Chapter 10
Involvement of AsA/DHA and GSH/GSSG Ratios in Gene and Protein Expression and in the Activation of Defence Mechanisms Under Abiotic Stress Conditions

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Abstract In a persistently changing environment there are many adverse abiotic stress conditions such as cold, heat, drought, salinity, heavy metal toxicity and oxygen deprivation, which remarkably influence plant growth and crop production. Plant cells produce oxygen radicals and their derivatives, so-called reactive oxygen species (ROS) during various processes associated with abiotic stress. Moreover, the generation of ROS is the main means for higher plants to transmit cellular signalling information concerning the changing environmental conditions. Therefore, plants have evolved inducible redox state-based sensing mechanisms that are activated or amplified in response to adverse environmental conditions. Ascorbate and glutathione, the key cellular redox buffers, are used for both detoxification of ROS and transmission of redox signals. In recent years, it has become clear that abiotic stress conditions induce changes in the reduction/oxidation (redox) state of signalling molecules, which in turn modulate gene and protein expression to increase plant acclimation to abiotic stress. This important redox state-related branch of science has given several clues in understanding the adaptive plant responses to different stressful regimes. In this chapter, an overview of the literature is briefly presented in terms of the main function of ascorbate and glutathione in plant cells. Further more, we describe how important forms of abiotic stress regulate the expression of genes and proteins involved in the ascorbate and glutathione redox sensing system.

Keywords Ascorbate • Glutathione • Redox state • Antioxidant enzymes • Gene expression • Abiotic stress • Reactive oxygen species

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1 Introduction

One of the major outcomes of the aerobic life of plant cells is the formation of reactive oxygen species (ROS). The term ROS defines the partially reduced forms of atmospheric oxygen. In general they result from the photodynamic excitation of oxygen, a process that ends with the formation of singlet oxygen ($^1O_2$) or during the partial reduction of oxygen through the transfer of one, two, or three electrons to its molecule that has as a result the birth of superoxide radical ($O_2 \cdot^-$), hydroxen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), respectively (Mittler 2002).

During the life cycle of the cell, ROS are formed in chloroplasts, mitochondria, plasma membrane, apoplastic space and peroxisomes. In chloroplasts ROS are produced during reactions that participate in the mechanism of photosynthesis, enhancing the concept that ROS are an unavoidable part of the aerobic metabolism of the cell. In mitochondria, $O_2 \cdot^-$ are produced in two segments of the electron transport chain: during respiration, in the flavoprotein NADH dehydrogenase (complex I) and in the ubiquinone zone. In the plasma membrane, the ROS-producing system is an NADPH oxidase. In the apoplastic space, cell wall-bound peroxidases have been shown to generate H_2O_2 and during stressful abiotic conditions ROS are formed in peroxisomes during photorespiration due to the formation of H_2O_2 by the enzymatic activity of glycolate oxidase (Corpas et al. 2001; Mittler 2002; Mittler et al. 2004).

Under stress-free growth conditions, the plant cell produces ROS, especially $O_2 \cdot^-$ and H_2O_2, in proportions that are kept at a rather low level (Asada 1994; Polle 2001). These molecules, apart from being toxic to the cell, act as second messengers and key regulators of growth, development and defence pathways, strengthening the dual role of ROS in cell biology (Mittler 2004; Foyer and Noctor 2005a). However, plants are not always cultivated under ideal conditions; in many cases various abiotic stress factors occur and the cellular homeostasis is disrupted resulting in the elevation of the level of ROS formation, a situation that is characterized under the heading of 'oxidative stress'. These abiotic stresses may include drought, salinity, high light, chilling, heavy metals, heat shock, ozone stress and others (Mittler 2002; Chalapathi and Reddy 2008).

During extreme abiotic stress conditions, high levels of ROS may lead to cell death; that event is the result of oxidative processes such as membrane lipid peroxidation, DNA/RNA nicking, protein oxidation and enzyme inhibition (Tanou et al. 2009c). For that case, the cell is equipped with a battery of antioxidant strategies, including enzymatic and non-enzymatic molecules. The latter facilitate the cell to be detoxified during extreme abiotic stress conditions, and also to keep ROS at an optimum level thus allowing all the necessary signalling cascades to take place in order to make all the appropriate modifications in gene/protein expression and cell structures in response to environmental and developmental stimuli (Foyer and Noctor 2005a). Among the enzymatic and non-enzymatic antioxidant pathways, ascorbate and glutathione are molecules with a regulatory role that participate in the redox signalling of the plant cell under abiotic stress conditions (Noctor 2006; Anjum et al. 2008a; Meyer 2008; Khan et al. 2009; Szalai et al. 2009).

Reduced glutathione (GSH) is a low molecular weigh tripeptide thiol with the formula γ-glu-cys-gly. This multifunctional molecule is a vital part of the antioxidant armory of the plant cell against oxidative stress, and contributes to the cellular defence and protection (Potters et al. 2002). Its pivotal role as an antioxidant derives from the fact that GSH participates in the ascorbate–glutathione cycle as the reducing agent of dehydroascorbate (DHA) while it also possesses the ability to protect the integrity of the cellular plasma membrane by maintaining a-tocopherol and zeaxanthine in the reduced state as well as protecting proteins from denaturation caused by the oxidation of protein thiol groups (Garnczarska 2005; Paradiso et al., 2008). In addition, glutathione exerts its antioxidant ability through the direct ROS scavenging as well as by acting as the substrate for glutathione peroxidase (EC 1.11.1.9; GPx) and glutathione-S-transferases (EC 2.5.1.18; GST), enzymes that participate in ROS removal. Finally, glutathione is involved in the formation of phytochelatines and together with its oxidized form (GSSG) they consist the redox couple (GSH/GSSG) which plays a drastic role in the maintenance of the cellular homeostasis and signalling system in plants (Rauser 1995; Ha et al. 1999; Clemens 2006; Srivalli and Khanna-Chopra 2008), leading to the suggestion that the GSH/GSSG ratio, indicative of the cellular redox balance, may be involved in ROS perception (Shao et al. 2008).

The concentration of GSH is increased under stressful conditions, and such an increase gives the ability to the cell to counteract the oxidation of glutathione and provokes alterations in gene expression directly or through interplay with regulatory proteins and/or transcription factors (Pasqualini et al. 2001; Ruiz and Blumwald 2002; Freeman et al. 2004). This elevation in GSH concentration is of vital importance, because it induces the signal transduction and defence against ROS which is achieved through a pathway with various control points, which include orchestrated activation of genes encoding enzymes related with glutathione metabolism (Sečenji et al. 2010). Therefore, high levels of glutathione provide the cell with the ability to counteract the negative effects of oxidative stress syndrome (Srivalli and Khanna-Chopra 2008).

Ascorbate (AsA) is considered by many as one of the most powerful and crucial antioxidants in the plant cell. Its presence has been detected in a wide range of cellular compartments like the cytosol, chloroplast, vacuoles, mitochondria and extracellular matrix (apoplast) (Kollist et al. 2000; Dipierro et al. 2005; Foyer and Noctor 2005b; Cheng et al. 2007). Under non-stress conditions ascorbate is detected mostly in its reduced form (Garnczarska 2005). Ascorbate is involved in the protection of a wide range of cellular compartments against oxidative attacks due to its ultra crucial ability to function as a donor of electrons in a broad range of enzymatic and non-enzymatic reactions (Noctor and Foyer 1998). Ascorbate possesses the ability to directly scavenge ROS and assist to the detoxification from H_2O_2 through its reduction via the ascorbate–glutathione cycle (Veljovic-Jovanovic et al. 2001; Anjum et al. 2008a; Khan et al. 2009). Other antioxidant abilities of AsA are the regeneration of the oxidized form of a-tochopherol as well as its ability to act as a cofactor of violaxanthine de-epoxidase (Blokhina et al. 2003). Specific cytosolic APX isoforms are significantly induced under the effect of abiotic stress and redox perturbation (Kubo et al. 1995; Karpinski et al. 1997; Ranieri et al. 2000; Murgia et al. 2004).
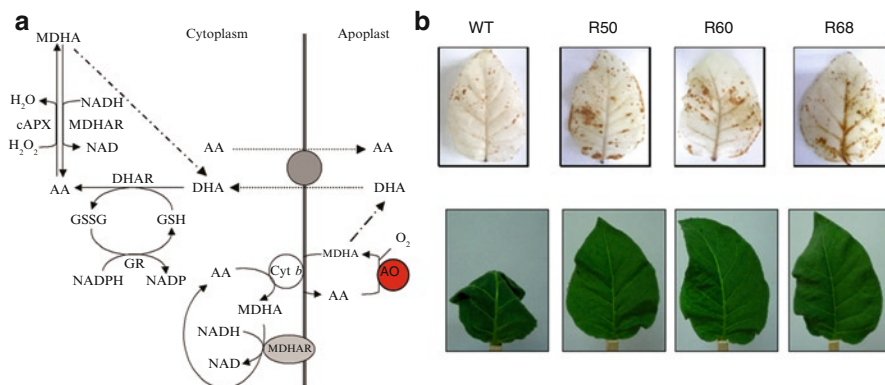
Fig. 1 (**a**) Schematic representation of the AsA–GSH cycle responsible for AsA recycling (Modified from Fotopoulos et al. 2006). Ascorbate oxidase which was targeted for over-expression in transgenic tobacco plants in the work by Fotopoulos et al. (2006, 2008) is *circled in red*. (**b**) Detached leaf assay and *in situ* localization of H_2O_2 in AO-over-expressing tobacco plants with reduced apoplastic AsA redox state (Taken from Fotopoulos et al. 2008). Fully-expanded leaves were detached from well-watered plants, immediately weighed, and held at room temperature for 1 h. Lower row displays images of representative leaves 1 h after detachment, while upper row displays leaves, (taken from plants grown under normal conditions, following incubation with 3,3′-diaminobenzidine (DAB) which polymerizes instantly (to form a reddish-brown complex which is stable in most solvents), as soon as it comes into contact with H_2O_2 in the presence of peroxidases. WT = wild-type, R50, R60 & R68 = transgenic tobacco lines over-expressing AO

Ascorbate and glutathione are linked through the ascorbate–glutathione cycle (outlined in Fig. 1a). During this cycle ascorbate is converted to the unstable radical monodehydroascorbate (MDHA) which rapidly disproportionates to yield dehydroascorbate (DHA) and AsA (Smirnoff, 2000). The latter is converted back to reduced ascorbate with the usage of reduced glutathione (GSH), acting as the electron donor in this reduction. During this process several enzymes coordinate their action in order to achieve the recycling of ascorbate, such as ascorbate peroxidase (EC 1.11.1.11; APX) and dehydroascorbate reductase (EC 1.8.5.1; DHAR) (Ushimaru et al. 1997). Efficient recycling of glutathione is ensured by glutathione reductase (EC 1.6.4.2; GR) activity (Noctor et al. 1998). Changes in turnover rates during the ascorbate–glutathione cycle may become manifested in altered redox ratios of AsA/DHA or GSH/GSSG (Tausz et al. 2004). Although DHA is reduced to ascorbate by DHAR, traces of DHA are always present in plant samples and the ratio of AsA/DHA is relatively lower compared to the ratio GSH/GSSG, especially under field conditions (Noctor et al. 1998).

During abiotic stress-driven oxidative stress, higher plants have the ability to sense, transduce and translate the ROS signals into specific cellular responses, a process that depends upon the presence of redox-sensitive proteins that possess the ability to accept reversible oxidation/reduction and may be functional or not according to the cellular redox state. ROS have the ability to oxidize redox-sensitive proteins directly or indirectly with the usage of molecules like glutathione. Cellular metabolism may be altered through the corresponding action of redox-sensitive metabolic

enzymes, whereas redox sensitive proteins exert their function via downstream signalling components such as kinases, phosphatases and transcription factors (Shao et al. 2008). A number of regulatory and structural genes which are under the orchestration of thiol–disulfate status and ROS signalling have been identified in transgenic and wild-type plants, contributing to the clarification of the function of the redox network (Tausz et al. 2004). This network manipulates the levels or ROS during abiotic stress conditions by interpreting signals received from various cellular compartments, with the redox couple GSH/GSSG having an exceptional role in its fine tuning (Szalai et al. 2009).

Although ascorbate and glutathione are united together through redox flux and coordinate their action during the metabolism of ROS, each one has specific roles in the regulation of the cellular status (Noctor 2006). Additionally, several studies conducted in a number of plant species under abiotic stress conditions have elucidated the fact that a high ratio of GSH/GSSG and/or AsA/DHA sustained by increased GSH and AsA or diminution of GSSG and DHA, may be the key element for efficient protection against abiotic stress-induced accumulation of ROS (Szalai et al. 2009). In this chapter, we present an up-to-date analysis of existing studies that reveal aspects of how ascorbate/glutathione-related responses are modulated by various envinromental stresses in plants.

2   Drought Stress

Drought is perhaps the most common abiotic stress limiting crop productivity world-wide. Different drought levels caused an oxidation of the glutathione pool in barley and pine leaves (Smirnoff 1993; Tausz et al. 2001), indicating that the maintenance of GSH/GSSG ratio appears to function as an important component of the plant's antioxidant defence system under drought stress. However, links between glutathione redox state and the drought tolerance are less clear. Lascano et al. (2001) found no clear differences between four different drought-tolerant wheat varieties following one month of drought exposure, but the two more resistant cultivars responded with an increase in total glutathione during the rewatering period. In the work of Loggini et al. (1999) two wheat cultivars were compared with different drought tolerance and the researchers found that both cultivars showed a higher (more reduced) GSH/GSSG ratio after a month of drought. Also, an increase in glutathione-related enzyme activities was only observed in the more susceptible cultivar. Moreover, Herbinger et al. (2002) reported an induction in total glutathione in wheat flag leaves exposed to drought stress, particularly evidenced in the more susceptible cultivar. In addition, in the work of Khanna-Chorpa and Selote (2007), susceptibility of Moti leaves during severe water stress was evident from the failure in H_2O_2 management and by a significant reduction in AsA/DHA and GSH/GSSG ratios. The high H_2O_2 level and/or oxidation of the ascorbate pool might have an inhibitory effect on antioxidant enzymes, particularly APX and GR, in the susceptible and in non-acclimated Moti leaves (Shigeoka et al. 2002). In the study of Sharma

and Dubey (2005) a decline in the concentration of total ascorbate as well as decline in AsA/DHA ratio was observed under drought stress in rice. Furthermore, the previous authors found that mild drought stress induced a significant increase in glutathione concentration which can be explained on the basis of significant increase in GR and DHAR activities. Results in this study further indicate enhanced activities of all the enzymes of the ascorbate–glutathione cycle, signifying a potential role of these enzymes in providing antioxidant defence under drought stress conditions.

It is known that an increase in AsA and an induction of ascorbate–glutathione cycle enzymes during water stress minimized the oxidative damage, but decrease in AsA content intensified oxidative processes during severe water stress conditions (Sgherri and Navari-Izzo 1995). AsA showed a reduction under drought stress in maize and wheat, suggesting its vital involvement in the oxidative response (Nayyar and Gupta 2006). Lascano et al. (2001) stated that lesser oxidative damage in the tolerant wheat cultivar during osmotic stress is due to higher AsA and induction of AsA–GSH cycle enzymes. Furthermore, Al-Ghamdi (2009) found that susceptibility of wheat leaves during severe water stress was evident from the failure in H_2O_2 management and by drastic oxidation of ascorbate–glutathione pool and significant reduction in AsA/DHA ratio. Finally, APX activity increased under drought stress in *Euphorbia escula* (Davis and Swanson 2001), *Zea mays* (Jiang and Zhang 2002), soybean (Riekert van Heerden and Kruger 2002), wheat (Dalmia and Sawhney, 2004), and *Populus accutifolius* (Turkan et al. 2005).

Several reports demonstrate the importance of the ascorbate–glutathione cycle in the regulation of the plant's response to drought stress, while numerous approaches have been made to produce transgenic crops tolerant to drought via the genetic manipulation of key enzymes in the ROS detoxifying and ascorbate recycling pathways. Examples include tall fescue and potato plants over-expressing SOD and APX (Tang et al. 2006; Lee et al. 2007 – for a comprehensive review see Cruz de Carvalho 2008).

Latest findings by Sečenji et al. (2010) who compared the responses of two wheat genotypes with differing capacity to withstand reduced water supply revealed major differences in ascorbate metabolism: both ascorbate oxidation and transcription levels of enzymes processing ascorbate were changed. Relative transcript levels of APX, monodehydroascorbate reductase (MDHAR), DHAR and GR isoenzymes showed different transcriptional changes in the two genotypes. Specifically, expression levels of two cytosolic APX isoenzymes and a thylakoid-bound variant increased significantly in the drought tolerant wheat cultivar while a cytosolic and a stromal APX coding transcript were found to be higher in the drought sensitive cultivar after a 4-week-long water deficit stress. In addition, mRNA levels of two cytosolic MDHAR isoforms were induced in the sensitive genotype, whereas only one was induced in the tolerant cultivar. An up-regulated chloroplastic DHAR was detected only in the sensitive cultivar. However, increased expression levels of a cytosolic and a chloroplastic GR were detected only in the tolerant wheat cultivar. Such a pattern of gene expression regulation following imposition of water stress was

accompanied by a significantly lower AsA redox state in leaves of the sensitive cultivar compared with the tolerant one, indicating that more robust transcription of ascorbate-based detoxification machinery may prevent an adverse shift of the cellular redox balance.

In a study by Chen and Gallie (2004), plants with an increased guard cell AsA redox state were generated by increasing DHAR expression, and these exhibited a reduction in the level of guard cell H_2O_2. In addition, a higher percentage of open stomata, an increase in total open stomatal area, increased stomatal conductance, and increased transpiration were observed. Guard cells with an increase in AsA redox state were less responsive to H_2O_2 or abscisic acid signalling, and the plants exhibited greater water loss under drought conditions, whereas suppressing DHAR expression conferred increased drought tolerance, thus suggesting that DHAR serves to maintain a basal level of AsA recycling in guard cells. The protective role of DHAR against oxidative stress was further supported by Eltayeb et al. (2006), who developed transgenic tobacco plants over-expressing cytosolic DHAR gene from *Arabidopsis thaliana*. Transgenic plants exhibited 2.3–3.1 fold higher DHAR activity and 1.9–2.1 fold higher level of reduced AsA compared with non-transformed control plants, resulting in enhanced tolerance to drought stress in terms of higher net photosynthesis.

Control of stomatal aperture is of paramount importance for plant adaptation to the surrounding environment. Work by Fotopoulos et al. (2008) reported on several parameters related to stomatal dynamics and performance in transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi) over-expressing cucumber ascorbate oxidase (AO), a cell wall-localized enzyme of uncertain biological function that oxidizes AsA to MDHA acid which dismutates yielding AsA and DHA. Transgenic tobacco AO-overexpressing plants exhibited significantly lowered AsA redox state in the apoplast. In comparison to wild type plants, leaves of AO over-expressing plants exhibited reduced stomatal conductance (due to partial stomatal closure), higher water content, and reduced rates of water loss on detachment. Transgenic plants also exhibited elevated levels of hydrogen peroxide and a decline in hydrogen peroxide-scavenging enzyme activity under normal growth conditions (see Fig. 1b). It should be noted that these transgenic tobacco plants also displayed enhanced sensitivity to various oxidative stress-promoting chemical agents, while RNA blot analyses suggested a correlation between such a response with a general suppression of the plant's antioxidative metabolism as demonstrated by lower expression levels of AsA recycling genes (Fotopoulos et al. 2006). Treatment of epidermal strips with either 1 mM DHA or 100 mM hydrogen peroxide resulted in rapid stomatal closure in wild type plants, but not in AO-over-expressing plants, therefore suggesting that signal perception and/or transduction associated with stomatal closure is altered by AO over-expression. These data support a specific role for cell wall-localized AsA in the perception of environmental cues, and suggest that DHA acts as a regulator of stomatal dynamics.

3 Salinity Stress

Salinity is a major abiotic stress that plants experience and greatly affects agricultural productivity. Due to changes in the osmotic balance, high salinity can cause water loss in cells and induce drought stress in plant tissues and results in ionic toxicity which can affect the physiological and biochemical function of the plant cell. Transgenic plants with low or high levels of AsA and GSH also exhibit an altered response to NaCl-induced oxidative damage (Huang et al. 2005; Yadav et al. 2005), demonstrating the crucial importance of a tightly orchestrated redox buffering capacity under salinity.

There is considerable evidence that GSH plays a protective role in salinity tolerance by maintaining the redox state (Gossett et al. 1996; Shalata et al. 2001). Salt stress induces increases in both GSH and γ-ECS activity (Ruiz and Blumwald 2002), which is known to be a key factor controlling the amount of GSH in leaves (Noctor et al. 1998). Additionally, Mittova et al. (2004) provided evidence that salt-dependent up-regulation of γ-ECS activity in tomato occurs at the level of transcription. In the study of Chaparzadeh et al. (2004), the increased foliar GR activity was accompanied by a decrease in GSH/GSSG ratio, suggesting that a predominant GSH oxidation took place under salinity. Furthermore, increases in GR activity during salt stress were reported in pea (Hernandez et al. 1993, 1995, 2000), cantaloupe (Fahmy et al. 1998), citrus (Gueta-Dahan et al. 1997), soybean (Comba et al. 1998), rice (Dionisio-Sese and Tobita 1998; Lin and Kao 2000; Vaidyanathan et al. 2003; Demiral and Turkan 2005; Tsai et al. 2005), tomato (Shalata et al. 2001; Molina et al. 2002; Mittova et al. 2003), *Arabidopsis thaliana* (Huang et al. 2005), wheat (Sairam et al. 2005), *Vigna radiate* (Sumithra et al. 2006), *Setaria italica* (Sreenivasulu et al. 2000), and *Helianthus annuus* (Davenport et al. 2003). On the other hand, over-expression of GR in plants leads to an increase in the antioxidant capacity and in the resistance to oxidative stress (Kocsy et al 2001).

It has been previously reported that salt stress leads to a decrease in the ascorbate–glutathione cycle components in salt sensitive cultivars and to an increase in salt tolerant ones (Hernández et al. 2001; Shalata et al. 2001; Mittova et al. 2003, 2004). Salt-induced oxidative damage led to necrotic lesions in the minor veins of pea leaves, as oxidative stress was higher in the apoplasts (Hernández et al. 2001). The GSH/GSSG ratio declined under salt stress, and there was no GR activity in the apoplasts of the sensitive pea cultivar Lincoln. Continuous exposure to salt stress in rice seedlings made them more tolerant when the GSG/GSSG levels returned to normal values after an initial decline (Fadzilla et al. 1997). The results of Mittova et al. (2003) indicate that salt tolerance is linked to the ability to up-regulate enzymes of GSH synthesis and utilization and that this is absent from the salt sensitive species. A comparison of the total amounts of GSH and the GSH/GSSG ratios in the tissues of both species under salinity indicates that the dramatic decrease in the GSH/GSSG ratio in salt-stressed *Solanum lycopersicum* roots is due to other factors such as NADPH deficits, rather than limitations on GR activity. A recent analysis in citrus plants revealed that pre-exposure to sodium nitroprusside (SNP), a donor of nitric oxide (NO), prior to salinity resulted in higher (less oxidized)

glutathione redox compared to NaCl-treated as well as control plants (Tanou et al. 2009b) providing a link between glutathione and nitric oxide during the establishment of salt tolerance. Additionally, proteome-wide analysis revealed the involvement of many ascorbate/glutathione-related proteins in the acclimation of citrus plants to high salinity (Tanou et al. 2009a).

Some researchers have reported that salt stress leads to a decrease in ascorbate content in salt-sensitive cultivars (Hernández et al. 2001; Shalata et al. 2001; Mittova et al. 2003, 2004). The biosynthetic capacity of ascorbate is impaired under stress conditions because the ascorbate pool is generally determined by its rates of not only regeneration but also synthesis (Song et al. 2005). It is also reported that regeneration of ascorbate under salinity is insufficient or that ascorbate synthesis is lower than ascorbate catabolism (Shalata et al. 2001; Amor et al. 2006). However, in some plants acclimated to salinity a significant increase in total ascorbate was found (Shalata et al. 2001). More important than the total AsA content is the AsA/DHA ratio that, in the case of roots in high and leaves in low salinity conditions, was found to be comparable to the values observed in salt-tolerant cotton plants and calli (Gossett et al. 1996). The low AsA/DHA ratio in leaves at high salinity might be an indication of APX participation in ROS scavenging. A proper increase of AsA, during H_2O_2 increase in conditions of high salinity, may be important for maintaining APX activity, being that APX is inactivated when ascorbate concentration drops dramatically (Asada 1999). Under salinity, AsA is mainly regenerated from MDHA (Shalata and Tal 1998; Mittova et al. 2000) or DHA (Meneguzzo et al. 1999). In marigold plants under salinity stress (*C. officinalis*), the decreasing trends of both MDHAR and DHAR activities may suggest that a non-enzymatic disproportionation of MDHA to AsA and DHA or a reduction of MDHA by reduced ferredoxin and b-type cytochrome (Noctor and Foyer 1998) could participate in AsA regeneration.

In view of the known links between oxidative stress and cell death, it is surprising that few studies have addressed the role of salt stress and cell death responses in plants. The results of a recent study by Tanou et al. (2009c) illustrate how the oxidation of ascorbate/glutathione pool participates in the oxyradical-mediated necrotic death-like destruction in salt-stressed leaves. The model of ascorbate/glutathione-mediated in salt dependent-oxidative cell death, based on components identified in strawberry response leaves, is outlined in Fig. 2.

Several approaches have been made in order to produce transgenic plants with an acquired tolerance to salinity stress by manipulating gene expression levels of various antioxidant enzymes involved in the ascorbate–glutathione cycle such as DHAR and APX (for a comprehensive review see Ashraf 2009). Enhanced expression of DHAR was achieved by the expression of rice DHAR in transgenic Arabidopsis plants (Ushimaru et al. 2006). This study further demonstrated that transgenic Arabidopsis plants showed enhanced tolerance to salt despite the fact that there had been a slight increase in DHAR activity and total ascorbate in the transgenic plants. The protective role of DHAR against oxidative stress was further supported by Eltayeb et al. (2006), who developed transgenic tobacco plants over-expressing cytosolic DHAR gene from *Arabidopsis thaliana*. Transgenic plants
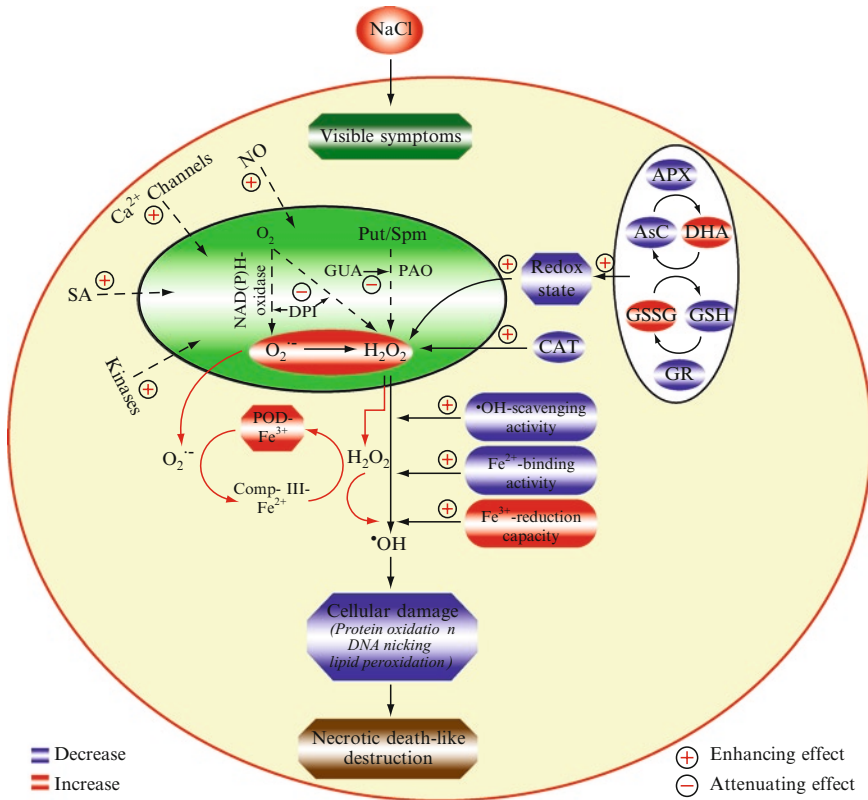
Fig. 2 Model schematizing some of the relationships among cellular redox state, ROS production and necrotic death-like destruction in leaves of strawberry plants exposed to salinity. NaCl ensures activation of membrane-bound NAD(P)H-oxidase produces H_2O_2 and $O_2 \cdot^-$. H_2O_2 also generated via the polyamine oxidase (PAO)-mediated catabolism of high Put and Spm. The low cell redox state, as indicated by the accumulation of the oxidized forms of ascorbate (DHA) and glutathione (GSSG), together with the inhibition in the activities of some H_2O_2-scavenging enzymes ensures H_2O_2 generation. Nitric oxide (NO), salicylic acid (SA), protein kinase and Ca^{2+} channel activity promote NaCl-dependent H_2O_2 production. NaCl-induced high Fe^{3+}-reduction capacity, and low Fe^{2+}-binding and $\cdot$OH scavenging activities provoke the conversion of H_2O_2 to $\cdot$OH via the Fe^{2+}-catalyzed Fenton reaction chemistry and favor the cellular oxidative damage and necrotic death-like destruction. It is possible that apoplastic peroxidase (POD), transformed into the Compound III by $O_2 \cdot^-$, catalyzes also the generation of $\cdot$OH in the presence of H_2O_2 (Reprint from Tanou et al. 2009c)

displayed enhanced DHAR activity and higher levels of reduced AsA compared with non-transformed control plants, maintaining redox state of AsA and ultimately resulting in enhanced tolerance to salt stress. Similarly, differential tolerance to salinity was achieved by over-expressing/suppressing the expression of ascorbate oxidase in tobacco and Arabidopsis (Yamamoto et al. 2005). AO activities in the transgenic tobacco plants expressing the gene in sense and antisense orientations were, respectively, about 16- and 0.2-fold of those in the wild type. At high

salinity conditions, the percentage germination and photosynthetic activity were higher in antisense plants. In addition, the redox state of apoplastic ascorbate in sense plants was very low even under normal growth conditions, whereas upon salt stress, the redox state of symplastic and apoplastic ascorbate decreased among the three types of plants, but was lowest in the sense plants.

Acquired tolerance to salinity has also been demonstrated via genetic manipulation of other gene targets not directly linked to the ascorbate–glutathione pathway. Yadav et al. (2005) developed transgenic tobacco plants over-expressing glyoxalase pathway enzymes that suppress accumulation of methylglyoxal (MG) in plants under salt stress. The transgenic plants showed enhanced activity of various gluta-thione-related antioxidant enzymes under both control and saline conditions. Additionally, these plants maintained high contents of reduced glutathione and overall increased glutathione redox state under salt stress, thus suggesting that hindrance in an increase in MG coupled with maintaining higher reduced glutathione levels can be considerably achieved by the over-expression of glyoxalase pathway enzymes for developing salt tolerant plants. Latest findings by G. Tanou et al. (unpublished data) provide significant evidence that NaCl application in citrus leaves results in the oxidation of the leaf ascorbate redox state, while the glutathione redox state remained unaltered. Antioxidant enzyme activity assays revealed that an array of redox-related proteins is induced as a result of high salinity stress, whereas transcript regulation was more variable.

4   Chilling-Low Temperature Stress

Chilling impairs all photosynthetic components provoking, for example, reduction of stomatal conductance, changes in pigment complexes and losses of photochemical efficiency, modifications in the biophysical properties of thylakoid lipids and restriction of electron transport, as well as decreases in enzyme activity and protein metabolism (Ensminger et al. 2006). The level of damage is dependent on the plant species, the developmental and phenological stage, the organ and tissues, and the degree of stress. ROS are also involved in freezing stress, participating in lipid peroxidation, protein destruction, and collapses of the antioxidant defence systems (Polle 1997). Thus, the improvement of chilling stress tolerance is often related to enhancement of activities of antioxidant systems in plants.

It has been documented that in order to prevent hydrogen peroxide accumulation to toxic levels during chilling stress, a high capacity of the AsA–GSH cycle is necessary (Kocsy et al. 2001). In the work of Zhang et al. (2008) a significant increase in ascorbate and glutathione concentration was observed during chilling stress. Higher glutathione concentrations in chilled maize plants are the result of an induction of key enzymes of glutathione synthesis, as well as sulphate reduction, which also increases cysteine levels (Kopriva et al. 2001). Increases in GSH levels and/or GR activity during chilling stress have been observed in many plant species such as tomato (Walker and McKersie 1993), sorghum (Badiani et al. 1997), wheat

(Kocsy et al. 2001), jack pine (Zhao and Blumwald 1998), and poplar (Foyer et al. 1995). A long duration chilling stress experiment in maize showed increased activities of various antioxidant enzymes, including GR (Hodges et al. 1997). In line, chilling stress and cold acclimation studies in rice showed an increase in GR activity (Oidaira et al. 2000; Kuk et al. 2003). In cotton, the increase of the activities of glutathione–ascorbate cycle enzymes in chloroplasts by genetic manipulation increased resistance to chilling-related photo-oxidative stress under laboratory conditions (Payton et al. 2001). When transgenic cotton overproducing GR was field-grown, there was no significant difference with wild plants as GR activity doubled in wild-type cotton during slow chilling exposure in the field (Logan et al. 2003). Genetic transformation studies for freezing tolerance in maize showed the up-regulation of three genes, including GSTs, under both normal and cold-acclimated conditions (Wang 2005). GSTs are involved in the oxidative signalling pathway and contribute to the genetic acclimation towards freezing tolerance in maize. Proteome analysis of chilling stress in rice showed the up-regulation of cysteine synthase (Yan et al. 2006). This enzyme is responsible for the final step in cysteine biosynthesis, a key-limiting step in GSH production. Thus, GSH and GR are important for resistance to chilling stress.

During chilling and cold acclimation, the maintenance of a high GSH/GSSG ratio is very important in order to ensure that GSH can function appropriately in the AsA–GSH cycle and other physiological processes. During chilling, the GSH content and the GSH/GSSG ratio were higher in tolerant genotypes of tomato compared with sensitive ones (Walker and McKersie 1993). Correspondingly, the GSH/GSSG ratio was generally higher in chilling-tolerant maize genotypes than in sensitive ones at 11°C (Hodges et al. 1996), demonstrating that the maintenance of a high GSH/GSSG ratio contributes to improved chilling tolerance. The higher GSH/GSSG ratio in the freezing-tolerant genotypes may keep the sulphhydryl groups of the proteins in reduced form, thus decreasing the possibility of intermolecular disulphide bridge formation when the plants are exposed to freezing temperatures (Levitt 1962).

In the works of Huang and Guo (2005) and Dai et al (2009), conducted using tolerant and sensitive rice and barley cultivars, respectively, the AsA content had a different pattern among the two cultivars in response to chilling. The tolerant one showed elevated amounts of AsA in contrast to lower levels in the sensitive one. In the same study the tolerant cultivar exhibited higher activities of APX and GR, an observation that is in accordance with the findings of Tao et al (1998) who used pine trees as a research model. These elevated activities of APX and GR facilitate the cell to cope with the oxidative stress due to chilling conditions. In addition, work by Guo et al. (2006) who tested four rice cultivars under chilling condition concluded that the chilling tolerance was well correlated with the enhanced antioxidant capacity of the cultivars, a capacity that was attributed to the higher AsA content and enhanced activity of antioxidant enzymes like APX and GR.

Freezing injury has been shown to involve the participation of ROS. Antioxidant enzymes can protect plant cells from oxidative stress imposed by freezing injury; therefore, cold acclimation may involve an increase in the expression of antioxidant

enzymes. In a work carried out by Baek and Skinner (2003), quantitative RT-PCR was used to measure the expression levels of a wide range of antioxidant enzymes during cold acclimation in near-isogenic lines (NILs) of wheat, differing in the Vrn1-Fr1 chromosome region that conditions winter versus spring wheat growth habit. The expression levels of several antioxidant enzyme transcripts were induced (Mn-SOD, MDAR, t-APX, DHAR, GPX, and GR), suppressed (CAT), or remained relatively constant (FeSOD and Cu/Zn–SOD). The Vrn1-Fr1 region appeared to have a role in regulating the expression level of some of the antioxidant enzyme genes because CAT, t-APX and Mn-SOD transcripts were expressed at significantly higher levels in the winter wheat NIL than the spring wheat NIL after 4 weeks' cold acclimation. More recently, Fortunato et al. (2010) performed the characterization of the antioxidant system of *Coffea* sp. genotypes with different cold acclimation abilities using an integrated biochemical and molecular approach. Cold-tolerant variety Icatu showed the greatest ability to control oxidative stress, as reflected by the enhancement of several antioxidant components (Cu/Zn–SOD and APX activities; ascorbate, α-tocopherol and chlorogenic acids contents) and lower reactive oxygen species contents (H_2O_2 and ·OH). Gene expression studies showed that GR and DHAR might also be involved in the cold acclimation ability of the cold-tolerant variety. The difference in the triggering of antioxidant components supports the hypothesis of their importance to cold tolerance in *Coffea* sp. and could provide a useful probe to identify tolerant genotypes.

5 Heat Stress

Although there have been several reports on oxidative stress and the response of antioxidant defence mechanisms in heat-stressed plants (Dat et al. 1998; Anderson and Padhye 2004), there have been fewer reports focusing on glutathione homeostasis. Treatment of maize roots to heat shock temperatures of 40°C resulted in decrease of cysteine levels and increase in GSH levels (Nieto-Sotelo and Ho 1986). There was an increase in the GSH-synthesizing capacity in maize root cells, which was related to the cell's capacity to cope with heat stress conditions. Accumulation of GSH has also been observed in heat-stressed tomato seedlings (Rivero et al. 2004). In wheat, it was established that heat stress induced accumulation of GSH levels and increased the activity of the enzymes involved in GSH synthesis and the GSH/GSSG ratio (Kocsy et al. 2001). Heat stress increased GSH levels during grain development in the flag leaf of two wheat genotypes with contrasting behavior under heat stress (Chauhan 2005). The GSH content was also higher in a heat-tolerant cabbage genotype compared to a sensitive one after heat stress.

There is increasing evidence for considerable interlinking between ascorbate redox state-related responses and heat stress. In the study of Ma et al. (2008), the contents of total ascorbate, AsA, total glutathione and GSH in apple leaves was increased during imposition of high temperature (40°C) for 2 h. The high thermal activation of GR observed in apple leaves after exposure to 40°C suggests that GR also

play an important role in the adjustment of metabolism to high temperature. APX and GR play an important role in the protection of plants from high temperature stress by preventing the oxidation of enzymes and membranes (Almeselmani et al. 2006). Furthermore, the study of Ma et al. (2008) demonstrated that, gene expression of APX, DHAR and GR in apple leaves in the high temperature (40°C) treatment was increased, compared with the control (28°C). In addition, cultivation of Arabidopsis plants at elevated but non-stress temperatures led to the increase of APX enzymatic activity and of foliar concentration of AsA (Panchuk et al. 2002). This suggests that the activation of AsA-dependent antioxidation system may be a pre-adaptive reaction to an enhanced production of ROS under severe heat stress. Song et al. (2005) stated in their study that the redox state of AsA is believed to play a pivotal role in influencing APX isoenzymes activities. Double Arabidopsis mutant *tylapx* and *capx1* mutant lacking thylakoid ascorbate peroxidase (tylapx) and cytosolic ascorbate per-oxidase1 (capx1) showed enhanced tolerance to heat stress (Muller et al. 2007).

Not an extremely wide body of evidence exists linking the gene expression involved in the regulation of the plant's response to heat stress with ascorbate–glutathione redox states. Work by Larkindale et al. (2005) investigated the importance of different processes to heat stress tolerance. Plants tested were reactive oxygen metabolism mutants with lowered ascorbate levels (*vtc1*, *vtc2*), and these were more defective in basal (heating to 45°C) than acquired thermotolerance (pre-exposure to 38°C, followed by acclimation to room temperature and re-heating at 45°C), especially under high light. All mutants accumulated wild-type levels of heat shock protein 101 and small heat shock proteins, which are typical markers detected under stressful conditions.

6 Heavy Metal Stress

Heavy metal pollution of soils and waters is a major environmental problem. Some of the heavy metals are essential for the plant growth when they are present in normal levels; however, when they are present in excess, they cause toxic effects on plant growth, ultimately resulting in decreased yields or even plant death. Heavy metals are also known to induce free radicals in plants and, consequently, oxidative damage (Dietz et al. 1999).

Cadmium (Cd) is a non-redox metal unable to produce ROS via Fenton and/or Haber–Weiss reactions. However, several lines of evidence have revealed that oxidative stress is a major component of Cd phyto-toxicity (Piqueras et al. 1999; Sandalio et al. 2001; Romero-Puertas et al. 2004; Cho and Seo 2005; Hsu and Kao 2007; Anjum et al. 2008a, b, c, d; Khan et al. 2009).

Phytochelatins (PCs) comprise one of the mechanisms involved in the chelation of heavy metals by a family of peptide ligands. Many trace metals in an environment are known to induce PC production by plants and Cd has been found to be the most effective inducer of phytochelatins (Nishikawa et al. 2006). Phytochelatins form a

family of peptides with a structure based on repetitions (2–11 times) of the γ-Glu-Cys dipeptide followed by C-terminal glycine (Gly), and they are structurally related to glutathione (GSH), which is a substrate for their synthesis (Rauser 1995; Ha et al. 1999; Clemens 2006). Thus, one of the mechanisms by which plants can withstand Cd toxicity is by maintaining high levels of phytochelatin or its precursor, GSH, which functions as a heavy metal ligand (Cánovas et al. 2004). Upon heavy metal exposure, GSH concentrations drop as a consequence of initiated PCs biosynthesis. This causes oxidative stress and in turn short-term toxicity (Schützendübel and Polle 2002; Nocito et al. 2006).

Cadmium was shown to induce a significant increase in the mRNA expression level of genes involved in GSH synthesis (*gsh1* and *gsh2*) and phytochelatin synthase (*pcs1*) in leaves of *Arabidopsis thaliana* (Semane et al. 2007). The authors observed a significant decrease of reduced GSH in Cd-treated plants, while Cd treatment increased the accumulation of GSSG, keeping the GSH/GSSG ratio lower than in control plants. The accumulation of GSSG was accompanied by suppressed GR mRNA levels, while the activity of GR was significantly enhanced. In addition, the authors observed a general increase of ROS-scavenging enzymes such as APX, CAT or SOD, indicating that the plants respond to Cd stress by activation of the AsA–GSH defence network at both transcriptional and enzymatic level. Studies of transgenic *Brassica juncea* plants, in which the expression of the GSH biosynthetic pathway enzymes was increased, have shown that PC biosynthesis and Cd tolerance have been correlated with over-expression of GSH (Zhu et al. 1999). Zhang and Ge (2008) found a close relationship between Cd level and GSH content as well as GST activity, suggesting that these two parameters of antioxidant defence system may be used as biomarkers of Cd-induced stress. Accumulated evidence suggests that GR plays an important role in the detoxification of Cd-induced ROS, possibly via the ascorbate–glutathione cycle. Increased GR activity in the roots exposed to Cd was reported in plants, including *Phaseolus vulgaris* (Chaoui et al. 1997), potato (Stroinski et al. 1999), radish (Vitoria et al. 2001), soybean (Ferreira et al. 2002), sugarcane (Fornazier et al. 2002), *Arabidopsis thaliana* (Skorzynska-Polit et al. 2003, 2004) and alfalfa (Sobrino-Plata et al. 2009). Moreover, GR activity was enhanced in shoots and roots of alfalfa plants exposed to Cd (Sobrino-Plata et al. 2009).

Notably, apart from its involvement in PC biosynthesis, GSH may contribute in several other ways to heavy metal tolerance. It may sequester toxic metal ions in the cytosol, and such complexes may activate PC synthase, transfer metal ions to newly synthesized PCs, or transport them to the vacuole (Howden et al. 1995; May et al. 1998; Xiang et al. 2001). Interestingly, recent findings by Wojas et al. (2008) suggest that the ability of high rate PC synthesis in transgenic tobacco plants over-expressing an *A. thaliana* PC synthase is insufficient to cope with the metal load if the functionality of the antioxidant system is simultaneously hampered. The authors demonstrated that transgenic plants were Cd-hypersensitive compared with wild-type plants, as manifested by strong depletion of GSH and higher oxidative stress.

Ascorbate concentrations were elevated following Cd exposure indicating that this antioxidant compound is necessary for redox cellular homeostasis under Cd stress (Sobrino-Plata et al. 2009). When AsA biosynthesis was enhanced by feeding plants with its last biosynthetic precursor, l-galactono-γ-lactone (GalL), Cd uptake was not affected (Paradiso et al. 2008). Chao et al. (2010) demonstrated that Cd toxicity of rice seedlings was accompanied by a decrease in the contents of AsA and AsA + DHA and in the ratios of AsA/DHA in leaves. Conversely, pre-treatment with AsA resulted in an increase in the contents of AsA and GSH, the ratios of AsA/DHA and GSH/ GSSG, and the activities of APX and GR in the leaves of rice seedlings, while several transcripts encoding APX and GR isoenzymes (*OsAPX2*-7 and *OsGR1*) were induced in rice leaves following AsA pre-treatment. Moreover, Hatata and Abdel-Aal (2008) found that AsA was markedly decreased to a very low level at high concentration (100 μM) of Cd stress, with a corresponding increase in the level of reduced ascorbate (DHA) indicating that DHA content was significantly enhanced probably through suppressed glutathione-dependent DHAR activity and/or due to a decrease in AsA synthesis. In addition, APX activity increased and a new basic root peroxidase isoform was found in Cd-treated alfalfa plants (Sobrino-Plata et al. 2009).

The role of AsA as an efficient scavenger for oxidative compounds is well-known (Polle and Rennenberg 1993). Furthermore, the effectiveness of AsA–GSH-regenerating enzyme system comprising MDHAR, DHAR and GR, and the maintenance of AsA, DHA, GSH and GSSG pools may contribute to controlling Cd-caused oxidative stress in plants (Paradiso et al. 2008; Anjum et al. 2010). The cellular concentration of AsA is, in fact, determined by the rate of its synthesis and decay. DHA is rapidly hydrolyzed into 2,3-diketogulanic acid if not reduced by DHAR. Anjum et al. noticed an enhancement in DHAR activity in Cd-exposed plants which could not maintain the AsA pool. Hence, they suggested that AsA generated by DHAR was utilized by some other metabolic function(s). It is also important to mention here that parallel to enhancement in AsA-regenerating enzyme activities, the activity of APX, an H_2O_2 scavenging enzyme, consumes/uses AsA as a reductant (Willekens et al. 1995). Besides, Anjum et al. (2010) reported Cd-induced increase in DHA with a corresponding increase in MDHAR activity and confirmed that this metabolite was chiefly formed by enzymatic action and not by non-enzymatic disproportionation which is in coincidence with results of Paradiso et al. (2008). In addition, Anjum et al. (2010) reported Cd-induced decrease in GSH pool in Cd-treated moongbean cultivars and suggested that the depletion of GSH pool due to Cd stress in spite of higher GR activity may indicate the mechanism of antioxidant defense through enhanced oxidation of GSH to GSSG by DHAR thus yielding AsA which was later utilized by APX for the detoxification of H_2O_2. In fact, GSH functions as an antioxidant by scavenging ROS, resulting in the oxidation of GSH to GSSG. It is well-established that not only the pool of GSH but also GSH/GSSG ratio is important to maintain the redox status of the cell (May et al. 1998; Paradiso et al. 2008). In the study of Anjum et al. (2010), the pool of GSH and also the ratio of GSH/GSSG (more, mainly due to Cd-induced decline in GSH pool) were significantly reduced. Furthermore, the reduced GSH/GSSG redox

state of glutathione under Cd stress also indicated that maximum metabolic load was exerted to maintain redox buffer status of the cells, suggesting a leading role of GSH in an adaptive response to Cd stress and the maintenance of redox status in physiological conditions to a greater extent in Cd-tolerant moongbean cv. Pusa 9531 than in Cd-susceptible cv. PS 16. Similar results have been reported earlier by Anjum et al. (2008a, c) in *Brassica campestris* and *Vigna radiata*.

Copper (Cu) is an essential micronutrient required by all living organisms. Nevertheless, the reactive nature of ionic Cu makes it a toxic metal if not properly handled by the cell. One of the main reasons for these disturbances is due to the generation of hydroxyl radicals by free copper, which readily oxidize disulfide bonds within proteins, destroying their secondary structure, and also causing catastrophic damage to lipids and nucleic acids (Hanna and Mason 1992). Like Cd, PCs were proved to play an important role in Cu toxicity. Cobbett and Goldsbrough (2002) found that Cu is also a strong activator of PC biosynthesis both *in vivo* and *in vitro* and can form stable complexes with PCs. However, GSH does not appear to be directly involved in Cu detoxification and tolerance in *A. thaliana* since its role as a substrate for PC synthesis in Cu-stressed plants is not fully deciphered (Wójcik et al. 2009). In earlier reports, a reduction in cellular GSH levels as well as GR activity was observed in plants exposed to toxic levels of Cu (Gallego et al. 1996; Mazhoudi et al. 1997; Patra and Panda 1998; Tewari et al. 2006). In the study of Drazkiewicz et al. (2003), the GSH/GSSG ratio played an important role in a short-time exposure of plants to Cu (after 1 and 3 days) as well as GSH after 7 days. Though the concentration of total ascorbate increased with the increasing supply of Cu, the ratio of the redox couple (DHA/AsA) increased in Cu-deficient or Cu-excessive mulberry plants (Tewari et al. 2006). The latter authors conclude that the disturbed redox state of the cellular environment due to increased DHA/AsA ratio could have caused accelerated senescence and poor growth of the Cu-excess plants. In addition, proteomic analysis of Cu-treated Arabidopsis seedlings (Smith et al. 2004) revealed a regulatory role towards enzymes such as GSTs and GPx which contribute to the redox poise of the cell and, thus reflect the modified redox state of the cells induced by Cu.

Aluminium (Al) is a major constituent of soil and, consequently, plants often grow in soil environments in which the roots are potentially exposed to high concentrations of aluminium. The possible connection between Al toxicity and oxidative stress had been previously suggested by Cakmak and Horst (1991) following the finding that the Al-induced inhibition of root elongation was correlated with enhanced lipid peroxidation. Oxidative stress is evidently involved in the development of Al-induced toxic symptoms. Thus, Al-tolerant lines of wheat developed by *in vitro* microspore selection produced lower amounts of ROS in response to Al and grew better in comparison with the Al-sensitive genotype. The Al tolerance could tentatively be ascribed to the higher activity of GST observed in the tolerant lines (Darkó et al. 2004). Oxidative stress caused by Al toxicity is an early symptom that can trigger cellular redox state as well as root growth inhibition in Macaca (Al-sensitive) and SMIC148-A (Al-tolerant) potato clones (Tabaldi et al. 2009). Furthermore, the increased level of hydrogen peroxide in pumpkin (*Cucurbita*

pepo) roots treated with aluminium sulphate was matched by both increased APX activity and ascorbate free radical reductase (AFRR) activity, while DHAR and GR did not change (Dipierro et al. 2005). Sharma and Dubey (2007) stated that the enhanced activities of enzymes of ascorbate–glutathione cycle MDHAR, DHAR and GR observed in Al^{3+} stressed rice seedlings appear to be due to the need of maintaining a favourable redox state, by maintaining a sufficient amount of reduced ascorbate and reduced glutathione and to overcome the possible problems of oxidation. Several approaches have been made in order to produce transgenic plants with an acquired tolerance to heavy metal stress including Al by manipulating gene expression levels of various antioxidant enzymes involved in the ascorbate–glutathione cycle, as well as genes involved in phytochelatin biosynthesis (for a comprehensive review see Sharma and Dietz 2009). Basu et al. (2001) demonstrated the importance of oxidative stress in Al tolerance by producing transgenic oilseed rape plants over-expressing a mitochondrial bread wheat Mn-SOD. Transgenic plants showed 1.5–2.5-fold greater SOD activity than wild type plants, resulting in reduced malondialdehyde accumulation and overall growth inhibition in response to Al. Yin et al. (2010) investigated the role of MDAR and DHAR in AsA regeneration during Al stress using transgenic tobacco plants over-expressing Arabidopsis cytosolic MDHAR or DHAR. Transgenic DHAR-over-expressing (but not MDHAR-over-expressing) plants showed better root growth than wild type plants after exposure to toxic Al level accompanied by lower hydrogen peroxide content, less lipid peroxidation and lower level of oxidative DNA damage. Furthermore, DHAR-over-expressing plants maintained a higher AsA level both with and without Al exposure when compared with wild type plants, in contrast with MDAR-over-expressing plants which maintained a higher AsA level only without Al exposure. These findings allowed the authors to suggest that the over-expression of DHAR, but not of MDAR, confers Al tolerance, and that maintenance of a high AsA level is essential towards acquiring Al tolerance.

Although nickel (Ni) is an essential element for plants, it is strongly phytotoxic at high concentrations (Eskew et al. 1983; Eskew et al. 1984). Nickel tolerance and hyperaccumulation in *Thlaspi* species is linked to the constitutive ability to accumulate higher concentrations of GSH, Cys and O-acetyl-L-serine (Freeman et al. 2004). The elevated GSH concentrations in *T. goesingense* were driven by constitutively elevated activities of serine acetyl transferase (SAT), which provides the C skeleton for Cys synthesis. The causality was proven in a transgenic approach: the overproduction of *T. goesingense* SAT in the non-accumulator *A. thaliana* led to the accumulation of O-acetyl-L-serine, Cys and GSH and coincided with strongly enhanced resistance to Ni-induced growth inhibition and oxidative stress (Freeman et al. 2004). On the other hand, the ascorbate recycling seems to be a mechanism of great importance in controlling the cellular redox state and the phenotypic performance of plants exposed to Ni. Exposure to 0.25 mM $NiCl_2 \times 6H_2O$ for 5 days resulted in toxicity symptoms, such as formation of reddish-brown mottled spots on the leaf blade in soybean seedlings (Saeidi-Sar et al. 2007). Exogenous addition of 1 mM AsA totally reversed these Ni-associated toxic symptoms. Also, the Ni-stressed seedlings exposed to AsA exhibited an improved growth as compared to Ni-treated

plants while AsA considerably reduced root-to-shoot translocation of Ni (Saeidi-Sar et al. 2007). Contrary to DHAR and GR, of which the activities were unaffected by Ni, MDHAR was stimulated by Ni treatment in maize (*Zea mays* cv. LG 23/01) plants; it appears, thus, that the regeneration of ascorbate was accomplished through an activation of MDHAR following toxic Ni exposure (Baccouch et al. 2001).

7 Light Stress

In the natural environment, plants are frequently exposed to fluctuating light intensities and often absorb more light energy than can be consumed by photosynthetic metabolism and thus require that excess excitation energy be dissipated. Failure to dissipate excitation energy results in over-reduction of the photosynthetic chain components that direct linear electron flux from water to NADPH (Baker et al. 2007). Part of the absorbed light energy is dissipated as heat in the light-harvesting complexes of photosystem II (PSII) through non-photochemical quenching (Müller et al. 2001). Additional dissipation of excitation energy is also achieved by photochemical quenching (Baker et al. 2007) and reflects that action of processes such as the reduction of molecular oxygen at photosystem I by the Mehler reaction (Ort and Becker 2002). In this reaction, the photoreduction of molecular oxygen via PSI leads to the formation of $O_2 \cdot^-$, which in turn becomes rapidly dismutated to H_2O_2, either spontaneously or enzymatically via SOD; H_2O_2 is then converted into water by APX ('water–water' cycle; Asada 1999). A soluble APX (sAPX) is present in the chloroplastic stroma, whereas another APX (tAPX) is anchored to the thylakoid membrane via a C-terminal transmembrane domain, its catalytic site facing the stroma (Shigeoka et al. 2002; Ishikawa and Shigeoka 2008). In recent years, it has become apparent that APX function is a key element in controlling many aspects of light stress in plants. The expression of both *Apx1* and *Apx2*, paralogous genes encoding cAPX in Arabidopsis, was found to be induced by H_2O_2 accumulation and redox changes in photosynthetic electron transport system through a plastoquinone pool within 15–30 min upon a low-light to high-light shift (Karpinski et al. 1997); this was found to be part of a systemic response to excess excitation energy (Karpinski et al. 1999), providing supporting evidence on the time frames in which acclimation responses occur. Studies were carried out using transgenic tobacco over-expressing tAPX or catalase in chloroplasts that showed a high degree of tolerance to photo-oxidative stress through a significant reduction in excess accumulation of H_2O_2 (Miyagawa et al. 2000; Yabuta et al. 2002).

Recently, Maruta et al. (2010) demonstrated that both chloroplastic APXs, but particularly tAPX, are important for photoprotection and gene regulation under photooxidative stress in Arabidopsis leaves. Additionally, the cAPX expression under light stress has been examined and the available data revealed that its regulation is directly associated with the heat shock transcription factors (HSFs). For example, transgenic Arabidopsis plants expressing a dominant-negative form of Hsf21 (encoded by HSFA4a), which lacks an activation domain, showed inhibition of

APX1 mRNA accumulation under H_2O_2-producing light stress conditions (250 mmol m^{-2} s^{-1}) (Davletova et al. 2005). In view of the physiological significance of APX against oxidative stress, transgenic plants over-expressing APX isoenzymes have been generated to improve stress tolerance, as in the case of other antioxidant enzymes. For example, transgenic tobacco and Arabidopsis plants over-expressing tAPX in chloroplasts showed remarkable tolerance against photo-oxidative stress following exposure to strong light and paraquat treatment (Yabuta et al. 2002; Murgia et al. 2004).

On the basis of the evidence previously presented it is not suprising that light signals influence leaf ascorbate accumulation (Gatzek et al. 2002). Leaves exposed to light contain more ascorbate than leaves in the shade (Grace and Logan 1996), and ascorbate levels in the leaves show a diurnal rhythm (Dutilleul et al. 2003). The AsA pool size decreased, in apple leaves and fruit peel, after the whole trees were shaded with shade-net for 20 days (Li et al. 2009). Also, Mieda et al. (2004) reported that spinach leaves grown for 3 days under dark condition showed a significant decrease of the mRNA expression and enzyme activity of L-galactose dehydrogenase and L-galactono-1,4-lactone dehydrogenase. Bartoli et al. (2006) provided proof that through an interplay of light and respiratory controls, the regulation of the activity of L-galactono-1,4-lactone dehydrogenase (L-GalLDH), an enzyme that catalyzes the last reaction of the AsA biosynthetic pathway, determines the extent of leaf AsA accumulation. Growth at low light has been found to lower the abundance of transcripts encoding enzymes involved in AsA synthesis such as L-GalLDH and GDP-mannose pyrosphorylase (Tabata et al. 2002). Comparative proteomics analysis revealed differential accumulation of a number of thylakoid-associated proteins between ascorbate-deficient mutant *vtc2-2* and wild type Arabidopsis plants after transition to high light (1,000 μmol m^{-2} s^{-1}). These proteins included Fe–SODs and Cu/Zn–SOD, HSP70, PsbS, and a chloroplast-localized glyoxalate I, all of which have been associated with stress–response functions. The differential accumulation of these proteins shows that the ascorbate deficiency does have a significance, albeit impacts on the chloroplast stress response under high light stress (Giacomelli et al. 2006). Furthermore, it was reported that Arabidopsis leaves grown under low-light condition also contained low activities of DHAR and MDHAR compared with those grown in high-light condition (Bartoli et al. 2006), further indicating that the rate of ascorbate turnover is tightly controlled by light conditions.

Findings by Chen and Gallie (2008) implicate the regulation of the plant's response to high light by the ascorbate redox state following a genetic engineering approach. The authors constructed transgenic tobacco plants over-expressing and suppressing DHAR expression, thus leading to an increase and decrease of ascorbate redox state respectively. Suppression of DHAR expression resulted in reduced amounts of chlorophyll and xanthophyll pigments, quantum yield of photosystem II, and CO_2 assimilation, whereas the level of reactive oxygen species increased. Significant photoinhibition was also observed following exposure to high light. Direct feeding with AsA reversed effects observed in DHAR-suppressed leaves. In contrast, DHAR over-expression increased the pool size of xanthophyll and

chlorophyll pigments as well as the rate of CO_2 assimilation, particularly at high light intensities, whereas the level of reactive oxygen species was reduced. Leaves of DHAR over-expressing plants experienced less photoinhibition than wild-type plants following exposure to high light, indicating the protective role of enhanced DHAR activity during high light stress.

Although significant evidence points to a central role of glutathione redox state-related responses in response to high light, the pattern of these responses is not uniform. Since the reduction of GSSG by GR is dependent on NADPH, which is mainly provided by the photosynthetic electron transport chain, a light-dependence of the GSH/GSSG ratio might be expected. Moreover, some studies reported a more reduced glutathione pool in the light, but in other cases the GSH pool became more reduced during a dark period (Tausz et al. 1999). Burritt and MacKenzie (2003) found that the pools of both glutathione and ascorbate in leaves of begonia plants grown under light were greater than those in shade. A different hypothesis concerning the role of light stress on GSH was addressed. For example, it was stated that GSSG might act as a molecule that inactivates light-modulated stromal enzymes such as fructose 1,6-bisphosphatase through an oxidation of their –SH groups (Alscher 1989; Vivekanandan and Edwards 1987). According to Baena-Gonzalez et al. (2001), high light intensity causes an elevation of the GSH/GSSG ratio, an effect that has as a consequence the inactivation of the plastid transcription kinase (PTK), an enzyme that was found to respond to changes in thiol/disulfide redox state mediated by glutathione (Link et al. 1997), and this inactivation causes changes in the phosphorylation of RNA polymerase subunits leading to an elevation of transcriptional activity. Similarly, Ogrzewalla et al. (2002) reported on a redox-sensitive plastid kinase that associates with the transcriptional complex and links transcriptional activity of chloroplast genes to the glutathione system (for excellent reviews on redox-regulated chloroplast gene expression in plants see Barnes and Mayfield 2003; Link 2003).

It is interesting to note that UV-B radiation was also shown to induce changes in glutathione metabolism and gene expression in pea plants (Kalbin et al. 1997). GSH and GSSG levels remained unchanged after exposure to the lowest UV-B dose rates; however, increased irradiation gave rise to 60- and 4.5-fold increases in GSSG and GSH respectively. Also, *cab* (Chlorophyll-a/b-binding protein) transcript levels decreased and *CHS* (chalcone synthase) and *PAL* (phenylalanine ammonia-lyase) transcript levels increased after shorter UV-B exposures to the higher dose rate of UV-B, and after exposure to an intermediate dose rate.

8 Low Oxygen Stress

Plants in the natural environment often experience limited oxygen availability due to transient or continuous flooding (reviewed by Drew 1997). Data on antioxidant levels and the activity of antioxidant-regenerating enzymes are somewhat contradictory, as both increases and decreases in tissue antioxidant capacity have been reported. An investigation on MDHAR and DHAR activities, and AsA and GSH

contents in various species exposed to anoxia revealed an increase in MDHAR and/ or DHAR in the anoxia-tolerant plants (Wollenweber-Ratzer and Crawford 1994). GSH decreased significantly during the post-anoxic period, while AsA showed increased values in the tolerant species. Induction of enzymes involved in the ascorbate–glutathione cycle (APX, MDHAR, DHAR and GR) has been demonstrated for anaerobically germinated rice seedlings and roots of wheat (*Triticum aestivum*) seedlings (Albrecht and Wiedenroth 1994; Ushimaru et al. 1997). Anoxia caused a significant inhibition of MDHAR, DHAR and GR activities (Biemelt et al. 1998) whereas inhibition of GR and APX was observed in corn leaves under prolonged flooding (Yan et al. 1996). Recent data have shown that the leaf and root cell ascorbate/glutathione systems react differentially to changes in oxygen concentration (Skutnik and Rychter 2009).

Immediately after the end of an anaerobic exposure, a rapid oxidation of ascorbate and glutathione pool took place in barley leaves, suggesting that these low-mass antioxidants are the "first line" of ROS detoxification under low oxygen stress (Skutnik and Rychter 2009). Conversely, ascorbate and glutathione pools became even more reduced in roots. Also, the imposition of anoxia and subsequent reoxygenation caused a decrease both in the content of ascorbate and in its reduction state in the roots of cereals and the rhizomes of *Iris* spp. (Blokhina et al. 2000). The highly reducing conditions prevailing under anoxia were also reflected in roots of wheat seedlings by increasing levels of AsA and GSH, leading to increased reduction states; however, the onset of re-aeration of plants caused enhanced oxidation of the reduced fractions, resulting in decreased AsA/DHA and GSH/GSSG ratios (Biemelt et al. 1998). Garnczarska (2005) observed increased content of total ascorbate in hydroponically grown lupine roots following oxygen stress, whereas total glutathione level decreased. However, a significant increase in the reduced forms of both metabolites was found directly after hypoxia.

Hypoxia triggers the induction of multiple transcripts responsible for ROS formation and utilization (e.g. genes associated with ascorbate and glutathione metabolism). The production of ROS is an integral part of the response to anoxic/ hypoxic conditions which encompasses several levels of metabolic regulation to sustain redox signalling and to prevent oxidative damage. Major examples of mRNAs directly related to ROS handling and utilization include SOD, MDHAR, APX and several GSH-related enzymes involved in H_2O_2 detoxification. Modern microarray analyses have allowed the profiling of gene transcripts that are regulated by hypoxic conditions: Klok et al. (2002) showed that some peroxidases are down-regulated in Arabidopsis root cultures, while Lasanthi-Kudahettige et al. (2007) demonstrated catalase to be suppressed or unaffected in anoxic rice coleoptiles under hypoxia. The authors went on to suggest that low catalase transcript abundance acts as a signalling factor and is of paramount importance for H_2O_2 build-up. Further microarray-based analyses examining transient expression patterns revealed that these may also reflect a temporal necessity for ROS accumulation for signalling purposes. In particular, transcripts coding for APX were induced transiently in Arabidopsis as early as 0.5 h after low O_2 treatment, followed by MDHAR and ATP4a peroxidase (Branco-Price et al. 2005; Liu et al. 2005).

9   Ozone Stress

The air pollutant, ozone (O_3), is a molecule that possesses the ability to cause extended tissue damage resulting in chlorosis, water-logging, premature loss of chlorophyll, leaf abscission, decrease of photosynthesis and accelerated senescence in asymptomatic leaves in a variety of plant species (Reich and Amundson 1985; Krupa and Manning 1988; Heath 1994; Heggestad 1991; Strohm et al. 1999). The ingress of ozone seems to depend on the number and size of stomata in different plant species (Conklin and Last 1995). Once ozone enters the stomata, it reacts instantaneously with the apoplastic cell structures and generates secondary oxyproducts like H_2O_2 and $O_2 \cdot^-$ (Pell and Dann 1991; Rao 1992; Baier et al. 2005).

Several differences in the effects of ozone on ascorbate/glutathione-related antioxidant mechanism have been reported in the literature for different species and also for the same species under treatment with different ozone concentrations. It has been suggested that changes in glutathione metabolism play a role in limiting damage to oxidative stress conditions induced by ozone (reviewed by Hausladen and Alscher 1993). In poplar, after an initial decline in GSH content, an overall increase in GSH, GSSG, and total glutathione content was observed in leaves in response to ozone fumigation (Sen Gupta et al. 1991). In an ozone sensitive cultivar of *Phaseolus vulgaris,* GSH levels were found to be lower than in the ozone-tolerant cultivars (Guri 1983), illustrating the importance of the capacity for reduction of the glutathione pool in ozone tolerance mechanism in plants. Futhermore, it has been shown that ozone exposure leads to up-regulation of sulphur assimilation in response to decreases in GSH/GSSG, via activation of adenosine 5ʹA-phosphosulphate reductase (Bick et al. 2001). On the other hand, Noctor et al. (1998) demonstrated that neither foliar glutathione accumulation nor the redox state of the GSH pool correlated with ozone sensitivity. The absence of an effect of GSH on ozone tolerance may be due to the compartmentalization of this antioxidant. Ozone enters the leaf through open stomata and produces ROS in the apoplastic fluid which therefore represents the first line of defence against ozone damage. In many plant species, however, the apoplast contains little or no glutathione (Polle et al. 1990). Therefore, increases in total foliar glutathione contents would be of little avail in improving defence against ozone. In this sense, it is proposed that increases in GSH synthesis or the GSH pool in the cytosol or chloroplast may not be sufficient for ozone stress tolerance, and enhanced tolerance may be achieved by increasing antioxidant capacity in the apoplast, which is likely to be the first target of ozone stress (Schützendübel and Polle 2002).

Available evidence suggests that increasing AsA levels via enhanced recycling has been implicated to be the first line of defence to ozone (Ranieri et al. 1999; Plochl et al. 2000; Ranieri et al. 2000, 2001; Moldau and Bichele 2002; Ranieri et al. 2003). In the work of Mahalingam et al. (2006) the inability of the ozone-treated plants to increase their AsA content to a level that is seen in the control plants suggests that AsA regenerative systems are adversely affected by ozone or ozone-derived ROS. Low AsA decreases the threshold for sensing stress and can trigger cell death and systemic acquired resistance (SAR), as seen in AsA-deficient

mutants (Pastori et al. 2003; Foyer and Noctor 2005a). However, in others works it was suggested that apoplastic AsA alone could not explain differential ozone tolerance in *Trifolium* clones and soybean leaves (D'Haese et al. 2005; Cheng et al. 2007). In support to this hypothesis, Kollist et al. (2000) provide experimental evidence that the direct removal of O_3 by apoplastic AsA is limited in various plant species because mesophyll cell walls are thin and the effective path length for the reaction is short. Padu et al. (2005) found that the cell wall/plasmalemma/cytosol system in birch (*Betula pendula*) had sufficient capacity to maintain AsA redox state in the apoplast, without necessity to restrict O_3 uptake by stomatal closure. Finally, Nali et al (2004) found that the content of reduced glutathione, but not that of reduced ascorbate, was significantly increased in the ozone-treated (110 ppb O_3, 5 h day^{-1}) leaves of strawberry and phillyrea, thus making it unlikely for AsA to have an involvement in the differential O_3-sensitivity exhibited by these species. Similarly, total ascorbate contents, APX, MDHAR and DHAR activities were not affected by acute ozone exposure (Noctor et al. 1998).

Several attempts have been made to produce transgenic plants tolerant to ozone stress by manipulating components of the AsA recycling pathway. The most common "target" for genetic engineering has been DHAR which is well-documented as a protective antioxidant (see previous chapters). Chen and Gallie (2005) carried out ozone exposure experiments with tobacco plants over-expressing DHAR which demonstrated increased endogenous levels of AsA. Interestingly, DHAR-over-expressing plants had a lower oxidative load, a lower level of oxidative-related enzyme activities, and a higher level of photosynthetic activity following exposure to ozone compared with control plants. Conversely, reducing the size of the AsA pool size through suppression of DHAR expression had the opposite effect. Similar findings were reported by Eltayeb et al. (2006) who constructed DHAR-over-expressing tobacco plants with higher levels of reduced AsA compared with non-transformed control and who observed enhanced tolerance to ozone in terms of higher net photosynthesis. The importance of apoplastic AsA for ozone tolerance was further supported by Yoshida et al. (2006) who characterized an Arabidopsis mutant with a deficient cytosolic DHAR. The mutant completely lacked cytosolic DHAR activity and demonstrated significantly lower amounts of apoplastic AsA, resulting in high ozone sensitivity. Notably, similar tolerance to ozone stress was observed following over-expression of MDHAR in transgenic tobacco plants, which exhibited higher levels of reduced AsA compared to non-transformed control plants (Eltayeb et al. 2007).

Ozone induced alteration of ascorbate/glutathione-related enzymatic anti-oxygenic activities as well as gene expression levels have been reported in various plants, indicating antioxidant systems linking to ozone tolerance (Creissen et al. 1994; Kangasjarvi et al. 1994; Willekens et al. 1994). Increase in GR activity during ozone stress was reported in pea (Madamanchi et al. 1992; Edwards et al. 1994), *Spinacia oleracea* (Tanaka et al. 1988), *Triticum aestivum* (Rao et al. 1995), and *Arabidopsis thaliana* (Kubo et al. 1995; Rao et al. 1996). Edwards et al. (1994) reported that ozone exposure induced two isoforms of GR in pea plants with no significant changes either in the GR protein or in the mRNA transcripts encoding

GR. Exposure to O_3 caused a sharp decline in chloroplastic GR mRNA levels in both tobacco cv. Bel B and Bel W3, known for their differential sensitivity to ozone (Pasqualini et al. 2001). Additionally, it is reported that the increased expression of APX activity was observed in the ozone-exposed Arabidopsis, while other antioxidant enzymes, such as MDHAR, DHAR and GR, were unaffected (Kubo et al. 1995). Analogous gene expression patterns were observed following exposure of *Arabidopsis thaliana* plants to ozone, in which transcript levels of genes coding for cytosolic APX, Cu/ZnSOD and GST increased while FeSOD and GR were suppressed (Conklin and Last 1995). Interestingly, over-expression of APX in the chloroplast of transgenic tobacco plants did not protect them from ozone stress (Torgethausen et al. 1997). Furthermore Price et al. (1990) observed a marked increase in GST activity in barley tissues as a result of ozone fumigation that was correlated with increased lipid peroxidation. Moreover, the relatively large increase in GST mRNA levels observed in ozone-treated plants suggests that GST may be an appropriate gene for detailed studies on ozone-mediated gene activation (Price et al. 1990).

Ranieri et al. (2000) found an enhancement in the apoplastic and symplastic activity of APX in sunflower plants exposed to 150 nL L^{-1} of O_3 (4 h day^{-1} for 4 days), while stromal and thylakoid-bound chloroplastic APX activity was unaffected. PAGE analysis revealed different apoplastic and cytoplasmic APX isoenzyme patterns between control and O_3-treated plants. Also, immunoreaction with a cytosolic APX antibody revealed stonger apoplastic and symplastic signal in the O_3-treated plants than in the control ones (Ranieri et al. 2000). APX was linked with NC-R (resistant) white clover clones treated with ozone (60 ppb for 56 days, 5 h day^{-1}) (Nali et al. 2005). An early decrease in APX activity was observed in tomato genotypes exposed to O_3, which, however, was recovered in the post-fumigation period. The activity of APX and MDHAR proved to increase in two clover species exposed to ozone (Scebba et al. 2003). Meanwhile, Sanmartin et al. (2003) showed that expression of cucumber ascorbate oxidase (AO) in the cell wall/apoplast of tobacco (*Nicotiana tabacum* L. cv. Xanthi) leaves resulted in the oxidation of apoplast AsA and shifts in the redox state of AsA and GSH, without affecting total AsA (AsA + DHA) and GSH (GSH + GSSG) contents. These effects were associated with an increased sensitivity of transformed lines to O_3-induced oxidative stress and clearly reveal the role of AO in the regulation of AsA in redox signalling under ozone-induced oxidative stress (Sanmartin et al. 2003).

An alternative pathway of protection against ozone-induced damage is achieved with the activity of alternative oxidase (AOX). Plant mitochondrial AOX transfers electrons from the ubiquinone pool to oxygen without energy conservation and prevents the formation of ROS when the ubiquinone pool is over-reduced. Pasqualini et al. (2007) constructed transgenic tobacco plants presenting differing AOX activity levels. Exposure of plants to acute ozone fumigation resulted severe damage in transgenic plants with increased AOX activity, contrary to transgenic plants with reduced AOX activity which remained undamaged. Further molecular and enzymatic analyses revealed a differential response between wild type and transgenic plants with increased AOX activity, in which gene expression and

enzymatic activity profiles of GPX and APX were suppressed in the latter, suggesting that AOX over-expression leads to increased O_3 sensitivity via disturbances of ascorbate/glutathione regulation.

10 Conclusions and Perspectives

In a constantly changing environment, the plant has to adapt primarily by quickly sensing all factors that could impose stressful conditions towards its function and development. It is therefore not surprising that a complex signalling system has evolved to provide protection against the many challenges of a harmful environment. This important role in orchestrating these diverse signalling pathways has been attributed to cellular redox regulation, which acts as a sensitive mechanism able to perceive even small changes in environmental conditions, and co-ordinate appropriate responses. Taking advantage of the latest advances in genomic, proteomic and transcriptomic technologies, researchers are focusing on the production and use of transgenic plants with altered levels of AsA/GSH content as well as of antioxidant enzymes and other protective metabolites, leading to enhanced antioxidant protection and ultimately to increased agricultural productivity. On the basis of the up-to-date findings outlined in this chapter, it is safe to conclude that redox regulation and antioxidants are one of the most promising areas of research for several years to come.

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