2

Environmental Factors Affecting Components of Ascorbate-Glutathione Pathway in Crop Plants

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ABSTRACT

Both plant breeders and crop producers have an interest in finding crops capable of tolerating environmental changes with as little damage as possible. In order to develop such crops, knowledge of plant defence mechanisms and regulatory processes is essential. Ascorbate and glutathione are involved in both the direct and indirect control of the concentration of reactive oxygen species. As components of the ascorbate-glutathione pathway, they take part in the removal of excess H_2O_2. Besides its role in the antioxidative defence line, glutathione also takes part in the detoxifying of harmful compounds generated in or taken up by the cells. The present chapter focuses on the regulation of ascorbate and glutathione synthesis under stress conditions, such as low temperature, heat or drought. Since glutathione is the precursor of phytochelatins, compounds responsible for detoxifying heavy metals, this role will also be discussed.

Keywords: Acclimation, ascorbate, environment, glutathione, oxidative stress.

3. CHANGES IN THE SIZE OF THE ASCORBATE AND GLUTATHIONE POOLS UNDER ABIOTIC STRESS CONDITIONS

The measurement of abiotic stress-induced changes in the glutathione levels and their comparison in several genotypes with different stress tolerance gave the first indication about the participation of GSH in the stress response. However, a better understanding of the role of GSH in stressed plants was only possible when GSH and GSSG contents were measured, since even at constant glutathione levels changes in the ratio of the two forms influence the redox state of the GSH/GSSG couple.

The redox state of the GSH pool was initially determined by the simultaneous spectrophotometric detection of GSH and GSSG (Mergel et al., 1979). Later on more sensitive high-performance liquid chromatography methods were introduced for monitoring the concentrations of GSH and GSSG and their precursors and homologues (Kranner and Grill 1996; Potesil et al., 2005; Rellán-Alvarez et al., 2006). In one HPLC method that detects the fluorescent monobromobimane derivatives of thiols, the levels of total and reduced thiols (Cys, γEC, GSH, hmGSH and hGSH) levels can be determined separately after reduction (Kranner and Grill 1996). The combination of the HPLC separation of thiols with electrochemical detection allowed the simultaneous determination of Cys, GSH, GSSG and phytochelatins (Potesil et al., 2005) and excludes possible mistakes originating from their separate detection. An HPLC electrospray/mass spectrometry method made the simultaneous measurement of GSH, GSSG, hGSH and homoglutathione disulphide (hGSSG) possible (Rellán-Alvarez et al., 2006) and a capillary zone electrophoresis approach ensured the simultaneous analysis of GSH and GSSG (Mendoza et al., 2004). One disadvantage of the latter three methods compared to fluorescent detection, is that the reduced and oxidized forms of the GSH precursors could not be determined.

The effect of abiotic stress on GSH synthesis can be monitored in various organs and cell compartments using selective antibodies against GSH and its precursors, Cys, glutamate and glycine (Zechmann et al., 2006; Zechmann and Müller 2008). Using this technique, 50% of glutathione was found in the mitochondria under control conditions and its relative level varied between 7 and 20 per cent in the other organelles in the leaves and roots of *Cucurbita pepo*, as calculated from the data of Zechmann and Müller (2008). Virus infection resulted in a 2-3-fold higher increase in Cys and GSH levels in plastids and nuclei compared to mitochondria, which also had altered ratios in the individual organelles (Zechmann and Müller 2008). Based on this observation, it can be anticipated that abiotic stresses also induce different changes in the concentration of GSH and its precursors in the various organelles. The current methods make it possible to detect GSH and changes in the redox state of GSH, even at subcellular levels, thus promoting a better understanding of its participation in stress responses. Experiments investigating the effects of abiotic stress on GSH synthesis in plant organs should be complemented in the future with studies at the cellular and subcellular levels.

3.1 Temperature Stresses

The role of GSH at low temperature is indicated by the greater glutathione contents

observed in spruce during the winter (Anderson et al., 1992) and in chilling-tolerant maize genotypes compared to sensitive ones during cool spring periods in the field (Leipner et al., 1999) indicated a possible protective role of GSH during low temperature stress. This assumption was corroborated in growth chambers by comparing maize and rice genotypes with different levels of stress tolerance (Kocsy et al., 2001a; Guo et al., 2006). The importance of GSH was also shown in the case of heat stress which resulted in higher glutathione content in wheat and maize (Nieto-Sotelo and Ho 1986; Dash and Mohanty 2002).

The cold-induced increase in the glutathione concentration in maize was the result of a greater synthesis rate, as demonstrated by the incorporation of ^{35}S from sulphate into GSH and the higher activity of the two enzymes involved in GSH synthesis (Kocsy et al., 2001a). In addition, chilling increased the γECS activity and γEC content in the bundle sheath cells of maize leaves (Gómez et al., 2004b). Either high temperature or cold treatments induced a greater increase in GSH and hmGSH synthesis in tolerant wheat genotypes than in sensitive ones, as shown in ^{35}S-labelling experiments (Kocsy et al., 2000; 2004c). Chemical manipulation of the GSH metabolism and mutants with altered GSH levels were suitable for proving the protective role of GSH and of the corresponding metabolic enzymes in the response to abiotic stresses. In a chilling-sensitive maize genotype the GSH content and chilling tolerance increased when herbicide safeners were added (Kocsy et al., 2001b), but when increasing concentrations of buthionine sulphoximine, a specific inhibitor of γECS, were simultaneously applied the plants gradually became sensitive again. These results were confirmed by the inhibition of GSH synthesis in a tolerant maize genotype, which became sensitive following buthionine sulphoximine treatment (Kocsy et al., 2001a). Chilling tolerance could be restored by the addition of exogenous γEC or GSH. In these studies a correlation was found between GSH level, GR activity and chilling tolerance in maize. The glutathione level is also affected by the transport processes. GSH is a long distance transport metabolite for transporting reduced sulphur between shoots and roots and into developing seeds (Herschbach and Rennenberg 2001; Cairms et al., 2006). The uptake of GSH into the cells was studied in rice, where a putative GSH transporter was cloned, the function of which may be the retrieval of GSSG and GS-conjugates from the apoplast into the cytosol under stress conditions (Zhang et al., 2004).

By comparison of 5 wheat genotypes after 3 weeks cold hardening a correlation was found between the level of freezing tolerance and components of the ascorbate-glutathione cycle, thus H_2O_2 concentration, ascorbate/dehydroascorbate and GSH/GSSG ratios in the crowns (Fig. 1). This observation indicates the involvement of this cycle in the cold acclimation process.

The accumulation of glutathione under environmental stress conditions could be due not only to increased synthesis, but also to less intense degradation. Whereas GSH synthesis is performed in the chloroplast and cytosol, GSH and GS-conjugate degradation is restricted to the vacuole and perhaps to the apoplast (Foyer et al., 2001). γ-Glutamyltranspeptidases (GGT) are essential for the degradation of GSH and GS-conjugates. GGT can also catalyse the degradation of GSSG (Ohkama-Ohtsu et al., 2007). The effect of abiotic stress on GGT activity was described in pine, where it decreased

during autumn, allowing the accumulation of GSH, which may be involved in the natural cold hardening process (Taulavuori et al., 1999). Recently a GGT-independent pathway of GSH catabolism to glutamate via 5-oxoproline was described in *Arabidopsis* (Ohkama-Ohtsu et al., 2008).

Besides synthesis and degradation, the conjugation of GSH with lipid peroxides, toxic metabolic products or xenobiotics also influences its concentration, as shown in various plant species (Dixon et al., 2002; Anderson and Davis 2004). This reaction is catalysed by GST, which is induced by abiotic stresses (Coleman et al., 1997). Conjugation takes place in the cytoplasm and the conjugates are transferred to the vacuole for further processing (Dixon et al., 2002).

Stress-induced changes in the GSH metabolism can be regulated at the transcriptional, translational and post-translational levels. The expression of the gene coding for ECS, the rate-limiting enzyme in GSH synthesis, was induced by chilling and ozone fumigation in maize and *Arabidopsis*, respectively (Gómez et al., 2004b; Sasaki-Sekimoto et al., 2005). Following various abiotic stress treatments the expression of *γECS* and *GR* genes increased in maize, and corresponding changes could also be detected at the level of GSH concentration and GR activity (Kellõs et al., 2008).

The post-transcriptional regulatory role of H_2O_2 was shown in maize subjected to various abiotic stresses, since the γECS and GR transcript levels either remained constant or decreased parallel to changes in the GSH synthesis, GSH/GSSG ratio and GR activity (Kellõs et al., 2008). The interaction between H_2O_2 and GSH in stress signalling was suggested in mungbean, where exogenous H_2O_2 increased both GSH levels and chilling tolerance (Yu et al., 2003).

The translational control of γECS activity was observed in *Arabidopsis* following H_2O_2 treatment and in maize subjected to chilling, since the amount of enzyme protein increased (Xiang and Bertrand 2000; Gómez et al., 2004b). The binding of the complex controlling the translation of γECS was induced by GSH and inhibited by GSSG under *in vitro* conditions (Xiang and Bertrand 2000). In pea higher GR activity was not accompanied by increased expression of the *GR* gene after heat stress (Kurganova et al., 1999; Escaler et al., 2000), suggesting the existence of translational or post-translational regulation.

Co-ordinated changes in the synthesis, degradation, transport and conjugation of GSH adjust its level and the GSH/GSSG ratio to stress conditions, allowing the effective participation of GSH in defence mechanisms.

Using HPLC separation and fluorescent detection, the importance of the high reduced/oxidised thiol ratio in the response to low temperature stress was shown in wheat, where higher GSH/GSSG and reduced/oxidized hydroxymethylglutathione (hmGSH/hmGSSG) ratios were found in freezing-tolerant genotypes compared to sensitive ones (Kocsy et al., 2001a). In addition, higher GR activity was detected in tolerant wheat and maize genotypes at suboptimal temperatures and in *Picea abies* during the winter indicating the involvement of GR in the maintenance of a high GSH/GSSG ratio in stressed plants (Esterbauer and Grill 1978; Leipner et al., 1999; Kocsy et al., 2001a). Besides the increase in total GR activity, the appearance of new, cold-specific GR-isoenzymes having high activity at low temperature is necessary for the efficient

reduction of GR, as described for cold-hardened spruce (Hausladen and Alscher 1994). As in the case of cold-hardened wheat, the GSH/GSSG ratio at high temperatures correlated with heat tolerance in wheat and maize (Kocsy et al., 2004a, 2004c). The effective removal of H_2O_2 by GSH during heat stress is also facilitated by the increased GR activity observed in mustard seedlings (Dat et al., 1998).

For the accurate description of the involvement of GSH in stress responses it is necessary to determine the temporal and spatial changes in its concentration and in the GSH/GSSG ratio. The rapid initial decrease in the GSH and hmGSH contents during the first day of cold hardening coincided with a similar change in the GSH/GSSG and hmGSH/hmGSSG ratios in shoots and crowns of cold-hardened wheat (Kocsy G., unpublished results). Later on, during the first week of the 21-day treatment there was a transient increase in both the parameters of the two thiols in the shoots, while there was no significant change in their levels in the crowns. When investigating the spatial changes in GSH content in maize and wheat at low temperature, similar changes were found in shoots and roots (Kocsy et al., 2001a). Chilling increased γECS transcript and protein levels in the bundle sheath but not in the mesophyll cells, which could be the reason for the different GSH levels in the two cell types (Gómez et al., 2004b).

Studies on the subcellular localization of GSH it showed that GSH synthesis is possible in the chloroplasts and cytosol, and that the degradation of GSH and GS-conjugates occurs in the vacuoles and perhaps in the apoplast. Therefore, large GSH redox gradients may exist between the various subcellular compartments (Foyer et al., 2001). Thus, it is important to monitor the GSH and GSSG levels in the individual organelles (Schafer and Buettner 2001). The fluorescent dyes monochlorobimane and mercury orange were successfully used for the detection of GSH in living cells of *Allium cepa* (Müller et al., 1999). A specific antibody raised against GSH was appropriate for monitoring its intracellular distribution in various cell compartments (Müller et al., 2005). However, these methods did not allow the detection of GSSG or of changes in the GSH/GSSG ratio occurring during the response to abiotic stress. In addition, the fluorescent dyes may not be able to penetrate all the organelles. These problems could be solved using redox-sensitive green fluorescent protein, which is able to sense the redox potential of the cellular GSH buffer via Grx as a mediator (Meyer et al., 2007). The usefulness of this system was demonstrated in *Arabidopsis* roots, where the GSH/GSSG ratio was modified using exogenous H_2O_2 or dithiothreitol, and in the detection of wounding-induced redox changes in *Arabidopsis*. The complete oxidation of GSH, typical of the endoplasmatic reticulum, could also be detected via the transient expression of redox-sensitive green fluorescent protein in tobacco (Meyer et al., 2007).

The tracking of compartment-specific changes in the redox state of GSH is very important in relation to the redox regulation of the proteins. For example, γECS is localised exclusively in the cytosol (Pasternak et al., 2008) and is active in the oxidised state (Jez et al., 2004), while the active form of the non-expressor of pathogen-related genes (NPR1) is reduced and localized in the nucleus (Mou et al., 2003). Since both proteins are involved in the stress response, their simultaneous activation may be necessary. This would require different redox environments, which could be ensured by the localization of γECS and NPR1 in different cell compartments. If proteins differing

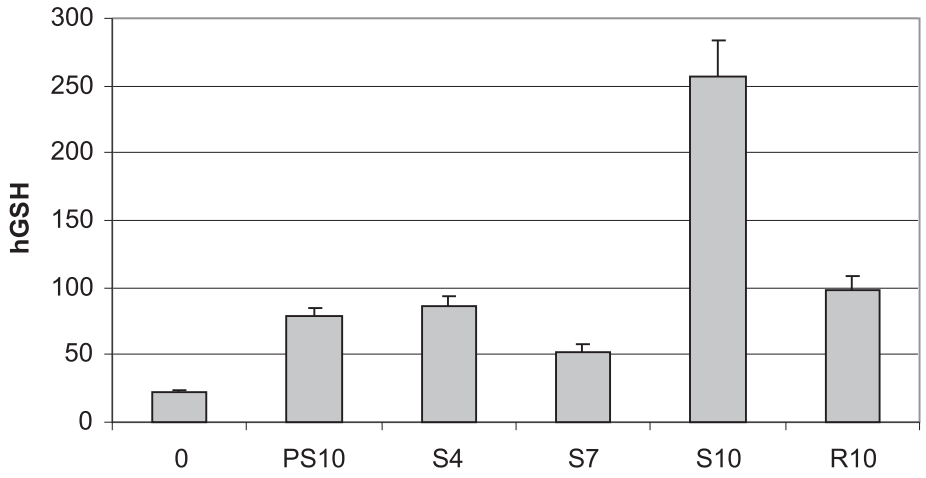

Fig. 2. Effect of drought on homoglutathione (hGSH) accumulation in soybean. The 6 weeks old plants were subjected to two subsequent 10 d long drought stress with one irrigation between them. Sampling was done before (0) and at the end of the first drought period (PS10), and after 4 (S4), 7 (S7) and 10 (S10) days during the second drought period and after 10 d recovery (R10). The youngest fully developed leaves were used for the biochemical analysis.

resulting in increases in the activities of the corresponding enzymes (Anderson and Davis 2004). Results obtained for pea show that the expression of the *GR* gene may be regulated by GSSG through its interaction with a possible GSSG binding site in the promoter region (Creissen et al., 1992).

Similarly to the low temperature, drought also resulted in an initial decrease in the GSH/GSSG ratio, but this change was followed by another decrease during the second half of the 23-day stress period (Tausz et al., 2004). After salt stress the GSH level and the GSH/GSSG ratio were lower after one week compared to the 3 d treatment in the maize inbred line Z7 (Kellõs et al., 2008). These results indicate that GSH may have an important role in ROS detoxification during the initial phase of various abiotic stresses. The decrease in the GSH/GSSG ratio could be due to the removal of ROS in the form of glutathione-conjugates (GS-conjugates) or due to GSH degradation. Following osmotic stress there was a similar increase in glutathione percentage in the shoots and roots in both drought-tolerant and sensitive wheat genotypes. However, the GSH/GSSG ratio was higher in the tolerant genotype under both control and stress conditions (Kocsy et al., 2004b). The GSH/GSSG ratio was similar in the shoots and roots under control and osmotic stress conditions. The changes in the size and redox state of the GSH pool at the cellular level could be even more important for the response to abiotic stress than the alteration of these parameters in various organs or tissues (Meyer 2008). Under control conditions different GSH levels were found in various cell types (Rennenberg et al., 2007), which may be the result of the compartmentation of the GSH metabolism (Kopriva and Koprivova 2005).

Besides GSH, AsA concentration and its redox state, is also important in the response to osmotic stress. In detached poplar leaves the amount of total ascorbic acid also

increased parallel to the water loss compared to the control leaves (Morabito and Guerrier, 2000). Drought stress resulted an increased AsA content and AsA/DHA ratio in soybean leaves, which changes were affected by proline level (Kocsy et al., 2005). The role of AsA during drought stress was also demonstrated in *Myrothamnus flabellifolia,* in which it was oxidized during dehydration, and it was reduced again during rehydration (Kranner et al., 2002). Following water loss the activity of APX and DHAR was greater in the drought-tolerant *Triticum durum* cultivar compared to the sensitive one (Sgherri et al., 2000). These results indicate that a coordinated regulation of the AsA concentration and the enzymes affecting its redox state is necessary in order to reduce the damaging effect of water shortage.

Besides drought, high salt concentration also results in changes of the AsA concentration. After salt stress the amount of TAA and the activity of APX increased in *T. durum* protoplasts (Meneguzzo et al., 1998). Salt treatment also resulted in higher APX activity in radish while the level of the corresponding mRNA remained unchanged (Lopez et al., 1996). Following salt stress the APX activity increased in seedlings of the salt tolerant *Setaria italica* genotype while it decreased in the sensitive ones (Sreenivasulu et al., 2000). In cotton genotypes there was also a relationship between salt tolerance and APX activity (Gossett et al., 1996).

An increase in the activity of antioxidant enzymes, superoxide dismutase, peroxidase, catalase and polyphenol oxidase was also demonstrated in water-stressed maize plants. Foliar application of ascorbic acid reduced the activities of stress-induced enzymes, and also the proline and malondialdehyde levels in water-deficit stressed plants (Dolatabadian et al., 2009). These results show that ascorbic acid application helps the plants for better resistance under the stress by inactivation and scavenging of free radicals; thereupon it may be effective for the improvement of stressed plants in arid and semi-arid regions.

3.3 Environmental Pollutants, Flooding

Contrary to the other abiotic stresses, toxic concentrations of copper shifted the GSH/GSSG couple to a more oxidized state in *Silene cucubalus*, which could be the result of the use of GSH for phytochelatin synthesis (de Vos et al., 1992). This shift was prevented in cadmium-treated soybean roots due to the induction of GR (Ferreira et al., 2002).

The refining process in the petrochemical industry generates oil refinery sludges, a potentially contaminating waste product, with a high content of hydrocarbons and heavy metals. Organic contaminants can be stabilized within a soil matrix, taken up by plants and transformed or stored in a non-phytotoxic form. The presence of sludge in soil induces in plants an oxidative stress, which could be provoked by the direct toxicity of the petroleum sludge in the soil and/or a result of the contaminant properties that alter the physical and chemical properties of the soil, potentially affecting oxygen transfer, available water uptake, and nutrient mobility. An increase in the AsA/DHA ratio was shown in alfalfa plants grown in soils contaminated with sludge, due to a decrease in their DHA mutant, which agrees well with the enhancement of MDHAR, DHAR, and GR activities found in plants (Marti et al., 2009). It was also shown that total glutathione

stress. This was confirmed using tobacco plants over-expressing SOD isoforms, which could tolerate flooding better than the wild type plants (Yu and Rengel 1999). Continuously flooded plants also showed an increase in the activity of APX (Lin et al., 2004; Yordanova et al., 2004, Hossain et al., 2009). Unaltered or decreased MDHAR and DHAR activities during the entire anoxic and post-anoxic periods, together with the lack of positive correlation between DHAR activity and AsA/DHA ratio suggest an increase in de novo synthesis of AsA under waterlogged conditions. Alternatively, non-enzymatic disproportionation of MDHA might be responsible for such high levels of AsA accumulation (Hossain et al., 2009). Studies with waterlogged citrus plants show that elevated APX activity might be insufficient to scavenge all H_2O_2 produced in excess underwaterlogging stress, and the recycling activity for AsA did not seem to be essential in the maintenance of a balanced redox status for AsA. Isolated increases in AsA/DHA or GSH/GSSG ratios do not seem to prevent oxidative damage, and release from waterlogging conditions does not seem to improve plant performance; rather, probably due to a sudden oxygen burst soon after release of water, it may enhance the incidence of oxidative damage (Hoaasin et al., 2009).

Treatment of green gram (*Vigna radiata* L.) plants with chromium may also cause antioxidative responses, such as temporary increase in the content of hydrogen peroxide and superoxide dismutase activity (Karuppanapandian et al., 2006). While catalase activities and the contents of reduced AsA and DHA were significantly decreased in Cr-treated plants, ascorbate peroxidase and guaiacol peroxidase activities showed a later increase suggesting that increased enzyme activities would be responsible for the removal of H_2O_2. The reduced glutathione content decreased at early stages of Cr-treatment; However, it was restored to the normal level as in controls thereafter. In contrast, the glutathione disulphide content showed a progressive increase during the initial hours of Cr-treatment. The non-protein thiol content was shown to increase during the first several hours, but it declines at later stages. These results demonstrate that Cr-induced oxidative stress is an important component of the plant's reaction to toxic levels of Cr (Karuppanapandian et al., 2006).

The involvement of GSH in the response to heavy metal stress was also demonstrated, since the glutathione content was reduced by cadmium in bread wheat (Lin et al., 2007) and by copper in *Silene cucubalus* due to increased phytochelatin synthesis (de Vos et al., 1992), which detoxifies heavy metals by forming complexes. Correspondingly, in the *Arabidopsis* mutants *rax1-1* and *cad2-1* (cadmium-sensitive) (mutations in the γECS gene resulting in decreased GSH content) it was shown that GSH affects the expression of several genes involved in protection against environmental stresses (Ball et al., 2004). Exposure of durum wheat plants to Cd led to an increase in the activity enzymes of the AsA-GSH cycle in the leaves without any symptom of the oxidative damage, suggesting that the whole plant improved its antioxidant defence, even in those parts which had not yet been reached by Cd. (Paradiso et al., 2008). When ascorbate biosynthesis was enhanced, by feeding plants with its last precursor, L-galactono-g-lactone, the oxidative stress induced in the roots by the heavy metal was alleviated. Results suggest that different strategies can successfully cope with heavy metal toxicity. This precocious increase in the enzymes of the ascorbate-glutathione cycle further highlight the tight

regulation and the relevance of this cycle in the defence against heavy metals, and highlights the interest in obtaining plants in which the ascorbate biosynthetic route has been enhanced, both for improving ROS scavenger systems and for removing heavy metals from metabolic reactions (Paradiso et al., 2008).

3.3.1 Phytochelatins

Following cadmium stress the enhanced synthesis of GSH and phytochelatins (PCs) was observed in various plant species (Mendoza-Cózati et al., 2005). Phytochelatins were first isolated by Grill et al. (1985) from a cell suspension culture of *Rauvolfia serpentina*. They have the structure [(γ-Glu – Cys)$_n$ – Gly], where n is the number of replications of the (γ-Glu – Cys) units, which is generally in the range 2–11. The primary structure of the cadmium phytochelatin complex is [$Cd_3(PC_3)_4$], which contains discrete $Cd(SCys)_4$ units (Strasdeit et al., 1991). The peptide SH : Cd mol ratio is 3.78, declining to 1.01 as the complex becomes saturated with cadmium (Rauser and Meuwly 1995). This latter ratio was also recorded in maize roots, indicating that the PC formed in the roots is capable of maximum Cd binding with a very small quantity of SH, while in maize leaves this ratio was 3.41–4.73, suggesting the over-production of phytochelatins (Drazkiewicz et al., 2003). The enzyme responsible for their synthesis is β-glutamyl cysteine dipeptidyl transpeptidase (phytochelatin synthase: PCS), the substrate of which is glutathione (Grill et al., 1989). The enzyme is also expressed without heavy metal stress, but is primarily activated by the presence of heavy metals (Cobbett, 2000).

Even a very low (0.05 μM) concentration of cadmium stimulated the synthesis of PCs in maize roots after a period of 24 hours; in the presence of 3 μM Cd phytochelatins (PC_2–PC_4) appear after 2 h, while after 6 h PC_2 is largely replaced by PC_3 (Tukendorf and Rauser 1990). The quantities induced are closely correlated with the cadmium concentration and the treatment duration, but also depend on the age of the leaf (Wójcik and Tukendorf 1999; Drazkiewicz et al., 2003). Low molecular weight phytochelatins were dominant in maize root tips one day after treatment with 3 μM Cd, while in the older parts of the root high molecular weight phytochelatins were dominant after two days (Rauser 2003). Cadmium stress reduced the accumulation of glutathione but increase the amount of PC in *Arabidopsis* seedlings. Supplementation with salt increased the glutathione and PC levels under Cd stress markedly. However, no significant difference was detected in the accumulation of transcripts of the phytochelatin synthase gene, AtPCS1 regardless of the salt/Cd treatment used (Xu et al., 2010).

Little is known about the expression of phytochelatin synthase or the tissue-specificity of phytochelatin biosynthesis. In tomato plants phytochelatin synthase activity was recorded in the roots and stems, but not in the leaves and fruit (Chen et al., 1997). The PCS activity measured in maize under *in vitro* conditions increased in response to cadmium treatment in the leaves, but declined in the roots (Szalai et al., 2002; Pál et al., 2005). Since the biosynthesis of PCs is self-regulated (Loeffler et al., 1989), it can be assumed that a sufficient quantity of PCs was synthesized in maize roots to bind the cadmium, so there were no heavy metal ions to activate the enzyme (Pál et al., 2005). It is possible, however, that the synthesis of PCs is differently regulated under *in vivo* conditions.

level of ROS by integrating signals from different cell compartments during abiotic stress, and the GSH/GSSG couple participates in its fine tuning (Meyer 2008).

In general, the protective and regulatory roles of GSH are based on changes in its redox state, which is defined by the reducing capacity of GSH (GSH concentration) and the half-cell reduction potential of the GSH/GSSG couple (Schafer and Buettner 2001). It differs in various organs, tissues, cells and compartments and also changes during the growth and development of the plants. While glutathione reductase (GR) uses NADPH to reduce GSSG to GSH, various free radicals and oxidants are able to oxidize GSH to GSSG. The proportion of glutathione in the reduced form reflects the relative rates of reduction and oxidation and is always greater than 0.9 under non-stress conditions. Since the concentration of GSH in the chloroplast stroma is thought to be close to 5 mM, the reduced form of glutathione may act as an important redox buffer, preventing enzyme inactivation by protecting potentially susceptible protein thiol groups (Noctor et al., 1998).

The stress-induced changes in GSH level and the GSH/GSSG ratio may derive from a higher rate of GSH synthesis. Abiotic stress-induced changes in the redox state of GSH precursors may also influence the synthesis and redox state of GSH, since there were similar differences in the ratios of GSH/GSSG, hmGSH/hmGSSG and their reduced/oxidized precursors between tolerant and sensitive wheat varieties subjected to osmotic or heat stress (Kocsy et al., 2004b; 2004c). Cystine reductase was described in pea (Romano and Nickerson 1954), but stress-induced changes in its activity were not studied. The accumulation of γ-glutamylcystine (ESSE), the oxidised form of the other GSH precursor in tobacco overexpressing γECS (Creissen et al., 1999), indicates that no enzyme exists for its efficient reduction in plants; therefore, it can only be removed by degradation or sequestration to the vacuole. The involvement of GSH in redox signalling is confirmed by the observation that inter- and intracellular GSH pools are linked by transport across the membranes, the rate of which could be similar to that of synthesis, as is the case for the chloroplast envelope (Noctor et al., 2002). The GSH transport in plants may also be regulated by antioxidants, since the promoter of the gene coding for a GSH transporter in mammals contains a functional antioxidant-responsive element (Wasserman and Fahl 1997).

The GSH/GSSG couple is able to modify the activity of various compounds (enzymes, regulatory proteins, etc.) directly through the reduction/oxidation of their disulphide bridges/sulfhydryl groups and through the (de)glutathionylation of sulfhydryl groups. The indirect regulation of proteins by the GSH/GSSG couple may occur due to crosstalk between GSH/GSSG and other redox systems through glutathionylation or thiol-disulphide transition, which may have a role in signalling and responses to abiotic stress (Rausch et al., 2007; Ying et al., 2007).

4.2 Interaction of Glutathione with Hormonal Systems

Both acorbate and the GSH/GSSG couple may interact with certain signalling pathways during the stress response. The effect of abiotic stresses on the H_2O_2, GSH and GSSG concentrations may be transmitted by various plant hormones. For example, the general

regulatory role of NO, an important regulatory molecule, in stressed plants was demonstrated in several studies (for review, see Arasimowicz and Floriszak-Wieczorek 2007). NO affected H_2O_2 concentration due to the inhibition of Cat and ascorbate peroxidase (Clark et al., 2000) while exogenous H_2O_2 activated NO synthesis in tobacco (de Pinto et al., 2006), suggesting a bidirectional interaction between the two compounds. NO may also influence the GSH synthesis, as demonstrated in *Medicago trunculata* roots, where the GSH level and gECS and GS gene expressions were increased by NO (Innocenti et al., 2007).

Components of the AsA-GSH cycle may interact with Ca^{2+}. H_2O_2 treatment alone or combined with low temperature increased the Ca^{2+} concentration in tobacco (Price et al., 1994), which could have a role in the Ca^{2+}-dependent regulation of the enzymes. In maize the interaction of Ca^{2+} and ROS was observed during the induction of the antioxidant system by ABA, and it was concluded that Ca^{2+} can be found both before and after ROS in the signalling pathway related to oxidative stress (Jiang and Zhang 2003). Yang and Poovaiah (2002) postulated a dual role for Ca^{2+} in the regulation of H_2O_2 homeostasis: (a) during positive regulation H_2O_2 will be produced due to the activation of NADPH oxidase; (b) during negative regulation the H_2O_2 concentration will decrease due to the activation of Cat. Interestingly, Ca^{2+} enhanced both the GSH concentration and the stress tolerance in rice (Lu et al., 1999). In tobacco, however, GSH and GSSG treatment resulted in a rapid, transient increase in the Ca^{2+} level, suggesting that GSH may be involved in the activation of Ca^{2+}-dependent protein kinases and in the early part of stress-induced signalling pathways (Gómez et al., 2004a). In plants, Ca^{2+} may interact not only with H_2O_2 but with other ROS, too. This assumption is based on observations on trout hepatoma cells, where the mobilization of Ca^{2+} was induced by $^{\cdot}OH$ (Burlando and Viarengo 2005). A review about interactions between ROS, Ca^{2+} and antioxidants were published recently (Noctor 2006).

Salicylic acid (SA) has long been known as a signal molecule in the induction of defence mechanisms in plants (Raskin 1992; Horváth et al., 2007). Although SA may also cause oxidative stress to plants, partially through the accumulation of hydrogen peroxide, the results published so far show that the preliminary treatment of plants with low concentrations of SA might have an acclimation like effect, causing enhanced tolerance toward most kinds of abiotic stresses due primarily to enhanced antioxidative capacity. The effect of exogenous SA depends on numerous factors such as the species and developmental stage of the plant, the mode of application, and the concentration of SA and its endogenous level in the given plant. SA increased the chilling tolerance of maize by inhibiting catalase, thus increasing the H_2O_2 concentration, which led to an increase in several protective mechanisms, including increase in the GR activity (Janda et al., 1999; Horváth et al., 2002). As also observed in chilled maize, SA stimulated the formation of ROS in *Arabidopsis* subjected to salt or osmotic stress (Borsani et al., 2001). GSH and GR were also affected by SA in a soybean cell suspension (Knörzer et al., 1999) and SA increased the GR activity in rice leaves (Ganesan and Thomas 2001). Pretreatment of barley seedlings with SA in the dark via the transpiration stream fully blocked the subsequent light-induced inhibitory effect of paraquat on photosynthesis and also decreased the paraquat-induced production of H_2O_2, lipid peroxidation, and

where the addition of oxidized ATGPX3 protein *in vitro* converted the protein phosphatase described in *ABA insensitive2* (*ABI2*) mutants to its oxidized form. *ABI2*, in turn, influences Ca^{2+} channels and stomatal closure (Miao et al., 2006). In addition, ABA influenced the GR activity in the cytosol of rice (Kaminaka et al., 1998). In two maize genotypes differing in their stress tolerance, ABA differentially affected the GSH content, GSH/GSSG ratio, GR activity and γECS transcript level (Kellõs et al., 2008). In *Vigna unguiculata* not only the GR activity, but also the expression of the corresponding gene was increased by ABA (Contour-Ansel et al., 2006). In summary, ABA was shown to affect the GSH/GSSG ratio and redox signalling in several plant species (Pastori and Foyer 2002).

Recent studies with maize plants differing in their stress tolerance indicate that SA and ABA may have different effects on GSH synthesis (Kellõs et al., 2008). Based on similarities in GSH synthesis following hormone and abiotic stress treatment, the possibility of a regulatory role for ABA during continuous darkness and salt stress, and for SA during H_2O_2 treatment and osmotic stress can be raised. These treatment- and genotype-specific differences in synthesis and reduction of GSH may be regulated at the post-transcriptional level.

Like SA and ABA, jasmonic acid (JA) also regulated gene expression through H_2O_2, as found in tobacco (Mur et al., 2006). In addition, JA influenced the GSH concentration and the genes involved in the GSH metabolism in *Arabidopsis* (Xiang and Oliver 1998; Sasaki-Sekimoto et al., 2005). Similarly to SA, ethylene and NO, JA also increased the transcript level of *GST* suggesting that the various plant growth regulators interact (Moons 2005).

The order of the components in the signalling pathway described above may vary, and some of them may be absent or additional ones may be present depending on environmental effects, plant species, organs and cell types. Multidirectional forward and backward interactions responsible for the regulation of metabolic pathways may exist between these compounds in order to ensure the most effective protection against environmental stress (Agarwal et al., 2005; Foyer and Noctor 2005; Noctor 2006; Dietz 2008; Miller et al., 2008).

CONCLUSION

In spite of the fact that the physiological role of the two main components of the AsA-GSH cycle, AsA and glutathione, have been extensively studied in several plant species, several questions remain to be clarified, for example the role of subcellular changes in the redox state of the redox pairs in stressed plants, and their interactions with other signalling molecules during the stress response (Szalai et al., 2009). Sequence comparisons of the related genes and proteins and the construction of phylogenetic trees could help to identify the evolutional events. Full knowledge of the genes involved in the L-galactose pathway of ascorbate synthesis in plants should facilitate a better understanding of the regulatory control of ascorbate accumulation and provide opportunities for increasing the vitamin C content of food crops. The majority of data on mechanisms related to these compounds were obtained using model plants. These results may provide a good

background for strategies aimed at manipulating plants for elevated vitamin C and or altered glutathione levels in order to develop crops capable of tolerating environmental changes with as little damage as possible.

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