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REVIEW

# Glutathione as an Antioxidant and Regulatory Molecule in Plants Under Abiotic Stress Conditions

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**Abstract** The glutathione (GSH)/glutathione disulfide (GSSG) redox couple is involved in several physiologic processes in plants under both optimal and stress conditions. It participates in the maintenance of redox homeostasis in the cells. The redox state of the GSH/GSSG couple is defined by its reducing capacity and the half-cell reduction potential, and differs in the various organs, tissues, cells, and compartments, changing during the growth and development of the plants. When characterizing this redox couple, the synthesis, degradation, oxidation, and transport of GSH and its conjugation with the sulfhydryl groups of other compounds should be considered. Under optimal growth conditions, the high GSH/GSSG ratio results in a reducing environment in the cells which maintains the appropriate structure and activity of protein molecules because of the inhibition of the formation of intermolecular disulfide bridges. In response to abiotic stresses, the GSH/GSSG ratio decreases due to the oxidation of GSH during the detoxification of reactive oxygen species (ROS) and changes in its metabolism. The lower GSH/GSSG ratio activates various defense mechanisms through a redox signalling pathway, which includes several oxidants, antioxidants, and stress hormones. In addition, GSH may control gene expression and the activity of proteins through glutathionylation and thiol-disulfide conversion. This review discusses the size and redox state of the GSH pool, including their regulation, their role in redox signalling and defense processes, and the changes caused by abiotic stress.

**Keywords** Abiotic stress · Glutathione · Glutathionylation · Reactive oxygen species · Redox signalling

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## Introduction

One consequence of the aerobic life form is the continuous formation of reactive oxygen species (ROS), a process enhanced by abiotic stresses. ROS levels need to be controlled and various antioxidants have evolved for this purpose. Glutathione (GSH) is involved in both the direct and the indirect control of ROS concentrations (May and others 1998; Noctor and Foyer 1998; Foyer and Noctor 2005). As a component of the ascorbate-glutathione pathway, it takes part in the removal of excess $H_2O_2$ (Noctor and Foyer 1998), in a reaction in which GSH is oxidized. The high ratio of GSH to its oxidized form, glutathione disulfide (GSSG), occurring under optimal growth conditions can be restored by means of higher glutathione reductase (GR) activity, increased GSH synthesis, decreased GSH degradation, or the transport of GSH and GSSG. Besides the GSH-ascorbate cycle, GSH may also participate in $H_2O_2$ degradation in a reaction catalyzed by glutathione peroxidase (GPx). However, the existence of GSH-specific peroxidases in plants is questionable because, according to Navrot and others (2006), plant GPxs do not react with GSH, but only with thioredoxins (Trxs). Besides $H_2O_2$, GSH removes lipid peroxides, methylglyoxal, and herbicides (Moons 2005; Rausch and others 2007; Yadav and others 2008). The reaction is catalyzed by

GSH S-transferases (GST) and the conjugates are transported to the vacuoles. GSH not only participates in the direct detoxification of ROS, it may also protect cells against unfavorable stress effects through the activation of various defense mechanisms due to its involvement in redox signalling (Foyer and others 1997; Apel and Hirt 2004; Mittler and others 2004; Foyer and Noctor 2005; Mullineaux and Rausch 2005; Pitzschke and others 2006). In this signalling pathway, GSH interacts with ROS, redox molecules [Trxs, glutaredoxins (Grxs)], and plant hormones [salicylic acid (SA), abscisic acid (ABA)]. Besides the control of ROS levels, GSH takes part in the regulation of growth, development, the cell cycle, gene expression, and protein activity due to its effect on the redox state of the cells (Noctor and others 1998; Aslund and Beckwith 1999; Ogawa 2005; Shao and others 2008). It is also involved in the transfer and storage of sulfur (Herschbach and Rennenberg 2001) and in the detoxification of heavy metals, which form complexes with GSH-derived phytochelatins (Blum and others 2007).

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 ($E_{GSSG/2GSH}$). The $E_{GSSG/2GSH}$ value can be calculated from the GSH and GSSG concentrations using the Nernst equation (Schafer and Buettner 2001). It differs in various organs, tissues, cells, and compartments and also changes during growth and development of the plants. In this review the stress-induced temporal and spatial changes in size and redox state of the GSH pool are discussed, including their regulation, their role in redox signalling, and defense processes.

## Stress-Induced Changes in the Size of the Glutathione Pool

The simplest way to obtain insight into the role of GSH in stress response is the measurement of total glutathione (TG) concentrations in stressed plants. However, this result gives no information about its redox state. The greater TG contents observed in spruce during the winter (Anderson and others 1992) and in chilling-tolerant maize genotypes compared to sensitive ones during cool spring periods in the field (Leipner and others 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 (Guo and others 2006; Kocsy and others 2001a). The importance of GSH was also shown in the case of heat stress which resulted in higher TG content in wheat and maize (Nieto-Sotelo and Ho 1986; Dash and Mohanty 2002). The TG content was increased not only by extreme temperatures but also by water deficit in sunflower seedlings (Sgherri and Navari-Izzo 1995) and detached poplar leaves (Morabito and Guerrier 2000), and by salt treatment in groundnut cell lines (Jain and others 2002). In addition, a comparison of various plant species revealed that salt tolerance was greater for those that had higher TG content (Tepe and Harms 1995). The involvement of GSH in the response to heavy-metal stress was also demonstrated because the TG content was reduced by cadmium in bread wheat (Lin and others 2007) and by copper in *Silene cucubalus* as a result of increased phytochelatin synthesis (de Vos and others 1992), which detoxifies heavy metals by forming complexes. The measurement of abiotic stress-induced changes in TG levels and their comparison in several genotypes with different stress tolerances gave a first indication about the participation of GSH in the stress response. However, a better understanding of the role of GSH in stressed plants was possible only when GSH and GSSG contents were measured because even at constant TG levels, changes in the ratio of the two forms influence the redox state of the GSH/GSSG couple.

## Redox State of Glutathione in Stressed Plants

The redox state of the GSH pool was initially determined by simultaneous spectrophotometric detection of GSH and GSSG (Mergel and others 1979). Later on more sensitive high-performance liquid chromatography (HPLC) methods were introduced for monitoring the concentrations of GSH and GSSG and their precursors and homologs (Kranner and Grill 1996; Potesil and others 2005; Rellán-Alvarez and others 2006). In one HPLC method that detects the fluorescent monobromobimane derivatives of thiols, the levels of total and reduced thiols [cysteine (Cys), $\gamma$-glutamylcysteine ($\gamma$EC), GSH, reduced hydroxymethylglutathione (hmGSH) and homoglutathione (hGSH)] can be determined separately after reduction (Kranner and Grill 1996). The combination of HPLC separation of thiols with electrochemical detection allowed the simultaneous determination of Cys, GSH, GSSG, and phytochelatins (Potesil and others 2005) and excludes possible mistakes originating from their separate detection. An HPLC electrospray/mass spectrometry method made simultaneous measurement of GSH, GSSG, hGSH, and homoglutathione disulfide (hGSSG) possible (Rellán-Alvarez and others 2006), and a capillary zone electrophoresis approach ensured the simultaneous analysis of GSH and GSSG (Mendoza and others 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.

Using HPLC separation and fluorescent detection, the importance of the high reduced/oxidized thiol ratio in

response to low-temperature stress was shown in wheat, where higher GSH/GSSG and reduced/oxidized hydroxymethylglutathione (hmGSH/hmGSSG) ratios were found in freeze-tolerant compared to freeze-sensitive genotypes (Kocsy and others 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 and others 1999; Kocsy and others 2001a). Besides the increase in total GR activity, the appearance of new, cold-specific GR isoenzymes with high activity at low temperatures is necessary for the efficient reduction of GSSG, 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 and others 2004a, c). The effective removal of $H_2O_2$ by GSH during heat stress is also facilitated by increased GR activity observed in mustard seedlings (Dat and others 1998), Besides extreme temperatures, maintenance of a high GSH/GSSG ratio and GR activity also play an important role in salt, desiccation, and drought tolerance as found in tomato, *Myrothamnus flabellifolia*, and wheat, respectively (Shalata and others 2001; Kranner and others 2002; Kocsy and others 2004b). 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 and others 1992). This shift was prevented in cadmium-treated soybean roots due to the induction of GR (Ferreira and others 2002).

Studies carried out on several plant species subjected to various abiotic stresses indicate that a high GSH/GSSG ratio, maintained by increased GSH synthesis and/or GSSG reduction, may be necessary for efficient protection of plants against abiotic stress-induced accumulation of ROS. Compared with the GSH/GSSG ratio, the half-cell reduction potential of the GSH/GSSG couple, the $E_{GSSG/2GSH}$ value, which can be calculated from the concentration of GSH and GSSG, is more closely correlated with the biological status of the cell (Schafer and Buettner 2001). At a value of around −240 mV the cells proliferate, at around −200 mV they differentiate, and at −170 mV apoptosis occurs. Kranner and others (2006) found this parameter to be a universal marker of cell viability, which could thus be used to monitor stress-induced damage. The $E_{GSSG/2GSH}$ value can be maintained not only by reduction of GSSG, but also by its export. However, because of the reduced size of the GSH pool, the buffering capacity of the GSH/GSSG redox couple will be smaller (Schafer and Buettner 2001). In maize, various abiotic stresses resulted in minor changes in the $E_{GSSG/2GSH}$ value, but there were substantial changes in the GSH concentration (Table 1). Although maize lines with different levels of stress tolerance had widely different GSH contents, the deviation in the half-cell reduction potential was much smaller, which may be based on the appropriate adjustment of GSSG concentrations. Thus, plants are able to maintain the $E_{GSSG/2GSH}$ value under moderate stress conditions and, depending on the $E_{GSSG/2GSH}$ value, physiologic processes are not disturbed.

**Table 1** Reduced glutathione (GSH) and half-cell reduction potential of the GSH/GSSG redox couple ($E_{GSSG/2GSH}$) in leaves of maize seedlings following 1 week of treatment

| Treatment | GSH [nmol (g FW)$^{-1}$] | | $E_{GSSG/2GSH}$ (mV) | |
|---|---|---|---|---|
| | Penjalinan | Z7 | Penjalinan | Z7 |
| C | 5.9 | 17.2 | −288.7 | −313.8 |
| ABA | 109.3 | 4.7 | −374.7 | −277.4 |
| SA | 92.1 | 126.7 | −369.0 | −378.9 |
| $H_2O_2$ | 82.7 | 119.6 | −372.4 | −373.5 |
| NaCl | 92.4 | 19.9 | −365.1 | −328.4 |
| PEG | 70.4 | 134.3 | −354.8 | −373.7 |
| Dark | 158.5 | 16.5 | −371.8 | −315.9 |
| SD | 42.3 | | 31.3 | |

Z7 = chilling- and drought-tolerant line; Penjalinan = chilling- and drought-sensitive line (Kocsy and others 2004a; Kellős and others 2008); ABA = 0.1 mM abscisic acid; SA = 0.5 mM salicylic acid; $H_2O_2$ = 1 mM hydrogen peroxide; NaCl = 200 mM sodium chloride; PEG = 15% polyethylene glycol 4000; Dark = continuous dark; SD = significant difference at the $p < 5\%$ level

## Temporal and Spatial Changes in the Glutathione Content and in the Ratio of its Reduced to Oxidized Form Under Stress Conditions

To accurately describe 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 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 (G. Kocsy, unpublished results). Later on, during the first week of the 21-day treatment there was a transient increase in both parameters of the two thiols in the shoots, whereas there was no significant change in their levels in the crowns. As with the cold treatment, 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 and others 2004). After salt stress the GSH level and the GSH/GSSG ratio were lower after 1 week compared to the 3-day treatment in the maize inbred line Z7 (Kellős and others 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 to GSH degradation.

When investigating the spatial changes in GSH content in maize and wheat at low temperatures, similar changes were found in shoots and roots (Kocsy and others 2001a). Following osmotic stress there was a similar increase in TG percentage in the shoots in both drought-tolerant and -sensitive wheat genotypes (Fig. 1). However, the GSH/GSSG ratio was higher in the tolerant genotype under both control and stress conditions (Kocsy and others 2004b, Table 2). The GSH/GSSG ratio was similar in the shoots and roots (Table 2) 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 and others 2007), which may be the result of the compartmentalization of GSH metabolism (Kopriva and Koprivova 2005). Chilling increased $\gamma$-glutamylcysteine synthetase ($\gamma$ECS) transcript and protein levels in the bundle sheath but not in the mesophyll cells, which could be the reason for the different

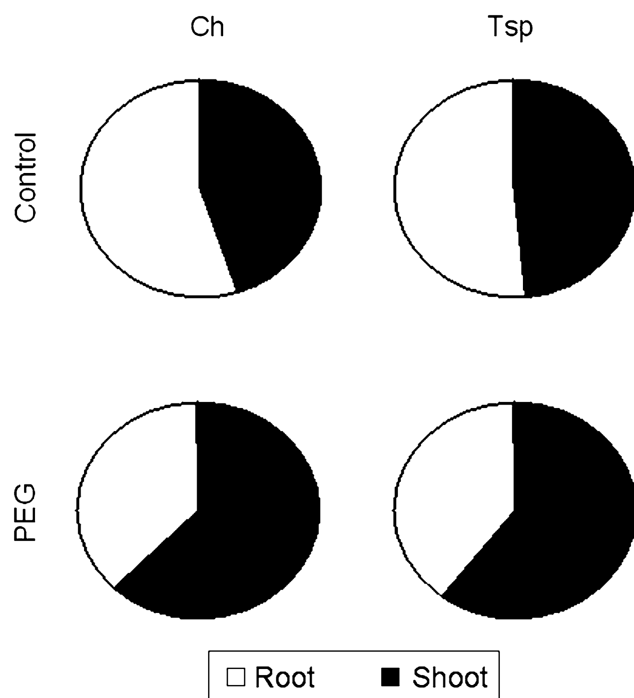


**Fig. 1** Relative amount of TG in shoots and roots of the drought-tolerant *Triticum aestivum* cv. Cheyenne (Ch) and drought-sensitive *Triticum spelta* wheat genotypes under control and osmotic stress conditions. Seedlings grown in hydroponics were treated with 11% polyethylene glycol (PEG) for 3 days

**Table 2** Comparison of the GSH/GSSG ratio in shoots and roots of drought-tolerant *Triticum aestivum* cv. Cheyenne (Ch) and drought-sensitive *Triticum spelta* wheat genotypes under control and osmotic stress (3-day 11% polyethylene glycol [PEG]) conditions

| | GSH/GSSG | |
|---|---|---|
| | Shoot | Root |
| Ch | | |
| Control | 14.9 | 15.4 |
| PEG | 13.5 | 14.3 |
| Tsp | | |
| Control | 10.6 | 9.2 |
| PEG | 7.8 | 6.2 |
| SD | 1.9 | |

GSH levels in the two cell types (Gómez and others 2004b).

Studies on the subcellular localization of GSH 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 and others 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 detection of GSH in living cells of *Allium cepa* (Müller and others 1999). A specific antibody raised against GSH was appropriate for monitoring its intracellular distribution in various cell compartments (Müller and others 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 and others 2007). The usefulness of this system was demonstrated in *Arabidopsis* roots, in which 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 and others 2007).

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, $\gamma$ECS is localized exclusively in the cytosol (Pasternak and others 2008) and is active in the oxidized state (Jez and others 2004), whereas the active form of the nonexpressor of pathogen-related genes (NPR1) is reduced and localized in the

nucleus (Mou and others 2003). Because 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 in their redox activation mechanism need to be activated successively during the stress response, their induction could be ensured by changes in the redox state of the relevant compartment.

Taken together, 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.

## Stress-Induced Changes in the Synthesis of Glutathione

The stress-induced changes in GSH levels and the GSH/GSSG ratio may derive from altered GSH synthesis. GSH is a tripeptide (γ-glutamylcysteinyl glycine), which is synthesized in two steps. First the formation of γEC is catalyzed by γECS, then a glycine is added to the dipeptide by GSH synthetase (GSHS). The regulatory enzyme of GSH synthesis is γECS (Rüegsegger and Brunold 1992; Rennenberg and others 2007). Besides the general occurrence of GSH, hGSH (γ-glutamylcysteinyl-β-alanine) is present in the *Fabaceae* family (Klapheck 1988) and hmGSH (γ-glutamylcysteinyl-serine) is present in the *Gramineae* family (Klapheck and others 1991).

The cold-induced increase in the TG concentration in maize was the result of a greater synthesis rate, as demonstrated by the incorporation of $^{35}$S from sulate into GSH and the higher activity of the two enzymes involved in GSH synthesis (Kocsy and others 2001a). In addition, chilling increased the γECS activity and γEC content in the bundle sheath cells of maize leaves (Gómez and others 2004b). Osmotic stress, high temperature, and cold treatments induced a greater increase in GSH and hmGSH synthesis in tolerant wheat genotypes than in sensitive ones, as shown in $^{35}$S-labeling experiments (Kocsy and others 2000, 2004b, c). After NaCl treatment the γECS, GSHS, and GST activity was much greater in a tolerant cotton cell line than in a sensitive one, indicating adaptation at the level of GSH synthesis, GSSG reduction, and GS-conjugate formation (Gossett and others 1996). Following cadmium stress enhanced synthesis of GSH and phytochelatins was observed in various plant species (Mendoza-Cózati and others 2005), and in maize was based on increased γECS activity (Rüegsegger and Brunold 1992). Data in the literature demonstrate that the abiotic stress-induced increase in the GSH content is due, at least partly, to a higher rate of GSH synthesis.

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 and Müller 2008; Zechmann and others 2006). Using this technique, 50% of TG was found in the mitochondria under control conditions and its relative level varied between 7 and 20% in the other organelles in the leaves and roots of *Cucurbita pepo*, as calculated from the data of Zechmann and Müller (2008) (Fig. 2). Virus infection resulted in a two- to threefold greater increase in Cys and GSH levels in plastids and nuclei compared with mitochondria (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.

Abiotic stress-induced changes in the redox state of GSH precursors may also influence the synthesis and redox state of GSH, because there were similar differences in the ratios of GSH/GSSG and hmGSH/hmGSSG and their reduced/oxidized precursors between tolerant and sensitive wheat varieties subjected to osmotic or heat stress (Kocsy and others 2004b, c). 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 oxidized form of the other GSH precursor in tobacco overexpressing γECS (Creissen and others 1999), indicates that no enzyme exists for its efficient reduction in plants; therefore, it can be

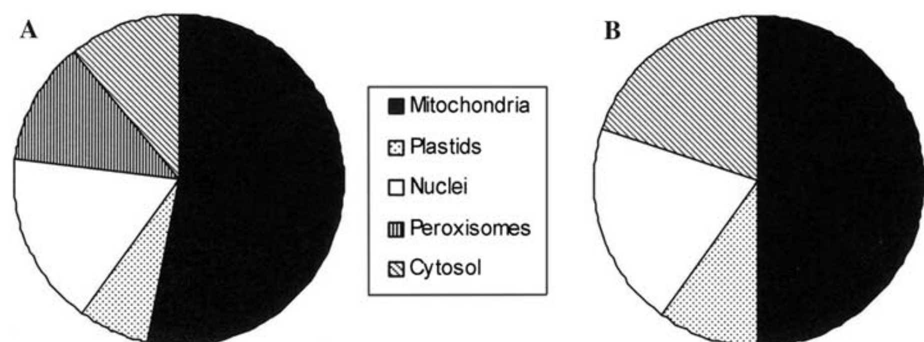


**Fig. 2** Relative subcellular amounts of TG in leaves (**a**) and roots (**b**) of *Cucurbita pepo* under control conditions. No glutathione could be detected in the vacuoles of either organ. Percentages were calculated from the data published by Zechman and Müller (2008)

removed only by degradation or sequestration to the vacuole.

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.

## Stress-Induced Changes in the Degradation, Conjugation, and Transport of Glutathione

The accumulation of TG 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 and others 2001). γ-Glutamyltranspeptidases (GGT) are essential for the degradation of GSH and GS conjugates. In *Arabidopsis* four GGT genes were identified and their temporal and spatial patterns in the degradation of GSH and its metabolites were determined, revealing an appreciable overlapping of the GGTs (Martin and others 2007). Recently, a GGT-independent pathway of GSH catabolism to glutamate via 5-oxoproline was described in *Arabidopsis* (Ohkama-Ohtsu and others 2008). In addition, phytochelatin synthase was found to play a role in the plant-specific degradation of GS conjugates (Blum and others 2007). GGT can also catalyze the degradation of GSSG (Ohkama-Ohtsu and others 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 and others 1999).

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 and others 2002; Anderson and Davis 2004). This reaction is catalyzed by GST, which is induced by abiotic stresses (Coleman and others 1997). Conjugation takes place in the cytoplasm and the conjugates are transferred to the vacuole for further processing (Dixon and others 2002).

The TG level is also affected by the transport of GSH and GSSG. GSH is a long-distance transport metabolite for transporting reduced sulfur between shoots and roots and into developing seeds (Herschbach and Rennenberg 2001; Cairns and others 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 and others 2004). GSH and GSSG are both taken up via proton symport, but the ion fluxes accompanying GSSG and GSH uptake are different, indicating that several parallel GSH transport mechanisms exist in plants (Foyer and others 2001). In contrast with the plasma membrane GSH transport system, tonoplast transporters have been studied more extensively (Foyer and others 2001). GSSG may be a substrate for ABC (ATP-binding casette) transporters on the tonoplast, and under oxidative stress conditions GSSG may be transported from the cytosol to the vacuole (Foyer and others 2001). The transport of GSH and its precursor γEC is important in the regulation of GSH synthesis. Pasternak and others (2008) demonstrated in transgenic *Arabidopsis* that consistent with the exclusive plastidic location of γECS, γEC is exported from the plastids to supply the cytosol with the immediate precursor for GSH synthesis. The effective control of GSH synthesis is ensured through feedback inhibition of γECS due to the efficient import of GSH into the plastids.

Coordinated 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.

## Regulation of the Glutathione Metabolism in Stressed Plants

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 γECS, the rate-limiting enzyme in GSH synthesis, was induced by chilling and ozone fumigation in maize and *Arabidopsis*, respectively (Gómez and others 2004b; Sasaki-Sekimoto and others 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 (Kellős and others 2008). Drought increased the expression of the *GR*, *GST*, and *GPx* genes, 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 and others 1992).

The translational control of γECS activity was observed in *Arabidopsis* following $H_2O_2$ treatment and in maize subjected to chilling because the amount of enzyme protein increased (Xiang and Bertrand 2000; Gómez and others 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 *GR* gene expression after heat stress (Kurganova and others 1999; Escaler and others 2000), suggesting the existence of translational or post-translational regulation.

The molecular mechanism of the post-translational regulation of γECS was recently described. According to the hypothesis of Jez and others (2004), γECS is a monomeric protein undergoing reversible conformational changes in response to oxidative stress in *Arabidopsis*. The $Cys^{186}$-$Cys^{406}$ disulfide bond has a dominant regulatory role, as shown by redox titration. γECS is active in its oxidized form and the ratio of the reduced to oxidized form of the enzyme changes under stress conditions (Hicks and others 2007). The midpoint redox potential of γECS (-318 mV at pH 7.0; Hicks and others 2007) is very similar to the value of $E_{GSSG/2GSH}$ in nonstressed cells (Meyer and others 2007). Hothorn and others (2006) came to the conclusion that the active enzyme in *Brassica juncea* is a dimer and that the reduction of the $Cys^{178}$-$Cys^{398}$ disulfide bridge (CC2) (present in the whole plant kingdom and in α-proteobacterial γECS) facilitates the formation of monomers, thus decreasing the activity. Further comparative studies on CC2 revealed that plant γECS are related to proteobacterial ones, but their redox regulation via CC2-dependent dimerization evolved later (Gromes and others 2008). The reduction of the $Cys^{341}$-$Cys^{356}$ disulfide bond (present only in the rosid clade) shields the active site. The relationship between high GSH levels and the inhibition of γEC synthesis can be explained by both assumptions. Mutation near the active site of γECS resulted in impaired Cys binding in *rax1-1* (regulator of ascorbate peroxidase) in *Arabidopsis* plants (Hothorn and others 2006). The activation of GSHS was not based on thiol-disulfide transition, as shown by the site-directed mutagenesis of active-site residues in *Arabidopsis* (Herrera and others 2007). The specific targeting of GSHS to the cytosol and the chloroplasts is achieved by multiple-transcription initiation (Wachter and others 2005). Unlike the two enzymes of GSH synthesis, the molecular mechanism of GR activation was not described. The post-translational regulation of GR was found in *Pinus sylvestris* L., where GSSG treatment increased enzyme activity without changing the amounts of GR mRNA and protein or the GR izoenzyme pattern (Wingsle and Karpinski 1996), thus indicating the activation of the existing GR protein. The induction of GR isozymes was not associated with an enhancement of the GR mRNA and protein levels in wheat, indicating the possibility of post-translational modification (Yannarelli and others 2007).

The articles cited above show that the enzymes involved in GSH metabolism are regulated at different levels in various plant species. Control of the corresponding enzymes may also depend on the organ and cell type and on the developmental stage. Future studies on the regulation of the enzymes involved in GSH degradation and conjugation are necessary.

## Study of Glutathione Metabolism Through its Manipulation and in Mutants Under Stress Conditions

The role of GSH in the stress response can be demonstrated by manipulating its level. In a chilling-sensitive maize genotype, the GSH content and chilling tolerance increased when herbicide safeners were added (Kocsy and others 2001b), but when increasing concentrations of buthionine sulfoximine, 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 that became sensitive following buthionine sulfoximine treatment (Kocsy and others 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.

In transgenic plants that overexpress genes involved in GSH metabolism, the role of GSH in response to abiotic stresses was also confirmed. Although Noctor and others (1998) observed higher GSH content and resistance to paraquat-induced oxidative stress in poplar transformed with the γECS gene, Bittsanszky and others (2006) were unable to detect elevated paraquat tolerance in transgenic poplar overproducing GSH. This discrepancy may have been due to differences in cultivation and treatment conditions. Transgenic poplars overexpressing γECS have increased tolerance to chloracetanilide herbicides and accumulate significantly more Cd than wild-type plants, suggesting that they could be used for the removal of herbicides and Cd from the soil for phytoremediation (Gullner and others 2001; Koprivova and others 2002). The overexpression of γECS in the cytosol and chloroplasts seems to affect GSH levels in a compartment-specific manner (Hartmann and others 2003). The overexpression of GR increased the antioxidant capacity and decreased the sensitivity to high light intensity and low temperature (Foyer and others 1995). The role of GST during salt stress was shown in transgenic tobacco, where the overexpression of the enzyme increased the GSH content and salt tolerance (Roxas and others 1997). Following application of paraquat, GST activity was significantly higher in transgenic rice overexpressing GST than in the wild type (Zhao and Zhang 2006). Interestingly enough, genetic manipulation of proline synthesis also affected GSH concentrations in soybean subjected to drought, which can be explained by the competitive use of glutamate, the common precursor of the two compounds (Kocsy and others 2005).

Besides the manipulation of GSH and GSSG contents, the use of mutants with an altered GSH metabolism is also a powerful tool for studying the role of GSH in the stress response. The GSH-deficient *Arabidopsis* mutant *cad2* (cadmium-sensitive γECS mutant, 75-80% reduction in

GSH content) was sensitive to cadmium, demonstrating the involvement of GSH in the detoxification of heavy metals (Cobbett and others 1998). The low GSH concentration in the *pad2-1* (phytoalexin deficient) mutant (mutation in the γECS gene, 80% reduction in GSH content) resulted in increased susceptibility to *Phytophthora brassicae* infection. In the *rax1-1* (γECS) mutant, a 50–80% decrease in the GSH level was found and the expression of the defense genes changed (Ball and others 2004). The more severe decrease in the GSH content in the *rml1* (root meristemless) γECS mutant arrested plant development even under optimal growth conditions (Vernoux and others 2000). These mutants could presumably also be used to study the role of GSH in the response to abiotic stress.

Chemical or genetic manipulation of 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.

## Involvement of Glutathione in Redox Signalling

The interaction between ROS and antioxidants may provide the metabolic contact point between signals originating from metabolic pathways and the environment, thus regulating the induction of adaptive or death processes (Foyer and Noctor 2005). The antioxidative system, including the GSH/GSSG redox couple, may have evolved for the adjustment of the cellular redox state and redox signalling and for the orchestration of gene expression (Noctor and Foyer 1998). Several regulatory and structural genes controlled by the thiol-disulfide status and ROS signalling have been identified in mutant and transgenic *Arabidopsis* and in wild-type plants treated with dithiothreitol or ROS-generating agents using transcript profiling, which could clarify the function of the redox network (Gadjev and others 2006; Kolbe and others 2006). This network controls the 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).

The redox state of the GSH/GSSG couple is altered under abiotic stress conditions because GSH is oxidized during the removal of the accumulating $H_2O_2$ under abiotic stress conditions. Stress-induced changes in the $H_2O_2$ content, and subsequently in the GSH/GSSG ratio, have a central role in signalling due to their effects on transcription, translation, post-translational modification of proteins, and metabolic processes (Fig. 3) (Foyer and others 1997; Neill and others 2002; Dietz 2008; Quan and others 2008). A histidine kinase was suggested as a putative $H_2O_2$ sensor in a cyanobacterium (Kanesaki and others 2007). The

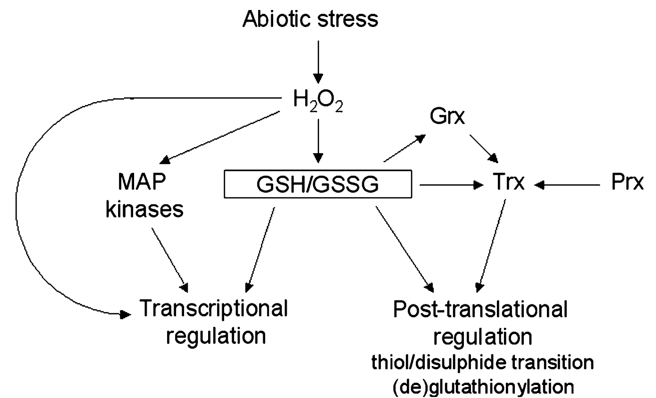


**Fig. 3** A general model for the involvement of the GSH/GSSG couple in redox signalling. Glutathione may have a direct or indirect regulatory role at the transcriptional or post-translational level due to its interaction with other redox systems. Grx, glutaredoxin; GSH, glutathione; GSSG, glutathione disulfide; MAP kinases, mitogen-activated protein kinases; Prx, peroxiredoxin; Trx, thioredoxin

influence of environmental conditions on redox signalling was shown in knockout mutants for catalase2 (Cat2), because the increased $H_2O_2$ content resulted in acclimation under short-day conditions and in death under long-day conditions (Queval and others 2007). In addition, the day length also affected the amount of GSH and the redox state of the GSH/GSSG couple and the expression of defense genes. The effect of alterations in the $H_2O_2$ level on transcription was also shown in ascorbate peroxidase-deficient mutants (Miller and others 2008). In addition, it was concluded after exogenous $H_2O_2$ application and from experiments with catalase-deficient mutants that $H_2O_2$ affects the expression of several regulatory and structural genes involved in the stress response (Desikan and others 2001; Vandenabeele and others 2003). The effect of $H_2O_2$ on gene expression may be transmitted through a mitogen-activated protein kinase cascade (Fig. 3) (Apel and Hirt 2004). A specific example of the regulatory role of $H_2O_2$ and the GSH/GSSG couple in transcription factors was described in the case of OxyR (oxygen radical-responsive), which is activated through the formation of disulfide bridges in *Escherichia coli* (Aslund and Beckwith 1999; Kim and others 2002). Among other things, OxyR increases the expression of genes coding for superoxide dismutase (SOD) and peroxidases. Similarly to OxyR, the oxidized form is also active in the case of γECS (Hicks and others 2007), whereas the reduced form is active for NPR1 (Mou and others 2003). Another example of the regulatory role of $H_2O_2$ in plants is the induction of catalase (*Cat*) and *GST* genes (Polidoros and Scandalios 1999). In maize, an antioxidant-sensitive element found in the promoter region of the *Cat* gene strongly bound proteins transported from the nucleus, but it did not interact with $H_2O_2$. This site was similar to one described in the promoter of γECS and

MnSOD in animals. The post-transcriptional regulatory role of $H_2O_2$ was shown in maize subjected to various abiotic stresses, as the γECS and GR transcript levels either remained constant or decreased parallel to changes in GSH synthesis, the GSH/GSSG ratio, and GR activity (Kellős and others 2008). Besides $H_2O_2$, other ROS such as $O_2^{\bullet-}$ and $^{\bullet}OH$ may also participate in redox signalling in plants, as previously described in human and animal systems. A signal transduction mechanism mediated by $O_2^{\bullet-}$ and by the participation of GSH was proposed for the regulation of a protein-tyrosine phosphatase in human epidermoid carcinoma cells (Barrett and others 1999), while $^{\bullet}OH$ was found to be involved in the activation of a $Ca^{2+}$-sensitive cation channel in epithelial cells (Simon and others 2004). Besides activating the genes involved in protection against environmental stresses, ROS have an important role in coordinating the expression of these genes during adaptation to unfavorable environmental conditions (Vranová and others 2002b).

The interaction between $H_2O_2$ and GSH in stress signalling was suggested in mungbean in which exogenous $H_2O_2$ increased both GSH levels and chilling tolerance (Yu and others 2003). The improvement of chilling tolerance could be a result of changes in the expression of many genes. Correspondingly, in the *Arabidopsis* mutants *rax1-1* and *cad2-1* (mutations in the γECS gene resulting in decreased GSH content), GSH was shown to affect the expression of several genes involved in protection against environmental stresses (Ball and others 2004). The low GSH concentration in the *pad2-1* mutant (mutation in the γECS gene) did not affect the transcript abundance of *γECS* and *GSHS*, but after inoculation with *Phytophthora brassicae*, their expression was much more strongly induced in *pad2-1* than in the wild type (Parisy and others 2007). 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 and others 2002). GSH transport in plants may also be regulated by antioxidants because the promoter of 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) directly through the reduction/oxidation of their disulfide 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 cross-talk between GSH/GSSG and other redox systems through glutathionylation or thiol-disulfide transition, which may have a role in signalling and responses to abiotic stress (Rausch and others 2007; Ying and others 2007). In *Arabidopsis,* oxidative stress-induced glutathionylation was described for a number of proteins, including Grx and several GSTs (Dixon and others 2005). Trxs are inactivated by glutathionylation, as shown in the case of chloroplastic Trx f, the activity of which was decreased by increasing ROS production (Michelet and others 2005). The formation of GSH adducts increases the redox potential, as described for plant Trx h2, which may affect the stress response (Gelhaye and others 2003). Trxs can be activated by deglutathionylation, for which Grxs, members of the Trx superfamily, are required (Nulton-Persson and others 2003). An interaction between the Trx and the GSH/Grx redox system was also suggested by Rouhier and others (2004). However, in their opinion the redox state of Trxs is independent of this system. Based on studies with *Arabidopsis* mutants deficient in the two NADPH-dependent Trx reductases (*ntra* and *ntrb*), it was assumed that in the absence of Trx reductases a GSH-dependent pathway reduces Trxs h (Reichheld and others 2007). The Grx-mediated reduction of Trxs was also suggested, but the reduction of Trxs by GR cannot be excluded either (Reichheld and others 2007). Grxs, which transfer electrons reversibly between GSH and target proteins (Meyer 2008) and may be glutathionylated (Dixon and others 2005), are reduced, in turn, by specific Grx reductase (Michelet and others 2005). A further possibility for the reduction of Trxs is a reaction involving GPxs and peroxiredoxins (Prxs). Redox changes in Trxs are important because they target the intercellular disulfide bonds of proteins, which are activated or inactivated (Besse and Buchanan 1997).

The possible interactions between glutathione, Trx, Grx, and Prx and the involvement of their redox changes in stress signalling will need to be clarified in further experiments.

## Cross-Talking with Other Signalling Pathways

$H_2O_2$ and the GSH/GSSG couple may interact with other signalling pathways during the stress response (Fig. 4). NO, an important regulatory molecule, affected $H_2O_2$ concentration due to the inhibition of Cat and ascorbate peroxidase (Clark and others 2000), whereas exogenous $H_2O_2$ activated NO synthesis in tobacco (de Pinto and others 2006), suggesting a bidirectional interaction between the two compounds. NO also influenced GSH synthesis, as demonstrated in *Medicago trunculata* roots in which the GSH level and γECS and GSHS gene expressions were increased by NO (Innocenti and others 2007). During the interaction of GSH with NO, S-nitrosoglutathione (GSNO) is formed in a reaction that may interconnect the ROS- and reactive nitrogen-based signalling pathways (Neill and others 2002).

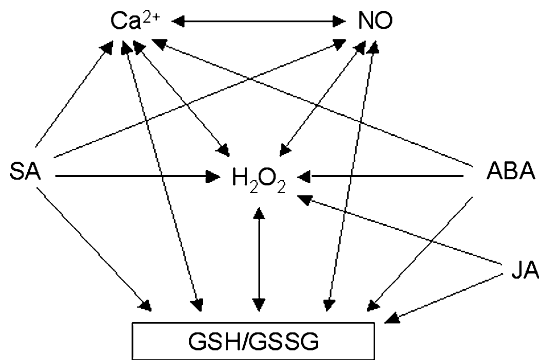


**Fig. 4** A model for possible cross-talking between redox and other signalling pathways. The various signal transducers ($Ca^{2+}$, NO) and plant hormones (ABA, abscisic acid; JA, jasmonic acid; SA, salicylic acid) may affect the GSH level and/or GSH/GSSG ratio directly or through $H_2O_2$

The participation of GSNO in the stress response was shown in Cd-treated plants (Barroso and others 2006). The general regulatory role of NO in stressed plants was demonstrated in several studies (for review see Arasimowicz and Floryszak-Wieczorek 2007).

Another possibility for the activation of protective mechanisms through $H_2O_2$ and the GSH/GSSG couple is based on their interaction with $Ca^{2+}$ (Fig. 4). $H_2O_2$ treatment alone or combined with low temperature increased the $Ca^{2+}$ concentration in tobacco (Price and others 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: (1) During positive regulation $H_2O_2$ will be produced due to the activation of NADPH oxidase, and (2) 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 and others 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 and others 2004a). In plants, $Ca^{2+}$ may interact not only with $H_2O_2$ but with other ROS. This assumption is based on observations of trout hepatoma cells, where the mobilization of $Ca^{2+}$ was induced by $^{\bullet}OH$ (Burlando and Viarengo 2005). Interactions between ROS, $Ca^{2+}$, and antioxidants were reviewed recently by Noctor (2006).

The effect of abiotic stresses on $H_2O_2$, GSH, and GSSG concentrations may be transmitted by various plant hormones (Fig. 4). SA increased the chilling tolerance of maize by inhibiting Cat, thus increasing the $H_2O_2$ concentration (Horváth and others 2002, 2007). In contrast, SA-induced $H_2O_2$ accumulation was not accompanied by the inhibition of Cat or ascorbate peroxidase in germinating wheat (Agarwal and others 2005), suggesting that the influence of SA on $H_2O_2$ levels depends on the plant species, the organ, and the interaction with environmental effects. As also observed in chilled maize (Janda and others 1999), SA stimulated the formation of ROS in *Arabidopsis* subjected to salt or osmotic stress (Borsani and others 2001). GSH and GR were affected by SA in a soybean cell suspension (Knörzer and others 1999) and SA increased the GR activity in rice leaves (Ganesan and Thomas 2001). SA induced various alterations in $\gamma$EC and GSH contents, GR activity, and $\gamma$ECS and GR transcript levels in two maize genotypes with different levels of stress tolerance (Kellős and others 2008); the $\gamma$EC and GSH concentrations were increased in both genotypes by SA. Consistent with this observation, the overexpression of a gene coding for an enzyme involved in SA degradation caused a decrease in both the GSH concentration and the resistance to oxidative stress in rice (Kusumi and others 2006). SA induces defense gene activation by NPR1, the cellular localization of which is regulated in turn by GSH; reduced NPR1 is transported to the nucleus, where it regulates gene expression (Mou and others 2003). SA-induced NO production was found in *Arabidopsis*, and $Ca^{2+}$ accumulation was a component of the signalling cascade (Zottini and others 2007).

Another stress hormone, ABA, induced changes in ROS concentration in *Arabidopsis*, activating the $Ca^{2+}$ channels of the cell membranes and increasing the $Ca^{2+}$ concentration in the cytosol (Murata and others 2001). The connection between the redox state of the cells and $H_2O_2$ and ABA was shown in the *Arabidopsis* mutant *glutathione peroxidase3* (*ATGPX3*) in which 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 and others 2006). In addition, ABA influenced GR activity in the cytosol of rice (Kaminaka and others 1998). In two maize genotypes differing in their stress tolerance, ABA differentially affected the GSH content, GSH/GSSG ratio, GR activity, and $\gamma$ECS transcript level (Kellős and others 2008). In *Vigna unguiculata*, not only GR activity but also expression of the corresponding gene was increased by ABA (Contour-Ansel and others 2006). Thus, ABA may affect the GSH/GSSG ratio and redox signalling (Pastori and Foyer 2002).

Like SA and ABA, jasmonic acid (JA) also regulated gene expression through $H_2O_2$, as found in tobacco (Mur and others 2006). In addition, JA influenced GSH concentration and the genes involved in GSH metabolism in

*Arabidopsis* (Xiang and Oliver 1998; Sasaki-Sekimoto and others 2005). As with SA, ethylene, and NO, JA also increased the transcript level of *GST*, suggesting that the various plant growth regulators interact (Moons 2005).

A simplified model for the interaction of $H_2O_2$ and the GSH/GSSG couple with other signalling molecules and plant hormones is summarized in Figure 4. The order of the components in the signalling pathway described above may vary, and some components 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 the compounds displayed in the figure to ensure the most effective protection against environmental stress (Agarwal and others 2005; Foyer and Noctor 2005; Noctor 2006; Dietz 2008; Miller and others 2008).

## Future Prospects

The following major challenges must be faced: (1) Clarification of the role of subcellular changes in the redox state of the GSH/GSSG couple in stressed plants, (2) study of the interaction of GSH/GSSG with other signalling molecules during the stress response, and (3) investigation of the effect of changes in the redox state of the GSH/GSSG couple on the transcript, protein, and metabolite profiles and on post-translational modification of proteins [thiol-disulfide transition, (de)glutathionylation]. In addition, it would be very interesting to discover how GSH evolved from being an antioxidant to being a key intermediate in multiple-signalling networks. To answer this question, the metabolism, compartmentalization, and transport of GSH, its interaction with other molecules, and the regulation of its redox changes should be compared in prokaryotes and in uni- and multicellular eukaryotes (animals and plants). Sequence comparisons of the related genes and proteins and the construction of phylogenetic trees could help to identify the evolutional events. In prokaryotes GSH/GSSG probably had a mainly antioxidant function, becoming a redox regulator during the evolution of multicellular organisms.

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