*, AtHMA3 transporter helps in sequestration of a wider range of HMs, and its overexpression increases the tolerance to HMs like Cd, Pb, Co, and Zn (Morel et al. 2009; Manara 2012). In ABC transporter family, AtPDR8 was discovered in the plasma membrane of *A. thaliana* root hairs and epidermal cells that helps in effluxing of Cd and Pb from plasma membrane (Kobae et al. 2006; Kim et al. 2007).
3. Oligopeptide transporters (OPTs) are a group of membrane-localized proteins. The OPT proteins belong to a small gene family in plants, named as the YSL

(yellow stripe-like) subfamily, taken its name from the maize Yellow stripe 1 protein (ZmYS1), and are involved in uptake of Fe by transporting Fe(III)-phytosiderophore complexes (Curie et al. 2000). Heavy metal ions like Fe, Zn, Cu, Ni, and to a lesser extent Mn and Cd are transported by ZmYS1 transporter (Schaaf et al. 2004). Based on sequence similarity with maize gene, eight presumed YSL transporters have been identified in *A. thaliana* (Colangelo and Guerinot 2006). AtYSL1 is expressed in the leaf xylem parenchyma, in pollen, and in young siliques, whereas AtYSL2 is expressed in shoot and root vascular tissues and is present in the lateral plasma membrane, steady with a role in the lateral movement of metals into the veins (DiDonato et al. 2004; Schaaf et al. 2004).

4. Metal Tolerance Proteins (MTPs) are metal efflux transporters in plants that belongs to CDF (cation diffusion facilitator) transporter family involved in the pumping divalent metal cations like Zn, Cd, Co, Fe, Ni, and Mn and transportation from the cytoplasm to the vacuole (Nies 1992; Krämer et al. 2007; Peiter et al. 2007; Montanini et al. 2007; Manara 2012). CDF transporters consist of six transmembrane domains, a C-terminal cation efflux domain, and a histidine-rich region between transmembrane domains IV and V (Mäser et al. 2001) which probably act as a sensor for heavy metal concentration (Kawachi et al. 2008).
5. Natural resistance-associated macrophage protein (NRAMP) transporters such as AtNRAMP3 or AtNRAMP4 are localized in the tonoplast and help in the transport of Fe from the vacuole (Thomine et al. 2003; Lanquar et al. 2005). Overexpression of AtNRAMP3 increases Cd sensitivity and prevents the accumulation of Mn, indicating a possible role in the homeostasis of metals other than Fe (Thomine et al. 2003).
6. The CAX (cation exchanger) proteins are one of five transporter families that constitute the Ca/cation antiporters (CaCA) superfamily (Shigaki et al. 2006; Emery et al. 2012). The CAX family members were first identified as Ca transporters but later it was revealed that they are capable of transporting more kinds of HMs. Typical CAX proteins contain 11 transmembrane domains. They facilitate the redistribution of cations across a membrane using electrochemical energy generated by a proton pump in order to maintain optimal ionic concentrations in the cell (Socha and Guerinot 2014).

Among these, the HM transporters which are involved in transport to vacuole or in exclusion at plasma membrane are effective in reducing HMs levels in the cytological active compartments including cytoplasm and plasmids in the cells, which reduce the toxicity exerted by HMs as free radicals or indirect inducers of ROS in the sites. There are strongly convincing proofs that many hyperaccumulator plants for various HMs are prevailed for these transportation mechanisms via xylem transport systems rather than their special detoxification mechanisms in the cells. For details about the respective functions of the transports in hyperaccumulators or HM-tolerant plants, see recent reviews cited above and others (Hossain et al. 2012b; Socha and Guerinot 2014).

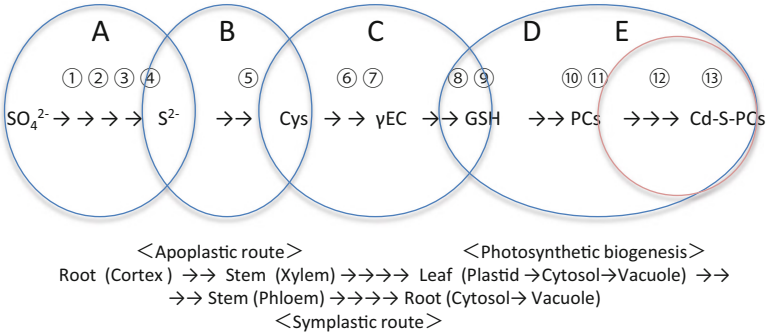
3.2.2 Redox Enzymes

Antioxidant system in plants is an intrinsic defense mechanism that regulates ROS levels according to the cellular requirements at a certain period. This system is actually governed under the catalytic activities by the several cooperative enzymes, named redox enzymes or detoxification enzymes (Fig. 1), which involve superoxide dismutase (SOD; EC 1.15.1.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.9), dehydroascorbate reductase (DHAR; EC 1.8.5.1), and glutathione reductase (GR; EC 1.6.4.2). These enzymes are regulated under HM stresses and hence consequently participate in the mechanism of protection against oxidative stress mediated by HMs. Glutathione S-transferase (GST; EC 2.5.1.18) that catalyzes the GSH-dependent conjunction with various types of substrate molecules to form thioether bond between them also contribute to detoxification of xenobiotics by conjugation reactions (Sherratt and Hayes 2001). There are many reports that support the positive effects of HM on the enzymic defense mechanisms expressed prior to or simultaneously with the enhanced tolerance characteristics to the stress. The water-soluble compounds such as AsA and GSH are used central substrates (Hossain et al. 2012b), but little is known for the role of PCs and h-PCs in the enzymic antioxidant systems at present. Gene expressions related with stress responses to quench directly ROS and develop further tolerance appear to be mediated by GSH and its oxidates (GSSG). In addition, abiotic stress tolerance through the glyoxalase pathway is widely been reported which consists of glyoxalase I (Gly I; lactoylglutathionelyase; EC 4.4.1.5) and glyoxalase II (Gly II; hydroxy-acylglutathione hydrolase; EC 3.1.2.6) (Hossain et al. 2009).

As mentioned earlier, ROS generation is evident in chloroplast, mitochondria, peroxisome, and apoplast adjacent to membrane. Here, several reports suggest that NADPH oxidase-dependent ROS induction can take place in response to Cd stress in *P. sativum* (Rodrıguez-Serrano et al. 2006), As stress in *A. thaliana* (Gupta et al. 2013a), Pb stress in *V. faba* (Pourrut et al. 2008), Cd and Cu stress in *A. thaliana* (Remans et al. 2010), and Ni stress in wheat (Hao et al. 2006). There is no evidence that cytoplasmic PCs have a role preventing the ROS induction at plasma membrane-associated ROS formation or at apoplast. However, it is acceptable that cell-wall-associated peroxidase catalyzes formation of membrane-permeable H_2O_2 in apoplast and then makes it possible to interact with cytosolic PCs and other thiol peptides. It is interesting that living with the appropriate concentration level of ROS like H_2O_2 can promote plant development and support resistance to environmental stressors by controlling the expression of genes and redox signaling (Neill et al. 2002). However, direct interactions between PCs and ROS that result in induction of any HM-tolerant mechanisms are not yet demonstrated; therefore, their actions might be independent but be synergistic.

3.2.3 Sulfur Assimilation

Sulfur (S) is an essential and ubiquitous element involved in a large number of vital biochemical and physiological processes. It is also responsible for developing hypersensitivity to HMs. Earlier studies on transgenic plants revealed that excessive PC levels helps the plant to accumulate more amounts of HMs without enhancing tolerance conferring HM-hypersensitivity (Lee et al. 2003; Pomponi et al. 2006; Manara 2012). Sulfur is known for its catalytic or electrochemical properties and having a capacity to react with a broad spectrum of agents, like cytotoxic electrophilic organic xenobiotics, HMs, and free radicals. GSH and PCs biosynthesis is highly regulated and coordinates to meet the demand for Cys consuming activities, which indirectly explain the overall S demand by plants. Sulfur requirement by plants vary under the diverse environmental conditions, biotic and abiotic stresses including HMs (Rausch and Wachter 2005). It has been previously observed that the withdrawal of S from the growing medium dramatically decreases the levels of S, Cys, and GSH in plant tissues (Lappartient and Touraine 1996; Lappartient et al. 1999; Saito 2004; Nocito et al. 2007). In addition, importance of PCs in homeostasis of metals, antioxidant property, and also in S metabolism was suggested (Dietz et al. 1999; Cobbett 2000). Furthermore, when more stable and massive Cd-PCs conjugates are formed in vacuole under Cd stress, large quantity of S^{2-} is incorporated to the complexes. All of these thiols and S^{2-} are originated through the common energy-dependent S assimilation using sulfate taken by roots. These processes are summarized in Fig. 3 (top), as a series of five process termed as A to E for convenience, where several transporters and/or enzymes with some intermediates as the key factors interconnecting functions among S-containing substances are also shown. Simultaneous or cooperative stimulation of these processes may result in total increase in the level of S-containing compounds as well as the total reducing power in the plants. The diversity of the components accumulated in the tissues also increases. Therefore, gross activation of the processes by HMs or other stressors may contribute to the stress tolerance mechanisms in many plants. However, it is important to note that such influences on the process or component are quite different in case and place, as shown in Fig. 3 (bottom) as tentative examples. Furthermore, it is already evident that diverse species of S-containing substances have diverse functions in plants. Briefly, Cys is the first organic product of S assimilation in plants and is notably used for synthesis of proteins (amino acids) directly or after converting to methionine (Leustek et al. 2000; Saito 2004). These amino acids are thought to be an important sink for reduced S, as well known as for GSH (Noctor et al. 2011). Such a supply or increased sink for reduced S in plants has been positively correlated with resistance to some pathogens, a phenomenon termed S-induced resistance (SIR) by some researchers (Bloem et al. 2007). Here, tissue contents of GSH or precursors are thought to be the factors linking S nutrition to the responses of plants to fungal and viral infection (Noctor et al. 2011).



① AST68 (SO_4^{2-} transporter), ② APS1 (ATP sulfurylase), ③ APR (APS reductase), ④ Sulfite reductase, ⑤ SAT (O-acetylserine (thiol) lyase), ⑥ Cys transporter, ⑦ γEC synthetase, ⑧ γEC transporter, ⑨ GSH synthetase, ⑩ GSH transporter, ⑪ PC synthase, ⑫ PC transporter, ⑬ PC-Cd-S complexation.

Case	Activation	Ideal Pool-Stock Reservation
1(Cd)	A,B,C,D,E	**Abundant S** (**S^{2-}**, Cys, GSH, **PCs**, **PC-S**)
2(As)	A,B,C,D	**Moderate S** (S^{2-}, Cys, **GSH**, **PCs**, PC-S)
3(Zn)	A,B,C	Shortage S (**S^{2-}**, Cys, **GSH**, PCs, PC-S)
4(Cu)	A (?)	Deficient S (S^{2-}, Cys, GSH, PCs, PC-S)

Fig. 3 Diagram of path for PCs, GSH, Cys, and S^{2-}. *Top*, examples for via root viz shoot circulate paths. (1) AST68 (SO_4^{2-} transporter), (2) APS1 (ATP sulfurylase), (3) APR (APS reductase), (4) Sulfite reductase, (5) SAT (O-acetylserine (thiol) lyase), (6) Cys transporter, (7) γEC synthetase, (8) γEC transporter, (9) GSH synthetase, (10) GSH transporter, (11) PC synthase, (12) PC transporter, (13) PC-Cd-S complexation. *Bottom*, ideal changes in the S and thiol pools as affected by representative HMs. The data are tentative ones and never cover all cases or previously reported respective evidence

Besides their roles as the major storage and transport forms of reduced S, the importance of GSH and Cys have been implicated in the regulation of S metabolism (Kopriva and Rennenberg 2004). GSH inhibits sulfate uptake and S assimilation by repressing the activities and expressions of several functional proteins mediating the earlier steps of the process, such as sulfate transporter (AST), ATP sulfurylase (APS1), and adenosine 5′-phosphosulfate reductase (APR) in plants (Lappartient et al. 1999; Leustek 2002; Noctor et al. 2011). In most studies, the increased

concentrations of Cys in response to oxidative stresses have been reported together with increased GSH concentrations, leading to the conclusion that Cys is mainly needed for the biosynthesis of S-rich compounds with antistress activity, such as GSH and stress-related proteins. However, it is generally thought that at concentrations above 50 μM, Cys is toxic for plants (Meyer and Hell 2005). Cys is a potent chelator of heavy metals ions, but the formed Cys-metal complexes can trigger the Fenton reaction, thereby producing the highly toxic $^{\bullet}OH$ radical, and free Cys is often irreversibly oxidized to different by-products. Sulfide is more toxic than sulfate but stabilized in the complex such as Cd-S-PCs. GSH is quickly turning over but the balance of it and its oxidized form (GSSG) are strictly regulated under enzymic redox systems. Therefore, like a general rule for stock chemicals, more stable S-containing substances (or ones under more strict control) may play a central role as a stable state S-donors in plants. Cd-S-PC complex may be a potential stable S donor as well as other S-containing storage proteins or polysaccharides.

3.2.4 Other Mechanisms: Hypothetical View

The detoxification and sequestration mechanisms driven by PCs may be restricted to the HMs and ROS invaded or occurring in the cell compartments in hyperaccumulator plants or tolerant plants grown under naturally metalliferous or artificially contaminated environments. Here, it is important to note that the direct precursor of PCs is GSH, which is multifunctional and thus required for the many other biochemical processes in response to or not to various HMs. In these plants, by blocking or inefficiency of or inefficiency of HMs and other oxidants to the synthesis of PC peptides and other S-containing oligo-/poly- peptides, can increase the small organic and inorganic S-substances such as S^{2-} or Cys, and thus GSH. This can be mimicked by the case if the final S-conjugated products are stored or immobilized in a certain compartment apart from cytoplasm. These increased S-substances are alone or in combination, capable of increasing the binding and quenching of the free radicals of HMs and ROS more. Furthermore, S can make more stable complex with PCs-Cd, and the core Cd-S formed inside of the PC-Cd-S complexes can act as the semi-conductance micro-devise to control electron flow or exchange between two or more different molecules in cytosol or protein complexes on the bio-membrane, while a direct association of PC peptides to other biopolymer/oligomer has not yet been proven. PC has been associated with many important functions in plants against HM ions, including intracellular binding, detoxification, transport, following compartmentalization in vacuole, and also as a substantial coordinator for the distinguished characteristics of several plant-hyperaccumulators of different HM species. However, relatively less attention has been paid on their potential roles in defense mechanism against ROS induced by various abiotic or biotic oxidative stresses, other than by HMs. This is mainly due to the smaller contents of PCs (less than 0.01 mM) than the other antioxidants under normal condition with little contaminated HMs. Intracellular levels of total PCs rise

drastically up to 1–10 mM and 0.1–0.5 mM orders as maximum so far reported, when exposed to 0.2–0.8 mM of metal Cd and 0.02–0.5 mM of metalloid As respectively; hence, the detoxification and sequestration mechanisms driven by PCs may be restricted to the HMs and ROS invaded or occurring in the cell compartments in hyperaccumulator plants or tolerant plants grown under naturally metalliferous or artificially contaminated environments. However, more interestingly, PC-HM complex has been detected in phloem sap rather than xylem sap, indicating that PC-functioning site is again intracellular well-reduced state or symplastic place but not oxidative places to produce ROS like apoplastic sites with high oxygen pressure and organelle having active electron transport system. Further assessments are needed but phloem loading and transport will be the attractive performance stage as nutritional PCs.

4 Conclusion and Future Prospective

PCs and related thiol peptides have been associated with many important functions in plants against HM ions, including intracellular binding, detoxification, transport, following compartmentalization in vacuole, and also as a substantial coordinator for the distinguished characteristics of several plant-hyperaccumulators of different HM species. However, relatively less attention has been paid on their potential roles in defense mechanism against ROS induced by HMs and/or other various abiotic or biotic stresses. This is mainly due to the trace levels of PCs (generally less than 10 μM) constitutively maintained in control plants and their trivial changes after either the treatment with HMs besides Cd or the influence of the other abiotic/biotic stresses, where instead evoked are drastic change and activation of the other antioxidants and redox systems. However, we need further consideration for the potential roles of PCs in the plants growing or habituated on the grounds with artificially or naturally HM-dense conditions, as core or polymeric absorbent for nutritional elements and especially S-coordinated substances, resulting also in a dominant storage bank for thio-mediated reduction power sources. As linking to a phenomenon SIR or a concept GSH/thiol pools, it can be urged for our reconsideration that these stocks will play an important role in the maintenance or increase of the robustious characteristics of the plants against various oxidative stresses under biotic and abiotic occasions with no further excess biochemical or metabolic costs. Potential roles of PCs as the key peptidic thiol and reducing agents in the interconnection to other antioxidant compounds and components, such as LWM soluble thiols, AsA, sugars and sugar alcohols, proline and other amino acids and compatible solutes, as well as membrane and cell-wall-associated redox cycle systems will be highlighted in future.

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Role of Polyphenols as Antioxidants in Native Species from Argentina Under Drought and Salinization

Mariana Reginato, Celeste Varela, Ana M. Cenzano, and Virginia Luna

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Abstract Plants inhabiting arid environments are exposed to drought and, in several cases, also to salt stress as usually occurs in different regions in Argentina. These plants have developed different strategies to avoid or tolerate the lack of water and/or the excess of toxic ions during their development. As drought and salt stress lead to increased production of reactive oxygen species (ROS) in plant cells, halophytic and xerophytic species have the ability to reduce these toxic ROS by means of a powerful antioxidant system that includes enzymatic and nonenzymatic components. Production of phenolic compounds is one of the strategies used by some native species of these adverse environments, principally to protect their cells from the oxidative damage caused by drought and salinity. This chapter provides an

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overview of the oxidative response and the polyphenols involvement as part of a tolerance mechanism in five native species from Argentina: the shrubs *Prosopis strombulifera*, *Larrea divaricata*, and *Lycium chilense* and the grasses *Pappostipa speciosa* and *Poa ligularis* which have shown to have a high polyphenol production correlated with a high antioxidant capacity. Also, two of these native species may be considered as important sources of antioxidants and biomolecules for biotechnological purposes.

Keywords Drought • Salinity • Native species • Oxidative stress • Polyphenols

1 Introduction

Oxidative stress is a central factor in abiotic and biotic stress phenomena and is defined as the chemical toxic effect of reactive oxygen species (ROS) on different plant structures. Oxidative stress occurs when there is a serious imbalance in any cell compartment between ROS production and antioxidant defense, leading to dramatic physiological challenges (Foyer and Noctor 2003). It was considered that ROS concentration needs to be maintained as low as possible, although this concept is changing because of the multiple functions that are currently being discovered for these molecules (Mittler and Blumwald 2010).

ROS production is known to be increased dramatically under different stressing conditions. A rapid increase in ROS production (known as "oxidative burst") has been reported in response to a variety of abiotic stresses including drought, salinity, flooding, heat, cold, ozone, heavy metals, air pollution, nutrient deprivation, excess of light, and UV radiation (Mittler 2002; Miller et al. 2008). Among the above mentioned stresses, drought and salinity are two of the most substantial factors affecting and limiting crop production worldwide. Oxidative stress induced by salinity and drought has been reported by numerous authors (Mittler 2002; Wang et al. 2003; Pang and Wang 2008; Tounekti et al. 2010).

To prevent damage caused by ROS, plants possess a complex antioxidant defense system that is generally able to balance these ROS and keep them at nontoxic levels. This system includes enzymatic and nonenzymatic components. Nonenzymatic antioxidants are vital because they can scavenge ROS that cannot be detoxified by enzymatic systems; polyphenols are an example of a group of compounds that exhibit a high antioxidant activity (Chanwitheesuk et al. 2005). These natural antioxidants are the result of the plant secondary metabolism; they occur in all plant organs and their enhanced synthesis under stressful conditions is believed to protect the cellular structures from oxidative effects (Jaleel et al. 2007). Among these compounds, mainly flavonoids play an important role in the defense against ROS.

Production of phenolic compounds is one of the strategies used by some native species that grow in adverse environments, principally to protect their cells from the oxidative damage caused by drought and salinity.

This chapter provides an overview of the oxidative response and the polyphenols involvement as part of a tolerance mechanism to drought and salinity in five native species from Argentina (*Prosopis strombulifera*, *Larrea divaricata,* and *Lycium chilense* as shrub and *Pappostipa speciosa* and *Poa ligularis* as grasses) that have shown a high polyphenols production correlated with high antioxidant capacity. Also, two of these native species may be considered as important sources of natural products.

2 ROS Production and Oxidative Damage in Plants

ROS is used as a collective term for several O_2-derived radicals like superoxide anion ($O_2^{\bullet -}$), peroxyl radicals (RO_2^{-}), alkoxyl radicals ($RO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), and also for non-radical singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). H_2O_2 is mostly produced as a result of dismutation of $O_2^{\bullet -}$, which can occur spontaneously or enzymatically in the cell or apoplast.

ROS integrate signaling pathways involved in plant growth, development, gravitropism, hormonal action, and many other physiological phenomena (Mittler 2002; Apel and Hirt 2004; Foyer and Noctor 2005; Miller et al. 2008). In many of these cases, ROS production is genetically programmed, and ROS are used as second messengers (Foyer and Noctor 2005). Thus, it appears that ROS have pleiotropic effects in plants as they do in animals (Storey 1996).

When ROS are produced in a controlled manner within specific cell compartments, they have key roles in plant growth and development. When ROS are produced in excess, an uncontrolled oxidation occurs, leading to cellular damage and eventual cell death. Thus, it is very important for cells to keep a tight control of ROS concentration (Schützendübel and Polle 2002). Under salinity and drought stresses, stomata are closed as a rapid response to reduce water loss. However, this response limits gas exchange, which is vital for effective photosynthesis. Limitation of gas exchange causes a variety of redox changes in the cell due to variations in the effectiveness of photosynthesis. Decreased availability of CO_2 can be dramatic in terms of ROS production when photosynthetic active radiation (PAR) is high. Uncoupling of light reactions and the Calvin–Benson cycle is the main cause of ROS production in chloroplasts. When electron transport chains are overloaded, $^1O_2$ can be produced in PSII (Asada 2006). Also, photorespiration is another ROS-producing process caused by limited gas exchange under salinity.

Besides photosynthesis related ROS, the production of these species can also occur during respiration in mitochondria; over reduction of the mitochondrial electron transport chain can cause $O_2^{\bullet -}$ production. Electrons can escape from complexes I and III to molecular oxygen if the other electron acceptors in the line are over reduced (Noctor et al. 2007). For more detailed information on the ROS production in chloroplasts and mitochondria, see chapter "Production Sites of Reactive Oxygen Species in Organelles from Plant Cells" by Corpas and colleagues.

Lipids and proteins are the main targets of damage caused by ROS in the cell. ROS can cause the oxidative decomposition of polyunsaturated lipids in membranes, known as lipid peroxidation, which starts a reaction chain that can also create other reactive products such as aldehydes, ketones, and hydroxyl acids and can modify proteins, by oxidation of some amino acid residues.

To prevent those oxidative processes caused by ROS, plants have developed a complex antioxidant defense system that includes enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX) and nonenzymatic antioxidants such as ascorbic acid, glutathione, a-tocopherols, and b-carotenes besides the polyphenols mentioned above. Also, other redox regulatory enzymes such as the ascorbate-glutathione cycle enzymes, monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DAR), glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione-S-transferases (GST), are also key components of the antioxidant defense.

3 Polyphenol Accumulation Under Stress Conditions

Plants may vary widely in their phenolic content and composition, with both genetics and environment affecting the type and level of these compounds (Awika and Rooney 2004; De Abreu and Mazzafera 2005). Generally, their accumulation is stimulated in response to ROS increases under biotic/abiotic stresses (Dixon and Paiva 1995; Roberts and Paul 2006; Julkunen-Tiito et al. 2015).

Phenolic compounds exhibit antioxidant activity in tissues exposed to a wide range of environmental stressors, by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals as mentioned above (Krishnaiah et al. 2011; Agati et al. 2012; Brunetti et al. 2013). In recent years, phenolic compounds research has focused the interest in their antioxidant role as nonenzymatic mechanism (Isabelle et al. 2010; Pollastri and Tattini 2011). Thus, in several species around the world, a role for polyphenols under different stresses has been proposed. To mention some examples, *Larrea tridentata*, a native plant from North America deserts, was found to have a dense resin in the leaves with high amount of phenolic compounds, providing drought tolerance through avoiding transpiration, protecting the photosynthetic system against UV radiation, preventing herbivores, and it can be also involved in allelopathy (Hyder et al. 2002; Martins et al. 2010). *Hypericum brasiliense*, a medicinal herb that produces several phenolic compounds with important pharmacological activity, increased the level of phenolic compounds under water stress (De Abreu and Mazzafera 2005). *Echinacea purpurea*, the purple-coneflower native from the dry hills of North America, enhanced total phenols content under brief drought stress periods (Gray et al. 2003). *Labisia pumila*, a small woody plant from the tropical forest of Malaysia, increases total phenols, anthocyanins, and flavonoids production under severe water stress (Jaafar et al. 2012). In tea plants, water stress increases the

production of two important flavan-3-ols (epicatechin and epigallocatechin gallate) with potent antioxidant properties (Hernández et al. 2006). Hawthorns native from North America showed an increase in polyphenols, majority flavonoids, under water and cold stress while under flooding and herbivores it showed a variable response, increasing some polyphenols and decreasing others (Kirakosyan et al. 2004). *Ligustrum vulgare*, a woody plant from the rain forest of Europe and Asia, increased flavonoids and hydroxycinnamates under drought and light stress (Tattini et al. 2004). Moreover, increases in total polyphenol content in different tissues under rising salinity has been reported in a number of plants (Agastian et al. 2000; Muthukumarasamy et al. 2000; Navarro et al. 2006). *Cakile maritima*, a native plant from the Atlantic coast, the Mediterranean Sea, and the Black Sea, enhanced phenolic compounds with high antioxidant capacity under salt stress (Ksouri et al. 2007). Also, different maize varieties showed an increase in phenolic compounds under water and salt stress (Hajlaoui et al. 2009).

4 The Importance of Polyphenols as Antioxidants

From the above reports, it can be deduced that plants produce thousands of phenolic and polyphenolic compounds. Polyphenols constitute one of the most numerous and widely distributed groups of natural products in the plant kingdom. It includes an ample variety of molecules with phenol ring structure. At present, there is not any universal classification for polyphenols, but the most current classification divides this type of molecules in different groups according to their chemical structure (Motilva et al. 2013). There are three main groups: non-flavonoids, flavonoids, and tannins. The non-flavonoids are molecules that have at least one phenolic ring with different reactive groups (hydroxyl, nitrosyl, SH, etc.); this group includes simple phenolic acids, phenyl alcohols, stilbenes, and chalcones (Fig. 1). The flavonoids are characterized by a phenyl chromane structure of C_{15} formed by two aromatic rings bound with a carbon chain (C_6–C_3–C_6) and include antocyanidins, flavonols, flavones, flavonons, isoflavons, flavan-n-ols, etc. The third group, called tannins, is subdivided into two groups: condensed tannins and hydrolysable tannins. Condensed tannins are flavonoid polymers type A (C_7–C_2 and an ether bond) and type B (C_4–C_8 or C_4–C_6) and hydrolysable tannins are phenolic acids polymers bound to a five or six carbon ring (Khanbabaee and Van Ree 2001).

Polyphenols are essential for the physiology of plants. These compounds have been implicated in diverse functional roles, including structure, pigmentation, lignification, plant resistance against microbial pathogens, and animal herbivores such as insects (antibiotic and anti-feeding actions), protection against solar radiation (screen against DNA-damaging UV-B light), and they were probably important during early terrestrial plant evolution (Hatier and Gould 2008; Lattanzio et al. 2008). The majority of polyphenols are synthesized by the highly branched phenylpropanoid pathway, which is responsible for the biosynthesis of a large

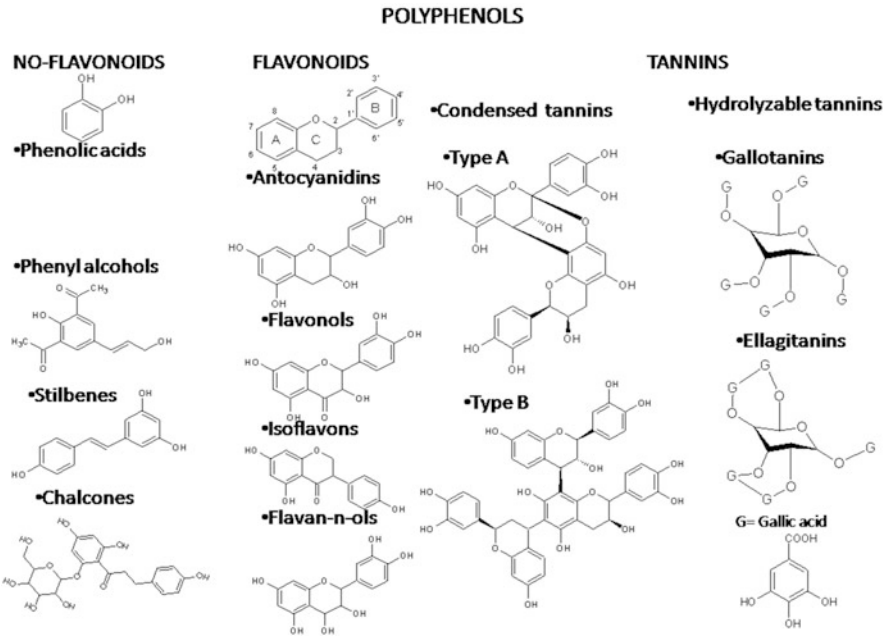

Fig. 1 Main groups of polyphenols according to their chemical structure. *G* gallic acid

number of chemical compounds with considerable structural diversity (Duthie et al. 2003).

Plant polyphenols exhibit a wide range of bio-physicochemical properties bundled within the phenol functional group, which makes them remarkably versatile metabolites and unique and intriguing natural products. The major characteristic of polyphenols is their capability to scavenge ROS, as well as oxidatively generate free radicals $RO^{\bullet}$ and $ROO^{\bullet}$ such as those derived from biomolecules like the low-density lipoproteins proteins (LDLs) (Neudörffer et al. 2004) and oligonucleic acids (DNA and RNA) (Li et al. 2000).

In its most elementary structural form, namely a benzene ring bearing a hydroxy group (PhOH), a phenol function constitutes an amphiphilic moiety that combines the hydrophobic character of its planar aromatic nucleus with the hydrophilic character of its polar hydroxy substituent, which can act either as a hydrogen-bond donor or as an acceptor. Hydrophobic p-stacking (van der Waals) interactions and the formation of hydrogen bonds are seemingly dichotomic, yet are often complementary effects that plant phenolics can use to interact physically with other biomolecules, among which proteins are often first in line (Dangles and Dufour 2008).

Polyphenols are also able to chelate transition metals through their multiple hydroxy groups on a phenyl ring open (Andjelkovic et al. 2008). By chelating metal ions, such as iron(II)/copper(I) and iron(III)/copper(II) that are involved in the

conversion of $O_2^{\bullet -}$ and H_2O_2 into highly reactive hydroxyl radicals (HOl), polyphenols can act as DNA protective agents from free radicals damages (Heim et al. 2002; Perron and Brumaghin 2009; Leopoldini et al. 2011). This capacity for metal chelating also contributes to plant pigmentation (Lattanzio et al. 2008) as well as cationic nutrient (e.g., Ca, Mg, Mn, Fe, Cu) cycling through plant–litter–soil interactions.

Polyphenols act as scavengers of free radicals and ROS, when these are unable to be subdued by the regular action of other antioxidants such as glutathione (GSH), glutathione peroxidase, or superoxide dismutase or by vitamins (e.g., vitamins E and C, carotenoids). Two main intimate antioxidative mechanisms have been proposed for polyphenols (Wright et al. 2001).The first is based on the capacity of the phenol functional group to donate a hydrogen atom to a free radical $R^{\bullet}$ such as peroxy radicals $LOO^{\bullet}$ generated during lipid (LH) autoxidation (peroxidation; $LH \rightarrow L^{\bullet}$, then $L^{\bullet} + {}^3O_2 \rightarrow LOO^{\bullet}$) such as peroxy radicals $LOO^{\bullet}$ generated during lipid peroxidation, acting as chain-breaking antioxidants. The second mechanism is the single-electron transfer from the phenolic antioxidant (ArOH) to a free radical $R^{\bullet}$ with formation of a stable radical cation $ArOH^{\bullet +}$.

5 Oxidative Stress and Phenolic Compounds in Native Species from Argentina

5.1 Xerophytic Species from the Patagonian Monte

Argentina has a large territorial extension with a variety of climates and plant species. The Phytogeographic Province of Monte is the most arid rangeland of Argentina located from 24°15′ S to 44°20′ S and 64° to 68° W (Fernandez and Busso 1999). The Southern part of Monte is characterized by strong water deficits in spring and summer, high evaporation enhanced by westerly winds, mean annual temperature of 13.9 °C, annual rainfalls between 200 and 260 mM, and unpredictable rainfall events (Fernandez and Busso 1999; Campanella and Bertiller 2008). Thus, in this region there are many native species exposed to severe drought periods, which are mostly evergreen or deciduous shrubs and perennial grasses. Within the shrubs we can find *Larrea divaricata, Chuquiraga hystrix, Lycium chilense, Junellia alatocarpa, Condalia microphylla, Prosopidastrum globosum, Schinus johnstonii, Monttea aphylla, Atriplex lampa, Chuquiraga avellanedae, Prosopis denudans*, *and Prosopis alpataco* and within the grasses *Stipa tenuis*, *Poa ligularis*, *Pappostipa speciosa* and *Stipa humilis* are the most common species (Beeskow et al. 1987; Ares et al. 1990). All these species have developed different strategies to survive and to avoid oxidative damage in their tissues through the production of secondary metabolites with antioxidant capacity.

Four of these native species were selected to carry out an ecophysiological study on the relationship between phenolic compound production, antioxidant capacity,

and lipid peroxidation throughout the annual seasons in year 2013. During this year, the rainfalls were concentrated in spring rendering the highest soil water content. The lowest soil water content was found in autumn. The highest temperatures were measured in summer and the lowest in winter. Taking into account the water availability in the soil and the evapotranspiration rate, it was assumed that plants were exposed to drought stress throughout the whole year being more severe during autumn and/or summer.

Among the dominating shrubs, *Larrea divaricata* (chaparral) and *Lycium chilense* (coralillo) are common species, and among grasses *Poa ligularis* (bluegrass) and *Pappostipa speciosa* (desert needlegrass) are common species (Ares et al. 1990). *Larrea divaricata* and *Pappostipa speciosa* are evergreen species grouped as xerophytic and resource-conservative species, whilst *Lycium chilense* and *Poa ligularis* are deciduous species grouped as mesophytic and resource-acquisitive species (Campanella and Bertiller 2008). From an ecophysiological point of view, species from the first group show morpho-functional traits typical of drought tolerance mechanisms and species from the second group show drought avoidance mechanisms (Campanella and Bertiller 2008; Cenzano et al. 2013, 2014).

In our study, the evergreen shrub *L. divaricata* increased total phenols and flavonoids production in autumn and consequently a major antioxidant capacity along with an increase in lipid peroxidation. *L. chilense*, a deciduous shrub which loses their leaves during autumn and winter, did not show differences in polyphenols production and antioxidant capacity among seasons, but the lipid peroxidation was higher in summer. In comparison with *L. divaricata*, the total phenols content by this species was two times smaller and flavonoids content was ten times smaller. These results were interpreted in the light of the drought avoidance mechanism displayed by the deciduous *L. chilense* which would have no need to waste carbon resources in polyphenols production. On the contrary, *L. divaricata* produces polyphenols as part of its tolerant mechanism to cope against drought.

P. speciosa, an evergreen grass, increased total phenols and flavonoids during autumn and winter in concordance with an enhanced antioxidant capacity and increased lipid peroxidation. *P. ligularis*, a deciduous grass, increased total phenols and flavonoids with high antioxidant capacity in the roots during autumn, accompanied by high lipid peroxidation. These results suggest that phenolic compounds, principally flavonoids, have an important antioxidant role during the dry seasons, especially in the species that maintain their leaves (*L. divaricata* and *P. speciosa*). Additionally, condensed tannins production was higher in roots than in leaves in the four species analyzed during the four seasons suggesting that this kind of molecules may have a structural role combined with an antioxidant role as reported by Ayres et al. (1997) and/or have an allelopathic role limiting growth of others plants, insects attacks, and/or microorganism invasion from the environment (Thelen et al. 2005; Li et al. 2010).

5.2 *Prosopis strombulifera*, a Native Halophyte

Several studies have established that most of the salt tolerance mechanisms used by halophytes are similar to those displayed by glycophytes (Sengupta and Majumder 2010; Bartels and Dinakar 2013). Nonetheless, although the salt tolerance mechanisms are similar, halophytes may either constitutively turn on salt tolerance mechanisms or exhibit changes in their transcriptional and posttranscriptional regulation, which causes large variation in the salt tolerance levels between glycophytes and halophytes (Oh et al. 2010; Kosová et al. 2013).

Halophytes could be defined as plants that are well adapted to live in soils containing a high concentration of salts and benefiting from it; thus, this group of plants represents a very interesting model to understand complex physiological and genetic mechanisms of salt tolerance.

P. strombulifera (Burkart 1976) is a halophytic spiny shrub widely distributed in America and particularly abundant in high-salinity areas of central Argentina. In these soils, proportions of two salts, NaCl and Na_2SO_4, are generally similar, although in previous studies we found that Na_2SO_4 was more abundant than NaCl (up to three times in several soil samples) (Sosa et al. 2005). Similarly, this situation occurs in many countries such as Pakistan, India, Egypt, China, Tunisia, and some regions of United States, where Na_2SO_4 is highly abundant, even more than NaCl in the soils and groundwater of some areas (Bie et al. 2004; Shi and Sheng 2005; Manivannan et al. 2008; Tarchoune et al. 2010; Peterson and Murphy 2015). However, most studies concerning salt tolerance of plants have been focused only in NaCl, and injury symptoms are often ascribed to the toxicity of Na^+ and Cl^- ions. For that reason, it is important to analyze the effects of different salts on plant growth, in order to have a complete view and to understand better the physiological responses of plants in natural environments.

In previous studies using NaCl, Na_2SO_4, and their iso-osmotic mixture, a great variability in the response of *P. strombulifera* plants depending on the type of salt (s) used and the osmotic potential (Ψo) generated in the culture medium was observed. *P. strombulifera* responded to NaCl treatment by increasing their growth up to 500 mM (−1.9 MPa) which is an interesting halophytic response, distinct from findings in other woody *Prosopis* species (Felker 2007). Moreover, our previous studies indicate that the NaCl tolerance of *P. strombulifera* exceeds the limits described for most halophytic plants (Catalán et al. 1994).

However, *P. strombulifera* has different response to Na_2SO_4, being much less tolerant and showing a strong growth inhibition from the beginning of salinization. These plants grown in the presence of Na_2SO_4 showed immediate and sustained reduction of shoot height and leaf number per plant that was accompanied by senescence symptoms such as chlorosis, necrosis, and leaf abscission (Reinoso et al. 2005; Reginato et al. 2012). Furthermore, treatment with Na_2SO_4 induced several structural alterations in cells and tissues and modification of growth patterns

in roots, stems, and leaves that were different to that induced by NaCl (Reinoso et al. 2004, 2005). *P. strombulifera* responds to progressive salt stress by changing leaf development, particularly Na_2SO_4-treated plants, leading to modifications in leaf size and micro-morphology of leaf cells (increase in stomata density and epidermal cell density) (Reginato et al. 2013). These anatomical modifications are consistent with our previous physiological studies which demonstrated that the adaptive success of *P. strombulifera* grown under high NaCl salinity seems to involve: (1) a delicate balance among Na^+ accumulation (and its use for osmotic adjustment) and efficient compartmentation in vacuoles (Reginato et al. 2014a); (2) the ability of the whole plant to maintain a sufficient K^+ supply by a high degree of K^+/Na^+ discrimination; (3) maintenance of normal Ca^{2+} levels in leaves (Reginato et al. 2014a); and (4) osmotic balance and protection by compatible solutes like proline, polyols (Llanes et al. 2013), and polycations such as polyamines under salt stress (Reginato et al. 2012).

Salt-induced damage to cellular membranes due to lipid peroxidation was also studied in *P. strombulifera*. This halophyte showed an important oxidative damage induced in tissues when the SO_4^{-2} anion was present in the medium, showing once again a greater sensitivity to Na_2SO_4 than to NaCl. The significant increase in malondialdehyde (MDA) that resulted from lipid peroxidation under Na_2SO_4 treatment (Reginato et al. 2014b) was correlated with the growth inhibition and several metabolic disorders mentioned above. Also, H_2O_2 concentration was significantly increased in roots of Na_2SO_4 and Na_2SO_4 + NaCl-treated plants at moderate salinity (−1.9 MPa) in correlation with increased lipid peroxidation in the former. Recently, several authors have demonstrated that H_2O_2 accumulation under highly saline concentrations acts as a signal for adaptive responses to stress (Miller et al. 2010). Therefore, a tight control of H_2O_2 concentration is critical for cell homoeostasis.

An interesting observation in our studies is that when SO_4^{-2} and Cl^- anions are both present in the growth medium (in the salt mixture treatment), ionic interactions occur possibly at the membrane level between both anions, causing a partial reversion of the oxidative damage caused by SO_4^{-2} in roots. This response is in agreement with the observation that NaCl + Na_2SO_4-treated plants showed intermediate values between those obtained with mono-saline treatments in growth parameters, compatible solute synthesis, and ion content, as previously demonstrated (Llanes et al. 2013; Reginato et al. 2014a). In regards to photosynthetic pigments, *P. strombulifera* showed a good ability to tolerate elevated NaCl concentrations without changes in chlorophyll levels, while Na_2SO_4 stress significantly reduced chlorophylls with respect to controls.

Carotenoids and vitamins are other typical compounds that exhibit high antioxidant activities besides polyphenols (Chanwitheesuk et al. 2005). Similar to the results reported by Ramani et al. (2006) in the halophytes *Sesuvium portulacastrum* and *Aster tripholium*, carotenoid levels remained unchanged in *P. strombulifera* plants (Reginato et al. 2014b). Carotenoids (β-carotene in particular), in addition to

their role as secondary light-absorbing pigments, are able to reduce the Chl triplet state and to prevent the formation of the harmful singlet oxygen or to scavenge it after its production by the interaction of triplet chlorophyll with O_2 (Ramani et al. 2006). In our experiments, Na_2SO_4-grown plants showed unchanged carotenoid level despite reduction in chlorophyll concentration, resulting in an increased carotenoid/chlorophyll ratio which may represent a strategy to protect photosystems against photooxidation. One of the most effective mechanisms of excess energy dissipation is de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin through the xanthophyll cycle (VAZ) (Demmig-Adams and Adams 1992). Such a protective mechanism seems to be carried out by salinized plants of *P. strombulifera*, principally those grown in the presence of Na_2SO_4 with the maximal de-epoxidation index (DEPS index), indicating the need to alleviate excessive excitation pressure (Reginato et al. 2014b). Concomitantly, these plants showed a remarkable decrease in the maximal photochemical efficiency (Fv/Fm) and electron transport rate at the end of the experiment (Devinar, PhD Thesis). If there were inactive units in photosystem II, there would be great potential for ROS formation.

Several studies showed that halophytes have higher constitutive antioxidant defense activity as compared with glycophytes. Some authors proposed that antioxidant enzymes in halophytes enhance their activity with increasing salt concentrations at which plants are exposed (Bose et al. 2014). In relation to antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD) activities in *P. strombulifera* exhibited differential responses in different organs to different salts. NaCl-treated plants showed an increase in CAT activity in roots under moderate and high salinity (−1.9 MPa, −2.6 MPa). Nevertheless, leaves showed an increase in CAT activity at moderate salinity followed by a marked decrease at high salinity. In Na_2SO_4-treated plants, a gradual decrease in CAT activity in roots was observed, while leaves showed a marked increase at high salinity. In contrast, a strong SOD activity was observed both in leaves and roots of these plants, with activity levels much higher than in NaCl-treated plants. In Na_2SO_4 + NaCl-treated plants, while CAT activity decreased with salinity, SOD activity decreased in roots but increased in leaves. From these results, it seems that CAT would play an important role in early detoxification of H_2O_2 in roots, while SOD would have a predominant role in leaves for anion superoxide detoxification. According to our previous results, we propose that at higher salt concentrations the antioxidant enzymatic activity allows H_2O_2 to act as a stress signal to trigger some adaptive physiological responses such as early suberification and lignification in roots (Reinoso et al. 2004, 2005). The intense activity of SOD in Na_2SO_4-treated plants indicates an effort of plants to counteract the severe oxidative stress caused by this salt (Fig. 2).

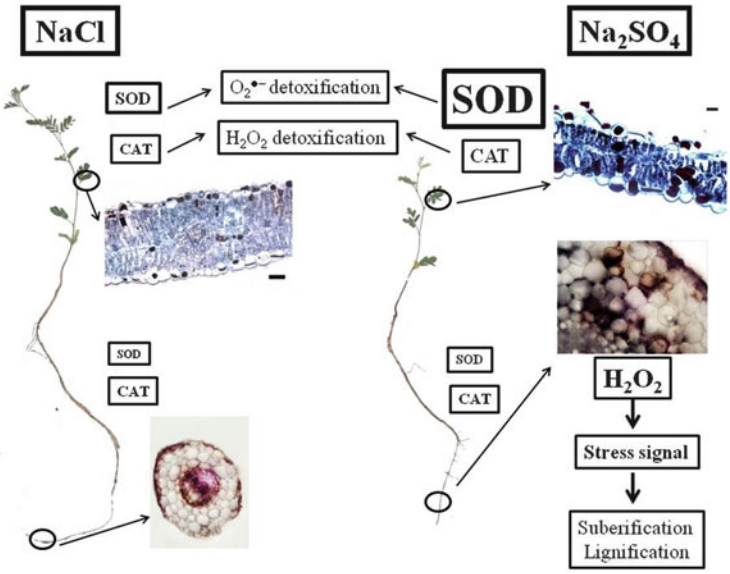

Fig. 2 Effects of NaCl and Na_2SO_4 on H_2O_2 accumulation, SOD and CAT activity, and polyphenol production in *P. strombulifera* plants ($\Psi o = -2.6$ MPa, 48 days). Areas of squares for each enzyme are approx. proportional to their activity. In leaves, cross section of leaflets of 48-days-old plants showing reduction in mesophyll thickness and polyphenols accumulation in Na_2SO_4-treated plants (scale = 50 μm). In roots, cross section indicating the H_2O_2 localization in cells (scale = 50 μm). In Na_2SO_4-treated plants, H_2O_2 can act as a stress signal to trigger some adaptive physiological responses such as early suberification and lignification in roots. In leaves of these plants, the intense accumulation of polyphenols may indicate a role for these compounds in counteracting the strong oxidative damage induced by severe salt stress when other ROS-detoxifying systems are not effective enough

5.2.1 Synthesis of Polyphenols: An Expensive Cost to Survive

As mentioned above, to counteract oxidative stress induced by salinity, plants have developed different strategies including the stimulation of the synthesis of secondary metabolites.

In the halophyte *P. strombulifera*, NaCl treatment did not increase the synthesis of polyphenols, differently from Na_2SO_4 treatment which induced a sharp increase in total polyphenols and consequently, in the antioxidant activity in both leaves and roots. Na_2SO_4 treatment sharply induced an accumulation of flavonoids and flavan-3-ols in leaves (40 % approx.) that accumulated mainly in mesophyll and epidermal cells, as evidenced by microscopical analysis (Reginato et al. 2014b) in coincidence with Tattini et al. (2005) who proposed that the main sites of flavonoid accumulation in plants (including glycosylated forms) are the mesophyll, epidermis, and subepidermis of photosynthetic tissues. A similar increase in flavonoids and flavan-3-ols was observed (30 % approx) in roots. HPLC analysis of extracts from leaves

of these plants showed high levels of different polyphenols as rutin, catechin, epicatechin, and proanthocyanidins.

The increase in total flavonoids and flavan-3-ols when SO_4^{2-} anion is present in the growth solution may indicate a role for these compounds in counteracting the strong oxidative damage induced by severe salt stress. Thus, when other ROS-detoxifying systems such as the xanthophyll cycle are not effective enough as it seems to be the case of Na_2SO_4-treated plants (Reginato et al. 2014b), polyphenols production is increased as an alternative detoxifying system. Accordingly, Agati et al. (2012) reported that antioxidant mesophyll flavonoids, at micromolar range, may effectively avoid reactive oxygen forms generation (e.g., by chelating transition metal ions).

Nevertheless, despite their protective functions, stress-induced increase of secondary metabolites was often counteracted by a corresponding decrease in biomass in several species (Selmar and Kleinwächter 2013). In coincidence with these observations, in *P. strombulifera*, the large increase in total polyphenols found in Na_2SO_4-treated plants was accompanied by a strong growth inhibition. In contrast, in NaCl-treated plants, polyphenols did not increase with salinization and growth was not affected; plants were healthy and without toxicity symptoms. It might be thought that under NaCl treatment, more efficient energy dissipation mechanisms such as the xanthophyll cycle or detoxification of the oxygen radicals by SOD would render unnecessary to invest resources in flavonoid synthesis (Reginato et al. 2014b). These observations lead to the proposal of a fundamental role of polyphenols in the protection of the photosynthetic apparatus under severe oxidative stress when other ROS-detoxifying systems are not sufficient, at the expense of growth.

6 *P. strombulifera* and *Larrea divaricata*: Natural Sources of Antioxidants and Biomolecules

In recent years, polyphenols have had an increasing recognition by the scientific community and by the general public because of their abundance in many fruits, seeds, and vegetables. Numerous epidemiological studies support the evidence that health-promoting effects of certain polyphenols are beneficial to human (World Health Organization 2003; Boeing et al. 2012). For this reason, regular consumption of vegetables and fruits with high polyphenol content is known to be beneficial for human health. It is their capacity to scavenge oxidatively generated free radicals, such as those derived from lipids and nucleic acids, that has been highlighted as the fundamental chemical event that underlies their utility in reducing the risk of certain age related degenerations and diseases.

Plants growing in severe environments as halophytes and xerophytes are well adapted to survive under extreme conditions by means of multiple tolerance mechanisms including synthesis of different compounds which help them grow in

conditions where other plants fail to survive (Khan and Qaiser 2006; Qasim et al. 2011). Several halophytic species are actually being used in folklore medicine, since their extracts have been proved to have activity against human, animal, and plant pathogens (Ksouri et al. 2012). A recent study of the ethno-botanical uses of medicinal plants from coastal areas of Pakistan showed that medicinal plants in this region were 54 % xerophytes and 40 % halophytes (including halophytes and xerohalophytes) and few of them (6 %) were glycophytes (Qasim et al. 2014). There is a growing interest in the use of plants as source of natural antioxidants (principally polyphenolic compounds) for many reasons, such as the replacement of synthetic antioxidants to enhance health and food preservation (Rice-Evans et al. 1996), search for natural healthy substances and additives, and the use of plant-derived therapeutics for cancer prevention and chemotherapy.

Plant polyphenols are one of the most important and extensively used classes of plant-derived therapeutics for cancer prevention and chemotherapy. Experimental evidence suggest that these protective effects could be in part explained by the capacity of plant polyphenols to act as antioxidants scavenging ROS which are involved in damaging mechanisms to DNA. Also, polyphenols can modulate proinflammatory and oncogenic signals acting as anti-invasive cancer agents (Pan et al. 2010; Vauzour et al. 2010).

Moreover, the cosmetic industry is currently exploiting polyphenols extracted from various plant parts to use them in the development of different products that aim to better protect the skin from damages caused by solar radiation and aging. For all these reasons, plant polyphenols can be considered as an important pool of bioactive natural products with potential benefits for human health.

In Argentina, the study of compounds obtained from regional plants is emerging. There are a lot of species for which "folkloric medicine" has described several uses to preserve and aid health (Roig 2002). Only a small number of them have recently been studied in detail to confirm their phytopharmaceutical properties. The halophyte *P. strombulifera* has been frequently used in folk medicine, mainly the fruits, which seem to have different properties as astringent, anti-inflammatory and odontalgic agent, and anti-diarrheic (Ariza Espinar et al. 2006; Ratera and Ratera 1980; Toursarkissian 1980). Recent scientific studies have confirmed part of these ethnopharmacological uses, describing the molecular mechanism involved in the analgesic effect of this plant (Saragusti et al. 2012) and its biological activity against several microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi* (Anesini and Perez 1993; Pérez and Anesini 1994a, b).

More recently, Hapon et al. (2014) reported for the first time the cytotoxic activity of *P. strombulifera* extracts against human tumor cell lines. These authors demonstrated that the crude aqueous extract obtained from leaves of this halophyte can be used without risk, avoiding the unexpected effects observed as a consequence of DNA injuries. For all mentioned above, the halophyte *P. strombulifera* is a promising native plant to obtain natural products and biomolecules with different purposes, for cancer research and treatment and other pharmacological uses.

The xerophytic species *L. divaricata* and *L. tridentata* have also been used in folk medicine by native populations due to the amount of bioactive compounds that

they contain, mostly in their leaves. Traditionally, leaves and twigs were used to prepare tea for treatment of stomach, kidney, or liver diseases and their extract as an ointment for skins or allergic problems (Arteaga et al. 2005). *L. tridentata* and L. *divaricata* are considerable similar species, the first one found in arid zones of North America and the second one in arid zones of South America, mostly in Argentina and Bolivia. Molecular studies have established that both are the same species with disjunctive distribution, even when taxonomically are classified as different (Lia et al. 2001).

L. divaricata is considered a source of natural products with approximately 50 % of the leaves (dry weight) as extractable matter. The resin that covers the leaves has large amount of flavonoids (principally kaemferol and quercetin), aglycones, lignans, sapogenins, essential oils, and nordihydroguaiaretic acid (NDGA) (Argueta 1994; Hyder et al. 2002). NDGA is considered a phenolic lignin with biological activity of interest in health area as antiviral, antifungal, antimicrobial, and antitumorgenic (Hwu et al. 2008). The therapeutic potential for tumors and cancer treatment of this compound has been demonstrated by the inhibition of cancer cell growth via an apoptotic mechanism (Zavodovskaya et al. 2008). Kaemferol and quercetin are the most abundant flavonoids in the resin of *L. divaricata* leaves (Palacio et al. 2012). These two flavonoids exist as a variety of glycosides or in aglycone form and are structurally similar, differing only by one hydroxyl group in the B-ring. Different researches have demonstrated important biochemical effects such as metal chelation and antioxidant properties (Brown et al. 1998; Boots et al. 2008). Interestingly, it has also been demonstrated that the interaction flavonoid–flavonoid enhances the antioxidant activity comparing with the individual activity (Hidalgo et al. 2010).

7 Conclusions and Perspectives

In recent years, a great deal of drought and salt stress research has been conducted, but knowledge about ecophysiology and stress tolerance mechanisms of native plants from Patagonian Monte is very scarce. Similarly, knowledge related with possible functions of secondary metabolites in drought and salinity responses is in its early phase.

Our studies show that native species adapted to semi-arid and saline environments display a variety of tolerance mechanisms to face these stressing conditions. The significant accumulation of phenolic compounds, mainly flavonoids, in tissues of these plants and their powerful antioxidant activity indicate an important role for these compounds in counteracting the oxidative damage induced by drought and severe salt stress. From a biotechnological point of view, the study of different wild species and their ability to produce different polyphenols, many of them of interest in the pharmacological and food industry, may help selecting plants species for the production of this bioactive compounds and knowing the better environmental conditions to enhance their production.

Halophytes and xerophytes are important sources of bioactive compounds, and more attention is needed to carry out detailed chemical and pharmacological evaluations. Such investigations may lead to the discovery of novel bioactive compounds that will help to assess the efficacy of herbal remedies. Particularly, the recent results obtained about biomolecules present in the leaves make *P. strombulifera* a promising plant as source of natural products for cancer research and treatment, as well as other pharmacological uses.

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Reactive Oxygen Species and Plant Disease Resistance

András Künstler, Renáta Bacsó, Yaser Mohamed Hafez, and Lóránt Király

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Abstract Plants may successfully limit or even kill pathogens at least in part by eliciting spatial patterns of ROS production in different parts of invaded plant cells, e.g., the cell wall and plasma membrane. Recent research also suggests a significant contribution to plant disease resistance by ROS-mediated processes in the plant cuticle and intracellular organelles. The role of temporal patterns (i.e., proper timing) of ROS accumulation in eliciting an effective plant disease resistance is also discussed. Essentially, defense against pathogens could be very effective if it is a rapid, symptomless process, eliminating the pathogen in due time and not overusing resources of the plant, a process likely mediated by ROS. On the other hand, a delayed and failed attempt by the host to elicit resistance may result in massively stressed plant tissues and a partial or almost complete loss of control over pathogen invasion. Thus, it seems that when plants encounter pathogens they need to defend themselves simultaneously against biotic and abiotic stresses (i.e., pathogen accumulation and excessive cell/tissue death) by turning on two different types of—partially overlapping—signaling pathways that may function in parallel. Very recent interesting data suggest a pivotal role of autopropagating ROS waves in these signaling processes.

Keywords Plant disease resistance • Reactive oxygen species • Antioxidants • Hypersensitive response • Symptomless resistance • Plant cell death

1 Introduction

1.1 Early Research on the Role of ROS in Plant Disease Resistance

When plants are able to successfully resist pathogenic invaders, the ultimate result is inhibition or killing of these pathogens. In past years, several theories have tried to explain the possible mechanisms of disease resistance in plants, including accumulation of antimicrobial compounds, cell wall reinforcement, localized cell and tissue death (hypersensitive response, HR), reactive oxygen species (ROS), etc. (Király et al. 1972; Goodman et al. 1986; Jones and Dangl 2006; Spoel and Dong 2012). In the last three decades, the role of ROS, primarily superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), has become a pivotal research topic in studies of both animal (human) immunity and plant disease resistance mainly because of two reasons. First, a cause-and-effect relationship between animal phagocytosis and the accumulation of ROS has been demonstrated (Morel et al. 1991; Segal 2008). Second, ROS accumulation has been also associated and functionally linked to numerous plant disease resistance events (Doke 1983a, b; Doke and Ohashi 1988; Ádám et al. 1989; Levine et al. 1994; Baker and Orlandi 1995; Delledonne et al. 2001; Torres et al. 2006; Shang et al. 2010; Torres 2010; Dubiella et al. 2013).

In 1980s, the pioneering discovery of Doke (1983a, b) demonstrated that resistance of potato to an oomycete pathogen (*Phytophthora infestans*) is specifically associated with the generation of superoxide that is rapidly transformed to hydrogen peroxide. Doke and Ohashi (1988) also described a similar phenomenon occurring during resistance of tobacco to a viral pathogen (*Tobacco mosaic virus*). Ádám et al. (1989) were the first to report ROS generation, i.e., the accumulation of superoxide, in plants (tobacco) during resistance responses to bacterial infection. A similar, rapid production of hydrogen peroxide was also observed during an incompatible interaction between another bacterium (*Pseudomonas syringae* pv. *tomato* DC3000) and the plant *Arabidopsis thaliana* (Alvarez et al. 1998).

These early observations set forth a series of studies on ROS production in plants exposed to live pathogens or their elicitors. It has become apparent that ROS in plants are produced during both abiotic and biotic stresses and are key components of plant disease resistance responses.

1.2 The Two Main Lines of Plant Defense to Pathogens and the Oxidative (ROS) Burst

The essence of disease resistance is the recognition of and protection against the foreign (the non-self). During plant–pathogen interactions, the first line of plant defense consists primarily of the pathogen-associated molecular pattern (PAMP) recognition system that has been shown to confer a so-called basal resistance to a wide range of pathogens (Jones and Dangl 2006; Boller and Felix 2009). PAMPs regularly occur in bacteria, fungi, and in several other microbes. Examples of PAMPs include conserved bacterial cell surface structures like lipopolysaccharides, flagellin, peptidoglucans, or fungal cell wall components like glucan or chitin. In plants, PAMPs are recognized by distinct pattern recognition receptors which result in the activation of basal (nonhost) defense responses also termed as PAMP-triggered immunity (PTI). Pathogens use effector proteins to block this first line of plant defense (PTI) leading to virulence (i.e., a successful infection). In this case, the effector is termed as a virulence factor and the pathogen is virulent. When the PAMP system fails to recognize the pathogen as an invader, a second line of plant defense is induced. Plants may produce specific surveillance proteins encoded by resistance (R) genes to recognize effectors (called then avirulence—avr—factors and the pathogens being avirulent) and mount this second line of defense called effector-triggered immunity (ETI).

ROS production in plants (primarily $O_2^{\cdot -}$ and H_2O_2) can be detected both at the first and second line of defense to pathogenic infections (i.e., during PTI and ETI). Pathogens eliciting PTI or ETI (i.e., resistance) typically induce a biphasic oxidative burst (ROS production) in plants, consisting of a rapid but weak and transient first phase, followed by a massive and sustained ROS accumulation during the second phase (Lamb and Dixon 1997; Bolwell et al. 2002; Torres et al. 2006).

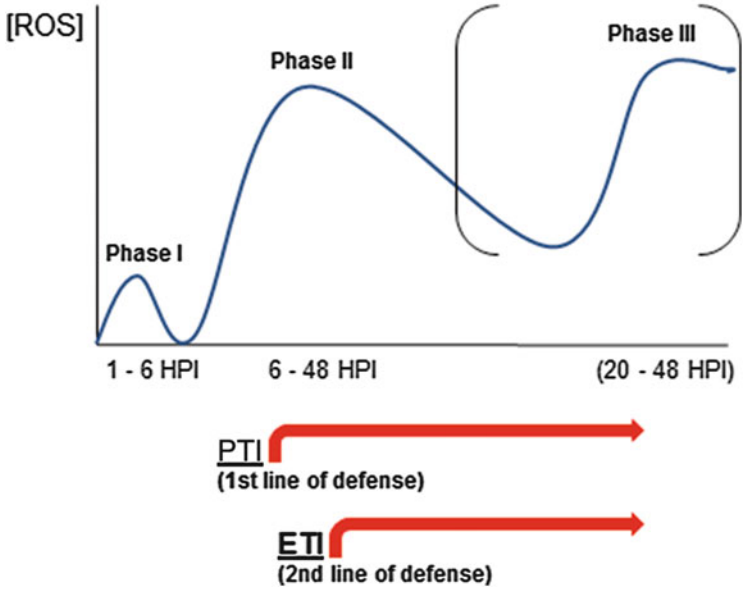

Fig. 1 The different phases of the oxidative (ROS) burst during elicitation of plant disease resistance (defense) by invading pathogens. Relationship of the ROS burst to the two lines of plant defense. *HPI* time (hours) after pathogen inoculation, *PTI* PAMP-triggered immunity, *ETI* effector-triggered immunity. Phase III of the ROS burst occurs only in certain host–pathogen combinations. For further explanations, see the text. Modified after Lamb and Dixon (1997), Hückelhoven and Kogel (2003)

In certain cases, a third phase of the oxidative burst has also been detected during plant disease resistance, e.g., to the powdery mildew pathogen (*Blumeria graminis* f. sp. *hordei*) attacking barley (Hückelhoven and Kogel 2003) and *Septoria tritici* infecting wheat (Shetty et al. 2003) (Fig. 1). These differences could be attributed to the complex development of these fungal pathogens and the influence of the host genotype, which might determine whether two or three phases of the oxidative burst may occur. Interestingly, in case of symbiotic plant–microbe interactions, ROS accumulation has also been observed but the second phase of the oxidative burst seems to be suppressed (Shaw and Long 2003; Lohar et al. 2007). Furthermore, usually only the transient first phase of ROS accumulation has been detected during plant interactions with virulent pathogens that avoid or suppress host recognition (Bolwell et al. 2002). These findings demonstrate that the first phase of the oxidative burst is a biologically nonspecific reaction. On the other hand, the second phase of ROS accumulation seems to play a pivotal role in the establishment of plant defense responses towards pathogens (Levine et al. 1994; Lamb and Dixon 1997).

The oxidative burst following pathogen recognition and initiation of resistance responses is indeed associated with a series of biochemical and pathophysiological events. ROS contribute to the establishment of physical reinforcements of plant cell

walls (papillae) that are formed at the site of pathogen ingress by cross-linking of cell wall glycoproteins (Bradley et al. 1992) or via oxidative cross-linking of precursors during the localized biosynthesis of lignin and suberin polymers (Thordal-Christensen et al. 1997). ROS also have a direct antimicrobial effect in vitro and also in planta (Aver'yanov and Lapikova 1988; Peng and Kuć 1992; Király et al. 1993). Evidence suggests, however, that ROS also have diverse signaling functions that mediate defense gene activation and establishment of additional defenses, e.g., by redox control of transcription factors (Mou et al. 2003) or by interaction with other signaling components like phosphorylation cascades (Kovtun et al. 2000; Dubiella et al. 2013), nitric oxide (Delledonne et al. 1998, 2001; Mur et al. 2006), and the plant hormone salicylic acid (Chen et al. 1993; Torres et al. 2006). ROS also contribute to the production of phytoalexins and other secondary metabolites that may play a role in arresting pathogen growth (Lamb and Dixon 1997; Thoma et al. 2003). Last but not least, ROS development is closely linked to the hypersensitive response (HR), a localization of invading pathogens to their entry site accompanied by programmed plant cell and tissue death (necrosis) (Doke 1983a, b; Doke and Ohashi 1988; Ádám et al. 1989; Levine et al. 1994; Alvarez et al. 1998). The HR not only contributes to the direct limitation of pathogens but is also a source of signals for the establishment of further defenses, both locally and systemically (e.g., priming, local, and systemic acquired resistance) (Chester 1933; Ross 1961a, b; Sticher et al. 1997; Conrath et al. 2001; Fu and Dong 2013; Pastor et al. 2013).

1.3 Expression of ROS-Related Genes and Their Functions in Plant Disease Resistance

As mentioned above, pathogen-induced ROS themselves are considered as signaling molecules. Elevated levels of ROS may be perceived by different receptors, proteins, or enzymes. However, it is not clear exactly how plant cells sense ROS, as specific ROS receptors have not been identified so far. One possibility is that ROS directly inhibit/modify redox-sensitive proteins like phosphatases (Apel and Hirt 2004) and heat shock transcription factors (HSFs; Miller and Mittler 2006). Overexpression of the *Arabidopsis thaliana* gene encoding HSF-A1b confers enhanced resistance to both drought and bacterial infection, and this response is dependent on H_2O_2 signaling (Bechtold et al. 2013). Transcriptome analysis of rice revealed that HSF binding sites were significantly enriched in promoters of up-regulated genes in response to cold, heat, and oxidative stress suggesting a role of HSFs as central regulators of plant stress responses that involve ROS accumulation, e.g., disease resistance (Mittal et al. 2012).

Expression of ROS-related, plant defense-associated genes is likely regulated by ROS through the oxidation of cysteine residues of transcription factors. For example, the zinc finger transcription factor ZAT6 positively regulates resistance to

abiotic stresses as well as resistance to bacterial infection by modulating gene expression related to ROS and salicylic acid (SA), a central component of plant defenses (Shi et al. 2014). Another family of transcription factors (WRKY) includes several members that play regulatory roles during plant disease resistance responses (see Pandey and Somssich 2009). For example, WRKY70 is a key regulator of plant disease resistance and a crossroads between two signaling pathways mediated by SA and jasmonic acid (JA), being induced by the former and inhibited by the latter (Li et al. 2004). Also, WRKY30 is rapidly induced by exposure to several pathogen-associated molecular patterns (PAMPs) and herbicide-induced oxidative stress, suggesting a ROS-dependent role in resistance to pathogens (Scarpeci et al. 2013).

One of the best characterized defense signaling pathways regulated by oxidation events (i.e., ROS accumulation) is the induction of SA-dependent resistance responses with the involvement of two key regulators, the SA receptor NPR1 and TGA transcription factors in *A. thaliana* (Liao et al. 2012; Fu and Dong 2013). In healthy plants, the NPR1 protein is S-nitrosylated at cysteine-156 by S-nitrosoglutathione (GSNO) and sequestered in the cytoplasm as an oligomer formed by disulfide bounds. SA accumulation upon pathogen attack alters the cellular redox state, causing a reduction of disulfide bonds in NPR1 by two thioredoxins, TRX-h3 and TRX-h5 (Tada et al. 2008). Furthermore, oxidation of cysteine residues of TGA transcription factors is also necessary to promote their interaction with NPR1 in the nucleus (Després et al. 2003). In fact, reduction of the NPR1 oligomer releases NPR1 monomers that translocate to the nucleus and interact with the oxidized TGA transcription factors, promoting the induction of defense response genes, primarily the so-called pathogenesis-related (PR) genes (Tada et al. 2008). Interestingly, however, ROS accumulation does not always result in the induction of these SA-dependent defense genes. During symbiotic infections in legume roots, a single phase oxidative burst (see above) suppresses the expression of PR genes/proteins (Peleg-Grossman et al. 2012), pointing to the complexity of ROS-dependent signal transduction in plant–microbe interactions.

PR proteins usually do not accumulate in healthy plants, but are induced by pathogen infection and abiotic stresses (Van Loon et al. 2006). In fact, an early, enhanced induction of PR proteins and their respective genes occurs during incompatible host–pathogen interactions (i.e., resistance), as compared to compatible interactions (Bell et al. 1986; Van Loon et al. 2006), suggesting a role during plant disease resistance. However, although some of these proteins exhibit potential in vitro antimicrobial activities and their accumulation is associated with plant resistance, a direct functional role in defense could not be demonstrated for all PR proteins (Van Loon and Van Strien 1999; Van Loon et al. 2006; Sels et al. 2008 and references within). Nevertheless, some PR proteins have activities that suggest a defense function via ROS generation. For example, PR-9 of tobacco has been shown to be a lignin-forming peroxidase with a likely role in producing H_2O_2 necessary for cell wall reinforcement during pathogen attack (Lagrimini et al. 1987). Also, it has been demonstrated that PR-15 and PR-16 in barley have oxalate oxidase and oxalate oxidase-like (i.e., superoxide dismutase) enzymatic

activities, respectively, resulting in H_2O_2-accumulation during resistance responses to powdery mildew (*B. graminis* f. sp. *hordei*) (Zhang et al. 1995; Wei et al. 1998). Thus, PR genes/proteins are ROS-related not only in the sense of their induction (see above) but some of them directly produce ROS associated with disease resistance.

Probably the most obvious ROS-related genes in plants are those encoding antioxidants. Pathogenic infections and abiotic stresses are frequently associated with ROS accumulation, eventually resulting in oxidative stress. Up-regulation of antioxidants is often the only evidence that oxidative stress has occurred in vivo (Halliwell and Gutteridge 1999). On the other hand, repression of antioxidant genes/enzymes in early stages of HR-type resistance (i.e., during formation of localized necrotic lesions) implies that a programmed, transient decline in antioxidant capacity could also contribute to enhanced ROS accumulation functional in, e.g., defense responses. This is supported by several observations showing that expression of antioxidant genes/enzymes is transiently suppressed during relatively early stages of viral HR. The activity of four antioxidant enzymes (glutathione reductase, glutathione S-transferase, superoxide dismutase, and ascorbate peroxidase) was found to be transiently suppressed within the first 24 h of HR induced by *Tobacco mosaic virus* (TMV) in tobacco (Fodor et al. 1997). Also, Mittler et al. (1998) reported the posttranscriptional suppression of cytosolic ascorbate peroxidase during systemically induced, HR-type cell death in TMV-infected plants. Furthermore, mRNA levels of a tobacco catalase gene (*CAT1*) and total catalase activity are transiently suppressed in HR-type necrotic lesions elicited by TMV and *Tobacco necrosis virus* (TNV) (Dorey et al. 1998; Yi et al. 1999, 2003; Künstler et al. 2007). It was also shown that *CAT1* is repressed only in the vicinity of necrotic lesions, while its expression is high in adjacent, healthy tissues (Dorey et al. 1998; Yi et al. 1999). These findings suggest that temporal and spatial control of antioxidant gene expression at sites of pathogen entry could drive localized, transient ROS accumulation leading not only to programmed plant cell/tissue death but also a significant limitation of pathogen movement and/or replication.

2 Pathogen Limitation in Plant Cells: The Contribution of ROS

2.1 Plant Cell Walls and Their ROS-Mediated Reinforcement: An Initial Barrier to Pathogen Ingress

A first line of spatial defense against pathogens directly penetrating plant cells operates at the plant cell wall (apoplast) (Schulze-Lefert 2004). These can be preformed barriers, determined by a thick cuticle or epicuticular wax layer or the composition and physical properties of the cell wall. Induced formation of cell wall barriers in response to pathogen attack may also occur by reinforcement of the cell wall at attempted penetration sites by deposition of callose and lignification. These

physical reinforcements of the plant cell wall are called papillae or cell wall appositions (Hückelhoven 2007).

ROS production has been shown to be associated with pathogen-induced cell wall reinforcements in several ways. *De novo* synthesized apoplastic class III plant peroxidases mediate ROS-dependent cross-linking of components of the cell wall including glycoproteins, lignin, and suberin (Almagro et al. 2009; O'Brien et al. 2012). Genetic evidence for this function was provided by *Arabidopsis* plants transformed with the antisense sequence of a class III peroxidase of french bean (*FBP1*). These plants showed a down-regulation of two related endogenous peroxidases and reduced levels of H_2O_2 production when challenged with a fungal phytotoxin (fumonisin B1). Furthermore, these plant lines displayed enhanced susceptibility against bacterial (*Pseudomonas syringae* pv. *maculicola* ES4326, *P. syringae* pv. *tomato* DC3000) and fungal (*Botrytis cinerea*, *Golovinomyces orontii*) pathogens (Bindschedler et al. 2006). It was suggested that down-regulation of apoplastic peroxidases might compromise resistance by preventing oxidative cross-linking, which could be effective in case of fungal pathogens that can penetrate plant cell walls. Interestingly, a similar apoplastic peroxidase in pepper ($CaPO_2$) plays a significant role in generating H_2O_2 and effective defense in response to the bacterium *Xanthomonas campestris* pv. *vesicatoria*, a further indication that apoplastic class III plant peroxidases may have additional roles in disease resistance besides contributing to cell wall reinforcement (Choi et al. 2007).

Indeed, it has been demonstrated that peroxidase-dependent H_2O_2 production (visualized by 3,3-diaminobenzidine staining) in barley infected with its powdery mildew pathogen (*Blumeria graminis* f. sp. *hordei*) leads to cross-linking (lignification) and protein immobilization in plant cell walls (Thordal-Christensen et al. 1997). The presence of H_2O_2 in papillae is in fact a biochemical marker of barley resistance to powdery mildew (Hückelhoven et al. 1999, 2000). A general role of H_2O_2 in resistance to pathogen penetration is supported by several observations. Overexpression of H_2O_2-generating enzymes (peroxidases, glucose oxidase) enhances resistance to penetration by wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) in transiently transformed wheat cells (Schweizer et al. 1999). Furthermore, enzymatic removal of H_2O_2 by catalase enhances fungal penetration of leaf epidermal cells in three different plant–fungus interactions (Mellersh et al. 2002). Interestingly, a catalase (CatB) secreted by *B. graminis* f. sp. *hordei* at penetration sites is involved in scavenging H_2O_2, demonstrating that a pathogen may actively interfere with penetration resistance (Zhang et al. 2004). In a similar experiment, it was shown that suppression of the oxidative burst in corn is facilitated by Pep1, a key virulence effector of the corn smut fungus *Ustilago maydis*. Pep1 inhibits the apoplastic oxidative burst by a direct interaction with, and inhibition of class III peroxidases (Hemetsberger et al. 2012). Another role of H_2O_2 in reinforcing the plant cell wall during pathogen attack besides lignification is the contribution to protein immobilization by cross-linking of proline-rich glycoproteins, thus conferring enhanced resistance to fungal cell wall-degrading enzymes (Bradley et al. 1992; Brisson et al. 1994). This process, together with lignification, may effectively entrap fungal penetration structures in a papilla.

Interestingly, research evidence suggests that H_2O_2 may also play a role in pathogen limitation upstream of papillae since compounds of papillae are oxidatively cross-linked on the way to the site of deposition. For example, Collins et al. (2003) showed that H_2O_2 was associated with vesicles containing cell wall components which were in transit to papillae. Similarly, An et al. (2006) demonstrated that multivesicular bodies, which might participate in the secretion of building blocks of papillae, are associated with H_2O_2 accumulation. Another interesting role of papillae besides arresting fungal penetration is the blockage of all plasmodesmata between intact cells and those undergoing localized necrosis during an HR, thereby containing hypersensitive cell death (Shetty et al. 2008).

A further remarkable role of ROS production in the first line of spatial defense against pathogens is the association of ROS with defects in the plant cuticle. A physical barrier to pathogen ingress that covers the outer surface of aerial plant parts, the cuticle consists mainly of the polyester cutin, polysaccharides, and waxes and is closely linked to the cell wall of underlying epidermal cells (Serrano et al. 2014). It is known that strong resistance to the infection of aggressive, virulent fungi like *Botrytis cinerea* can be observed both in wounded plants and in plants with cuticular defects (Chassot et al. 2008). L'Haridon et al. (2011) have observed that the production of ROS ($O_2^{\cdot -}$ and H_2O_2) and a permeable cuticle is common to all these situations. For example, the *A. thaliana* cuticular mutants, *bdg* and *lacs2*, constitutively produce ROS as shown by fluorescence assays and display enhanced resistance to *B. cinerea* (L'Haridon et al. 2011; Serrano et al. 2014). However, treatment of wild type leaf surfaces with fungal cutinase also results in ROS accumulation and resistance. It is possible that cutin monomers accumulating in mutants with a defective cuticle are perceived by the plant as a danger signal (danger-associated molecular pattern, DAMP) leading to the production of ROS and induction of further defense responses and resistance. In case of fungal pathogens that penetrate plant cell walls, this illustrates why plants that are in fact permanently exposed to external pathogen elicitors do not constantly induce resistance responses; this only takes place once the cuticle has been permeabilized, for example, after a successful pathogen attack.

From the above discussed data, it seems evident that ROS production associated with plant cell walls and the cuticle govern diverse pathophysiological processes that ultimately lead to the inhibition of microbial invaders in the initial stages of pathogenesis.

2.2 *Plant Stomatal Immunity: A Barrier to Pathogen Ingress Through Natural Openings is Mediated by ROS*

Openings in the epidermal layer of terrestrial plants are called stomata. These pores are surrounded by two guard cells regulating opening and closure in order to establish gas exchange between the leaf and the environment. This regulation

system allows the control of transpiration. For example, during drought stress, the regulation of anion channels in guard cells is coordinated by abscisic acid (ABA). In fact, in *A. thaliana* the perception of ABA activates the guard cell-specific, ABA-related protein kinase OST1 (also called SNF1-RELATED KINASE 2.6) followed by ROS production and activation of Ca^{2+}-signaling (Mustilli et al. 2002). This results in activation of anion channels and K^+ efflux both through Ca^{2+}-independent and Ca^{2+}-dependent pathways, and a decrease in guard cell turgor leading to stomatal closure (Sawinski et al. 2013). Interestingly, stomatal closure is also tightly controlled during plant resistance responses, and it may have a crucial role in prevention of pathogen invasion. This phenomenon is referred to as stomatal immunity or stomatal defense response and functions as a physical barrier against the infection of pathogens preferring stomatal entry, e.g., bacteria (Melotto et al. 2008; Sawinski et al. 2013). An active role of guard cells in plant defense was first demonstrated when inoculation of leaf surfaces with *Pseudomonas syringae* pv. *tomato* DC3000 was found to decrease stomatal aperture within 1 to 2 h after inoculation (Melotto et al. 2006).

However, the guard cell response to biotic stress seems to differ from the response to abiotic stress in several aspects. First, pathogen-induced stomatal closure primarily depends on the elicitation of basal resistance, i.e., perception of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR) in guard cells (Sawinski et al. 2013). For instance, FLS2, the PRR detecting the PAMP flg22 (a conserved peptide of the bacterial flagellum), is indeed expressed in guard cells. In fact, mutant *fls2 Arabidopsis* plants are impaired in stomatal closure in response to flg22 and show increased susceptibility to *P. syringae* pv. *tomato* DC3000 when sprayed onto the leaf surface—as opposed to infiltration to the leaf intercellular space (Zipfel et al. 2004; Zeng and He 2010). Second, although ROS function in both ABA- and pathogen-induced stomatal closure, ROS production in response to PAMPs and pathogens seem to rely on RBOHD, a NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase in plant cell membranes with a pivotal role in the generation of $O_2^{\cdot -}$ during plant disease resistance (discussed below), as opposed to the role of RBOHF during ABA-induced stomatal closure (Mersmann et al. 2010; Sawinski et al. 2013). Indeed, flg22- and elf18-dependent stomatal closure is abolished in the *rbohD* mutant of *Arabidopsis* (Mersmann et al. 2010; Macho et al. 2012). Third, mitogen-activated protein kinases like MPK3 and MPK6 are activated in *Arabidopsis* by flg22 downstream of ROS production, and these kinases are also highly responsive to drought stress (Kovtun et al. 2000; Liu et al. 2010b; Tsugama et al. 2012). Although this suggests an overlap in pathogen- and ABA-activated kinase signaling pathways, guard cell-specific *mpk3* antisense lines are affected in closing stomata when exposed to bacteria or the PAMP LPS but not ABA (Gudesblat et al. 2009), suggesting a specific requirement for MPK3 in pathogen-induced stomatal closure.

Based on the above data, it was suggested that stomatal closure during pathogen defense is controlled by an ABA-independent signaling pathway distinctly different from that activated during abiotic stress (Montillet et al. 2013). A stomatal defense

response is induced upon perception of PAMPs like flg22 resulting in pathogen-induced oxidative stress (ROS production, oxidative burst) and induction of various defenses including activation of MPK3 and MPK6. These kinases would induce a guard cell-specific lipoxygenase, LOX1, that is required for signaling and the peroxidation of poly unsaturated fatty acids to oxylipins like *cis*-OPDA. Montillet and coworkers (2013) found that in guard cells of *Arabidopsis*, the accumulation of *cis*-OPDA in response to flg22-elicitation is followed by an increase in levels of salicylic acid (SA), a hormone playing a central role in regulating plant defenses. Finally, downstream of SA accumulation, the activation of the anion channel SLAC1 is required for stomatal closure to occur.

In summary, it seems that ROS play an instrumental role in mediating plant resistance at natural openings (stomata) to the infection of pathogens that prefer this mode of host entry, e.g., bacteria.

2.3 *Pathogen Limitation by ROS at the Plasma Membrane: A Possible Role of NADPH Oxidases*

Early research by Doke and coworkers (Doke 1995; Doke and Miura 1995) showed that an NADPH-dependent $O_2^{\cdot-}$-generating system is present in plasma membrane fractions of late blight-infected potato tubers. Incubation of tuber slices with an incompatible *Phytophthora infestans* race or treatment with pathogen-derived elicitors stimulated NADPH-dependent $O_2^{\cdot-}$-generating activity, while no stimulation of NADPH oxidase activity was detected in membrane fractions from tissues inoculated with a compatible race of the pathogen or from control tissues. The ROS burst could also be observed in elicitor-treated protoplasts (Doke 1983a), indicating that NADPH oxidase-mediated ROS generation does not necessarily require the presence of the cell wall or apoplastic enzymes. $O_2^{\cdot-}$ release was extracellular which is expected, since $O_2^{\cdot-}$ is hardly diffusible across membranes (Doke 1983a; Lamb and Dixon 1997). It has been shown in different experimental systems that this pathogen-elicited, plasma membrane-derived, NADPH-oxidase generated $O_2^{\cdot-}$ is rapidly dismutated to H_2O_2 via enzymatic catalysis by superoxide dismutases (Levine et al. 1994; Tenhaken et al. 1995).

Plasma membrane NADPH oxidases and their corresponding genes in plants have been discovered on the basis of their sequence similarity (homology) to the mammalian respiratory burst NADPH oxidase subunit gp91phox (Groom et al. 1996; Keller et al. 1998; Torres et al. 1998), hence the abbreviated name of these genes/proteins: RBOH. These NADPH oxidases are closely associated with ROS formation during plant–pathogen interactions (Suzuki et al. 2011; Marino et al. 2012). For example, two RBOH genes have been described in *Nicotiana benthamiana*: *NbRBOHA* being expressed constitutively at low levels and *NbRBOHB* induced by the elicitor INF1 from *Phytophthora infestans*. Transient silencing of these genes resulted in a reduction in ROS accumulation and

INF1-induced cell death, along with a loss of resistance to *P. infestans* (Yoshioka et al. 2003; Asai et al. 2008). In general, the absence of expression of NADPH oxidase (RBOH) genes confers enhanced susceptibility to biotrophic pathogens (preferring live host tissues, e.g., powdery mildews), as shown, e.g., in the pathosystems *Arabidopsis thaliana/Golovinomyces cichoracearum* (Berrocal-Lobo et al. 2010) and *Hordeum vulgare/Blumeria graminis* f. sp. *hordei* (Proels et al. 2010). This indicates that NADPH oxidase-derived ROS indeed has a role in limiting these pathogens, by inducing pathogen and host cell death (Király et al. 1993; El-Zahaby et al. 2004) and/or promoting cell wall reinforcements in attacked host cells (Proels et al. 2010). On the other hand, infection of NADPH oxidase mutant plants by necrotrophic pathogens often results in enhanced resistance, as demonstrated for *A. thaliana* infected by *Alternaria brassicicola* (Pogány et al. 2009) and *N. benthamiana* attacked by *Botrytis cinerea* (Asai and Yoshioka 2009). Apparently, RBOH-dependent ROS facilitate colonization of host tissues by necrotrophic pathogens by promoting death of attacked host cells (for a review see Barna et al. 2012).

However, the role of particular RBOH genes in pathogen limitation may be different. As mentioned above, the *NbRBOHA* gene of *N. benthamiana* is expressed constitutively, while another RBOH gene in the same host, *NbRBOHB*, is induced only upon elicitor treatment, although both genes are required for elevated ROS accumulation and disease resistance. Interestingly, a dual role during infection by *A. brassicicola* was shown for *AtRBOHD*, but not *AtRBOHF*: a functional RBOHD protein triggers death in cells that are damaged by fungal infection but simultaneously inhibits death in neighboring cells (Pogány et al. 2009).

Taken together, accumulated research evidence implies that, during plant–pathogen interactions, the regulatory mechanisms conferred by NADPH oxidases and ROS produced by these enzymes are highly sophisticated and may function not only in limitation of pathogens at the plasma membrane but also in maintenance of the normal physiological state of plant hosts.

2.4 Subcellular Localization of Intracellular ROS and Pathogen Limitation

Bacterial and fungal plant pathogens do not enter the intracellular space of attacked plant cells. In fact, fungi can only penetrate through plant cell walls, but not the plasma membrane, while bacteria multiply only in the intercellular space. However, plant viruses do enter plant cells and are intimately associated with the cytoplasm and cellular organelles during viral pathogenesis (i.e., they are obligate biotrophic pathogens). Nevertheless, limitation of all these pathogens is influenced by ROS-related biochemical processes that occur within plant cells (as opposed to the cell wall and plasma membrane).

2.4.1 Mitochondria

Both earlier and recent research suggests that plant mitochondria may play an important role in host defense responses to biotic stresses. Mitochondrial respiration is one of the most important metabolic processes of plant cells, and a pronounced increase in the respiratory rate of a host can be a marker of resistance during plant–pathogen interactions (Goodman and Novacky 1994; Hanquing et al. 2010 and references within). In mitochondria of higher plants, electrons produced by the respiratory oxidation of NADH (reduced form of nicotinamide adenine dinucleotide) can flow through the usual cytochrome respiratory pathway or the alternative respiratory pathway. It is well known that the alternative respiratory pathway is catalyzed by alternative oxidases (AOX), located in the mitochondrial inner membrane and acting as terminal oxidases in the mitochondrial electron transport chain (mtETC). The primary function of AOXs in mitochondria is to prevent overreduction of the cytochrome pathway and the resulting accumulation of ROS during abiotic stresses and pathogen attack (Chivasa and Carr 1998; Maxwell et al. 1999; Robson and Vanlerberghe 2002). In fact, mitochondria are a main source of ROS generation in plant cells contributing to 20–30 % of the cytoplasmic steady-state concentration of H_2O_2 (Hanquing et al. 2010). Therefore, AOX may potentially control ROS levels in the plant cytoplasm. This is supported by observations that inhibition of antioxidant (catalase) activity can enhance AOX mRNA expression. Furthermore, a lack of AOX is accompanied by an increase in cytoplasmic antioxidant defenses (Rizhsky et al. 2002; Mizuno et al. 2005; Amirsadeghi et al. 2006).

Pathogen elicitors and toxins can increase the production of mitochondrial (mt) ROS, suggesting that these organelles are a likely source of ROS in biotic stress, in particular, during the plant defense-related oxidative burst (Bolwell and Wojtaszek 1997; Krause and Durner 2004; Rhoads et al. 2006). Furthermore, defense-related compounds (e.g., salicylic acid and methyl jasmonate) associated with plant disease resistance responses are inhibitors of mtETC, and their application can cause an increase in mtROS (Norman et al. 2004; Zhang and Xing 2008). Indeed, it has been shown recently that salicylic acid may inhibit the cytochrome pathway of mtETC through binding to alpha-ketoglutarate dehydrogenase E2 (α-kGDH E2), a rate limiting enzyme controlling NADH supply for efficient mitochondrial electron transport. This results in limiting systemic infection and accumulation of *Tobacco mosaic virus* (TMV), a process that requires mRNA expression of AOX1a (Liao et al. 2015). The authors suggest that inhibition of α-kGDH E2 could result in elevated ROS generation that activates AOX and virus resistance. Accordingly, *Nicotiana sylvestris* mitochondrial mutants with elevated AOX protein levels display a higher degree of resistance to TMV (Dutilleul et al. 2003). Furthermore, AOX transcript and protein levels increase in tobacco that displays hypersensitive resistance (HR, local necrotic lesions) during TMV infection, but do not change during a compatible tobacco–TMV interaction (Lennon et al. 1997; Chivasa and Carr 1998). On the other hand, an *A. thaliana* mutant

disrupted in stress responses (*dsr1*) exhibits lower mitochondrial ROS production and enhanced susceptibility to bacterial and fungal root pathogens, e.g., *Rhizoctonia solani* AG8 (Gleason et al. 2011). This indicates a potential role of plant mitochondria in resistance to pathogens that do not enter the cytoplasm, e.g., through ROS-mediated signaling. In general, increases in AOX gene and protein expression have been noted in plant resistance responses to viral and other pathogens but not during compatible infections (Chivasa et al. 1997; Lennon et al. 1997; Chivasa and Carr 1998; Lacomme and Roby 1999; Simons et al. 1999; Király et al. 2008). The above-discussed results indicate that increased production of mitochondrial ROS and its control by AOX could be a key factor of plant resistance to certain pathogens. This might be especially the case with viruses that could be in close contact with mitochondria and therefore might be directly inhibited by high concentrations of, e.g., mtROS during HR-type resistance. In fact, loss of HR-type resistance to TMV in tobacco at high temperatures (30 °C) is associated with a down-regulation of AOX mRNA expression and $O_2^{\cdot -}$ levels suggesting that AOX indeed contributes to the regulation of ROS levels during HR in order to prevent excessive plant cell death (Király et al. 2008). Importantly, AOX-mediated ROS regulation during HR-type resistance also involves a transient suppression of AOX gene expression with a likely role in transiently increasing ROS in, e.g., mitochondria to limit viral and bacterial pathogens. The transient nature of ROS increase would ensure the control of plant cell death during an HR-type resistance (Lacomme and Roby 1999; Künstler et al. 2007).

2.4.2 Chloroplasts

In photosynthetic organisms, chloroplasts transform light into reducing power resulting in CO_2 fixation. However, excess reducing power is inevitably produced during photosynthesis, since most plants use only ca. 50 % of the absorbed light energy for this process. Excess reducing power can increase the leakage of electrons from the photosynthetic electron transport chain of chloroplast thylakoid membranes (photosystems/PS/I and II) leading to ROS generation and oxidative damage to the photosynthetic apparatus (Dat et al. 2000). Environmental and pathogen stress may further decrease the CO_2 fixation of photosynthesis, therefore leading to enhanced ROS accumulation in chloroplasts. In fact, the plant defense-related oxidative burst occurs in several cellular locations, including chloroplastic PSI and PSII (Sharma et al. 2012), implying that elevations of chloroplastic ROS may be exploited by plant hosts for successful defense against pathogens, especially viruses that are often associated with chloroplasts. Remarkably, it has been shown that compatible tobamovirus infections facilitate the inhibition of PSII electron transport by disturbing the oxygen-evolving complex (OEC), a potential site of ROS generation (Lehto et al. 2003). The levels of two PSII OEC proteins, PsbP and PsbQ, were lower in plants infected by *Pepper mild mottle virus* (PMMoV), as compared to healthy controls, and virus infection also affected oxygen evolution rates of thylakoid membranes (Rahoutei et al. 2000). Proteomic analyses have

shown specific down-regulation of the PsbP protein not only by PMMoV (Perez-Bueno et al. 2004) but also by CMV (Di Carli et al. 2010) during compatible infections. Interestingly, Balasubramaniam and coworkers (2014) have recently demonstrated that PsbP interacts with the coat protein (CP) of *Alfalfa mosaic virus* (AMV) in the cytoplasm and in high concentrations PsbP may inhibit virus replication, suggesting a function in antiviral defense. The authors suggest that in compatible infections, AMV CP may act as an effector protein by interacting with PsbP to interfere with host defense signaling. AMV CP may sequester the chloroplast-targeted PsbP in the cytoplasm, thereby inhibiting the biogenesis of PSII and the consequent ROS generation and activation of other plant defense responses by chloroplasts. Such a strategy has been suggested for the interaction of chloroplast proteins with the TMV replicase protein and the *Plum pox virus* CI protein (Abbink et al. 2002; Jimenez et al. 2006) and, recently, for the interaction of *Rice stripe virus* SP protein with rice PsbP (Kong et al. 2014). However, modulation of chloroplast-controlled defense responses like ROS generation by pathogen effectors is not a virus-exclusive strategy for evading host defense responses. HopN1, an effector protein of the bacterium *P. syringae* pv. *tomato* DC3000, was shown to localize to the thylakoids of chloroplasts where it bounds the PSII OEC protein PsbQ and promotes its degradation (Rodriguez-Herva et al. 2012). The resulting interruption of PSII activity and suppression of ROS production compromised cell death during HR-type resistance and points to the role of chloroplastic PsbQ as a potential factor of antibacterial defense.

2.4.3 Peroxisomes

Excess reducing power generated in chloroplasts through photosynthesis can be diverted, e.g., to peroxisomes by the export of glycolate. In peroxisomes, glycolate is first metabolized to glyoxylate by glycolate oxidases, a reaction that generates H_2O_2 during a process called photorespiration. Recent evidence suggests that peroxisome-generated H_2O_2 indeed has a role in defense against virus infections. Mathioudakis et al. (2013) have demonstrated an in planta interaction between the triple gene block protein 1 (TGBp1) of *Pepino mosaic virus* (PepMV) and tomato catalase 1 (CAT1). The primary function of class 1 catalases is the removal of H_2O_2 produced during photorespiration in leaf peroxisomes (Willekens et al. 1995). Interaction of the viral TGBp1 and tomato CAT1 was observed in nuclei and the cytoplasm suggesting that during a compatible PepMV infection, virus limitation is evaded at least in part by scavenging peroxisomal H_2O_2 (Mathioudakis et al. 2013). Both PepMV infection and in planta overexpression of TGBp1 increased CAT activity and lowered H_2O_2 levels, while silencing of *CAT1* conferred a reduced accumulation of PepMV. The role of peroxisome-generated H_2O_2 in resistance to plant viruses is also supported by an earlier study (Talarczyk et al. 2002). Overexpression of a yeast peroxisomal catalase A1 gene (*CTA1*) in tobacco revealed that the CTA1 protein is indeed localized in peroxisomes of tobacco

cells and likely confers a reduction in resistance to TMV. Interestingly, the size of HR-type (localized) necrotic lesions was significantly larger in the infected leaves of these transgenic plants coupled with lower H_2O_2 levels around lesions. This suggests that peroxisomal H_2O_2 is indeed functional in limiting virus replication and/or movement during HR-type virus resistance, which is also supported by observations that—similarly to AOX (see above)—plant *CAT1* genes and catalase activity are transiently suppressed in TMV-elicited HR-type necrotic lesions (Dorey et al. 1998; Yi et al. 1999, 2003; Künstler et al. 2007). Remarkably, peroxisome-generated H_2O_2 can also significantly limit plant pathogenic bacteria. Chamnongpol et al. (1998) showed that excess H_2O_2 produced in *CAT1* antisense tobacco dramatically suppresses levels of *P. syringae* pv. *syringae* within 24 h following inoculation.

The data discussed above point to a central role of ROS produced by various intracellular organelles (mitochondria, chloroplasts, peroxisomes) in the suppression of pathogens, even in case of bacteria and fungi that do not enter plant cells.

3 Temporal ROS Accumulation and the Efficiency of Pathogen Limitation in Plant Tissues: Timing is Everything?

3.1 ROS Accumulation may Result in Disease Resistance and Plant Cell/Tissue Death During the Hypersensitive Response

Recognition of a pathogen as an invader may result in the elicitation of localized programmed cell death associated with pathogen restriction at the infection site. This phenomenon often culminates in the formation of macroscopically visible localized necrotic lesions (hypersensitive response [HR], see, e.g., in Klement 1982; Goodman and Novacky 1994; Greenberg and Yao 2004). HR is known to occur during both the first and second line of plant defense (i.e., basal/nonhost resistance /PAMP-triggered immunity/ and R gene-mediated resistance /effector-triggered immunity/). Although macroscopic cell and tissue death often seems to be tightly linked to the onset of pathogen resistance, it is possible to uncouple cell death from resistance during certain fungal, bacterial, and viral infections (Király et al. 1972; Mittler et al. 1996; Schiffer et al. 1997; Yu et al. 1998; Bendahmane et al. 1999; Cole et al. 2001; Gassmann 2005; Coll et al. 2010; for a review see Király and Király 2006). However, the existence of independent resistance and cell death responses within an HR does not exclude the possibility that cell death has a role in reinforcing or stimulating the induction of defenses and pathogen localization (Lamb and Dixon 1997; Hatsugai et al. 2004; Greenberg and Yao 2004).

A substantial evidence indicates that the HR is associated with an oxidative burst, i.e., a rapid and localized accumulation of ROS like $O_2^{\cdot -}$, H_2O_2, and the

hydroxyl radical ($OH^{\cdot}$) (Doke 1983a, b; Doke and Ohashi 1988; Ádám et al. 1989; Alvarez et al. 1998; Allan and Fluhr 1997; Lamb and Dixon 1997; Torres et al. 2006). Furthermore, it has been shown that artificial, external ROS treatments are indeed sufficient to induce cell death and plant resistance (an HR). For instance, it was possible to induce HR-type resistance to bacterial and fungal pathogens (powdery mildews and rusts) in originally susceptible plants by external application of 50 mM H_2O_2 or treatments with ROS-generating agents (riboflavin–methionine, xanthine–xanthine oxidase) (Hafez and Király 2003; El-Zahaby et al. 2004). These observations are supported by analysis of transgenic antisense tobacco plants with reduced expression of antioxidant genes (e.g., catalases) that display enhanced HR-type resistance during bacterial infections (Chamnongpol et al. 1998; Takahashi et al. 1997). All of the above cited reports demonstrated that ROS accumulate in resistant hosts that display an HR. However, certain data indicate that in some cases elicitors and pathogens may trigger a strong oxidative burst and associated defense mechanisms without causing an HR (Glazener et al. 1996; Jabs et al. 1997). This indicates that (1) cell and tissue death is not always a necessary component of ROS-induced resistance during an HR and (2) ROS could play other roles during plant disease resistance as direct antimicrobial agents.

3.2 ROS as Antimicrobial Agents in Plants

ROS were first regarded as antimicrobial agents during plant defense responses by demonstrating an in vitro sensitivity of bacterial and fungal pathogens to externally produced $O_2^{\cdot -}$ (Aver'yanov and Lapikova 1988; Tzeng et al. 1990; Jordan et al. 1992; Király et al. 1993; Ouf et al. 1993) and H_2O_2 (Peng and Kuć 1992; Király et al. 1993; Wu et al. 1995; Shetty et al. 2007). For example, micromolar concentrations (25 μM) of H_2O_2 may inhibit spore germination of a number of fungal pathogens in vitro (Peng and Kuć 1992). Also, 100 μM H_2O_2 completely hinders growth of cultured bacteria (*Erwinia carotovora* ssp. *carotovora*) and results in >95 % suppression of the growth of *Phytophthora infestans* (Wu et al. 1995). Interestingly, 5 mM H_2O_2 inhibited fungal development in young (4-day-old) *Septoria tritici* cultures while a much higher concentration (50 mM H_2O_2) was required to inhibit growth in older (16-day-old) cultures, reflecting the differing ability of a pathogen to tolerate H_2O_2 during different stages of its life cycle (Shetty et al. 2007).

The hypothesis that pathogens are also sensitive to ROS produced during infection of their plant hosts was first suggested and tested by Aver'yanov and Lapikova (1988) and Jordan et al. (1992). The former authors demonstrated that in rice, the toxicity of leaf diffusates towards the fungal pathogen *Pyricularia oryzae* is mediated by ROS, while the latter group showed that potato (*Solanum tuberosum* cv. Kennebec) pretreated with an $O_2^{\cdot -}$-generating riboflavin–methionine mixture develops fewer diseased leaves in response to *Phytophthora infestans*. Accordingly, high, sustained levels of ROS (H_2O_2) achieved by transgenic approaches in

different crops also caused resistance to disease symptoms and pathogen multiplication at least in part by direct killing and/or inhibition of plant pathogens. Overexpression of a H_2O_2-producing fungal glucose oxidase gene in potato, tobacco, and cabbage conferred not only a reduction in necrotic disease symptoms in response to bacteria and the oomycete *P. infestans* but, in case of potato infection by *E. carotovora* ssp. *carotovora*, also a suppression of pathogen multiplication (Wu et al. 1995; Lee et al. 2002). Expression of the *BvGLP-1* gene of sugar beet encoding a germin-like protein in *A. thaliana* increased the H_2O_2 content in transgenic plants and conferred resistance of roots to the fungal pathogens *Verticillium longisporum* and *Rhizoctonia solani* (Knecht et al. 2010). Also, transgenic tomato expressing a wheat oxalate oxidase displayed oxalate oxidase activity (H_2O_2-generation) and reduced symptoms after inoculation with *Botrytis cinerea* (Walz et al. 2008). It must be noted here that oxalate (oxalic acid) is a major pathogenicity factor of necrotrophic fungi like *B. cinerea* and a suppressor of ROS (Cessna et al. 2000). Therefore, elevated oxalate oxidase expression in these tomato plants is not only an extra source of in planta ROS generation but ensures normal ROS production in the plant host by eliminating the ROS-suppressor activity of the attacking pathogen in due time. In fact, another necrotrophic fungus (*Sclerotinia sclerotiorum*) also confers an early, oxalic acid-mediated ROS-suppression in the host during initial stages of pathogenesis, as demonstrated by the elegant experiments (real-time GFP redox sensing, histological staining, and reverse fungal genetics) of Williams et al. (2011). Thus, when pathogens, including necrotrophic fungi, are exposed to an oxidative burst early, during the initial phase of infection, their invasion seems to be weakened.

3.3 *Timing is Everything: Early ROS Accumulation Seems to Confer Efficient, Symptomless Disease Resistance in Plants*

The fact that a constitutive overproduction of H_2O_2 in, e.g., transgenic plants confers resistance by killing/limiting pathogens (see above) indeed points to the role of the temporal patterns of plant ROS production in eliciting a successful defense. It was possible to induce resistance to powdery mildew (*B. graminis* f. sp. *hordei* A6) in susceptible barley plants (*Mlo* genotype) by external application of 50 mM H_2O_2 or treatment with ROS-producing agents (Hafez and Király 2003). Symptomless resistance occurred if ROS were administered early (ca. 1 day) after inoculation, while HR developed when ROS application was late (ca. 3 days after inoculation). Interestingly, a barley line expressing the resistance gene *mlo5* (resistant without HR) also developed HR upon late application of ROS, while the line expressing *Mla12* (resistant with HR symptoms) displayed a symptomless resistance upon early ROS application but an enhanced HR (i.e., an earlier development of a higher number of necrotic lesions) following a late ROS exposure. A similar

phenomenon (induction of symptomless resistance following early ROS treatments and HR following later ROS exposure) was also demonstrated to occur during infection by fungal and bacterial pathogens in barley and other plants (El-Zahaby et al. 2004). Importantly, the antimicrobial and cell death-inducing effects of these ROS treatments were completely eliminated by an initial infiltration of antioxidant enzymes (superoxide dismutase and catalase) into leaves.

The above-mentioned studies imply that an early in planta ROS accumulation may efficiently kill/limit pathogens, while a later development of higher ROS levels may only partially hinder pathogen replication and spread, accompanied by death of attacked plant host cells (an HR). Therefore, defense against pathogens could be very effective if it is a rapid, symptomless process, eliminating the pathogen in due time and not overusing resources of the plant. In fact, our preliminary results suggest an important role of early ROS accumulation during symptomless (Type I) non-host resistance (i.e., resistance to pathogens adapted to other host plants, mechanistically the same process as basal resistance /PAMP-triggered immunity). For example, during symptomless non-host resistance of barley to wheat powdery mildew (*B. graminis* f. sp. *tritici*) a rapid, early accumulation of $O_2^{\cdot-}$ is already detectable 1 day after inoculation, much earlier than during HR-type host resistance of barley to its own powdery mildew (*B. graminis* f. sp. *hordei*), as assayed by nitro blue tetrazolium (NBT) tissue staining (Király et al. 2013).

The pivotal role of early ROS production in eliciting plant disease resistance responses also seems likely during plant–virus interactions. We have shown that not only HR-type resistance to TMV but NADPH oxidase-dependent $O_2^{\cdot-}$-generation is also suppressed in tobacco plants carrying the resistance gene *N* and exposed to higher (30 °C) temperatures (Király et al. 2008). However, the early, external application of ROS—2 h after inoculation—confers a symptomless resistance of tobacco otherwise susceptible to TMV, while ROS treatments 3 days after inoculation do not elicit resistance, only HR-like cell and tissue death in the host (Király et al. 2008; Bacsó et al. 2011). In fact, a rapid symptomless plant resistance to virus infections indeed occurs in nature and is termed extreme resistance. This is a very efficient resistance response of plant hosts conferring almost total immunity against viruses like, e.g., *Potato virus X* (PVX), *Soybean mosaic virus* (SMV), or *Turnip crinkle virus* (TCV) (Bendahmane et al. 1999; Cooley et al. 2000; Hajimorad and Hill 2001). Extreme resistance to PVX in potato is conditioned by the *Rx1* and *Rx2* resistance genes (Bendahmane et al. 1999, 2000). Interestingly, during transient expression of the PVX coat protein (the viral avirulence gene product) in *Rx1* plants, the coat protein elicits HR (Bendahmane et al. 1999). This implies that the resistance conditioned by *Rx* genes to PVX occurs so rapidly that the PVX coat protein cannot attain a concentration sufficient to elicit HR. A similar model has been proposed to explain the resistance conferred by the *HRT* gene in *Arabidopsis thaliana* to TCV. *A. thaliana* that carries *HRT* responds to TCV with HR (Cooley et al. 2000). Transgenic plants with moderately elevated *HRT*-expression exhibit a “micro-HR” (i.e., death of individual plant cells), while transgenic *A. thaliana* overexpressing *HRT* to high levels develops no HR upon TCV infection. In fact, the latter plants display extreme resistance to TCV because they respond rapidly to the

virus (Cooley et al. 2000). These studies also suggest that the efficiency of disease resistance depends on the speed of host response: a rapid reaction of the plant host ensures early elimination of the pathogen without any disease symptoms. Although the biochemical mechanism of symptomless (extreme) resistance is unknown, Bendahmane and coworkers (1999) proposed the possible involvement of ROS. Thus, the extremely rapid induction of resistance governed by *Rx* genes would cause an early accumulation of ROS ($O_2^{\cdot -}$, H_2O_2, or perhaps $OH^{\cdot}$ that is considered to be one of the most reactive ROS) and an almost complete inhibition of virus replication in the initially attacked plant cells.

If early ROS accumulation is crucial for plant disease resistance, then the often very intensive in planta ROS production during the advanced stages of pathogenesis might be regarded, at least in part, as a late and failed attempt by the host to limit pathogens. For example, the correlation of high ROS levels with susceptibility to fungal pathogens that are hemibiotrophic (i.e., having an early biotrophic and late necrotrophic phase of pathogenesis) could also be explained by this phenomenon. This is the case with barley infected with *Bipolaris sorokiniana*, where $O_2^{\cdot -}$ can be detected in chloroplasts of cells surrounding necrotic spot blotch lesions (Schäfer et al. 2004). Also, an enhanced accumulation of H_2O_2 in the apoplastic space of systemically infected leaves is a characteristic of apricot cultivars susceptible to *Plum pox virus* (PPV) (Diaz-Vivancos et al. 2006; Hernández et al. 2006). The strong systemic burst of H_2O_2 in these plants that display severe chlorosis might also be attributed to a delayed and failed attempt by the host to elicit resistance in systemic tissues. It is likely that a similar phenomenon occurs in tobacco systemically infected by *Cucumber mosaic virus* (CMV), where Shang and coworkers (2010) have demonstrated that absence of the virus in so-called "dark green islands" of systemically infected leaf tissues correlates well with the presence of $O_2^{\cdot -}$.

From the above, it seems evident that the proper timing of ROS accumulation is a pivotal factor of plant disease resistance. Thus, even during advanced stages of pathogenesis, plant hosts may repeatedly try to limit pathogens by processes that involve ROS accumulation.

4 ROS-Mediated Signaling During Plant Disease Resistance: Regulating Abiotic Stress and Pathogen Levels in Concert

4.1 The Dual, Concentration-Dependent Role of ROS in Plant Disease Resistance

When plants encounter pathogens, a properly timed ROS accumulation seems to contribute to the development of disease resistance, either without any obvious side effects in the host (extreme resistance) or with the concomitant development of controlled and limited cell and tissue death (HR). Consequently, a delayed and

failed attempt by the host to elicit resistance responses would result in massively stressed plant tissues (pathogen-elicited systemic chlorotic/necrotic symptoms) and a partial or almost complete loss of control over pathogen invasion.

Obviously, however, ROS produced by plants in response to pathogenic infections promote not only the direct killing/limitation of pathogens and cell/tissue death in the host but participate in various signaling pathways that elicit (1) further defense responses to pathogens (accumulation of antimicrobial compounds, induction of transcription factors and so-called pathogenesis-related genes, etc.) and (2) defenses to control unwanted cell/tissue death occurring during pathogen invasion (induction of plant antioxidants). Thus, it seems that when plants encounter pathogens they need to defend themselves simultaneously against biotic and abiotic stresses (pathogen accumulation and excessive cell/tissue death) by turning on two different types of—partially overlapping—signaling pathways that may function in parallel. These ROS-related plant signaling pathways have been thoroughly discussed in past years by several excellent reviews (Lamb and Dixon 1997; Dat et al. 2000; Mittler 2002; Torres and Dangl 2005; Hanquing et al. 2010; Barna et al. 2012; Barrios Perez and Brown 2014; Gilroy et al. 2014). Briefly, the main features that qualify ROS as important signaling molecules are (1) a dynamic control of ROS levels through scavenging by antioxidants in a cell autonomous manner, (2) ROS accumulation in different subcellular organelles, resulting in efficient intracellular signaling, (3) the chemical nature (reactivity and short half-life) of ROS confers targeted interaction/modification of proteins, (4) ROS-induced signaling is rapidly propagated from the origin of stimuli to other plant cells (Mittler et al. 2011). Indeed, plants exploit this versatility of ROS when mediating defenses to both abiotic stresses and pathogenic infections.

NADPH oxidase-dependent ROS ($O_2^{\cdot -}$ and H_2O_2) have been shown to have such a dual action, i.e., a participation in defense to both abiotic (excessive cell/tissue death) and biotic (pathogen accumulation) stresses. In fact, low concentrations of H_2O_2 act as a diffusible signal for the induction of antioxidant and pathogenesis-related genes in adjacent plant tissues. However, high concentrations of H_2O_2 are related to the induction of death (necrosis) of plant as well as invading pathogen cells. This was first demonstrated by inoculation of soybean cells with avirulent *P. syringae* pv. *glycinea;* NADPH oxidase-derived H_2O_2 mediated cell death and resistance in bacteria-infected plant cells, while accumulation of transcripts of, e.g., glutathione-S transferase and glutathione peroxidase occurred in adjacent healthy cells separated by dialysis membranes (Levine et al. 1994). These results were later confirmed by analysis of H_2O_2-producing transgenic plants (Wu et al. 1997; Chamnongpol et al. 1998; Vandenabeele et al. 2003). Furthermore, suppression of two NADPH oxidase genes (*AtRBOHD* and *AtRBOHF*) in *lsd1* (lesion simulating disease 1) mutants of *Arabidopsis* that form spontaneous, localized lesions gave unexpected results. Torres et al. (2005) have shown that suppression of these RBOH genes in the *lsd1* mutants leads to plants that develop enhanced, spreading necrotic lesions. The authors speculated that ROS produced by these RBOH genes could limit the spread of cell death around pathogen infection sites by, e.g., activating antioxidant enzymes. Indeed, Pogány et al. (2009) have

convincingly demonstrated that during infection by the fungus *Alternaria brassicicola,* a functional *Arabidopsis* RBOHD protein triggers death in plant cells damaged by fungal infection but simultaneously inhibits death in neighboring plant cells.

The concentration-dependent role of ROS in mediating plant defenses to abiotic stresses and pathogens was further demonstrated by experiments analyzing the effects of exogenously applied H_2O_2. Indeed, an exogenous application of relatively low concentrations (5–20 mM) of H_2O_2 to tobacco leaves and pea seedlings stimulated the antioxidant capacity of plants, increasing thereby tolerance to abiotic stresses and enhancing seedling growth (Gechev et al. 2002; Barba-Espin et al. 2010). We have shown, however, that pretreatment of tobacco with 5 to 10 mM of H_2O_2 also induces a suppression of pathogen-induced necrotic symptoms associated with either HR-type resistance to viruses and bacteria or a compatible infection by a fungus (Hafez et al. 2012). This "immunization" of plant hosts was associated with the induction of transcription and activity of antioxidant enzymes and was shown to operate indeed by the enhancement of plant antioxidant capacity, since artificial, simultaneous application of superoxide dismutase and catalase suppressed necrosis caused by viral, bacterial, or fungal pathogens similarly as H_2O_2 pretreatment. Remarkably, however, pathogen multiplication did not change in "immunized" tobacco plants clearly differentiating this H_2O_2-mediated suppression of pathogen-induced necrosis from plant disease resistance responses (Hafez et al. 2012).

Besides mediating plant defenses to abiotic stresses and pathogens locally, at the site of plant stress, ROS are also involved in the induction of systemic defenses. This means acquired resistance responses to both abiotic stresses (systemic acquired acclimation, SAA) and pathogens (systemic acquired resistance, SAR, and induced systemic resistance, ISR). SAA is induced by usually mild abiotic stresses and confers enhanced resistance to a later exposure of distal tissues to the same or similar (stronger) stresses. SAA is accompanied by H_2O_2 accumulation, systemic redox changes, and the induction of antioxidant defenses (Karpinski et al. 1999; Miller et al. 2009). On the other hand, SAR is typically induced by pathogenic infections resulting in tissue necrosis (Ross 1961b; Sticher et al. 1997), although there are exceptions (see Liu et al. 2010a), while ISR is elicited by nonpathogenic plant growth-promoting rhizobacteria (PGPR) (Pieterse et al. 1996). In both cases, an initial microbial challenge will result in resistance against subsequent challenge by a pathogen in distal plant parts. ROS may be involved in ISR (Barna et al. 2012), while the contribution of ROS to SAR is much more evident. HR-type resistance is often associated with the activation of SAR (Chen et al. 1993; Gaffney et al. 1993; Grant and Lamb 2006). ROS, together with salicylic acid (SA), a central regulator of plant disease resistance, were suggested to play a pivotal role in the establishment of SAR (Durrant and Dong 2004). Specifically, SA potentiates the oxidative burst and facilitates programmed cell death. However, enzymatic, and nonenzymatic antioxidants are also activated during the development of SAR (Fodor et al. 1997) but down-regulated in a transgenic tobacco that fails to develop SAR following virus infection (Király et al. 2002). In fact, these results demonstrate

that the dual role of ROS (concentration-dependent mediation of plant defenses to abiotic stresses and pathogens) is also functional during SAR.

4.2 ROS Waves in Plant Disease Resistance: An Integration of Signaling Pathways

Although ROS involvement in systemic defenses seems obvious, previously, the involvement of ROS molecules traveling as long distance signals during systemic acquired resistance responses (SAA, SAR/ISR) was questioned since most ROS are very sensitive to scavenging enzymes (antioxidants). Remarkably, a new mechanism for rapid, long-distance cell-to-cell signaling during SAA was described that utilizes ROS (Miller et al. 2009; Mittler et al. 2011; Gilroy et al. 2014). Exposure of a particular plant tissue to different abiotic stresses (high light, heat, salinity, cold, mechanical injury) initiates enhanced ROS production in the affected local tissue and triggers a systemic, autopropagating ROS producing wave traveling from the affected tissue to distal plant parts at a rate of up to ca. 8.4 cm min^{-1}. This ROS wave is dependent on the presence of the NADPH oxidase RBOHD and is accompanied by H_2O_2 accumulation in apoplast of cells along the systemic signal path. The autopropagating nature of the signal suggests that each cell along the way activates its own RBOHD and generates ROS that induce all adjacent cells to undergo this same process.

The existence of autopropagating systemic ROS waves seems also likely during elicitation of plant disease resistance responses. One of the first evidences was provided by Park et al. (1998) who demonstrated that the application of a fungal elicitor in potato tubers induced an oxidative burst in distal non-treated parts of the tissue. It was also shown that a primary inoculation of lower leaves with avirulent pathogens induces a secondary oxidative burst in distant upper leaves, leading to low-frequency systemic micro-HRs (single cell HRs) required for the establishment of SAR (Alvarez et al. 1998; Fodor et al. 2001). Operation of a ROS wave functional during plant disease resistance has been recently suggested by Dubiella et al. (2013). A Ca^{2+}-dependent protein kinase 5 (CPK5) in *Arabidopsis thaliana* was activated upon stimulation by pathogen-associated molecular patterns (PAMP) or ROS and phosphorylated (activated) RBOHD in vivo. This CPK5 signaling resulted in elevated SA-mediated resistance to the bacterial pathogen *P. syringae* pv. *tomato* DC3000, enhanced plant defense gene expression in distal plant parts, and ROS synthesis (Dubiella et al. 2013). These results suggest a model of ROS-mediated cell-to-cell communication, where a self-propagating activation circuit (the protein kinase CPK5 and the NADPH oxidase RBOHD) facilitates rapid signal propagation necessary for activation of defenses and pathogen resistance at distal plant sites.

As discussed above, autopropagating ROS waves may participate in eliciting plant defenses to both abiotic stresses and pathogens. In fact these two types of

signaling pathways (conferring inhibition of abiotic and biotic stresses) could significantly overlap, as shown, e.g., by their requirement for NADPH oxidase (RBOHD)-dependent ROS production (Miller et al. 2009; Mittler et al. 2011) and the necessity of proper antioxidant responses during SAR (Fodor et al. 1997; Király et al. 2002). Nevertheless, the fact that plant pretreatments with low concentrations of exogenous ROS cause a reduction of necrotic disease symptoms but not of pathogen levels (Hafez et al. 2012) points to the existence of different types of ROS waves activated in response to abiotic and biotic stresses, respectively. In order to confer an efficient, simultaneous plant defense to pathogen infection and abiotic stress, these independently developed ROS waves should act in concert and be integrated.

5 Conclusions

ROS production associated with plant cell walls and the cuticle may lead to the inhibition of microbial invaders in the initial stages of pathogenesis. As a second line of spatial defense, ROS produced by various intracellular organelles (mitochondria, chloroplasts, peroxisomes) may also play a central role in the suppression of pathogens, even in case of bacteria and fungi that do not enter plant cells.

The proper timing of ROS accumulation is a pivotal factor of plant disease resistance. Thus, a very early and rapid ROS accumulation seems to contribute to the development of symptomless (extreme) resistance, while a moderately early ROS accumulation results in resistance with the concomitant development of controlled and limited cell and tissue death (HR). Accordingly, a delayed and failed attempt by the host to elicit resistance responses would result in massively stressed plant tissues (systemic necrosis) and a partial or almost complete loss of control over pathogen invasion. Thus, it seems that when plants encounter pathogens they need to defend themselves simultaneously against biotic and abiotic stresses (pathogen accumulation and excessive cell/tissue death) by turning on two different types of—partially overlapping—signaling pathways that may function in parallel.

From the above, it seems logical that autopropagating ROS waves are required for eliciting plant defenses to both abiotic stresses and pathogens. It is likely that these two signaling pathways are significantly overlapping, as shown by their requirement for NADPH oxidase (RBOHD)-dependent ROS production (Miller et al. 2009; Mittler et al. 2011) and the necessity of proper antioxidant responses during SAR (Fodor et al. 1997; Király et al. 2002). On the other hand, the fact that low concentrations of exogenous ROS confer a reduction of necrotic disease symptoms but not of pathogen levels (Hafez et al. 2012) suggests that different types of independent ROS waves could also be activated in response to abiotic and biotic stresses. Accordingly, these ROS waves should act in concert and be integrated to confer efficient defense to pathogen infection with the simultaneous control of abiotic stress.

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Modulation of the Ascorbate–Glutathione Cycle Antioxidant Capacity by Posttranslational Modifications Mediated by Nitric Oxide in Abiotic Stress Situations

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Abstract Environmental stresses cause a rapid burst of second messengers belonging to reactive oxygen (ROS) and nitrogen (RNS) species, mainly hydrogen peroxide (H_2O_2) and nitric oxide (NO), respectively. H_2O_2 can act as a signal molecule or become toxic at high levels. Plants have developed different antioxidant tools, such as the ascorbate–glutathione (Asa–GSH) cycle, a key antioxidant system involved in the finely tuned regulation of H_2O_2 in cells, in order to control H_2O_2 overproduction. In recent years, a growing body of evidence points to the existence of a link between NO and physiological and stress responses in plants.

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NO activity is mainly conveyed through posttranslational modifications (PTMs) such as S-nitrosylation and/or tyrosine nitration. Over the last 10 years, the number of S-nitrosylated and nitrated proteins subjected to physiological and a stress condition has been observed to increase significantly in higher plants, suggesting that NO-PTMs are involved in plant physiology. Emerging evidence shows that ROS and NO interact during plant responses to (a)biotic stress, and proteins linked to ROS metabolism have been reported to be regulated by NO-related PTMs. Furthermore, using proteomic analytical techniques, enzymes involved in the Asa–GSH cycle have been identified as NO targets. However, little information exists on the specific impact of NO-PTMs on the structure and activity of these antioxidant enzymes. In this chapter, we will discuss recent findings concerning the regulation of the Asa–GSH cycle antioxidant capacity by NO-PTMs, particularly in relation to the role played by the NO target residues identified under stress conditions.

Keywords Ascorbate–glutathione cycle • Nitric oxide • Tyrosine nitration • S-nitrosylation • Abiotic stress

1 Introduction

In plants, (a)biotic stress situations trigger signaling pathways that produce secondary messengers, the most important being hydrogen peroxide (H_2O_2) and nitric oxide (NO). A growing body of evidence suggests a link between both pathways through a regulation of the antioxidant systems by NO, including the ascorbate–glutathione cycle (Asa–GSH) (Corpas et al. 2011; Groβ et al. 2013; Yu et al. 2014).

The Asa–GSH cycle is a powerful antioxidant system, essential for the detoxification and regulation of hydrogen peroxide in plant cells (Asada 1992; Noctor and Foyer 1998). This cycle is composed of monodehydroascorbate reductase (MDAR), glutathione reductase (GR), ascorbate peroxidase (APX), and dehydroascorbate reductase (DHAR) in addition to the antioxidant metabolites ascorbate and glutathione, and NADPH as a key electron donor for the oxidoreductase catalyzed reactions taking place in this cycle. In this system, H_2O_2 is reduced to water by APX, using ascorbate as the electron donor and generating monodehydroascorbate (MDA). Having a relatively short lifetime, if MDA is not rapidly reduced, it disproportionates to ascorbate and dehydroascorbate (DHA) (Noctor and Foyer 1998). MDA can also be reduced to ascorbate by MDAR using NADPH as the electron donor. Furthermore, DHA is reduced by DHAR and regenerates ascorbate using GSH as a reducing substrate. This reaction generates oxidized glutathione (GSSG) which is finally reduced by GR with the aid of NADPH as electron donor. Thus, GR is involved in maintaining intracellular levels of GSH. At the subcellular level, these enzymes have been demonstrated to be located in cellular compartments such as the cytosol, chloroplasts, peroxisomes,

and mitochondria (Asada 2006; Foyer and Halliwell 1976; Groden and Beck 1979; Jimenez et al. 1998; Romero-Puertas et al. 2006; Reumann and Corpas 2010). The enzymes of the Asa–GSH cycle enhance plant tolerance under various stress situations (Diaz-Vivancos et al. 2013; Liu et al. 2014; Zhang et al. 2013, 2015).

Nitric oxide (NO) has emerged as an essential signaling molecule associated with the modulation of physiological processes (Shapiro 2005; del Río et al. 2004; Lamattina et al. 2003) and plant responses to (a)biotic stress conditions (see Corpas et al. 2011; Siddiqui et al. 2011). NO is a lipophilic-free radical-type molecule with an unpaired electron in its outer orbital, which facilitates its diffusion across cell membranes, and also reacts with macromolecules such as proteins, lipids, and nucleic acids. NO belongs to a family of related molecules known as reactive nitrogen species (RNS) capable of directly or indirectly mediating posttranslational modifications (PTM) such as protein S-nitrosylation and tyrosine nitration. These NO-PTMs may be involved in cell signaling under physiological and stress conditions (Corpas et al. 2011).

As mentioned above, the close relationship between NO and the Asa–GSH cycle in plants has been studied in recent years. Endogenous and exogenous NO has been reported to modulate the function of antioxidant plant systems, such as Asc–GSH cycle enzymes, in stress situations (see Groβ et al. 2013). In addition, proteomic studies have identified certain enzymatic components of the ascorbate–glutathione cycle as potential PTM targets mediated by NO-derived molecules (Reumann et al. 2007). However, little is known about the specific impact of NO-related PTMs on the activity and structure of proteins involved in antioxidative systems (Holtgrefe et al. 2008; Chaki et al. 2011a; Astier and Lindermayr 2012; Begara-Morales et al. 2013a, b).

This chapter presents an overview of the state of the art concerning the modulation by NO of the Asa–GSH cycle's antioxidant capacity, particularly in relation to the physiological significance of NO-PTMs and their role in plant responses to stress situations.

2 S-Nitrosylation and Tyrosine Nitration Under Stress Conditions

As mentioned above, NO exerts its biological function mainly through posttranslational modifications such as tyrosine nitration and S-nitrosylation. Tyrosine nitration adds a nitro group ($-NO_2$) to one of two equivalent ortho-carbons of the tyrosine residue aromatic ring that generates 3-nitrotyrosine (NO_2-Tyr) (Gow et al. 2004; Radi 2004). This converts tyrosine into a negatively charged hydrophilic nitrotyrosine moiety and causes a marked shift in the hydroxyl group's local pK_a from 10.1 in tyrosine to 7.5 in nitrotyrosine (Turko and Murad 2002; Abello et al. 2009). In recent years, a growing body of evidence has shown that tyrosine nitration occurs under physiological conditions and/or in response to abiotic and

biotic stress situations regulating key processes in plants (see Corpas et al. 2015). This PTM is an irreversible process which can lead to a loss, gain, or no change in the protein's function (Souza et al. 2008; Radi 2013), although up to now, most studies have shown that nitration usually causes function loss (Álvarez et al. 2011; Chaki et al. 2011a, 2013; Begara-Morales et al. 2013a; Corpas et al. 2013a, b).

On the other hand, S-nitrosylation is a reversible process which adds a NO group to a specific cysteine thiol in the target protein, giving rise to *S*-nitrosothiols (SNOs), which can alter the function of a wide range of proteins (Astier et al. 2011). In recent years, the *S*-nitrosothiol metabolism has become an important issue, with considerable evidence suggesting that protein S-nitrosylation may play a key role in plant biology (Belenghi et al. 2007; Romero-Puertas et al. 2007, 2008; Lindermayr and Durner 2009; Astier et al. 2011; Hu et al. 2015). Moreover, the importance of *S*-nitrosothiols in plant responses to both pathogens (Feechan et al. 2005; Rusterucci et al. 2007; Chaki et al. 2009a) and abiotic stresses (Valderrama et al. 2007; Corpas et al. 2008; Airaki et al. 2011a; Chaki et al. 2011a, b) has recently been demonstrated in several plant species. Among SNOs, *S*-nitrosoglutathione (GSNO), formed by the S-nitrosylation of the antioxidant tripeptide glutathione (GSH), is a key low-molecular-weight *S*-nitrosothiol regarded as an endogenous NO reservoir in cells (Gaston et al. 1993; Durner et al. 1999; Leitner et al. 2009; Airaki et al. 2011b). Furthermore, being phloem mobile, GSNO is regarded as a long-distance NO vehicle that is important for redox signaling mechanisms (Malik et al. 2011). GSNO can also generate transnitrosylation reactions in which a NO group is transferred from an *S*-nitrosothiol to a target protein's cysteine thiol group (Astier et al. 2011). GSNO is at present being used to study the S-nitrosylation of proteins in vitro and is associated with plant responses to biotic stress (Feechan et al. 2005; Chaki et al. 2009a). Recently, the number of S-nitrosylated proteins has been demonstrated to increase considerably (Hu et al. 2015). Interestingly, a link between S-nitrosylation and the ROS metabolism has been observed in reports on NADPH oxidase (Yun et al. 2011), catalase (Ortega-Galisteo et al. 2012), and peroxiredoxin IIE (Romero-Puertas et al. 2007) and IIF (Camejo et al. 2015), among others. A connection between NO and the ROS pathway under different physiological and stress conditions has also been observed (Corpas et al. 2011; Groβ et al. 2013; Procházková et al. 2014).

NO can enhance plant tolerance to stress situations by directly regulating the antioxidant capacity of plant tissues involving Asa–GSH cycle enzymes under aluminum (Sun et al. 2014), cadmium (Wang et al. 2015), and drought (Shan et al. 2015) stress conditions, among others (see Corpas et al. 2011; Procházková et al. 2014). However, although many nitrated and S-nitrosylated proteins have been identified in stress situations in plants, little is known about the role of the NO target residues identified or the specific impact of NO-PTMs on the structure and activity of proteins involved in antioxidant systems such as the ascorbate–glutathione cycle whose APX structure and activity have been most fully characterized (see below).

3 Glutathione Reductase (GR) is Unaffected by NO in Pea Plants

Glutathione reductase (GR) is a key enzyme in the cellular redox metabolism due to its ability to reduce oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as a cofactor. Thus, GR enables GSH/GSSG to be maintained at high levels which is very important as GSH is considered to be the most abundant soluble antioxidant in plants. GR, with isoenzymes located in different compartments (Edwards et al. 1990; Romero-Puertas et al. 2006; Wu et al. 2013), has been reported to be involved in maintaining and regenerating reduced glutathione (GSH) in response to biotic and abiotic stress in plants (Creissen et al. 1992; Foyer and Noctor 2011; Leterrier et al. 2012; Gill et al. 2013; Zhang et al. 2015). NO modulation of GR activity in abiotic stress situations is the subject of a previous study (see Groβ et al. 2013) which highlighted the capacity of this enzyme to respond to (a)biotic stress conditions. In addition, a nitroproteomic study of sunflower hypocotyls has identified GR as a target for tyrosine nitration (Chaki et al. 2009b) and for S-nitrosylation in rice (Lin et al. 2012). Human and bovine GR activity has also been reported to be inhibited by tyrosine nitration. This suggests that Tyr106 and Tyr114, located close to the GSSG binding zone, are responsible for the decrease observed in GR activity after treatment with peroxynitrite (Francescutti et al. 1996; Savvides et al. 2002). Furthermore, GSNO inactivates human GR activity through the S-nitrosylation of two key cysteines Cys63 and/or Cys58 (Becker et al. 1995) involved in catalytic GR mechanisms (Francescutti et al. 1996). However, to our knowledge, little information exists on the effect of NO-PTMs on the GR structure in higher plants. In contrast to animals, chloroplastic and cytosolic GR in pea plants is S-nitrosylated and nitrated by GSNO and peroxynitrite, respectively, although these NO-PTMs do not significantly affect recombinant protein activity. This is unusual in higher plants as most studies show that nitration causes function loss in all proteins identified up to now (Astier and Lindermayr 2012; Corpas et al. 2013a). This could be a mechanism for maintaining GSH regeneration in order to sustain the ascorbate–glutathione cycle's resistance to nitro-oxidative cell conditions.

4 Monodehydroascorbate Reductase (MDAR) is Inactivated by NO-Related PTMs

Monodehydroascorbate reductase (MDAR) is a key enzyme in the ascorbate–glutathione cycle involved in the regeneration of the reduced ascorbate. In plants, MDAR has been reported to be localized in subcellular compartments, such as chloroplasts (Hossain et al. 1984), cytosols, mitochondria (Dalton et al. 1993; Jimenez et al. 1997; Mittova et al. 2003), and peroxisomes (Bowdicht and

Donaldson 1990; Leterrier et al. 2005; Lisenbee et al. 2005). MDAR plays a crucial role in plant responses to situations that trigger nitro-oxidative stress. Pea MDAR activity has been reported to increase under high light intensity and cadmium conditions but is reduced by herbicide 2,4-D (Leterrier et al. 2005). In tomato, MDAR activity is increased by salinity (Mittova et al. 2003) and high light intensity (Gechev et al. 2003), by low temperature in rice (Oidaira et al. 2000), and by UV-B radiation in *Arabidopsis* (Kubo et al. 1999). However, in *Arabidopsis*, stresses such as high temperature (30 °C), enhanced light intensity (200 μE m^{-2} s^{-1}), water deficiency (water deprivation for 2d), and low temperatures (5 °C) did not affect MDAR activity (Kubo et al. 1999). As mentioned above, several proteomic studies have identified MDAR as a potential target for both S-nitrosylation and nitration (Lin et al. 2012; Tanou et al. 2012). However, the specific effects of these NO-PTMs on MDAR functions are unclear. Begara-Morales et al. 2015 have shown that S-nitrosylation and tyrosine nitration of MDAR by GSNO and peroxynitrite, respectively, cause a reduction in peroxisomal recombinant MDAR protein activity. With the aid of mass spectrometry, Tyr213, Tyr292, and Tyr345 were identified as nitration targets following peroxynitrite ($ONOO^-$) treatment. In addition, site-directed mutagenesis confirmed that Tyr345 is the primary nitration site responsible for the inactivation of MDAR activity by $ONOO^-$. Localization analysis of these residues in the pea MDAR structure reveals that Tyr345 is found at 3.3 Å of His-313, which is involved in the NADP-binding site.

5 Effect of NO-Related PTMs on Dehydroascorbate Reductase (DHAR)

Dehydroascorbate reductase (DHAR) is the other enzyme in the ascorbate–glutathione cycle involved in the regeneration of reduced ascorbate for which GSH is used as electron donor. Researchers have devoted less attention to this enzyme which, however, can also be modulated in stress situations and in response to NO donors (Groβ et al. 2013). Using proteomic analyses, DHAR has recently been identified as a S-nitrosylation target in potato, demonstrating that S-nitrosylation at Cys20 and Cys147 inactivates DHAR activity (Kato et al. 2013). DHAR has also been reported to be S-nitrosylated in *Arabidopsis* under physiological conditions at Cys 20 (Fares et al. 2011; Puyaubert et al. 2014), although this protein has not been observed to be S-nitrosylated during cold stress (Puyaubert et al. 2014), a condition which increases DHAR activity (Eltelib et al. 2011). Taking these results together, it is possible to speculate that cold stress could cause the denitrosylation of DHAR and thus increase its activity in response to these environmental conditions.

6 Dual Regulation of Ascorbate Peroxidase (APX): Inactivated by Nitration and Enhanced by S-Nitrosylation

APX is one of the key antioxidant enzymes involved in regulating H_2O_2 levels during plant development and adverse stress conditions (Jiménez et al. 1998; Gomez et al. 2004; Palma et al. 2006). Different APX isoenzymes are distributed in subcellular compartments including the cytosol, chloroplasts, peroxisomes, and mitochondria (see Shigeoka et al. 2002). In higher plants, APX is an essential element in the finely tuned regulation mechanism of H_2O_2 during plant development and under environmental stress conditions. NO is able to modulate APX activity through a process of either inactivation (Clark et al. 2000) or activation (Keyster et al. 2011; Lin et al. 2011). With the aid of proteomic analysis, APX has been identified as a potential NO-PTM target through tyrosine nitration in *Arabidopsis* (Lozano-Juste et al. 2011) and *Citrus aurantium* (Tanou et al. 2012) and through S-nitrosylation in *Arabidopsis* (Fares et al. 2011), *Antiaris toxicaria* (Bai et al. 2011), and *Solanum tuberosum* (Kato et al. 2013). In the next section, we will review the effect of tyrosine nitration and S-nitrosylation on the APX structure in different plant species and will suggest that APX is regulated by both NO-PTMs.

6.1 APX Is Inactivated by Nitration of Tyr235

APX has been identified as a tyrosine nitration target (Lozano-Juste et al. 2011; Tanou et al. 2012). However, little information exists on the specific impact of tyrosine nitration on the activity and structure of APX or on the proteins involved in antioxidative systems (Radi 2013). With regard to the effect of tyrosine nitration on the APX structure, a recent study has shown that pea cytosolic APX can be nitrated and inactivated by the peroxynitrite donor SIN-1 (Begara-Morales et al. 2013b). Mass spectrometry shows that tyrosine residues Tyr5 and Tyr235 are nitration targets, with Tyr235 appearing to be the most reliable candidate for causing APX inactivation as this residue is located at the bottom of the catalytic pocket and only 3.6 Å from the heme group (Patterson and Poulos 1995; Jespersen et al. 1997; Mandelman et al. 1998; Begara-Morales et al. 2013b). The addition of the nitro group may therefore disrupt heme group properties and result in a loss of activity. With respect to Tyr5, it has been reported that nitration of this tyrosine, which is conserved in plants, could have physiological consequences, although, given its location, it is difficult to demonstrate that tyrosine nitration affects enzymatic activity or protein structure (Begara-Morales et al. 2013b). Thus, determination of the role played by the nitration of Tyr235 in vivo under physiological conditions and in plant responses to different stress situations could be a useful starting point for future analysis.

6.2 APX Is Enhanced by S-Nitrosylation of Cys32

With the identification of the cysteine(s) and their potential role in different stress situations, information concerning the effect of S-nitrosylation on the APX structure has recently been provided. Correa-Aragunde et al. (2013) have reported that APX1 is S-nitrosylated in vivo and that auxins cause its denitrosylation and partial inhibition in *Arabidopsis* roots. To corroborate these findings, the APX1 recombinant protein was treated with increasing concentrations of a NO donor (CysNO) which showed that S-nitrosylation of APX1 produced a gain-of-function mutation (Correa-Aragunde et al. 2013). This increase in APX activity due to NO has previously been described by Keyster et al. (2011) and Lin et al. (2011). *Arabidopsis* APX1contains five Cys residues, with Cys32 and Cys168 being the most conserved cysteine in plants. Using in silico analysis, it has been suggested that, among the cysteine residues present in the *Arabidopsis* APX1, the Cys168 residue located near the heme group could be the S-nitrosylation target. However, there is no clear evidence to suggest that Cys168 rather than Cys32, located near one of the two ascorbate-binding sites (Correa-Aragunde et al. 2013), is responsible for S-nitrosylation and increased APX activity. Proteomic analysis has also identified Cys32 as an S-nitrosylation target in *Arabidopsis* (Fares et al. 2011). Finally, using *apx1* mutants, the authors suggest that the counterbalance of APX1 S-nitrosylation/denitrosylation mediated by auxins could help to control root development and to determine root architecture (Correa-Aragunde et al. 2013).

In contrast to *Arabidopsis* roots, APX S-nitrosylation during programmed cell death (PCD) induced by H_2O_2 or heat shock (HS) inactivates enzyme activity which is corroborated using partial purified APX and GSNO as NO donor (de Pinto et al. 2013). These findings are in line with the inhibition of APX observed after treatment of cell culture tobacco leaves with GSNO (Clark et al. 2000). It is worth noting that S-nitrosylation has been found to induce cytosolic APX ubiquitination and subsequent proteasomal degradation, suggesting that S-nitrosylation may act as part of a signaling pathway that leads to PCD (de Pinto et al. 2013). Tobacco APX also has five cysteine residues including Cys32 and Cys168 (Correa-Aragunde et al. 2013). Based on the changes observed in the kinetic properties of APX enzymes following S-nitrosylation, it has been suggested that S-nitrosylation of Cys32 could be responsible for the decrease in APX activity as the high-affinity site for ascorbate binding is in adjacent to Cys32 (de Pinto et al. 2013). However, Clark et al. (2000) report that, after treatment with GSNO, tobacco APX enzymes are inhibited by the formation of an iron–nitrosyl complex between NO and the heme group's iron atom. Thus, as Cys168 is located close to the heme group (Correa-Aragunde et al. 2013), it cannot be ruled out that S-nitrosylation of Cys168, and not Cys32, inactivates APX activity.

To determine the way in which APX is affected by NO-derived molecules, another study, using cytosolic APX from pea plants, demonstrated that this antioxidant enzyme is subjected to dual regulation by NO-related PTMs, i.e., inactivated by Tyr235 nitration (see above) and enhanced by S-nitrosylation mediated by

GSNO (Begara-Morales et al. 2013b). The treatment of recombinant APX with increasing concentrations of GSNO causes S-nitrosylation and increased protein activity, while treatment with GSH did not have any effect. In order to evaluate the potential physiological role played by S-nitrosylation under stress conditions, APX activity under salinity stress has been analyzed. The data show that APX activity increases after treatment with 150 mM NaCl, with a concomitant increase in the levels of H_2O_2, malondialdehyde (MDA), NO, and SNOs (Begara-Morales et al. 2013b). In addition, APX was found to be S-nitrosylated in vivo, process that was increased under salinity conditions as a consequence of an increase in both NO and SNOs. Under these circumstances, it has been suggested that a rise in APX activity caused by salinity stress could be partly due to S-nitrosylation where SNOs may alleviate oxidative damage induced by salinity stress. It is worth noting that the sequence of pea APX only contains Cys32, making this cysteine an S-nitrosylation target, which is responsible for the effects, observed under in vivo and in vitro conditions. As mentioned above, Cys32 is close to the heme group's propionate side chain and has been reported to form thiyl radicals through the interaction of APX with H_2O_2 (Kitajima et al. 2008) which would indicate a direct reaction with NO (Martínez-Ruiz and Lamas 2007). Interestingly, Cys32 oxidation causes enzyme deactivation, suggesting that glutathionylation protects the enzyme from irreversible oxidation (Kitajima et al. 2008). Furthermore, it has been hypothesized that Cys32 S-nitrosylation may be protected from deactivation by H_2O_2 oxidation (Begara-Morales et al. 2013b), as a relationship between cysteine oxidation and S-nitrosylation in plants has been observed (Lounifi et al. 2013). In this respect, S-nitrosylation of mouse galectin-2 has recently been reported to prevent oxidative inactivation by hydrogen peroxide (Tamura et al. 2015).

This finding has been corroborated in a very recent study by Yang et al. (2015) who show that S-nitrosylation induces APX activity in *Arabidopsis* seedlings. Cys32 and Cys49 have been identified as S-nitrosylation targets, which, with the mutation of these cysteines, confirm that Cys32 is responsible for the increased activity observed following S-nitrosylation. They also demonstrate that the S-nitrosylation of Cys32 plays a role in plant immunity and plant resistance to oxidative stress. All this data taken together appears to show that S-nitrosylation of Cys32 is involved in plant responses to stress situations and may prevent the oxidation of this cysteine and therefore protect the enzyme from inactivation under adverse conditions.

7 Conclusions

Our knowledge of NO involvement in physiological and stress situations in plants has increased in recent years. It has been established that a link exists between ROS and NO pathways through the control of proteins involved in the ROS metabolism caused by posttranslational modifications associated with NO (NO-PTMs) such as S-nitrosylation and tyrosine nitration. Enzymes in the ascorbate–glutathione cycle,

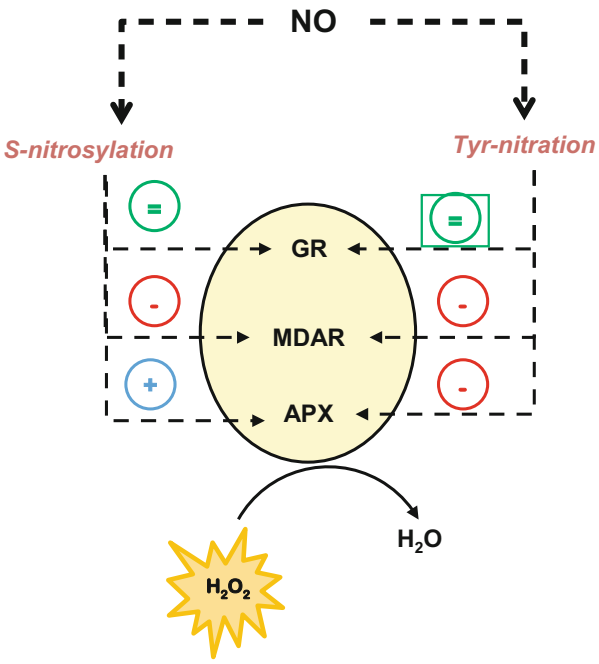

Fig. 1 Regulation of the ascorbate–glutathione cycle by nitric oxide (NO). NO can modulate the antioxidant capacity of the ascorbate–glutathione cycle by posttranslational modifications such as S-nitrosylation and tyrosine nitration. In this sense, during a nitrosative stress situation, tyrosine nitration could inhibit MDAR and APX activity, while S-nitrosylation enhances APX function by S-nitrosylation of Cys32. DHAR is also inactivated by S-nitrosylation. In addition, GR is not affected by these NO-PTMs in an attempt to maintain the cellular redox state under the adverse conditions. *MDAR* monodehydroascorbate reductase, *APX* ascorbate peroxidase, *DHAR* dehydroascorbate reductase, *GR* glutathione reductase

one of the most important H_2O_2-removing antioxidant systems in cells, are regulated by NO. However, little information exists on the impact of NO-PTMs on the structure of these proteins and the role of NO targets in abiotic stress situations. Figure 1 summarizes existing data regarding the effect of NO-PTMs on the ascorbate–glutathione cycle and shows that APX is inactivated by tyrosine nitration and enhanced by S-nitrosylation of Cys32 (dual regulation). MDAR is also inactivated by S-nitrosylation and tyrosine nitration, which may compromise the cycle's antioxidant capacity. However, GR is not affected by any of these modifications in order to maintain GSH levels, and thus, the cellular redox state which could be crucial in nitro-oxidative stress situations. Future research on the identification of endogenous NO targets in the ascorbate–glutathione cycle under (a) biotic stress conditions will be necessary in order to understand how NO modulates this antioxidant system.

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ROS–RNS–Phytohormones Network in Root Response Strategy

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Abstract Roots are considered as a "brain" of a plant, with apical meristems pointed as command centers of an organism. Besides nutrient and water uptake, roots act as organs decisive and responsive for environmental factors. Dynamic reorganization of root growth and architecture in reaction to various stressors is commonly observed. Self-recognition and plants' ability to identify neighbors are manifested by root movement. Root physiology is strictly controlled by diverse agents including phytohormones, reactive oxygen species (ROS), and reactive nitrogen species (RNS). ROS and RNS as molecules of bimodal function are known as cellular messengers crucial for regulation of fundamental physiological processes. Most of them depend on auxins; thus, auxin–ROS–RNS cross talk seems to be a typical pattern in root response to different stimuli. This chapter presents information on ROS and RNS contribution in regulation of root movement, growth, and development, described on the basis of auxin and abscisic acid action.

Keywords Auxin • Growth • Nitric oxide • Root apex • Root movement

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1 Roots as the Administrative Center of Plant Response to Environmental Signals

Some parts of plant studies refer to the controversial idea called "plant intelligence." This aspect of plant physiology, although questionable, is connected with recognition of the root as the most important organ of the plant body responsible for signal perception. The root apical meristem covered by a root cap is considered as a sensory structure, called also as command/decision center (Baluška et al. 2010) (Fig. 1). During evolution, plants were forced by terrestrial environment to develop well-organized root system. The anatomy of the root apical meristem, constantly producing new cells, depends on plant species (evolutionary or environmental changes in structure) and alteration in its organization, e.g., haustorial roots of parasitic plants are frequently observed. Haustorium penetrates the host tissues to absorb nutrients and water (De Tullio et al. 2010). In plant ontogeny, during the process of seed germination, elongation growth of embryonic root is of great importance, linked to breakage of seed coats. This process also terminates seed *sensu stricto* germination and initiates the next phase—development of seedling (Gniazdowska et al. 2010a, b). Root as a soil-localized fast-growing organ of the entire plant body is responsible for water and nutrient uptake (related to development and growth of lateral roots and root hairs) as well as recognition and response to neighbors (other plants or microorganisms) or the environment (abiotic factors of positive or negative character) (De Tullio et al. 2010; Roy and Bassham 2014). A single root is not sufficient to ensure the survival of the entire plant organism; therefore, one plant produces many roots, able to recognize themselves (self-recognition and communication between roots) and able to grow in different directions to accomplish life strategy. As roots of individual plant and/or roots of many plants share the same soil territory, they developed a course of action to produce, secrete, and respond to chemical compounds (signaling molecules) widespread in the environment (plant–plant communication and communication between plants and other organisms). The response and acclimation/adaptation of roots to environmental factors (concentration of nutrients) determine architecture of this organ. Availability of nutrients concentration of nitrogen and/or phosphorous, strongly affects root growth and development, related particularly to auxin regulation (Correa-Aragunde et al. 2004; Sun et al. 2014). High concentration of nitrate ions affects the nitrate sensor—NITRATE TRANSPORTER1·1 (NTR1·1) protein-dependent auxin transport. As a result, higher auxin accumulation in lateral roots initiates and stimulates their growth (Kazan 2013). Moreover, nutrients, nitrogen or phosphorous, are involved in strigolactone production and exudation from the roots, acting as a part of the nutrient acquisition strategy also linked to the modulation of auxin transport (Ruyter-Spira et al. 2011; Sun et al. 2014). In this context, diverse environmental factors bring into alterations in root architecture length of the primary root, density of lateral roots, and root branching.

Motion sensing of roots is regulated by phototropism (negative) and gravitropism (positive). In primary roots, commonly orthogravitropism is observed.

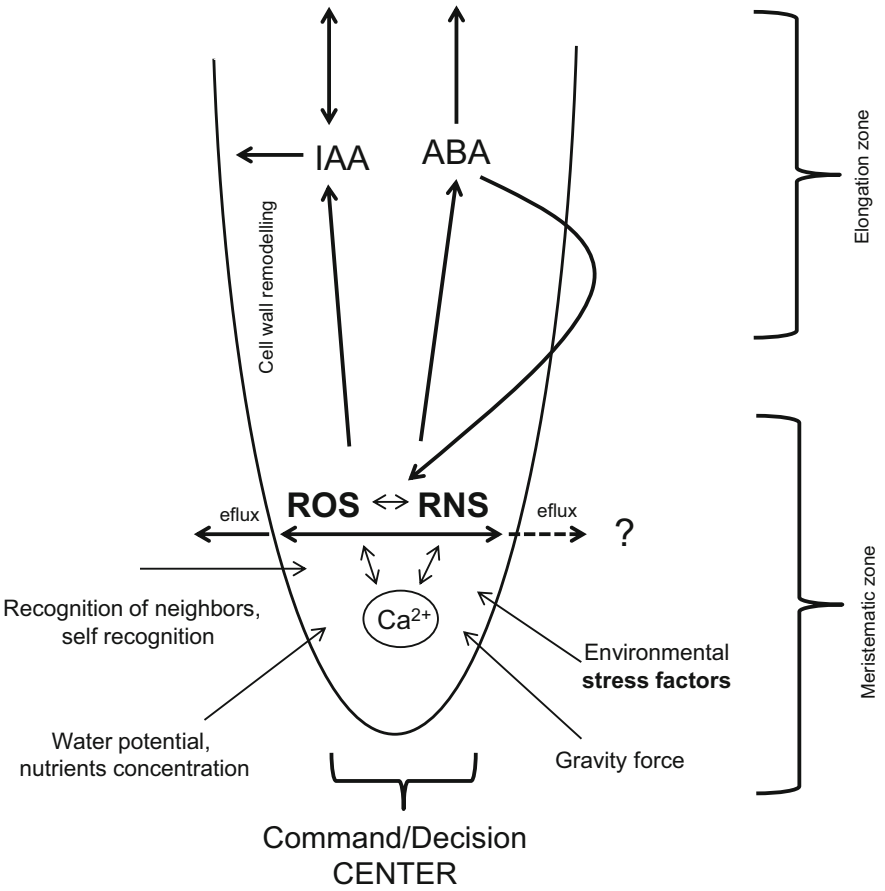

Fig. 1 Schematic view of the root apex. Complex action of ROS and RNS with auxin and Ca^{2+} indicates dependence of root growth on balance between ROS/RNS formation, scavenging, and efflux. Arrows indicate importance of direct and indirect action of ROS/RNS in processes of cell differentiation and elongation in reaction to environmental stimuli

Lateral roots, during maturation process, exhibit temporal variations in the direction of growth. Reorientation of the course of organ growth toward the gravity vector is described as plagiogravitropism (Kuya and Sato 2011). Gravitropic movements of roots strongly impact root architecture, as is associated with asymmetric growth of cells located on opposite sides of the organ. This response starts from seed germination which provides proper orientation of the young plant in the environment. Gravity signal initiates characteristic curvature at the distal elongation zone of the primary root as was shown for germinating seeds of grass pea (*Lathyrus sativus*) (Jiang et al. 2012). Gravitropic response is linked to a root cap, especially to the columella cells, where sedimentation of amyloplasts (gravity susceptors) depends on the gravity vector (Kuya and Sato 2011). Gravitropism is connected with the

auxin polar (cell-to-cell) transport. This process is mediated by different auxin carriers: influx carriers AUXIN RESISTANT1 (AUX1) and LIKE AUX1 (LAX1) and efflux carriers PIN-FORMED (PIN) and ATP-binding cassette type B (ABCB) (Kazan 2013). Among them, especially PINs (particularly PIN2) determine root gravitropic movements. Arabidopsis (*Arabidopsis thaliana*) mutants lacking PIN2 have roots that poorly recognize gravity force and are more sensitive to different environmental stress conditions (Baluška et al. 2010). Moreover, modification of PIN2 content allows roots to avoid stress factors, e.g., salinity. In cells of salt-stressed roots, PIN2 selective degradation correlating to root growth alteration was observed (Li and Zhang 2008). PIN phosphorylation is an important modification that regulates membrane binding. Phosphorylation state is modified by accumulation of reactive oxygen species (ROS) (Bartoli et al. 2013). Root apices are characterized by the presence of two pathways of the polar auxin streams, symmetry of which governs the root growth direction. Any terrestrial factor (gravity, light, humidity) influences this symmetry and forces root apices to initiate the response (tropism) (Baluška et al. 2010). In natural conditions, roots are not exposed to light, as they grow in darkness or shadow/twilight maintained by soil layers. Light exposure alters auxin transport and stimulates reaction of light avoidance called as light-escape tropism (Baluška et al. 2010). Roots are also able to sense (and respond) mechanical stimuli (thigmotropism) (Roy and Bassham 2014). Root movement (reaction) is under auxin control (with indole-3-acetic acid (IAA) accepted as the most important metabolic active form). Concentration of this hormone and its undisturbed polar transport regulate various tropisms as described in detail for Arabidopsis plants (Rashotte et al. 2000; Baluška et al. 2010). Auxins are shoot synthesized, then transported to the root tips (root caps), and later relocated laterally. This IAA stream plays a potent role in root development and branching (Ruyter-Spira et al. 2011). Local auxin's gradient in the root elongation zone is mediated by acropetal transport, but accompanied also by basipetal one. Authors of the idea of "plant neurobiology" proposed that auxins could be considered as plant neurotransmitters (Baluška et al. 2010; Ruyter-Spira et al. 2011).

Root growth is under the control of various signaling molecules (ROS, reactive nitrogen species (RNS), Ca^{2+}), mode of action of which will be explained in more details in the next part of this chapter. Besides auxins, other phytohormones and growth regulators (such as ethylene, abscisic acid (ABA), brassinosteroids, and polyamines) affect growth and physiology of roots (Baluška et al. 2010; Yu et al. 2014). These organs are also liable for perception of variations in local water potential (hydrotropism) although hydrotropic movement is independent of gravitropism (Roy and Bassham 2014). Moreover, temporal water deficit in soil impacts root-to-shoot signaling. Information on water stress is mediated by ABA, phytohormone influencing also root growth and root hydrotropic movement (Roy and Bassham 2014; Tracy et al. 2015). ABA is commonly regarded as inhibitory and a stress-related regulator. The mode of action of ABA, including cross talk with ROS and RNS, is described in detail in guard cells and the regulation of stomata movement in stress response. ABA stimulates activity of NADPH oxidase (known also as respiratory burst oxidase homologues—Rbohs), resulting in activation of

Ca^{2+}-permeable channels, and leads to modification in Ca^{2+} concentration (Bartoli et al. 2013). Nevertheless, during stress conditions (water deficiency), ABA acts as stimulator of root growth (Zhang et al. 2014). This stimulatory effect provides a stress avoidance strategy of root growth. Root and nodule development in *Medicago truncatula* is regulated by a nitrate transporter encoded by the *LATERAL ROOT ORGAN DEFECTIVE/NUMEROUS INFECTIONS AND POLYPHENOLICS* (*LATD/NIP*) gene. Mutation of this gene (*latd*) led to disruption in elongation of primary and lateral roots and formation of nodules (Liang et al. 2007). ABA application preserved viability of *latd* apical root meristem (Liang et al. 2007). ABA (10 μM) treatment of mutants lacking *MtLATD/NIP* resulted in declined *RbohC* expression (Zhang et al. 2014) and modulated expression of *Cu/Zn SOD*. Thus, authors proposed that in the regulation of root elongation, ROS may act downstream of ABA signaling. Experiments carried out on wild-type and ABA-deficient mutant of tomato (*Solanum lycopersicum*) confirmed importance of ABA (at physiological level) in root growth and movements. ABA influenced the number of lateral roots providing soil exploration, especially in compacted soil (Tracy et al. 2015). Involvement of ABA in the regulation of redox potential is connected with ABSCISIC ACID INSENSITIVE4 (ABI4) transcriptional factor required for ascorbate-dependent regulation of plant growth (Kerchev et al. 2011). Modification of the redox potential of Arabidopsis ascorbate *vtc1* and *vtc2* mutants led to alteration of gene expression comparable to *abi4* mutants (Kerchev et al. 2011).

Among plant growth regulators, polyamines (PAs) are known as moderators of diverse physiological processes. PAs are polycationic, low molecular weight compounds with positive charge. Due to their structure, they can bind to the biomacromolecules such as proteins, lipids, or nucleic acids. PAs participate in the regulation of gene expression and stabilization (or destabilization) of proteins and membrane structures and are crucial for acclimation to abiotic stresses (Kusano et al. 2008; Tisi et al. 2011). An indirect impact of spermine (Spm) on root growth occurs *via* products of its catabolism. Polyamine oxidases (PAOs), enzymes catalyzing Spm degradation, produce hydrogen peroxide (H_2O_2), as a coproduct of the reaction. H_2O_2 as other ROS is known to contribute to the regulation of the cell wall integrity (Kusano et al. 2008). However, Spm at mM concentration inhibited root growth of Arabidopsis seedlings. Spm is covalently bound to the hydroxycinnamic acid often conjugated with components of the cell wall (hemicelluloses) and acting in lignin–polysaccharide cross-linking in the wall, leading finally to stiffening of the cell wall. Also, overaccumulation of spermidine (Spd) resulted in the restriction of root growth (Tassoni et al. 2000). In maize seedling treated with Spd, inhibition of elongation growth of cells of primary roots and enhanced accumulation of phenolic compounds was shown (Tisi et al. 2011). Development of a primary root is connected with differentiation of the xylem depending on H_2O_2 concentration. This ROS compound regulates also cell wall maturation observed as generation of cross-linking of polysaccharides and proteins. Enhanced cell wall maturation in primary roots of maize seedlings was accompanied by increase of amine oxidase activity (Tisi et al. 2011).

As newly identified phytohormones, strigolactones (SLs) or their derivatives belong to the family of plant regulators participating in the regulation of root growth (Umehara et al. 2008; Sun et al. 2014). Different environmental and developmental stimuli lead to SLs synthesis in roots, resulting in alterations in root branching and development (Rasmussen et al. 2012). There is also evidence for SL–auxin cross talk. In rice (*Oryza sativa*), SLs produced in Arabidopsis affected root growth by decreasing auxin transport from shoots (Ruyter-Spira et al. 2011; Sun et al. 2014). SLs took part in plant nutrient acquisition strategy, detected in soil deficient in phosphate and nitrate ions (Sun et al. 2014). It has been shown that in roots of Arabidopsis, SLs' mode of action was linked to modification of ascorbate and glutathione concentration, influencing cellular redox state. In contrast, SLs had no effect on the activity of antioxidant enzymes such as catalase (CAT) or superoxide dismutase (SOD) (Bartoli et al. 2013). As was mentioned above, root apical meristem can be evolutionarily modified depending on a plant life strategy. In some parasitic plants, induction of haustorium formation depends on SLs presence (De Tullio et al. 2010).

Ca^{2+} plays not only nutritional but also signaling role as a second messenger in signal transduction pathways (Xiong et al. 2006). It has been indicated that Ca^{2+} wave is part of the systemic communication (from one cell or tissue within the entire plant). Thus, Ca^{2+} wave is spread through the entire plant when local environmental factors are recognized by a root tip (signaling recognition) (Gilroy et al. 2014). Development, growth, and plant responses to various stimuli depend on alteration of Ca^{2+} concentration in cellular compartments (so-called Ca^{2+} signature of the stimulus). Cell wall stability also requires the presence of Ca^{2+}, and gravitropic bending is regulated by redistribution of Ca^{2+} (Xiong et al. 2006; Gilroy et al. 2014). Activity of many proteins depends on Ca^{2+} binding ability, e.g., NADPH oxidase, that is the example of protein–ROS–Ca^{2+} interaction. Moreover, Ca^{2+}-dependent protein kinase 5 (CPK5) plays a beneficial role in the regulation of ROS wave (Causin et al. 2012; Gilroy et al. 2014). On the other hand, ROS impact on Ca^{2+} signaling in root growth is observed. It was reported that radical forms of ROS rather than H_2O_2 activated Ca^{2+}-permeable channels (Foreman et al. 2003).

Root organogenesis is connected with cell division and differentiation and is originated from a stem cell center at the tip. Differentiation of the root cells is initiated by the transition from cellular proliferation to elongation. After elongation, cells differentiate into various cell types in the maturation zone. However, the final volume/dimension of the root meristem depends on ROS availability (Tsukagoshi et al. 2010). The shape of the root differs in mono- and dicotyledon plants. In Arabidopsis, belonging to the dicot plants, root system consists of a primary root and lateral roots. Monocots, e.g., maize (*Zea mays*) or rice roots, have a specific shape, recognized as shoot-borne and lateral roots (Tian et al. 2014). Lateral root primordium is formed from cells of the root pericycle that pass differentiation (Correa-Aragunde et al. 2004). It is suggested that the role of administrative center in roots is linked to the part of the root apex which is defined as the transition zone (TZ). Cells of this zone are characterized by the highest rate of auxin transport. This

allows integration of the sensory–motoric pathways necessary for various root tropisms (Baluška et al. 2010).

2 Reactive Oxygen Species in Root Responses

Aerobic conditions and existence of O_2 molecule as a component of metabolic pathways, especially electron transport chains, are associated with activation of oxygen atoms and generation of ROS (Demidchik 2015). Singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), superoxide radical ($O_2^{\bullet -}$), and H_2O_2 are most frequently studied ROS. Other ROS involved in alterations of the redox potential are peroxyl, alkoxy, and hydroperoxyl radicals. Strong oxidative activity is attributed to peroxynitrite ($ONOO^-$), the molecule belonging to the RNS family. This compound is generated by the reaction of nitric oxide ($^{\bullet}NO$) and $O_2^{\bullet -}$ (Yu et al. 2014; Demidchik 2015; Corpas and Barroso 2014).

It is commonly accepted that ROS are molecules of bimodal function. ROS generated at high levels disturb cell metabolism. Toxicity of ROS relates to their ability to react with a high range of biological molecules: nucleic acids, proteins (including enzymes and transcription factors), lipids, and sugars. Uncontrolled oxidation of cell components leads to oxidative stress. Such pathophysiological conditions accompanied by low activity of antioxidant system, in the long run, cause the death of cells. On the other hand, low concentration of ROS is necessary to maintain metabolism at the accurate level. ROS are known as signaling molecules involved in all physiological processes including immune responses, stomata movements, seed germination, seedling growth, root gravitropic response, and control of root and root hair growth (Foreman et al. 2003; Liszkay et al. 2004; Krasuska and Gniazdowska 2012; Demidchik 2015). ROS induce posttranslational modifications (PTM) of protein structure that could be regarded both as positive and negative. Some of ROS-modified (carbonylated) proteins (or peptides) probably function as organellum-specific signals (inter-organellar communication) (Møller and Sweetlove 2010). These peptides acting as messengers from mitochondria could participate in the control of root growth (Causin et al. 2012). These findings strongly highlight that the entire cell metabolism depends on ROS concentration which must be kept at the optimal hormetic window.

At physiological conditions, ROS homeostasis is maintained by the presence and activity of an antioxidant system: enzymatic and nonenzymatic. Enzymes involved in ROS scavenging (modulation) are various isoforms of superoxide dismutase (SOD); catalase (CAT); peroxidases (POx), especially glutathione peroxidase (GPX) and ascorbate peroxidase (APX); and thioredoxins, glutaredoxins, and peroxiredoxins (Krasuska and Gniazdowska 2012; Demidchik 2015). Nonenzymatic antioxidant system involves reduced form of ascorbic acid (ASA) and glutathione (GSH). PAs, proline, betaine, carotenoides, and α-tocopherol are also included in the group of ROS scavengers (Krasuska and Gniazdowska 2012; Demidchik 2015). GSH is not only an antioxidant but also plays an important role

in protein PTM by glutathionylation, resulting in modification of signaling transduction by alteration of MAP kinase activity or influencing Ca^{2+} concentration/waving (Bartoli et al. 2013). Elongation of root cells is under the control of ASA and dehydroascorbic acid (DHA). Gravitropic response of Arabidopsis roots depends on oxidation of ASA that leads to the formation of DHA in the reaction catalyzed by ASA oxidase (Lee et al. 2011). Moreover, the gravistimulation enhanced the expression of gene coding ASA oxidase (*AAO1*). Expression of this gene was stimulated by application of brassinosteroids and correlated to growth expansion (Lee et al. 2011).

ROS are molecules of a short half-life. It is well documented that various ROS have diverse physiological properties, for example, $O_2^{\bullet -}$ by itself is not able to modify macromolecules, but reacting with H^+ forms highly reactive hydroperoxyl radical ($HO_2^{\bullet -}$). Both compounds in reaction of dismutation generate H_2O_2, molecule of relatively long half-life. Also, mobility of H_2O_2, which is able to pass through membranes *via* aquaporins, makes this compound a good candidate for systemic signaling molecule (Mori and Schroeder 2004). H_2O_2 at low level (below 1 μM) participates in the creation of balanced physiological metabolic state of the cells. On the other hand, at high (mM) level H_2O_2 initiates and points out on oxidative stress acting as one of the markers of oxidative stress (Demidchik 2015). Apoplastic space (cell wall surrounding) is one of the cellular sites of H_2O_2 generation. H_2O_2 can be synthesized also by reactions depending on the activity of NADPH oxidases, extracellular heme-containing Class III peroxidases (POx), PAO, and oxalate oxidase (Tsukagoshi et al. 2010; Demidchik, 2015). In barley (*Hordeum vulgare*) roots, POx were indicated as essential components for initiation of root hair formation (Kwasniewski et al. 2013). Moreover, it was proposed that root hair-specific POx and root hair-specific NADPH oxidase work in a team and are necessary to provide production of $^{\bullet}OH$ in the cell wall of trichoblast required for root hair formation. Treatment of barley roots with inhibitors of POx drastically decreased root ability for the formation of root hairs (Kwasniewski et al. 2013). Moreover, negative impact of H_2O_2 on root hair formation and growth was shown for willow (*Salix nigra*) seedlings; 72 h of H_2O_2 (1 mM) treatment led to complete blockage of root hair formation (Causin et al. 2012). Treatment of Arabidopsis seedlings with H_2O_2 (0.1 mM) for 24 h resulted in decline of root growth and shortened root meristem (Tsukagoshi et al. 2010). Addition of H_2O_2 (30 or 40 mM) into the germination medium of grass pea seeds resulted in further disturbance in gravitropic response of the primary root (Jiang et al. 2012). Prolonged exposure of these roots to H_2O_2 led to root curling, maintenance of horizontal curvature, and finally blocked growth. Removal of ROS donor from root medium resulted after 24 h in the recovery of growth consistent with the action of the gravity vector. Thus, it can be assumed that exogenous H_2O_2 negatively but temporarily and reversibly controls primary root growth (Jiang et al. 2012). Similarly, inhibition of embryonic root growth was observed in apple (*Malus domestica*) seedlings developed from embryos shortly (3 h) pretreated with H_2O_2 (1 mM) just after seed coat removal (Gniazdowska et al. 2010b). Jiang et al. (2012) suggested that restriction in growth and root

horizontal curvature is rather a response to exogenous H_2O_2 (at high level) application than the result of excessive accumulation of this compound. Gravitropic bending of the roots is dependent on different endogenous H_2O_2 concentrations in the lower and upper cortex of roots (Joo et al. 2001). But it should be mentioned that the horizontal curvature of primary root growth depends on the plant species and was observed in faba bean (*Vicia faba*), pea (*Pisum sativum*), and dwarf chickling (*Lathyrus cicera*) but not in *Arabidopsis*, soybean (*Glycine max*), rice, maize, tobacco (*Nicotiana tabacum*) (Jiang et al. 2012). Inhibition of root growth of maize and tomato caused by cyanamide—allelopathic compound produced by hairy vetch (*Vicia villosa* Roth.)—was due to overaccumulation of H_2O_2 (observed particularly in the apical region) inducing earlier differentiation of cells and restriction in cells' division and elongation (Soltys et al. 2012, 2014).

Radical forms of ROS react with polysaccharides (including pectin and hemicelluloses) of the cell wall, resulting in loosening of their structure (Carol and Dolan 2006; Müller et al. 2009). Thus, root growth is associated with elongation of cells, as the consequence of initially loosening and finally stiffening of the cell wall. H_2O_2 acts as an important stiffening agent which leads to cross-linking of cell wall polymers (Müller et al. 2009). Cell wall loosening enables radicle protrusion during seed germination and was deeply examined in cress (*Lepidium sativum*). Incubation of cress seeds with H_2O_2 inhibited endosperm rupture (Müller et al. 2009). As was mentioned above, one of the important apoplastic sources of H_2O_2 is catabolism of PAs mediated by the activity of amine oxidases. Among them, copper-containing amine oxidases (DAO, CuAO), oxidizing diamines, flavin-containing PAO, and oxidizing Spm and Spd should be distinguished. High level of H_2O_2 produced by enhancement of DAO or PAO activity correlates with cell wall stiffening and lignification (Delis et al. 2006; Kusano et al. 2008; Tisi et al. 2011). Putrescine (diamine) catabolism mediated by DAO seems to be involved in cell expansion as was proposed for soybean seedlings. These findings are supported by organ-specific gene (coding DAO) localization, mainly in regions of cell elongation in primary and secondary roots. The highest level of transcripts of *GmCuAO1* was identified in the root tips, the root hair region, and the lateral root emergence region (Delis et al. 2006).

It was reported in maize, cucumber (*Cucumis sativus*), or Arabidopsis that the root apex is rich in radical forms of ROS (Liszkay et al. 2004; Renew et al. 2005; Dunand et al. 2007). The growing zone (narrow zone behind the apical meristem) of primary maize roots was identified as the region of $O_2^{\bullet -}$ production. Generation of this molecule is localized in the epidermis and vascular tissues of the growing zone. These findings come from experiment conducted on Arabidopsis, tomato, and soybean and indicated that the apoplastic ROS production in the growing zone of the roots is a common feature of seed plants (Liszkay et al. 2004). In addition, restriction of root elongation correlating with decline in $O_2^{\bullet -}$ concentration was observed as a result of application of IAA (Liszkay et al. 2004).

NADPH oxidases lead to increased production of oxygen free radicals. The catalytic domain of plant Rbohs shows homology to the mammalian phagocyte respiratory burst oxidase subunit $gp91^{phox}$ (Müller et al. 2012). In Arabidopsis, an

amount of ten *Rbohs* (*AtrbohA–AtrbohJ*) was identified. Physiological importance of this enzyme was shown in *Rboh* knockout mutants characterized by reduction of their size (Jones et al. 2007). Rbohs seem to participate not only in seed germination (Müller et al. 2009) but also in regulation of growth of cells of root tips (Foreman et al. 2003), mechanosensing (Monshausen et al. 2009), Casparian strip formation (Lee et al. 2013), and root response to phytotoxins/allelochemicals (Oracz et al. 2012). ROS action in allelopathic and phytotoxic interaction (defined as root–root communication) was recently described in detail (Gniazdowska et al. 2015). The importance of Rboh in the regulation of root growth in cress seedling was demonstrated using transgenic plants with reduced expression of *Rboh* genes. Roots in mutants were shorter compared to the wild-type plants (Müller et al. 2012). Initially, roots had thickened root tips, and then abnormal (irregular) formation of lateral root was observed. Moreover, these malformations were also characteristic of auxin mutants (e.g., auxin overproducer's phenotypes). Based on these results, authors suggested that there is a link between Rboh activity and auxin signaling. Morphologically intact architecture of roots and their proper function are associated with the ability of plant for precise control of ROS generation and maintenance of redox state in specific roots' zones. This is in agreement with findings in Arabidopsis that *RbohB* is specifically expressed in the root tip below the elongation zone (Vernoux et al. 2000; Müller et al. 2012). Activity of NADPH oxidases is also regulated by ROP (Rho of plant) GTPases (Jones et al. 2007). This particular ROS modulation is linked to the stimulation of the Ca^{2+} influx via plasma membrane hyperpolarization-activated Ca^{2+} channels (HACC). A high intracellular Ca^{2+} gradient is necessary for growth of root hair tips. Elongation of root hair is also connected to ROS formation *via* activity of ROP GTPase (Jones et al. 2007). The differences in Ca^{2+} gradients play a role not only in the control of growth of root hairs but also in the growth of the whole root system. Importance of Ca^{2+} in root growth was proved by application of the Ca^{2+} channel blocker—lithium ($LaCl_3$) (Demidchik et al. 2003; Foreman et al. 2003). Moreover, it was demonstrated that NADPH oxidases also undergo *S*-nitrosylation (reversible PTM) in the presence of nitric oxide (NO) (Yun et al. 2011). This finding points out the strong correlation of ROS and RNS in the growth process. ROS participate in the regulation of Ca^{2+} concentration (by indirect creation of Ca^{2+} wave) and also stimulate mitogen-activated protein kinase (MAPK) cascades (Pitzschke and Hirt 2006).

Fenton reaction depending on the simultaneous presence of the reductant, transition metal ions, and H_2O_2 is the source of $^{\bullet}OH$ in the apoplastic space. $^{\bullet}OH$ is also generated in peroxidase-mediated Haber–Weiss reactions (Liszkay et al. 2003; Müller et al. 2009; Demidchik, 2015). Significance of $^{\bullet}OH$ in the weakening of endosperm and finally growth of embryo axis in germinating cress seed was proven using a method corresponding to puncture force measurement. It is not the random production and high reactivity of $^{\bullet}OH$ with cell wall polysaccharides that suggest its specific action in apoplastic space. Reactivity of this compound is temporal (of narrow time window) and depends on tissue, developmental

progress, and ABA concentration, which inhibits $^{\bullet}OH$ production (Müller et al. 2009). On the other hand, growth of roots of willow seedlings was associated with $O_2^{\bullet-}$ rather than $^{\bullet}OH$. These findings were confirmed by experiment with ROS scavengers. Inhibition of root growth of willow was observed after application of $O_2^{\bullet-}$ scavenger, while scavenging of $^{\bullet}OH$ had no effect (Causin et al. 2012). The authors postulated that generation of $O_2^{\bullet-}$ in the meristematic root zone is probably related to some other physiological processes including growth.

Transcription factor UPBEAT1 (UPB1), repressing a set of POx and modulating ROS concentration and also H_2O_2/O_2^{-} ratio, affects the size of the root meristem (Tsukagoshi et al. 2010). Thus, differentiation and proliferation of root cells depends on accumulation of specific ROS. Increase in H_2O_2 level is required for cell differentiation, whereas high $O_2^{\bullet-}$ concentration is necessary for cell proliferation. In consequence, cell elongation in the root elongation zone occurs after $O_2^{\bullet-}/H_2O_2$ ratio reaches a certain level. In the elongation zone, UPB1 repressed POx expression. Moreover, *UPB1* expression is controlled by H_2O_2 *via* feedback loop. It was shown that H_2O_2 application caused upregulation of *UBP1* expression (Tsukagoshi et al. 2010). It was also demonstrated that one of the direct targets of UPB1 was the gene coding thioredoxin reductase (*NTRA*). The involvement of UPB1 in lignin synthesis, thus in cell wall modification, is also suggested.

Cellular redox potential impacts development of the root apical meristem. It depends on homeostasis of ASC/DHA and GSH/GSSG (oxidized form of GSH) (De Tullio et al. 2010). The balance between reduced/oxidized states is mediated by ROS modulating system. It is commonly accepted that both ASA and GSH regulatory roles in meristem development refer to the impact of these cellular antioxidants on mitotic activity (Sanchez-Fernandez et al. 1997). As was mentioned above, ASA content regulates cell cycle progression. Modification in ASA/DHA ratio is central in root gravitropic response that correlates to ROS level (Lee et al. 2011). Cellular redox homeostasis is controlled by thioredoxins. In root meristem, NTR impacts POx activity and thus the transition from cell proliferation to cell differentiation (Tsukagoshi et al. 2010).

There are suggestions of ROS involvement in auxin metabolism. ROS accumulation influences auxin response probably due to its conjugation or catabolism to inactive products (Peer et al. 2013).

A link of H_2O_2 to hormone signaling was also reported for ethylene (ET). This phytohormone is known as root growth regulator acting by protein kinases (MAPK3 and MAPK6) which are also elements of ROS transduction cascades (Bartoli et al. 2013). It is well known that this gaseous hormone modifies root architecture. Characteristic growth responses of Arabidopsis roots to ET are the enhancement of the thickness of the organ and reduction of cell elongation (Huang et al. 2013). Mung bean (*Vigna radiata*) seedlings treated with ET donor showed shortened length of primary roots and lowered number of lateral roots. These results were correlated with increased lignin content and increased POx activity (enhanced H_2O_2 level) (Huang et al. 2013). ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) acts synergistically to auxin in inhibition of cell expansion in the elongation zone of roots (Muday et al. 2012). Any modification of ET concentration

in root tips affects auxin transport and signaling. However, in lateral root initiation, antagonistic action of auxin and ET is observed. Also, this hormone negatively impacts gravitropic response (Muday et al. 2012). There is evidence that increased ET nonenzymatic formation by oxidation of ACC can be achieved in conditions of enhanced ROS (high H_2O_2 concentration) (Gniazdowska et al. 2010a). Inhibition of elongation of roots of apple seedling after embryo treatment with H_2O_2 could be due to elevated ET production. Also, increase of ET emission from seedlings was observed after NO treatment, suggesting contribution of RNS in biosynthetic pathway of this hormone.

3 Reactive Nitrogen Species Contribution in Root Responses

RNS, in addition to ROS, are considered as important morphogens. Nitric oxide (NO) at different redox states modulates various physiological and morphological processes including also root growth and development or molding of root architecture. NO is generated via enzymatic or nonenzymatic pathways and at low concentration acts as a signaling molecule (Yu et al. 2014). This gaseous compound takes part in signal transduction cascades as activator of cyclic guanosine 3′,5″-monophosphate (cGMP) synthesis, modulator of Ca^{2+} signaling, and activator of protein kinase cascades and participates in posttranslational modification of proteins (e.g., *S*-nitrosylation and/or nitration) (Pagnussat et al. 2004; Kasprowicz et al. 2009; Yu et al. 2014). As was recently presented for roots of pepper (*Capsicum annuum*) seedlings, their growth and development corresponded to the decrease of the level of nitrated proteins (Airaki et al. 2015). Important modulators of developmental processes are also fatty acid amides, e.g., alkamides (Méndez-Bravo et al. 2010). It was shown that NO treatment of *Arabidopsis* seedlings mimics variation of root system architecture typical to alkamides. In addition, NO acts as a second messenger in signaling pathway initiated by alkamides (Méndez-Bravo et al. 2010). NO affects actin cytoskeleton and thus can influence root architecture (Kasprowicz et al. 2009). The experiments carried out on maize root apices indicated a strong, but reversible, effect of different NO donors on the actin cytoskeleton assembly and organization (Kasprowicz et al. 2009). NO treatment influenced endocytosis, formation of endocytic vesicle, and trafficking in cortex cells of the transition zone of the maize root apex. Moreover, NO affected membrane trafficking and recycling of cell wall polysaccharides. NO synthesized in the pericycle cells (during lateral root formation), similarly to auxin-induced transcription of genes coding cyclins *CYCD3;1* and *CYCA2;1* responsible for G1-to-S phase transition (Correa-Aragunde et al. 2006). It is also known that NO can act as antioxidant by prevention of $^{\bullet}OH$ generation or by affecting activity of antioxidant enzymes (Tewari et al. 2008; Krasuska and Gniazdowska 2012). As observed in mountain ginseng (*Panax ginseng*), NO application during adventitious root

formation enhanced activity of SOD, CAT, POx, APX, and glutathione reductase (GR). In the contrary, application of NO stimulated activity of NADPH oxidase and increased $O_2^{\bullet -}$ level (Tewari et al. 2008). However, NG-Nitro-L-arginine methyl ester (L-NAME, an inhibitor of mammalian NO synthase) did not alter $O_2^{\bullet -}$ level and did not affect the number of proliferated ginseng rootlets (Tewari et al. 2008) that points out NO origin in this process. On the other hand, in the literature, it can be found that *S*-nitrosylation of NADPH oxidase due to high concentration of *S*-nitrosothiols is coupled rather to inhibition of enzyme activity. This inhibition was observed in plant immunology (Yu et al. 2014). These findings indicate the regulatory role of NO, which modulates concentration of specific ROS in particular root regions. Impact of NO on ROS metabolism was analyzed also in roots of young apple seedlings, wherein stimulation of CAT, POx, and GPX activity after NO treatment was observed (Krasuska and Gniazdowska 2012). APX utilizing ASA as electron donor is involved in ROS scavenging and maintenance of redox homeostasis. Cytosolic isoform of APX (cAPX) was shown to be modified by ROS and NO; it underwent carbonylation, nitration, or *S*-nitrosylation (Begara-Morales et al. 2014). Although carbonylation and nitration which are irreversible inhibited cAPX activity, it was reported that *S*-nitrosylation prevented carbonylation of cAPX and even stimulated its activity (Correa-Aragunde et al. 2015).

With increasing amount of data focusing on NO action in plant morphogenesis, much more attention was focused on its interaction with auxins. It was shown that during adventitious root, lateral root, and root hair formation, auxin treatment led to NO accumulation (Pagnussat et al. 2002; Correa-Aragunde et al. 2004; Lombardo et al. 2006; Terrile et al. 2012). Moreover, conversion of indole-3-butyric acid (IBA), localized in peroxisomes, into IAA was followed by NO formation, an important process of IBA-induced lateral root formation (Schlicht et al. 2013). In soybean root tips, gravitropic bending mediated by NO was due to its asymmetric localization similarly as noted for auxins, accompanied by enlargement of cGMP (Hu et al. 2005). NO as the "molecule of the 1992 year" was shown to participate in the initiation of trichoblast differentiation of developing root hairs of lettuce (*Lactuca sativa*) and Arabidopsis seedlings. NO application increased number of root hairs and promoted their elongation (Lombardo et al. 2006). Similarly as observed for other plant growth regulators or signaling compounds, NO concentration has to fit in a range of hormetic window, characteristic for the maintenance of the specific physiological process (Monzón et al. 2014). Application of NO scavengers, including 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide (cPTIO), unblocked inhibition of primary root growth and inhibited lateral root development (Correa-Aragunde et al. 2004; Monzón et al. 2014). Reduction of the number of lateral roots in sunflower (*Helianthus annuus*) seedlings after cPTIO application was accompanied by upregulation of POx, cytochrome P450, or GR gene expression (Monzón et al. 2014). NO takes part in lateral root development but rather negatively impacts their length. Application of cPTIO resulted in enhancement of lateral root length by 95 % compared to the control (Monzón et al. 2014). This data point out NO–ROS cross talk in the design of root architecture.

Involvement of NO in mediation of the randomization of cortical microtubules that impacted cell polarity during the morphogenic events is strongly suggested. In Arabidopsis roots, NO altered auxin interaction with the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) proteins (auxin receptors) and AUX/IAA proteins (transcriptional repressors) that regulated expression of auxin-induced genes. This NO-related modulation referred to *S*-nitrosylation of TIR1 protein at two specific Cys residues, probably promoting its interaction to auxin/IAA protein (Terrile et al. 2012). PIN1-dependent acropetal auxin transport was also considered as the process controlled by NO (Fernández-Marcos et al. 2011). Elevated NO concentration resulted in reduction of PIN1 protein level. Moreover, it is believed that PIN1 loss was connected with PTM (Fernández-Marcos et al. 2011).

As was mentioned before, Class III POx activity is linked to root shaping. NO application altered expression of genes coding several extracellular POx involved in lignin polymerization, as well as some other genes involved in lignin biosynthesis (Monzón et al. 2014). Moreover, NO donors can inhibit POx activity (Krasuska and Gniazdowska 2012; Monzón et al. 2014). In sunflower roots, it was proposed that NO is a transcriptional regulator of the lignin biosynthesis (Monzón et al. 2014). Modification of lignification (lignin content and activity of related enzymes) after NO treatment was also demonstrated for soybean seedlings (Böhm et al. 2010).

Recently, NO action in root development was confirmed using NO-deficient Arabidopsis mutants. Reduced length of the primary root and shortened meristem with abnormal pattern of cell division were characteristic features of NO-deficient mutants (*nitric oxide associated1* [*noa1*], *nitrate reductase1* [*nia1*], and *nia2*) (Sanz et al. 2014). In Arabidopsis, accumulation of ROS and flavonol, mostly quercetin, enhanced by NO-deficient mutants in root region of developmental transition was reported (Sanz et al. 2014). These findings strongly indicate RNS–ROS cross talk in the mode of action of root response to different (positive or negative) environmental stimuli.

4 Conclusions

Plants, as other organisms, are able to modify their metabolism and development according to the conditions of the environment, including also stimuli of negative or positive nature. Believable examples of adaptation of plants modifying their movement, size, and growth rate in the presence of various stress factors by activation of complicated signaling network led to the rise of the concept of plant intelligence (Trewavas 2005). Perception of gravity, light, nutrient, and soil moisture allows to point on roots (particularly root tips) as space-extended guide center—"a brain" of the plant (Darwin's view of the plant body) (Baluška and Mancuso 2009). The ability of plants to integrate in real time the environmental data that are simultaneously provided and are of a variety of parameters and strength depends

on root function and results in modification of root growth and architecture or movement toward or against the stressor. Such behavior of plant organism needs complex network of phytoneurotransmitters with auxins accepted as key messengers (Fig. 1). A detailed explanation of plant susceptibility implies the existence of short-life and membrane-permeable signaling molecules acting in orchestra with classical phytohormones. Thus, ROS and RNS occur as natural candidates; all the more, their mode of action requires oxidative window and "nitrosative door," respectively (Krasuska et al. 2015), or similarly to Ca^{2+} signature obliges formation of waves, special zones, or puffs, but this is another story.

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Relationship Between Changes in Contents of Nitric Oxide and Amino Acids Particularly Proline in Plants Under Abiotic Stress

David W.M. Leung

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Abstract Studies on the physiological response of plants to abiotic stress have identified an array of changes including nitric oxide (NO) generation, accumulation of free proline, reactive oxygen species, antioxidants and oxidative damages. Little is known about the relationships between two of the concurrent changes, NO and proline metabolism. Here, the insights obtained so far and the important research gaps about this were explored.

Keywords Crop productivity • Morphological alterations • L-proline • Sodium nitroprusside • Stress tolerance

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1 Introduction

The agricultural productivity of crop plants could be severely limited by adverse environmental factors or abiotic stressors. Drought and elevated soil salinity are the two main abiotic stressors that can threaten crop production worldwide (Farooq et al. 2012; Munns et al. 2006). Research into the physiological impacts of these and other adverse plant growth conditions (Deinlein et al. 2014; He et al. 2014; Iqbal et al. 2014; Li et al. 2014; Shin et al. 2015; Talbi et al. 2015) has yielded information about a diverse array of changes in the plant body under abiotic stress (Fig. 1). It is frequently thought that abiotic stress-induced oxidative damages including unfavourable alterations to protein structure and activity and destabilisation of cell membranes (Gill and Tuteja 2010) are closely associated with the stress-induced visual symptoms or morphological alterations to the plant body (Potters et al. 2009; Leung 2015).

The goal of developing crop plants with enhanced adaptation to abiotic stress (via improved avoidance or tolerance mechanisms) remains an important grand research challenge in contemporary plant science. A way toward this goal is to gain a comprehensive understanding of the interrelationships among all the changes already shown to take place in plants reacting to different abiotic stressors (Fig. 1). Here, the few insights already gained and the important research gaps about the relationship between nitric oxide (NO) and accumulation of amino acids particularly proline in plants under abiotic stress were explored.

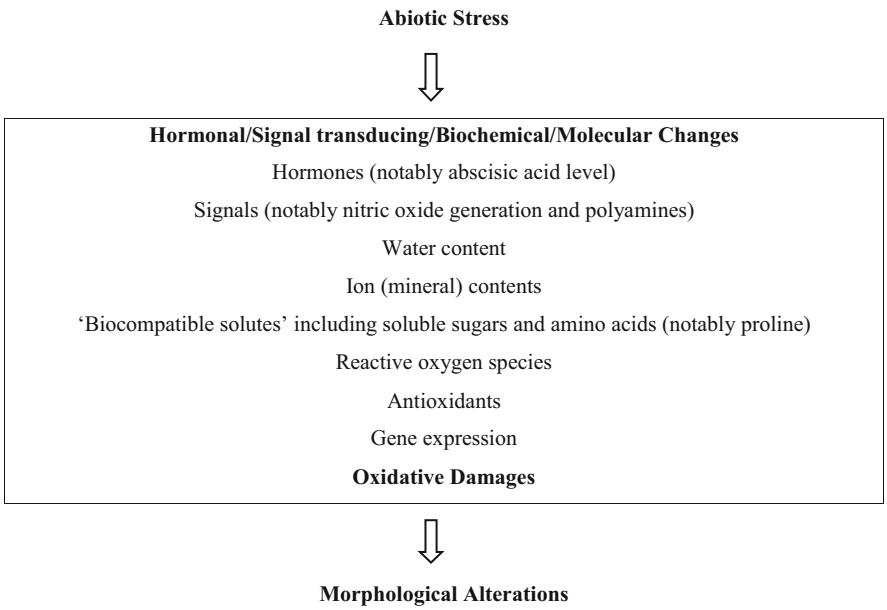

Fig. 1 Scheme of major changes in plants under abiotic stress

2 Nitric Oxide Generation and Proline Accumulation are Concurrent Biochemical Changes

Proline accumulation is frequently observed as a response of plants to different stresses (Table 1). For example, in response to a low temperature treatment, a cold-resistant sugarcane cultivar exhibited a higher level of free proline than a cold-sensitive cultivar (Huang et al. 2015). This was one of the many biochemical differences found between the two contrasting sugarcane cultivars.

In comparison, there are relatively fewer studies that determined NO generation in plants under abiotic stress (Table 1). For example, increased NO emission by poplar roots, leaves and stems was found under oxygen-deficit conditions (Liu et al. 2015a). Changes in NO levels were also found in response to cold (Cantrel et al. 2011), high temperature stress (Hasanuzzamann et al. 2014), salinity stress (Gould et al. 2003), water-deficit stress (Signorelli et al. 2013b; Planchet et al. 2014) and copper (heavy metal) stress (Hu et al. 2015). Therefore, it would seem that similar to free proline level, NO level in plants is also likely modulated in response to different stresses. In addition, there are many more studies implicating NO as a signal molecule involved in the response of plants to a variety of abiotic stresses (Table 1; Hu et al. 2015; Leung 2015). These were based on experiments involving (a) inhibition of nitric oxide synthase (NOS, an enzyme that is associated with NO synthesis in plants) with a NOS inhibitor such as *N*-nitro-L-arginine methyl ester (L-NAME) or manipulating the nitrate reductase pathway for NO production (Boldizsar et al. 2013), (b) exogenous application of a nitric oxide donor such as sodium nitroprusside (SNP) (He et al. 2014) and (c) a NO-specific scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide (cPTIO) (Planchet et al. 2014).

It was clearly confirmed in a recent study that endogenous NO generation and free proline accumulation were concurrent, correlated changes in roots of *Lotus japonicus* (Signorelli et al. 2013b) and the embryo axes during seedling establishment in *Medicago truncatula* under water-deficit stress (Planchet et al. 2014). However, in a literature survey, it was found that proline accumulation and nitric oxide generation in plants under abiotic stress have been mainly studied separately (see the few sample studies shown in Table 1). Little is known about the relationships or precise mode of interaction, if any, between nitric oxide and proline in contributing to stress-induced oxidative damages and morphological alterations. This is hardly an ideal situation on the road map to genetic improvement of crop plants with enhanced abiotic stress tolerance.

Table 1 Examples of investigations into nitric oxide generation and free proline accumulation in plants under abiotic stress

Abiotic stressor	Plant material	NO generation	Proline accumulation	Reference
Cold	Sugarcane seedlings	n.d.	Stimulated	Huang et al. (2015)
Cold	*Arabidopsis thaliana* L. Heynh. wild type (WT) in the Columbia (Col-0) background 4-week-old plants	Measurement based on fluorometric and chemiluminescence methods. A significant increase in leaves after exposure of plants at 4 °C for 1–4 h	n.d.	Cantrel et al. (2011)
Cold	Different plants	Measurement based on a variety of methods: fluorometric NO determination using the fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA) haemoglobin assay, chemiluminescence sensor-coupled NO metres, most studies showing an increase in NO level following treatment of plant materials at low temperatures	n.d. But recognised as a critical component in stress response	Puyaubert and Baudouin (2014)
High temperatures	Leaf peels and mesophyll cell suspensions of *Nicotiana tabacum* cv. Xanthi	Increased Determined using DAF-2DA, a NO-specific fluorophore	n.d.	Gould et al. (2003)
High temperature stress	10-day-old rapeseed (*Brassica napus* L. cv. BINA Sarisha 3) seedlings treated at 38 °C for 24–48 h	n.d.	Increased	Hasanuzzamann et al. (2014)
Hypoxia	Roots of intact 4-month-old poplar (*Populus* × *canescens*) cuttings	NO gas emission measure using NO analyzers (chemiluminescence) NO emission from stems of young tress if the roots experienced a deficit in oxygen supply	n.d.	Liu et al. (2015a)

Drought	2-month-old *Oudneya africana* (a Saharian plant), grown in pots under greenhouse conditions	Determined using a NO-specific fluorophore (diaminofluorescein) Increased with duration and severity of drought conditions; returned to lower levels upon 5 days of re-watering	n.d.	Talbi et al. (2015)
Drought	Two adult field-grown tea [*Camellia sinensis* (L.) O. Kuntze] plant genotyes including drought-susceptible 'Zhuyeqi' (T1) and drought-tolerant 'Ningzhou 2' (T2)	n.d.	Increased significantly as water deficit progressed and then rapidly decreased following re-watering in both drought-susceptible and drought-tolerant genotypes	Liu et al. (2015b)
Water stress	Roots of cucumber plants (*Cucumis sativus* cv. Dar) Plants treated at the third fully expanded leaf stage	NO-specific fluorescence was enhanced more than eightfold after 17 h from start of water withdrawal in root segments above the elongation zone using confocal laser scanning microscopy	Progressive water stress caused more than fourfold increase at 17 h 100 μM SNP caused a significant decrease in free proline content by about 30 % 200 μM cPTIO + SNP did not reduce free proline content Implication is that SNP pretreatment protected the roots, but this was accompanied by a reduction in free proline content, suggesting that the osmolyte might not be essential in water stress tolerance	Arasimowicz-Jelonek et al. (2009)
Water stress	*Lotus japonicus* plants	NO content was increased in roots under water stress compared to well-watered plants	23-fold increase in proline content of roots under water stress compared to well-watered plants	Signorelli et al. (2013b)
Water-deficit stress (PEG induced)	During seedling establishment in *Medicago truncatula*	Based on fluorometric NO determination NO fluorescence increased in the embryo axes of 3-day-old seedlings	Increased with duration of PEG-induced stress	Planchet et al. (2014)

(continued)

Table 1 (continued)

Abiotic stressor	Plant material	NO generation	Proline accumulation	Reference
		with the increased duration (2–24 h) of PEG-induced stress		
Salinity (150 mM NaCl)	Maize seedlings (*Zea mays* L. cv. Silverking) at the 3-leaf stage were grown hydroponically Measured in the youngest fully developed leaves	n.d. But the study involved the use of inhibitors of NO synthesis (5 μM nitro-L-arginine (L-NNA)) and exogenous application of NO donors (5 μM DETA/NO [*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate])	Proline accumulation increased during NaCl treatment	Boldizsar et al. (2013)
Salinity (50 mM NaCl)	Cucumber seedlings (cv. Jinchun 2) at the 2-leaf stage or 21 days old	n.d. But NO donor (100 μM sodium nitroprusside or SNP) was used	Increased in leaves and roots with increasing time under salt stress (0–8 days) A greater increase in response to SNP application	Fan et al. (2012)
Salinity (200 mM NaCl)	Spinach (*Spinacia oleracea* L.) seedlings with three true leaves grown hydroponically	n.d. But application of 200 nl/l NO gas partially counteracted salt-induced growth inhibition	Increased in the leaves after plants were under salt stress for 21 days compared to control (absence of NaCl); there was no difference in proline level in leaves of the plants grown in the absence of NaCl with or without exposure to NO In the NaCl + NO treatment, the proline content was more than twice to that in the NaCl treatment alone	Du et al. (2015)
Cadmium (Cd)	2-week-old wheat seedling (*Triticum aestivum* L.) variety 'Inqalab-91' grown in liquid nutrient culture or	Determined using a chemiluminescence NO analyser NO production in cut root sections	n.d.	Mahmood et al. (2009)

	exposed to 1 μM Cd for 4 weeks Fairly Cd resistant with ninefold increase in Cd accumulation (2.53 mM) without exhibiting any visual stress-related growth reduction	more than double; respiration about halved after 4 weeks of Cd treatment compared to the control (in the absence of Cd)		
Cd stress	*Brassica juncea* L. (Czern. & Coss.) (25-day-old Indian mustard plants) Treated with 200 mg/l $CdSO_4 \cdot 8H_2O$	n.d.	Increased significantly and almost doubled the level of the control (no Cd stress) Even enhanced more by a calcium treatment which reversed the Cd toxicity implication Proline was involved in salt stress protection	Ahmad et al. (2015)
Cu ($CuSO_4$) stress	3-day-old seedlings of Hulles barley (*Hordeum vulgare* L. var. nude)	NO generation based on haemoglobin assay Peaked at 24 h after start of Cu stress and then began to decline	n.d.	Hu et al. (2015)
Pb [0–200 μM Pb $(NO_3)_2$]	*Potamogeton crispus* L. (Potamogetonaceae), a submerged macrophyte	n.d.	Increased in response to 50 μM Pb but decreased at higher Pb concentrations	Qiao et al. (2015)

n.d. = not determined

3 Involvement of NO and Proline Interactions in Abiotic Stress Tolerance Mechanisms

Accumulation of free proline is determined by activation of proline biosynthesis and catabolic degradation in plants under stress, and the details of the metabolic pathways and regulation in relation to reactive oxygen species (ROS) have been discussed in a recent review (Rejeb et al. 2014). The immediate precursor for proline biosynthesis is glutamate. A key enzyme involved in the formation of proline is pyrroline-5-carboxylate synthetase (P5CS), while proline dehydrogenase (ProDH) is a key proline catabolic enzyme. The expression of these genes has been shown to be altered in plants under stress (Planchet et al. 2014).

It has been frequently speculated that accumulation of free proline in plants under abiotic stress compared to absence of stress has physiological significance. Specifically proline is thought to be a critical component of abiotic stress tolerance mechanistic network (Deinlein et al. 2014). Osmotic adjustment seems to be an important adaptive reaction to stress. Elevated accumulation of 'compatible solutes' such as free proline and soluble sugars has been frequently observed, and they are thought to be important osmolytes (Deinlein et al. 2014). In addition, in in vitro assays, proline seems to be capable of free radical scavenging (Kaul et al. 2008). However, the physiological significance of increased accumulation of free proline in antioxidant defence of plants under abiotic stress has been considered to be unlikely (Signorelli et al. 2013a, 2014).

There are studies with findings putting the notion of proline involvement in abiotic stress tolerance in doubt, however. For example, the impact of osmotic stress (induced by treatment with 5 % PEG) on winter oilseed rape (*Brassica napus* L.) seedlings (26 d from sowing seed) was different between the drought-sensitive and drought-resistant genotypes (Hatzig et al. 2014). In response to osmotic stress, the sensitive genotypes exhibited significant shoot and root growth inhibition which was accompanied by typical accumulation of free proline. However, these effects of osmotic stress were not observed in the resistant genotypes, suggesting that accumulation of proline for being an osmolyte involved in osmotic adjustment is not the critically important mechanism underpinning osmotic stress tolerance.

The controversy surrounding the role of proline accumulation in abiotic stress tolerance can be examined from another line of investigations by probing the relationship between nitric oxide level and proline metabolism in plants under abiotic stress. For example, in response to 50 mM NaCl for 8 days, the proline content in the cucumber leaves was 356 % that of the leaves of cucumber plants grown in the absence of NaCl (Fan et al. 2012). In treatment of cucumber plants with 50 mM NaCl and 100 μM SNP (a NO donor), leaf proline content was about 12 % higher than that in NaCl alone (Table 1). It was suggested that the higher proline content was associated with protection of the SNP treatment against salinity stress. However, it should be noted that there were no data showing any growth improvement with the SNP treatment. Moreover, SNP treatment or exogenous application of other NO donor solutions which would presumably supply more

bioactive NO molecules to the experimental material under question may not necessarily change or lead to an increase in proline accumulation (Planchet et al. 2014). Exogenous SNP application in the absence of abiotic stress was found to result in a reduction in proline content in the calli of a halophyte *Nitraria tangutorum* Bobr. instead (Yang et al. 2014).

In spinach, there was more than a fourfold increase in proline content under NaCl stress (Du et al. 2015; Table 1), and the plant biomass was concomitantly reduced. However, application of NO gas promoted about a tenfold increase in proline content and counteracted partially reduction in plant biomass under NaCl stress. Based on manipulations of the response of maize seedlings under NaCl stress using NO donors and a NOS inhibitor (Table 1), it was concluded that the much higher levels of free proline content were associated with NO-induced salt tolerance (Boldizsar et al. 2013).

Water-deficit stress induced by treatment of *Medicago truncatula* seedlings with polyethylene glycol (PEG 8000 at −0.75 MPa) had a substantial inhibitory effect on embryo axis (hypocotyl and root) elongation. A correlative temporal and localisation relationship was found between inhibition of axis elongation and NO production which was increased about 2.2-fold in the axis after 24 h under PEG-induced water-deficit stress (Table 1). Both nitrate reductase-dependent pathway and NOS activity were involved in NO generation under water-deficit stress. However, a scavenger of NO production (cPTIO) used at a concentration that was very effective in eliminating endogenous NO level in the seedling axis could not counteract the inhibition of seedling growth induced by the PEG treatment (Planchet et al. 2014). Therefore, NO generation in the embryo axis of *M. truncatula* was just coincidental with inhibition of axis growth under water-deficit stress. Interestingly, unlike many other studies, exogenous application of a NO donor also did not protect the axis under PEG-induced water stress.

During seedling establishment of *M. truncatula* in the absence of drought stress, total amino acids or proline content in the embryo axis decreased, but the opposite pattern was exhibited under PEG-induced water stress (Planchet et al. 2014). Elevation in proline content induced by PEG was contributed by an up-regulation of *P5CS2* gene expression and a concomitant downregulation in *ProDH* gene expression. Interestingly, the content of glutamate (the precursor for proline biosynthesis) in the embryo axis remained unchanged during 24 h of water stress. Altered NO and proline metabolism were correlative biochemical changes, but application of cPTIO was not able to alter the response of the *M. truncatula* seedlings to PEG treatment as far as total amino acid or proline content elevation in the embryo axis under water stress is concerned. Therefore, elevation in proline content and NO production can occur in the embryo axis at the same time induced by a 24-h PEG treatment, but they are independent biochemical responses to water stress. This is a variation to the observations leading to the following proposed sequence of changes under NaCl or cold stress (Boldizsar et al. 2013; Puyaubert and Baudouin 2014): abiotic stress → modification in NO level → changes in amino acid contents, notably free proline.

4 Other Amino Acids

Levels of total amino acids are often elevated under abiotic stress (Boldizsar et al. 2013; Planchet et al. 2014), although most studies have only put emphasis on free proline levels. In maize seedlings under NaCl stress, for example, besides proline, amino acids of different properties or chemical categories including the nonprotein amino acid γ-aminobutyric acid (GABA) were generally increased to levels of several-fold higher than in the absence of stress (Boldizsar et al. 2013). The effects of a NO donor and a NOS inhibitor on several amino acids besides free proline during stress and upon recovery from stress clearly warrant a closer and further examination in maize and other plants. For example, exogenous application of a NO donor to maize seedlings under NaCl stress for 11 days increased GABA content more than those under NaCl stress alone. Interestingly, exogenous application of a NOS inhibitor to the seedlings under NaCl stress for 11 days brought about an even higher level of GABA than in the treatment of NO donor + NaCl. Based on these findings, the precise interaction between NO and the metabolism of this amino acid remains unclear until endogenous NO generation is determined as well.

Soil drench with 0.3 mM β-aminobutyric acid was found to improve drought tolerance of potato plants (Sos-Hegedus et al. 2014). This was accompanied by an increase in generation of NO and ROS in the roots but not in the leaves. The protective effect, however, appeared to last for a few weeks only.

5 Concluding Perspectives

Generally, changes in NO generation and signal transduction and changes in amino acid metabolism are concurrent events in plants under stress. The paucity of studies on their precise interactions is clear. In particular, the implications and connections of the following are in need of urgent further research: (a) application of NO donors is frequently linked to the effects of antioxidants and (b) the effects of ROS signals inducing proline accumulation and the influence of proline metabolism on ROS generation (Rejeb et al. 2014).

Our understanding of the changes in NO level and accumulation of individual amino acids apart from free proline in plants under stress is in a highly unsatisfactory state. Given that free proline accumulation may or may not be associated with abiotic stress tolerance, the idea that modulation of NO levels could change the accumulation of other amino acids which might be associated with abiotic stress tolerance is worthy of further investigations.

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Transgenic Plants and Antioxidative Defense: Present and Future?

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Abstract Environmental stresses considerably limit plant growth, distribution, and productivity. Biological systems generate a range of different reactive oxygen species (ROS) like superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2), during the course of normal metabolic reactions. If it is not effectively and rapidly removed, ROS damages a wide range of macromolecules, ultimately leading to cell death. ROS are also generated in response to various biotic and abiotic stresses. Cells have evolved both enzymatic and nonenzymatic defense mechanisms to protect cells from lethal effects of free radicals. ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) that play a crucial role in regulating ROS accumulation in cells. Transgenic plants expressing specific gene candidates have been proven to

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increase the tolerance to environmental stresses significantly. In recent years, several efforts have been made to improve the oxidative stress tolerance in plants by over-expressing plant or bacterial genes coding either for ROS-scavenging enzymes or for enzymes modulating the cellular antioxidant capacity. In this chapter, we have discussed some of the significant reports on transgenic plants with altered antioxidant capacity mainly focusing on the new insight into the antioxidant defense mechanisms. Finally, future focus of transgenic research to combat oxidative stress has been briefed.

Keywords Reactive oxygen species • Antioxidants • Superoxide dismutase • Ascorbate peroxidase • Glutathione peroxidase • Glutathione reductase • Catalase • Alternative oxidase • Uncoupling protein

1 Introduction

The rise in global population along with reduction in agricultural practices and diminishing availability of resources poses serious challenges to present community for continuous supply of agricultural products. To feed 9 billion people that is expected by 2050, a significant increase in grain yield of approximately 44 million metric tons per year is required (Tester and Langridge 2010). Global warming and other stress factors negatively influence the plant growth and yield, that is making it even more challenging (Reguera et al. 2012). Thus, the environmental stress is one of the major limiting factors that determine the crop yield. Stress injuries on plants cause oxidative damages both at the cellular and molecular levels.The critical role of reactive oxygen intermediates (ROI) in cellular damage has been well studied (Rennenberg and Polle 1994).

Over the last few decades, deep understanding of the role played by of ROS lead to discovery of signaling molecules regulating various physiological processes in plants (Mylona and Polidoros 2010) (Fig. 1). Recently, several reports proved that low concentration of ROS is acting as redox signal molecules playing a crucial role in various signal transduction pathways in plant cells (Foyer and Noctor 2005a, b). Environmental stresses like salinity, temperature, or drought result in a marked increase in ROS level leading to oxidative damage in plant cells. During the course of evolution, plant system also developed potential antioxidant defense system to protect itself from oxidative damage (Asada 1999; Mittler et al. 2004). Till date, different ROS-scavenging enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), and catalase (CAT) have been discovered, which are localized in different cellular compartments (Table 1). In general, there are more than one type of enzyme in a cell for scavenging different kind of ROS (Mittler et al. 2004).

ROS are continuously generated as by-products of various cellular processes like photosynthesis, photorespiration, fatty acid oxidation, electron transport chain, etc.

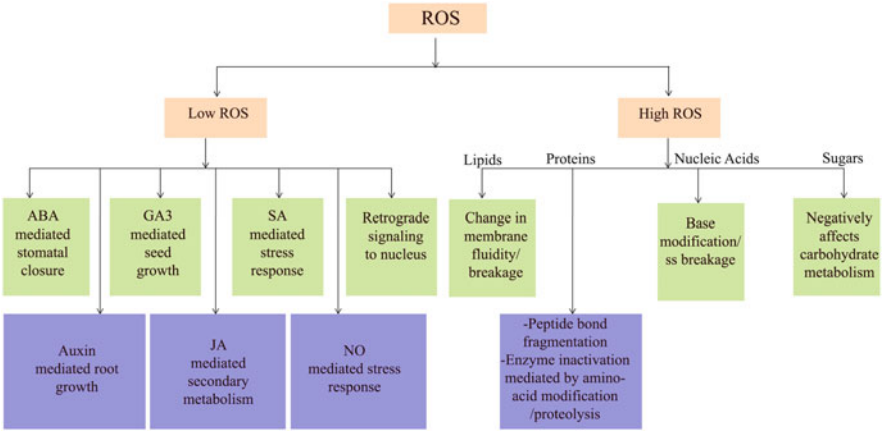

Fig. 1 Reactive oxygen species (ROS) at low levels act as secondary messengers in several physiological processes, whereas at high levels they negatively act on biomolecules

Table 1 Major ROS-scavenging enzymes, reaction mechanism, and cellular localization

Sl. No	ROS-scavenging enzymes	Mechanism	Localization	References
1	SOD	$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow 2H_2O_2 + O_2$	Cytosol, golgi, plastids, and mitochondria	Mittler et al. (2004)
2	APX	$AA + H_2O_2 \rightarrow DHA + 2H_2O$	Cytosol, plastids, peroxisomes, glyoxysomes, and mitochondria	Foyer and Noctor (2005a, b)
3	GPX	$H_2O_2 + 2GSH \rightarrow H_2O + GSSG$	Cytosol, plastids, nucleus, peroxisomes, and mitochondria	Ursini et al. (1995), Rodriguez et al. (2003)
4	GR	$GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^+$	Cytosol, plastids, peroxisomes, and mitochondria	Edwards et al. (1990), Creissen et al. (1995)
5	CAT	$2H_2O_2 \rightarrow O_2 + 2H_2O$	Peroxisomes, glyoxysomes	Mhamdi et al. (2010)

Up to 1 % of the molecular oxygen consumed by plant is metabolized to form ROS in different cellular compartments (Bartoli et al. 2000). ROS molecules include free radicals like superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), etc., and non-radical molecules like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), etc. (Shafi et al. 2015). Under normal conditions, ROS generated is efficiently metabolized by the multicomponent antioxidant system and protects them from potentially deleterious effects of ROS (Fig. 2). However, during stress (both biotic and abiotic),

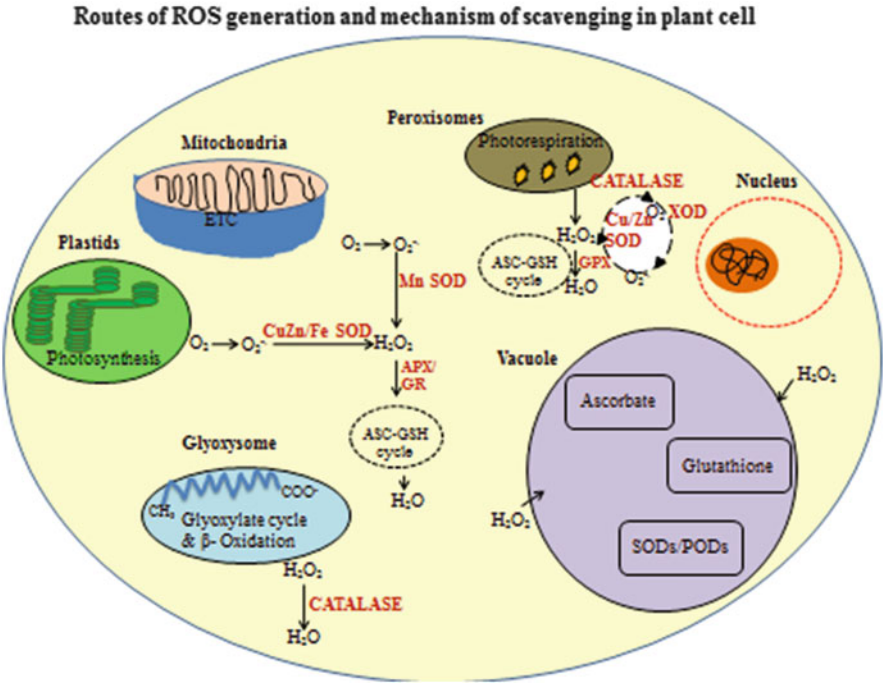

Fig. 2 Overview of cellular process generates ROS in different compartments and their scavenging in cells [Modified from Kuzniak (2002)]

ROS levels are elevated and thus the cellular antioxidant capacity can be overwhelmed leading to oxidative stress (Robinson and Bunce 2000). It is well known that much of plant injury as a result of environmental stresses are related to ROS-initiated oxidative damage. Hence, enhancing tolerance to several environmental stresses could be achieved through modulation of gene expression to different ROS-scavenging enzymes ultimately reducing ROS population in cells (Fig. 3).

Transgenic approaches are widely used for crop improvement programs in recent years. Gene discovery and functional genomics have revealed infinite mechanisms and led to the identification of many potential gene families, which could confer adaptation and improved productivity during adverse environmental conditions (Kumar et al. 2012). To improve oxidative stress tolerance in plants, overexpression of genes encoding either ROS-scavenging enzymes or enzymes modulating the cellular antioxidant capacity from various sources has been proved effective (Liu et al. 2013, 2014; Zhai et al. 2013; Zhang et al. 2013). The rationale behind these approaches is to minimize massive loss in productivity of crop plants by various environmental constraints. The study of transgenic lines expressing antioxidant genes revealed a complete understanding of the roles of individual enzymes in scavenging free radicals and imparting tolerance to various stress factors.

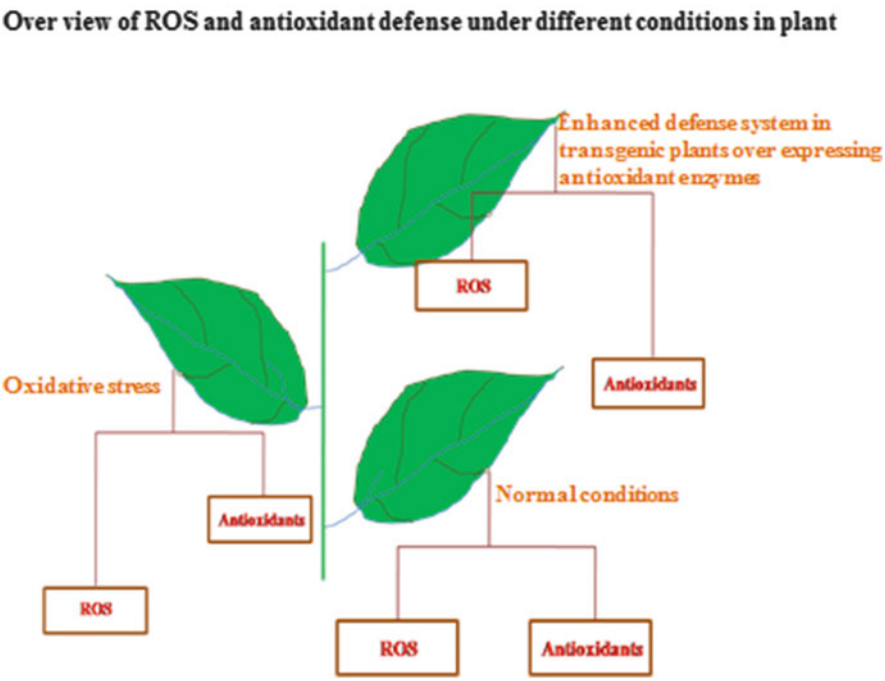

Fig. 3 Modulating ROS scavenging in transgenic plants by overexpression of antioxidant enzymes

Besides, transgenic lines with altered antioxidant capacity also provided new insights into the interrelations between various enzymatic and nonenzymatic antioxidant components and their signaling network. It has been already reported that the transgenic lines generated through gene pyramiding or co-expression of several antioxidant defense genes imparted more stress tolerance than the plants overexpressing either of genes alone (Lee et al. 2007; Wei et al. 2011; Diaz-Vivancos et al. 2013; Xu et al. 2014). In this chapter, we focus mainly on recent studies on transgenic plants with altered ROS-scavenging capacity by overexpression of antioxidant genes; readers are requested to refer to Gill and Tuteja (2010) and reference therein for transgenic plants for ROS scavenging by non-enzymatic antioxidants. Future focus is on the new insight in antioxidant defense mechanism by expressing genes like alternative oxidase, uncoupling protein has also been discussed briefly. In the end, future of transgenic research under field conditions has been emphasized.

2 Superoxide Dismutase (SOD)-Expressing Transgenic Plants

SOD and APX constitute the first line of defense response against ROS (Alscher et al. 2002), thus playing an important role in protecting cells against superoxide radicals. SODs are metallo-enzymes with different isoforms localized in various cellular compartments like cytosol, plastids, and mitochondria. Irrespective of their compartmentalization, they all catalyze dismutation of superoxide ions ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2) (Gill and Tuteja 2010). The endogenous plant genes encoding different SOD isoforms have been used for plant transformation studies. Initial attempts to express SOD in heterologous systems were not successful. *Petunia hybrida* plastidial Cu/ZnSOD when over-expressed in tobacco did not increase the photosynthetic efficiency after methyl viologen (MV) treatment (Tepperman and Dunsmuir 1990). Also, over-expression of *Petunia* SOD in transgenic tobacco lines did not alter the oxidative stress tolerance (Pitcher et al. 1991). Later, several reports revealed reasons for failure of initial attempts using plastidial Cu/Zn/Fe SOD are due to inactivation of SOD by peroxides, whereas MnSOD is not susceptible to peroxide inactivation (Allen et al. 1997). A chimeric MnSOD (native mitochondrial transit peptide was replaced by plastidal peptide) from *Nicotiana plumbaginifolia* when over-expressed in tobacco lead to reduced levels of membrane damage following exposure to MV, ozone, and photooxidative stress (Van Camp et al. 1994). Transgenic alfalfa lines expressing same chimeric MnSOD enhanced freezing tolerance and water deficit (McKersie et al. 1993, 1996).

Sen Gupta et al. (1993a, b) reported some interesting results with respect to expression of different isoforms of SOD in tobacco. Transgenic tobacco lines over-expressing plastidial Cu/ZnSOD or a chimeric chloroplastic MnSOD showed different tolerance level to MV. MnSOD-expressing lines showed superior protection after MV exposure, whereas Cu/ZnSOD-expressing plants revealed low level of tolerance. Since expression levels of SOD in both lines were similar, it was reported that peroxide sensitivity of Cu/ZnSOD could account for low level of tolerance relative to the chloroplastic MnSOD-expressing plants. It was also shown that tobacco lines expressing plastidial Cu/ZnSOD were least susceptible to photo-oxidative damage induced by excess light and also chilling temperatures; however, tobacco lines expressing chloroplastic MnSOD were susceptible. Adding to this, chloroplastic MnSOD-expressing lines did not protect MV-induced damage to photosystem II (PSII). Since plastidial Cu/ZnSOD is located on the thylakoid membrane, close association with photosystem I (PSI) enzyme rapidly scavenges $O_2^{\bullet -}$ before significant damage occurs. Since MnSOD is not native to the chloroplast, it is possible that it may not form a productive association with PSI. Hence, expression of peroxide insensitive MnSOD in plastids alleviate the damage to cellular membranes possibly by reducing the leakage of ROI from plastids to cytosol, but MnSOD does not appear to provide considerable protection to photosynthetic components.

Over-expression of Cu/ZnSOD from *Kandelia candel* in tobacco resulted in reduced ROS generation in plastids and enhanced tolerance to salinity stress (Jing et al. 2015). Transgenic *Arabidopsis* co-expressing *Potentilla atrosanguinea SOD* and *Rheum austral APX* enhanced lignin deposition along with higher biomass production and yield under salinity stress (Shafi et al. 2015). The involvement of SOD and peroxides in cell wall lignification has been also previously reported (Karpinska et al. 2001; Kim and Barbara 2008). It was also reported that H_2O_2 signaling enhanced genes involved in lignin biosynthesis indirectly through other secondary messengers like MAP kinases and transcription factors like NAC, MYB, and WRKY (Fujita et al. 2006). The same combination of genes (*PaSOD* and *RaAPX*) also conferred tolerance to chilling stress in *Arabidopsis* and had higher levels of total antioxidant enzymes and chlorophyll content and lower levels of ROS (Shafi et al. 2014). Transgenic rice either over-expressing or knocked down with *OsMnSOD* was tolerant and susceptible to heat stress, respectively. Plants over-expressing MnSOD upregulated ROS-scavenging enzymes, chaperone, and quality control systems in rice grain under heat stress (Shiraya et al. 2014). It was also shown that over-expressing lines had better quality rice grains compared to knock down lines revealing that constitutive expression of golgi/plastid targeted MnSOD is effective in regulating formation of perfect grains under unfavorable conditions like heat stress. Over-expression of both yeast MnSOD and pea mitochondrial MnSOD in plastids of rice conferred tolerance to salt and oxidative stress (Tanaka et al. 1999; Wang et al. 2005). Tolerance to oxidative and chilling stresses in transgenic Cassava plants co-expressing Cu/ZnSOD and APX was mediated through improved ROS scavenging and resulted in reduced peroxide accumulation and improved chilling stress resistance (Xu et al. 2014).

Transgenic potato lines over-expressing tomato plastidal Cu/ZnSOD had reduced levels of damage after MV treatment (Perl et al. 1993). Transgenic rice transformed with mangrove cytosolic Cu/ZnSOD showed superior tolerance to drought stress (Prashanth et al. 2008). *E. coli* MnSOD gene with a soybean-derived leader sequence over-expressed in tobacco chloroplasts showed superior tolerance to photooxidative stress (Van Assche et al. 1989). A FeSOD over-expressed in transgenic tobacco chloroplasts conferred protection from MV damage as well as PSII inactivation (Van Camp et al. 1996). However, expression of FeSOD did not enhance tolerance to chilling-induced photoinhibition in Poplar (Arisi et al. 1998) and salinity stress in tobacco (Van Camp et al. 1996). This could be due to that response of different stress factors is mediated through different routes, and superoxide anion scavenging capacity of the chloroplasts may not be rate limiting for each of them. Further physiological, biochemical, and molecular analyses of transgenic lines along with transgenic lines with silenced SOD activities will provide deeper insight into the functions of SOD in plant cells.

3 Ascorbate Peroxidase (APX) Expressing Transgenic Plants

Ascorbate peroxidase (APX) is the primary H_2O_2-scavenging enzyme in both cytosol and plastids (Asada 1992). APXs have also been reported from glyoxysome, where it is presumed to act with catalase to remove peroxides (Yamaguchi et al. 1995; Bunkelmann and Trelease 1996). Transgenic tobacco lines expressing either pea cytosolic APX or pea cytosolic APX fused with pea chloroplastic transit peptide of Cu/ZnSOD to target plastids showed 3 and 16-fold increase in SOD activity, respectively, with enhanced membrane stability. Also, both transgenic lines were tolerant to photooxidative stress, which is attributed to increase in scavenging of peroxides (Pitcher et al. 1994; Webb and Allen 1995, 1996). *Arabidopsis* over-expressing rice *OsAPXa* or *OsAPXb* enhanced salinity tolerance to different levels. Over-expression of *OsAPXb* enhanced and maintained APX expression to a much higher extent than *OsAPXa* (Lu et al. 2007). Transgenic tobacco lines expressing *C. annuum APX*-like 1 gene enhanced tolerance to oxidative stress and resistance to fungal pathogen *Phytophthora nicotianae*. Interestingly, transgenic lines showed different levels of susceptibility to other pathogens like *Pseudomonas syringae* and *Ralstonia solanacearum*. Tolerance to oxidative stress and *Phytophthora* is associated with overproduction of APX, which could have further increased the POD activity further strengthening an ROS-scavenging system (Sarowar et al. 2005).

Transgenic *A. thaliana* over-expressing peroxisomal *APX* from *Puccinellia tenuiflora* enhanced salt tolerance by reducing H_2O_2 accumulation (Guan et al. 2015). Similarly, peroxisomal *APX* from *Salicornia brachiata* imparted salinity tolerance when over-expressed in *Arachis hypogaea* (Singh et al. 2014a, b). Peroxisomal-specific *Sb APX* not only conferred salinity and drought tolerance but also enhanced vegetative growth and germination rate in transgenic tobacco lines (Singh et al. 2014a). A cytosolic APX from *Lycium chinense* enhanced salinity tolerance and photosynthetic rate and reduced peroxide accumulation in transgenic tobacco lines during salt stress (Wu et al. 2014). Transgenic tobacco expressing thylakoid targeted APX from *Jatropha curcas* enhanced ROS-scavenging system by reducing malondialdehyde (MDA) and H_2O_2 accumulation during salt stress (Liu et al. 2014). *Anthurium andraeanum APX* conferred chilling tolerance in transgenic tobacco lines, which corroborated with enhanced APX activity, reduced MDA content, and enhanced membrane stability (Liu et al. 2013). Rice *OsAPX 2* mutant negatively affected growth and development of rice seedlings and was sensitive to abiotic stress like cold, salinity, and drought. ROS metabolites like H_2O_2 and MDA levels were high in *OsAPX2* mutants; however, they were low in transgenic lines over-expressing *OsAPX2* after stress treatments (Zhang et al. 2013). Similarly, *Arabidopsis* cytosolic *APX* knockout mutants were highly sensitivity to wounding and MeJA treatment. In control plants, peroxide accumulated only in the vicinity of wound, but in leaves of *APX* knockouts, it accumulated both in damaged and undamaged regions. Transgenic *Medicago sativa* over-expressing rice *APX2* had

longer roots; ~3-fold more APX activity, less MDA content as compared to control plants (Guan et al. 2012). All the above studies clearly indicate that over-expression of different isoforms of *APX* improved detoxification of ROS and imparted enhanced tolerance in transgenic host system.

4 Glutathione Peroxidase (GPX) Expressing Transgenic Plants

GPX in mammals reduces H_2O_2 and other organic hydroperoxides to water and corresponding alcohols using glutathione (GSH), thus protecting cells from oxidative damage. Plant GPX family shares the highest sequence homology to animal phospholipid hydroperoxide GPXs (PHGPXs) and possess a cysteine (Cys) residue in catalytic site rather than Se-Cys seen in PHGPXs (Zhai et al. 2013). In plants, GPXs are localized in subcellular organelles like cytosol, nucleus, chloroplast, mitochondria, peroxisome, etc., and use thioredoxin (Trx) as a reducing agent (Ursini et al. 1995; Rodriguez et al. 2003). Plant GPXs are expressed in response to different kinds of abiotic factors like salinity, mechanical injury, high-light stress or treatment with Paraquat, etc. (Holland et al. 1993; Avsian-Kretchmer et al. 2004; Herbette et al. 2011; Gaber et al. 2012). *Arabidopsis* knockout mutants of *AtGPX8* were highly sensitive to oxidative damage by Paraquat treatment in roots, and transgenic lines over-expressing *AtGPX8* were less sensitive to oxidative damage than the wild-type controls (Gaber et al. 2012). *Mus musculus* GPX5 over-expressing transgenic tomato lines were more tolerant to oxidative damage after chilling exposure (Herbette et al. 2005). *MmGPX5* transgenic tomato lines were resistant to mechanical injury; however, transgenic plants were sensitive to biotic agents like *Oidium neolycopersici* and *Botrytis cinerea* (Herbette et al. 2011). Two wheat *GPXs* when expressed in plastids of *Arabidopsis* enhanced growth and survival rate during salt stress. Transgenic lines also had increased peroxide-scavenging capacity and enhanced tolerance to H_2O_2 (Zhai et al. 2013). Over-expression of a tobacco glutathione S-transferase having both GST and GPX activity lead to enhanced activities of both enzymes (GST/GPX) in transgenic tobacco seedlings. Also transgenic plants had elevated levels of monodehydro-ascorbate reductase activity and higher levels of glutathione and ascorbate compared to control plants (Roxas et al. 2000). Transient over-expression of tomato phospholipid like GPX (PHGPx) in tobacco leaves suppressed apoptotic-like features during severe salt and high-temperature stresses, and stable tobacco lines expressing *LePHGPx* also conferred protection against the fungus *B. cinerea* (Chen et al. 2004).

5 Glutathione Reductase (GR) Expressing Transgenic Plants

Maintenance of ascorbate and glutathione levels in a reduced state is a crucial factor in ROS-scavenging system in which glutathione reductase (GR) plays a critical role. A bacterial GR over-expressed in tobacco enhanced GR activity to ~4-fold, further reduced visible damage after MV exposure (Aono et al. 1991). Targeting of the *E. coli* GR to plastids in transgenic tobacco also showed similar level of resistance to MV and sulfur dioxide (which was not observed in above study), but not to ozone in both cases (Aono et al. 1993). Transgenic poplar plants over-expressing plastid directed *E. coli* GR resulted in ~500-fold transgene activity with close to two fold higher levels of both glutathione and ascorbate pools compared to control and cytosolic GR expressing lines. Transgenic lines were only resistant to photoinhibition by high-light intensity and chilling temperature (which is attributed to increased level of both GSH and ascorbate) and did not increase MV tolerance (Foyer et al. 1991). A pea cDNA when over-expressed in tobacco elevated GR activity in different sites like cytosol, plastid, and mitochondria, which was attributed to leaky targeting of GR due to presence of N-terminal transit peptide (Creissen et al. 1995). Transgenic lines with pea GR showed different levels of tolerance to ozone; however, none of them significantly reduced MV-induced damage (Broadbent et al. 1995). Surprisingly, few of transgenic lines with pea GR either with the native transit peptide or without any signal sequence showed tolerance to MV; again there was no correlation with the level of protection with the levels of GR expression. Payton et al. (1996) reported that tobacco over-expressing *Arabidopsis* GR had higher levels of GSH and also enhanced MV tolerance; however, some lines showed up to 20% reduction in GR activity, which could be due to sense suppression. Antisense glutathione reductase tomato lines accumulated more peroxides, leaked more electrolyte, and showed lower net photosynthetic rate as compared to WT plants (Feng Shu et al. 2011). Aono et al. (1995) generated antisense tobacco with reduced GR activity, which increased their susceptibility to MV-induced damage. Co-expression of both GR and SOD in cytosol of tobacco plants enhanced protection from MV treatment than transgenic lines with either gene expressed individually (Aono et al. 1995). Hence, it is clear that expressing combinations of different antioxidant enzymes can scavenge different ROS with synergistic effects further enhancing stress tolerance in transgenic lines.

6 Catalase Expressing Transgenic Plants

Catalases (CAT) are tetrameric heme containing enzymes, which directly dismutate H_2O_2 into water and molecular O_2, and they are indispensable for ROS detoxification during stressed conditions (Garg and Manchanda 2009). CAT has high

turnover rates with one molecule of CAT converting approximately 6 million molecules of peroxide per minute (Gill and Tuteja 2010). CAT plays an important role in scavenging peroxides generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration, and purine catabolism. Transgenic rice over-expressing endogenous type 1 metallothionein *OsMT1a* showed enhanced CAT activity and tolerance to drought stress (Yang et al. 2009). *B. juncea CAT 3* (*BjCAT3*) enhanced cadmium tolerance with two fold higher activity when compared to control plants (Guan et al. 2009). Over-expression of maize CAT and SOD in plastids of *B. campestris* not only enhanced tolerance to sulfur dioxide and salinity stress but also enhanced endogenous K^+, Mg^{2+}, and Ca^{2+}, which could maintain ion homeostasis and secondary signaling molecule (Tseng et al. 2007). Co-expression of *E. coli CAT* and Mn*SOD* in *B. campestris* also enhanced tolerance to sulfur dioxide and showed increased activity of APX and GR, which could reduce ROS accumulation. Peroxide accumulation in rice as a result of dismutation of $O_2^{\bullet -}$ is accelerated during chilling stress as SOD activity is normally enhanced during low temperature (Saruyama and Tanida 1995). A rapid increase in generation and accumulation of peroxides during chilling exposure was reported in wheat (Okuda et al. 1991). Although peroxides themselves are not lethal, they become highly toxic when they react with $O_2^{\bullet -}$ to form the highly reactive $OH^{\bullet}$ radical, which negatively act on various cellular components like proteins, DNA, and membrane lipids (Bowler et al. 1992). Expression of wheat CAT in rice reduced peroxide accumulation during chilling stress, and its superior tolerance is attributed to effective detoxification of peroxides in transgenic lines (Matsumura et al. 2002). Transgenic Cassava expressing endogenous catalase and Cu/ZnSOD had higher catalase, SOD activities, and lower MDA content than control plants after cold stress (Xu et al. 2014). Also, after harvest, the storage roots of over-expressing cassava lines showed a delay in postharvest physiological deterioration (PPD) response for approximately 10 days, which was accompanied by less mitochondrial oxidation and H_2O_2 accumulation. This was attributed to the combined expression of catalase and Cu/ZnSOD leading to an improved synergistic ROS-scavenging capacity in roots of transgenic plants (Xu et al. 2013). The physiological responses of tobacco with catalase-deficient plants revealed higher SOD activity (less catalase activity) in response to cadmium treatment (Iannone et al. 2015). On the other hand, a type 3 catalase from *B. juncea* enhanced tolerance to cadmium stress in tobacco. At 100 μM Cd, control plants became chlorotic, while *BjCAT*3 transgenic tobacco lines remained green and phenotypically normal. Transgenic lines had lower level of Cd-induced peroxide accumulation (Guan et al. 2009). In catalase-deficient *A. thaliana*, reduced peroxisomal catalase expression leads to increased sensitivity to ozone and photorespiratory peroxide-induced cell death (Vandenabeele et al. 2004). Transgenic tobacco expressing maize catalase 2 gene exhibited ~1.3-fold higher catalase activity and was not negatively affected by MV damage at moderate and high MV concentrations (Polidoros et al. 2001).

7 Current Limitations and Future Directions for the Usage of Transgenic Plants to Combat Oxidative Stress

It is clear from the above studies that modulating the expression of genes coding for ROS-scavenging enzymes should provide a better way to study their specific role, in stress tolerance, and interactions with other enzymes or nonenzymatic antioxidants or osmolytes. Although, modification of ROS-scavenging systems can cause considerable changes in oxidative stress tolerance, there are also several reports on the variance between levels of enzymes and plant tolerance to oxidative stress, which indicate that other cellular factors might be also involved in achieving such increased oxidative stress tolerance in transgenic plants. Also, reports of different transgenic plants expressing single enzymes has achieved only a slight increase in stress tolerance and failed to tolerate different kinds of stress factors. One of the major reason is the need for a balanced interaction of different ROS enzymes at similar levels. Besides, some of initial attempts did not increase stress tolerance (Tepperman and Dunsmuir 1990; Pitcher et al. 1991) due to enzyme sensitivity to products or lack of proper knowledge in targeting. Hence, from the enormous set of data on expression of ROS-scavenging enzymes known till date, it is obvious that an appropriate physiological balance of all the components of the antioxidative defense is crucial to achieve stress tolerance without affecting plants' normal physiological function. Moreover, studies with SOD and glutathione system have clearly indicated that in the future, enzyme should be engineered to overcome feedback inhibition or inhibition by-product.

Plants with the ability to scavenge and/or control the level of cellular ROS may be useful in the future to withstand harsh environmental conditions. Hence, alternate approaches like engineering of plants to reduce ROS levels using proteins like mitochondrial alternative oxidase (AOX), uncoupling protein (UCP), plastid targeted DnaJ protein, etc., are few of the promising genes, which have been proven already to reduce ROS in plants. AOX plays a role in lowering ROS formation in plant mitochondria. Besides function of thermogenesis in plants, the possible explanation for reduced ROS by AOX is explained below. AOX lowers ROS levels by acting on a second oxidase downstream of the ubiquinone pool maintains upstream components of electron-transport chain in a more oxidized state and hence lowering ROS generation by over reduced electron carriers (Maxwell et al. 1999; Umbach et al. 2005; Sugie et al. 2006; Zidenga et al. 2012; Vanlerberghe 2013).

Mitochondrial UCP is a specialized protein that uncouples electron transport from ATP synthesis (Nicholls and Rial 1999). Although thermogenesis was initially attributed to UCPs also, their ubiquitous presence suggests that this protein may have other functions including reducing ROS generation or maintaining its level. It has been already reported that UCP expression in transgenics positively regulated important physiological process like reduced ROS, stomatal conductance, transpiration rates, net photosynthesis, enhance seed viability, improved water balance, higher biomass, etc. (Begcy et al. 2011).

DnaJ proteins act as molecular chaperones (along with Hsp 70) and play a crucial role in maintaining protein conformation and cellular protein homeostasis under harsh environments like heat, high light, MV, chilling stresses, etc. (Piippo et al. 2006; Scarpeci et al. 2008; Rajan and D'Silva 2009). *Arabidopsis* knockout lines of DnaJ proteins induced global response to oxidative stress (Chen et al. 2010). Transgenic tomato lines over-expressing DnaJ accumulated lower ROS level during chilling stress (Kong et al. 2014).

8 Conclusion

New approaches using systems biology are opening doors to generate an all-inclusive transgenic line that are able to maintain its crop productivity even under stressed and changing environmental conditions. As already described above, numerous genes associated with plant response(s) to oxidative stress have been identified and used for generation of transgenic plants to overcome the stress. However, most of these studies were conducted only under controlled laboratory conditions applying artificial stress conditions using model plants like tobacco, *Arabidopsis*, *Medicago*, etc., with a main focus only on recovery from a particular type of stress. In fact plants in real time situations are exposed to different stresses under field conditions. Therefore, in the future, more emphasis should be on the development of transgenic crops (rather than model plants), and the testing should mimic the field condition (i.e., combination of environmental stresses) and focus should be on the crop yield.

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