Chapter 9

Biosynthesis, Compartmentation and Cellular Functions of Glutathione in Plant Cells

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Summary

Glutathione is the most abundant low molecular weight thiol in all plant cells with the only exception of some plant species that produce and accumulate homologous tripeptides to similar levels. The broad range of functions of glutathione in terms of detoxification of heavy metals, xenobiotics and reactive oxygen species (ROS) has been highlighted in numerous reviews before. Glutathione *S*-conjugates formed during detoxification of electrophilic xenobiotics are immediately sequestered to the vacuole for degradation. This degradation is initiated by cleavage of the two terminal amino acids of glutathione. The cleavage of the γ-peptide bond between glutamate and cysteine involves a specific γ-glutamyl transpeptidase. Other members of this gene family are suggested to be involved in glutathione catabolism in the apoplast and linked to long-distance transport of glutathione. Recent findings on the biosynthesis and compartmentation now begin to illuminate how the biosynthesis of glutathione is regulated at the molecular level and how different subcellular pools of glutathione are interconnected. Glutamate-cysteine ligase (GSH1) is

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the key regulatory enzyme of glutathione biosynthesis. Redox-dependent modulation of GSH1 activity also makes GSH1 a key factor in cellular redox homeostasis. Current work indicates that the redox state of the cellular glutathione redox buffer can be read out and directly transferred to target proteins by glutaredoxins. In this way glutathione is both, a scavenger for toxic compounds and a sensor for environmental signals which impact on the cellular redox state. This review aims at describing the important recent results on the cellular glutathione homeostasis in plant cells and highlighting the implications for glutathione-based redox sensing and signaling.

I. Introduction

Thiol-containing compounds are key players in a broad range of significant metabolic reactions and are essential for redox signaling. The tripeptide glutathione (reduced form: GSH; oxidized form: GSSG) is the most abundant low molecular weight thiol in almost all eukaryotic and many prokaryotic cells. Glutathione is essential for detoxification of xenobiotics and, being the precursor of heavy metal binding phytochelatins, also for the detoxification of heavy metals. Furthermore glutathione is an essential part of the glutathione–ascorbate cycle (GAC) and thus also involved in the detoxification of reactive oxygen species (ROS). Detoxification of ROS through GAC goes along with reversible oxidation and reduction of glutathione and thus immediate effects on the glutathione redox potential. Because glutathione is present in low millimolar concentrations in plant cells and thus the dominating redox buffer besides ascorbate, changes of the redox potential of the glutathione pool will have important effects on other cellular redox systems and thiol containing proteins in particular.

To understand the broad range of functions of glutathione and the integration of glutathione in the complex cellular signaling network it is essential to investigate the biosynthesis and the compartmentation of glutathione metabolism. The focus of this work is thus to review recent progress on different factors affecting glutathione homeostasis in plants and thereby outlining the foundations of glutathione-dependent signaling events during stress reactions.

Abbreviations: APS – adenosine 5′-phosphosulfate; BSO-L-buthionine-(S,R)-sulfoximine; ER – endoplasmic reticulum; DHAR – dehydroascorbate reductase; GAC – glutathione–ascorbate cycle; γ-EC – γ-glutamylcysteine; GGT – γ-glutamyl transpeptidase; GR – glutathione reductase; GRX – glutaredoxin; GSB – glutathione *S*-bimane; GSH – reduced glutathione; GSSG – oxidized glutathione; GST – glutathione *S*-transferase; MCB – monochlorobimane; OPT – oligopeptide transporter; PC – phytochelatin; PCS – phytochelatin synthase; ROS – reactive oxygen species; TRX – thioredoxin

II. Biosynthesis of Glutathione

A. Evolution of GSH Biosynthesis

Glutathione and its homologues are the most prominent low molecular weight thiols in virtually all eukaryotic cells (with the exception of cells that lack mitochondria; Fahey et al., 1984; Newton et al., 1996) and in most Gram-negative bacteria, including cyanobacteria and purple bacteria (Fahey, 2001). In Gram-positive bacteria glutathione is less frequently found and in many cases replaced by other redox active thiol compounds (Fahey, 2001). Glutathione is generally synthesized in two ATP-dependent steps from its constituent amino acids glutamate, cysteine and glycine. In some plant species glycine is replaced by other residues (see below). The two enzymes involved in this biosynthetic pathway are glutamate-cysteine ligase (GSH1; GSHA in bacteria) and glutathione synthetase (GSH2; GSHB in bacteria). GSH1 catalyzes the formation of the atypical peptide bond between the γ-carboxylic group of glutamate and the amino group of cysteine. GSH2 subsequently catalyzes the formation of the peptide bond between the carboxylic group of cysteine and the amino group of glycine. Both enzymes are highly conserved between different plants, but surprisingly plant GSH1 was shown to be highly divergent from other eukaryotic organisms (May and Leaver, 1994).

Generally it is assumed that the need for a stable cellular redox buffer and compounds keeping ROS under control arose with the evolution of oxygenic photosynthesis about 2.6 billion years ago (Des Marais, 2000). From

cyanobacteria in which GSHA originally evolved the gene was transmitted to other species including archaea and proteobacteria. In proteobacteria that made use of the increasing atmospheric oxygen concentration by developing aerobic metabolism the presence of GSH would have been advantageous because of increasing amounts of ROS.

Direct comparison of sequences for GSH1 from a broad range of organisms from all kingdoms showed that the sequences cluster in three distinct groups (Copley and Dhillon, 2002). Group 1 comprises primarily γ-proteobacteria including *Escherichia coli*, group 2 comprises most eukaryotic organisms including human and *Saccharomyces cerevisiae* but excluding plants. Plant GSH1 sequences form a third group together with α-proteobacteria and archaea. Despite the highly divergent forms of the same enzymatic function, careful analysis of small blocks of conserved sequence motifs indicated that the three groups of sequences are indeed distantly related (Copley and Dhillon, 2002). While non-plant eukaryotic organisms might have received their GSH1 genes from α-proteobacterial progenitors of mitochondria (Fahey et al., 1984; Fahey and Sundquist, 1991; Fahey, 2001), plants were assumed to have received their GSH1 genes from the cyanobacterial progenitor of chloroplasts. It is puzzling, however, that the plant GSH1 sequences are very similar to a number of α-proteobacterial sequences (see below). In this context it remains unknown whether the α-proteobacterial progenitor gene in plants replaced an already present gene or whether the ancestral plastidic genes were lost first, the α-proteobacterial genes subsequently filling the functional gap (Copley and Dhillon, 2002).

Evolutionary relationships are even less clear for GSH2. In this case all known eukaryotic sequences are related, but none of them show any significant homology with bacterial GSHB (Copley and Dhillon, 2002). Polekhina et al. (1999) suggested that eukaryotic GSH2 did not evolve from a bacterial ancestor. Instead, both bacterial GSHB and eukaryotic GSH2 might have evolved independently from ancestors that had the characteristic fold of the ATP-grasp superfamily. The members of this family exhibit a distinct carboxylate-amine/thiol ligase activity (Galperin and Koonin, 1997).

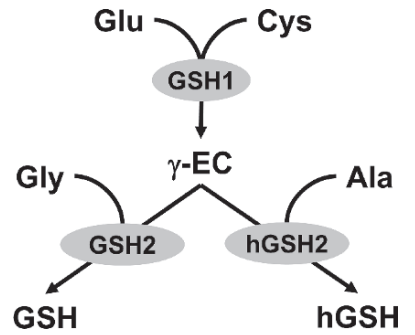

Fig. 1. Pathways for biosynthesis of GSH and hGSH. All depicted biosynthetic steps consume one ATP per synthesized molecule.

Many members of the Fabaceae contain homoglutathione (γ-Glu-Cys-β-Ala, hGSH) besides glutathione (Fig. 1) (Price, 1957; Klapheck, 1988). Analysis of the molecular basis of homoglutathione biosynthesis in *Medicago trunculata* showed that homoglutathione synthetase (hGSH2) is closely related to glutathione synthetase. A gene duplication event for GSH2 after divergence of Fabales from Solanales and Brassicales and subsequent substitution of two highly conserved alanines by Leu-534 and Pro-535 in one of the two copies gave rise to synthesis of hGSH (Frendo et al., 2001).

B. Biochemistry of GSH1 and GSH2 Enzymes: From Structure to Regulation

Although plant GSH1 proteins and GSHI from E. Coli share no statistically significant similarities in their sequences (May and Leaver, 1994; Copley and Dhillon, 2002), the recent elucidation of the GSH1 protein structures from *E. coli* (Hibi et al., 2004) and *Brassica juncea* (Hothorn et al., 2006) has revealed overall similarity in the protein fold with some additional plant GSH1-specific properties. The primary plant GSH1 sequence folds into a six-stranded antiparallel β-sheet forming a bowl-like structure, which is flanked by helical regions. While the catalytic residues were conserved between the plant and *E. coli* enzymes, an unexpected β-hairpin was discovered in the plant enzyme, which is absent in the *E. coli* enzyme. While a detailed description of the structure of plant GSH1 is beyond the scope of this review, it is important to note that for the *B. juncea* enzyme, the protein structure has revealed the presence of two intramolecular disulfide bridges (Hothorn et al., 2006). One of these disulfide bridges,

CC1, has been proposed to position the β-hairpin (see above) in a way that the access of cysteine to its binding site is allowed, whereas reduction of CC1 possibly shields the Cys binding site. In agreement with this assumption, mutating these Cys residues resulted in a 10-fold reduced enzyme activity of the mutant recombinant protein. Using the mutant *Bj*GSH1 protein, the possible role of the second disulfide bridge (CC2) could be addressed. Again, reduction of this disulfide bridge resulted in a further decrease of enzyme activity (about fourfold; Hothorn et al., 2006).

How exactly the previously reported redox control of the plant GSH1 enzyme activity (Hell and Bergmann, 1990; Jez et al., 2004) relates to the roles of CC1 and CC2 for regulating GSH1 enzyme activity in vivo remains to be convincingly shown. In their seminal paper on the plant GSH1 enzyme, Hell and Bergmann (1990) revealed that upon addition of DTT the tobacco enzyme apparently underwent a profound structural change, resulting in inactivation and changed mobility in gel filtration chromatography (i.e. from approx. 60 to 30 kDa). In their recent study on recombinant Arabidopsis GSH1 protein, Jez et al. (2004) could basically confirm this observation. As under reduced conditions, the tobacco GSH1 enzyme (Hell and Bergmann, 1990) and the Arabidopsis enzyme (Jez et al., 2004) showed similar behavior, it can be excluded that CC1 is relevant for this structural change, as the presence of CC1 Cys residues is not conserved in plant GSH1 enzymes (i.e. absent in tobacco). While Hothorn et al. (2006) confirmed for the enzyme from *B. juncea* the occurrence of a profound structural change in response to the redox environment, these authors advocate a redox-regulated monomer-homodimer switch, based on their results from size exclusion chromatography and analysis of *Bj*GSH1 crystal structure. Interestingly, this redox-induced monomer-homodimer switch is not observed for recombinant GSH1 proteins from the structurally closely related α-proteobacteria (R. Gromes, M. Hothorn and T. Rausch, unpublished). Also, the amino acid residues forming the interface of the homodimer in the *Bj*GSH1 enzyme are conserved in GSH1 proteins across the plant kingdom but not in α-proteobacteria. It is intriguing, that the enzyme catalyzing the rate-limiting step in the biosynthesis of one of the cells major antioxidants should be itself under tight redox control. In fact, the results discussed above suggest that, under normal conditions (i.e. reducing milieu in the chloroplast stroma) the plant GSH1 enzyme would operate "with brakes on", being fully activated only under oxidizing conditions as encountered upon stress exposure. While definite proof for this attractive hypothesis remains to be provided, Jez and colleagues have recently presented first supportive evidence for in vivo operation of the proposed redox switch (Jez et al., 2006; Hicks et al., 2007).

In contrast to GSH1, plant GSH2 enzymes belong to one large family with all other eukaryotic GSH2 enzymes, which shows no similarity with bacterial GSH2 enzymes (Wang and Oliver, 1996; Copley and Dhillon, 2002). While GSH2 from *E. coli* is a functional tetramer of approximately 300-residue subunits (Hara et al., 1996), the larger eukaryotic GSH2 subunits from mammals and yeast (approx. 470 residues) form a homodimer (Polekhina et al., 1999; Gogos and Shapiro, 2002). Likewise, the analysis of recombinant *At*GSH2 protein indicated that the enzyme operates as a homodimer (Jez and Cahoon, 2004). While no plant GSH2 structure has as yet been reported, the fairly high conservation of eukaryotic GSH2 proteins (approx. 40% sequence identity) supports the assumption that the kinetic mechanism is conserved among eukaryotic GSH2 enzymes. Despite the lack of sequence conservation, their difference in oligomer structure and molecular masses of subunits, GSH2 enzymes from bacteria and eukaryotes share a common protein fold and belong to the ATP-grasp structural family (Hara et al., 1996; Galperin and Koonin, 1997; Polekhina et al., 1999; Gogos and Shapiro, 2002).

Only recently have the catalytic properties of plant GSH1 and GSH2 enzymes been studied with recombinant enzymes from *Arabidopsis thaliana* after expression in *E. coli* (Jez and Cahoon, 2004; Jez et al., 2004). In both enzymes, the three substrates form ternary complexes in the active site, with all substrates mutually affecting their binding. Detailed kinetic analysis of GSH1 and GSH2 has revealed that both enzymes appear to operate via a random ter-reaction mechanism with a preferred order of substrate addition. Thus, in *At*GSH1, binding of the substrates ATP or glutamate increases the affinity for the other substrate

2.5-fold, whereas the positive interaction between cysteine and glutamate results in an even higher reciprocal increase of binding affinities (16-fold; Jez et al., 2004). Similarly, in *At*GSH2 binding of γ-glutamylcysteine or ATP increase the affinity of the enzyme for the other substrate 10-fold (Jez and Cahoon, 2004), whereas binding of either glycine or γ-glutamylcysteine decrease binding of the other substrate by almost sevenfold.

Since the cloning of the first plant cDNAs encoding GSH1 and GSH2 (May and Leaver, 1994; Ullmann et al., 1996; Wang and Oliver, 1996), numerous studies have addressed the control of their expression at the transcript level. The results of these studies have largely confirmed that under conditions where GSH biosynthesis is upgraded in response to various developmental or environmental cues, GSH1 expression appears to be more affected, corroborating its proposed role as catalyzing the limiting step (Schäfer et al., 1998; Xiang and Oliver, 1998; Xiang et al., 2001). However, some reports also document a coordinate increase of GSH1 and GSH2 mRNAs. Thus, in *B. juncea* transcript amounts were increased for both genes (Schäfer et al., 1998). Likewise, in *A. thaliana* the infection with *Phytophthora brassicae* caused a more than twofold coordinate increase of GSH1 and GSH2 transcripts (Parisy et al., 2007).

Recently, Wachter et al. (2005) reported on different transcript populations for GSH1 in *A. thaliana* and *B. juncea*. In both species, two TATA-boxes located in proximity give rise to two distinct transcript populations, differing in their length of 5′UTR sequence. The quantitative ratio of long to short 5′UTR mRNAs was dependent on the developmental stage (Wachter et al., 2005) and was also affected by several stress-related cues (Wachter, 2004). The biological significance of different 5′UTRs is not yet known, however, it may be speculated that transcript stability and/or binding of protein factors to the 5′UTR could be affected (Xiang and Bertrand, 2000; Wachter, 2004). Mapping of transcript start sites revealed that mRNAs were initiated about 30 bp downstream of both TATA-boxes, indicating that both were operative. Thus it can be assumed that two closely spaced, overlapping promoters differentially regulate the formation of short and long 5′UTR transcripts in these species. Both transcript classes code for the same GSH1 protein with functional transit peptide, rendering an effect of different 5′UTR structure on GSH1 targeting unlikely (Wachter et al., 2005).

Transcriptional control of GSH1 expression in response to hormonal (e.g. jasmonic acid) and stress-related cues (e.g. heavy metal exposure) undoubtedly contributes to the observed changes in GSH1 activity, however, earlier work has already indicated that other regulatory mechanisms are likely to be involved. Thus, in Arabidopsis suspension-cultured cells, a stress-induced increase of GSH1 enzyme activity was observed in the absence of transcript increase (May et al., 1998). While several independent studies support the existence of a post-transcriptional control of GSH1 expression (May et al., 1998; Xiang and Bertrand, 2000), our factual knowledge about the underlying molecular mechanism(s) is fragmentary at best. Clearly, there is an urgent need for further detailed analysis of the observed interactions of GSH1 transcripts with 5′UTR-binding proteins, as work has not progressed beyond initial observations (Xiang and Bertrand, 2000; Wachter, 2004). The postulated redox-control of such an interaction would provide a feedback mechanism to assure cellular glutathione homeostasis. It is noteworthy that such a control would have to operate in the cytosol and would therefore respond to the cytosolic redox poise, whereas the GSH1 enzyme is exclusively targeted to the plastids (Wachter et al., 2005). Therefore, such a mechanism would constitute a conduit for redox communication between both compartments. In addition to the postulated redox-mediated translational control, the post-translational control of GSH1 activity via a redox-mediated monomer-homodimer switch, as observed in vitro with recombinant GSH1 enzyme (see above; Hothorn et al., 2006), may account for an additional level of regulation.

The Cd-sensitive Arabidopsis *cad2-1* mutant, which shows a 2 amino acid deletion in the GSH1 protein, provided the first genetic evidence for a causal link between GSH biosynthesis and a specific GSH function in planta (Cobbett et al., 1998). The recent elucidation of plant GSH1 structure (Hothorn et al., 2006) indicated that this deletion most likely affects the position of residues involved in glutamate binding. As a result of reduced GSH1 activity, the *cad2-1* mutant has a significantly decreased

GSH content (45% as compared to wild-type). Conversely, and as expected, its cysteine content is increased about twofold as compared to wild-type plants (Cobbett et al., 1998). The second GSH1 mutant, *rml1 (r*oot-*m*eristem-*l*ess), depicts a strong developmental phenotype, its root meristem being nonfunctional (Vernoux et al., 2000). This mutant documented for the first time a direct link between GSH content and GSH function during plant development, and revealed its essential role in initiating and maintaining cell division during post-embryonic root development. The *rml1* mutation, in which an aspartate is exchanged for an asparagine (D250N), is most likely affected in adenine nucleotide binding (Hothorn et al., 2006). This mutant has only about 3% GSH as compared to the wild-type and shows a threefold increased cysteine content. The *rax1-1* mutant was initially identified as showing a constitutive expression of an otherwise stress-inducible ascorbate peroxidase (Ball et al., 2004). While this GSH1 mutant showed a reduction of GSH content (about 30% of the wild-type) similar to *cad2-1*, its cysteine content was unaffected, in marked contrast to *cad2-1* and *pad2-1* (see below). Surprisingly, the molecular basis of the *rax1-1* mutation is a single conservative amino acid exchange (R229K). The functional analysis of recombinant *rax1-1* protein revealed an about fivefold higher K_m towards cysteine (with V_{max} reduced by about 50%), in agreement with the position of R^{229} being proximal to the cysteine-binding pocket (Hothorn et al., 2006). Thus, this mutant also supports the widely held assumption that cysteine availability may affect GSH biosynthesis. Recently, a fourth GSH1 mutant, *pad2-1*, has been shown to be impaired in resistance towards *P. brassicae* and *Pseudomonas syringae* (Parisy et al., 2007). The Arabidopsis *pad2-1* mutant was originally shown to exhibit a reduced accumulation of the phytoalexin camalexin (Glazebrook et al., 1997); however, later work revealed that this was not the cause for the observed phenotype (Zhou et al., 1999). The *pad2-1* mutant shows a S298N substitution, located close to the cysteine binding site. While its GSH content is only about 20%, this mutant exhibits an about fivefold increased cysteine content. While it is tempting to speculate that the decreased GSH content is causally related to the reduced resistance towards pathogens, it cannot be excluded that the strong increase in cysteine content may also contribute to the observed phenotype. In summary, the analysis of several GSH1 mutants has provided proof of the (direct or indirect) role of GSH in several vital plant functions, including plant development and tolerance against abiotic and biotic stress.

III. Compartmentation of Glutathione Metabolism in Plants

A. Transport of GSH and Precursors Across the Chloroplast Envelope

A key parameter in cellular glutathione homeostasis is efficient transport of glutathione and/or its precursors between different organelles. For a long time, the abundant low molecular weight thiols cysteine and especially GSH have been assumed to fulfill the function as a transport metabolite. In terms of glutathione biosynthesis the differential subcellular localization of GSH1 and GSH2 in Arabidopsis highlights the importance for consideration of subcellular compartments in GSH metabolism. At the same time exclusive plastidic localization of GSH1 and dual targeting of GSH2 to both cytosol and plastids (Wachter et al., 2005) points away from cysteine and GSH transport and rather implies export of γ-EC from plastids to provide cytosolic GSH2 with its substrate. Export of γ-EC from plastids was suggested by Meyer and Fricker (2002) after in situ labeling of Arabidopsis cell cultures with monochlorobimane (MCB) for several hours. This fluorescent dye has been shown to predominantly label glutathione in live cells (Meyer et al., 2001; Cairns et al., 2006). Long-term labeling of Arabidopsis cells for several hours resulted in a steady increase in fluorescence which could be blocked by the GSH biosynthesis inhibitor L-buthionine-(*S,R*)-sulfoximine (BSO) indicating demand-driven de novo synthesis of GSH und conditions of extended exposure to MCB as an electrophilic xenobiotic. Because this synthesis phase could also be abolished by removal of sulfate from the external medium this implied that the steady increase in fluorescence was not only due to GSH biosynthesis but in fact mir-

rored flux through the entire sulfur assimilation pathway starting from external sulfate and running down to GSH. The first step of sulfate reduction is catalyzed by adenosine 5′phosphosulfate (APS) reductase, an enzyme that contains a sub-domain homologous to glutaredoxins (GRXs) at its carboxy-terminus. Therefore APS reductase is assumed to use electrons delivered by GSH for reduction of APS (Bick et al., 1998). This demand for plastidic GSH as electron donor for continued sulfur assimilation indicates that the plastidic GSH pool is not depleted during the incubation with MCB (Meyer and Fricker, 2002). Conversely, this implies that not GSH but rather its precursor γ-EC is exported from the plastids to cover cytosolic demand for reduced sulfur. This hypothesis was further supported through the isolation of null-mutants for *GSH2* from Arabidopsis T-DNA insertion collections. Homozygous *gsh2* null-mutants hyperaccumulate γ-EC to levels 200-fold greater than wild-type GSH and 5,000-fold greater than wild-type γ-EC (Pasternak et al., 2008). In situ labeling with MCB showed that in this case the extreme concentration of γ-EC led to partial labeling of this pool. The label was predominantly in the cytosol and thus indicated that γ-EC was exported. This result is also corroborated by biochemical in vitro assays on GSH1 showing that this enzyme would quickly be inhibited by accumulating γ-EC albeit with 50% efficiency compared with the normal feedback inhibitor GSH. Furthermore, cytosol specific complementation of the *gsh2* knockouts with wild-type *GSH2* restored the wild-type phenotype and low molecular weight thiol levels almost similar to wild-type.

The cytosolic complementation of *gsh2* knockouts strongly suggests efficient transport of GSH from the cytosol into the plastid. Preliminary data indicating uptake of radioactive GSH into isolated wheat chloroplasts have been presented (Noctor et al., 2000; Noctor et al., 2002). From their data, Noctor and colleagues concluded that the rate of GSH uptake is sufficient to play a significant role in determining the GSH pool on either side of the membrane. Further analysis of the cytosolic complementation of *gsh2* knockouts and quantitative analysis of plastidic GSH in these mutants is expected to provide final evidence for the efficiency of this transport. Despite the increasing evidence for γ-EC as the major metabolite for export of reduced sulfur from the plastids, export of GSH from the plastids can still not be ruled out. The fact that plastid-specific complementations of a *gsh2* knockout are fully viable indicates that GSH can also be transported from plastids to the cytosol (M. Pasternak and A.J. Meyer, unpublished).

The accumulating evidence for efficient export of γ-EC from the plastids now immediately raises the question for a γ-EC transporter on the chloroplast envelope. Because such a transporter when knocked out should severely affect cellular GSH homeostasis and thus result in clear phenotypes candidate proteins should eventually appear in genetic screens. However, none of the solute transporters on the plastid envelope membrane characterized to date has been linked to GSH metabolism (Weber et al., 2005).

B. Glutathione in Other Organelles

Besides photosynthesis mitochondrial respiration is the second main source of ROS in plant cells and at the same time mitochondria house a range of different redox pathways, utilized for protection from oxidative damage and assembly of the organelle (Koehler et al., 2006; Logan, 2006). Thus, glutathione is required within mitochondria to buffer against ROS production and to avoid oxidative damage. Similar to chloroplasts, the most important pathway for ROS detoxification is the GAC (see below), which has been shown to be present in mitochondria with all participating enzymes (Chew et al., 2003b). Given that GSH is not synthesized in mitochondria it has to be imported from the cytosol. Putative transporters for GSH have been identified based on homology with a high affinity glutathione transporter (hgt1p) from *S. cerevisiae* (Bourbouloux et al., 2000). The identified family of nine genes in Arabidopsis is designated as *OPT1–OPT9* (Koh et al., 2002) and at least *AtOPT6* as well as some homologues form other species have been shown to complement the yeast *hgt1* knockout mutant (Bogs et al., 2003; Cagnac et al., 2004; Zhang et al., 2004). Both, OPT3 and OPT6 are predicted to be targeted to the mitochondria by different bioinformatics tools. However, restricted tissue-specific expression of these genes (Stacey et al., 2006) suggests that these proteins are not the essential GSH-transporters for

uptake of GSH into the mitochondria. Transport studies with isolated mitochondria from rats suggest that GSH is taken up into mitochondria via dicarboxylate and 2-oxoglutarate carriers (Chen and Lash, 1998). The fact that the glutathione reductase 1 (GR1, AT3G54660) is dually targeted to both chloroplasts and mitochondria (Chew et al., 2003a) together with the fact that a knockout of this mutant is embryo lethal (Tzafrir et al., 2004) might indicate that GSSG cannot be exported from mitochondria for reduction in the cytosol.

The first reaction of the conversion of glycolate to glycine during photorespiration produces the toxic intermediate glyoxalate and the toxic by-product H_2O_2. Containment of these reactions in the peroxisomes avoid that the toxic products can harm other reaction in the chloroplasts. The GAC was also shown to be present in peroxisomes of pea (*Pisum sativum* L.) (Jimenez et al., 1997). As a result of senescence, the pools of GSH and GSSG were considerably increased in peroxisomes while the oxidative damage in mitochondria was significantly accelerated already. This observation lead to the suggestion that peroxisomes may function longer than mitochondria in the oxidative mechanisms of senescence (Jimenez et al., 1998). The presence of the GAC in peroxisomes implies the presence of glutathione reductase(s) (GR) in this compartment. While ascorbate peroxidase has bee found in proteomic studies of Arabidopsis peroxisomes (Fukao et al., 2002) there is currently no genetic nor proteomic evidence for a GR in peroxisomes (Heazlewood et al., 2007). Nevertheless, glutathione is required as a redox buffer and is used by a number of enzymes as a substrate. Glutathione-depending enzymes shown to be present in peroxisomes of non-plant organisms include glutathione peroxidase in rat peroxisomes (Singh et al., 1994) and glutathione *S*-transferase in *S. cerevisiae* (Barreto et al., 2006). Expression of a novel glutathione specific redox sensor in tobacco peroxisomes suggests that the redox potential of the glutathione redox buffer in peroxisomes is highly reducing and thus similar to that in the cytosol (M. Schwarzländer et al., submitted)[1]

The endoplasmic reticulum (ER) is the compartment in which proteins destined for the secretory pathway are folded. Attainment of their native structure often includes the formation of intramolecular disulfide bridges. Glutathione is known to be the principle redox buffer in the ER, but contrary to the highly reduced state of cytosolic glutathione the ratio of GSH to GSSG in the ER is between 1:1 and 3:1 (Hwang et al., 1992). Targeting of the glutathione dependent redox-sensitive GFP (roGFP) to the ER recently allowed to directly showing the highly oxidized state of the luminal glutathione pool in tobacco (Meyer et al., 2007). Maintenance of such steep gradients across the ER membrane for both GSH and GSSG can only be achieved through active transport mechanisms, but to date no such transporter has been identified. A large fraction of the luminal glutathione pool was shown to be present in the form of mixed disulfides with proteins, which may play a role as a glutathione reserve and a component of the luminal redox buffering system, but may also play a more active role in the process of native protein disulfide bond formation (Bass et al., 2004). Due to its high degree of oxidation glutathione in the ER lumen has long been suspected the prime source of oxidative power for protein folding, but this hypothesis was refuted by the observation that in yeast oxidizing equivalents are provided by the protein Ero1 (Cuozzo and Kaiser, 1999). Despite being present mainly in the oxidized form, glutathione still acts as a source of reducing equivalents by playing a direct role in the isomerization of luminal oxidoreductases and maintenance of theses enzymes in their reduced state (Jessop and Bulleid, 2004; Sevier et al., 2007).

Nuclear compartmentalization of glutathione with ATP-dependent maintenance of the nucleoplasm/cytosol concentration gradient has been shown for hepatocytes based on specific labeling of GSH with MCB (Bellomo et al., 1992). This observation is highly surprising as the only gateway for exchange of macromolecules between cytosol and nucleoplasm is the nuclear pore complex (NPC). This complex has a 9 nm aqueous pore and allows passive diffusion of molecules up to about 60 kDa, molecules over 50 kDa at a very slow rate (Meier, 2007). It was also shown that MCB adducts injected into cells immediately accumulated in the nucleus (Briviba et al., 1993). Labeling of GSH in living plant cells with MCB also very quickly gives rise to very strong nuclear labeling, but even under conditions where normal vacuolar sequestration of glutathione-bimane conjugates is prevented no indication of differential labeling between cytoplasm and nucleus could be observed (Gutiérrez-Alcalá et al., 2000;

Meyer et al., 2001; Hartmann et al., 2003). Tight redox control within the nucleus is essential for normal functioning and it has been shown that the members of the TGA family of transcription factors need to be in the reduced state to allow efficient DNA binding (Despres et al., 2003). A specific role of the glutathione redox buffer in this control is strongly supported by the observation that TGA transcription factors interact with a subclass of GRXs in Arabidopsis (Ndamukong et al., 2007).

C. Compartmentation Between 'Sink' and 'Source' Tissues

Glutathione is the major form of systemically transported reduced sulfur (Foyer et al., 2001). Glutathione is thought to be synthesized in vegetative shoot tissues and transported to generative tissues and developing seeds in particular as well as in the opposite direction to the roots. Long-distance transport of glutathione and/or the precursor γ-EC along the phloem has been shown (Lappartient and Touraine, 1996; Herschbach and Rennenberg, 2001; Li et al., 2006). Split-root experiments in which one half of the root system was exposed to low sulfate conditions indicate that a systemic signal, most likely glutathione, controls sulfate uptake in the roots (Lappartient et al., 1999). High glutathione levels are correlated with decreased sulfate uptake and thus glutathione might contribute to a well-balanced feedback control mechanism for sulfate uptake. Conversely, artificially reduced GSH levels during growth on BSO relieved the expression of APS reductase, the key enzyme for sulfate reduction (Vauclare et al., 2002). It is not clear, however, whether this control of sulfate uptake in the roots and further assimilation is solely controlled by glutathione or whether other components of the glutathione biosynthesis pathway upstream of GSH are also involved. Other possible control mechanisms might include O-acetylserine, sucrose and different phytohormones (Kopriva, 2006).

Homozygous *gsh1* knockouts are not capable of synthesizing γ-EC and hence GSH. Due to this defect these mutants show an embryo-lethal phenotype (Cairns et al., 2006). Despite not being able to synthesize GSH the homozygous knockout embryos develop to their normal size indicating that cell division during seed development can progress without any GSH or, alternatively, supply with GSH from maternal tissues. In situ labeling of GSH with MCB failed to detect significant amounts of GSH (Cairns et al., 2006), but it can not be excluded that limited amounts of GSH are indeed supplied by the mother plant. Very intense fluorescent labeling at the chalaza suggested release of GSH from the phloem at this point. Because embryonic tissues are symplastically isolated from maternal tissues, organic and inorganic compounds supplied to the embryo have to cross three apoplastic borders on the way from the phloem to the embryo (Stadler et al., 2005). Transport of GSH would thus require highly efficient transport systems on membranes along this pathway. Proteins of the OPT family have been suggested to facilitate the membrane transport of GSH (Bourbouloux et al., 2000; Cagnac et al., 2004), but no information is available on the involvement of OPTs in seed supply. Alternatively, GSH supplied to the seed via the phloem might be largely degraded to the amino acids, which are then taken up by the embryo. In animals, GSH present in the extracellular space is degraded by subsequent action of γ-glutamyl transpeptidases (GGTs) and dipeptidases to the respective amino acids, which can then be taken up (Meister, 1988; Lieberman et al., 1996). Arabidopsis also contains a family of 4 GGT genes with homology to human and mouse GGTs (Storozhenko et al., 2002). Two of the GGTs have recently been assigned to the apoplast (Storozhenko et al., 2002; Martin et al., 2007; Ohkama-Ohtsu et al., 2007a). The fact that the apoplast does not contain significant amounts of glutathione (Foyer and Noctor, 2005) might be indicative of high catabolic activity of GGTs towards GSH and GSSG.

D. Catabolism of Glutathione

Inhibition of GSH biosynthesis by BSO leads to almost complete depletion of the entire GSH pool within 3 days indicating a turnover of the GSH pool in normal metabolism (A.J. Meyer, unpublished). It is less clear, however, which metabolic pathways are responsible for this turnover or in which cellular processes GSH is being used up. GSH is well known to form mixed disulfides with a large number of proteins (Shelton et al., 2005) and it might be possible that a certain amount of GSH is continuously lost during protein turnover. To date, however, the effect of protein degradation on the GSH pool has not been studied.

Direct metabolic turnover of the GSH pool in the cytosol is unlikely because the distinct γ-amide bond between glutamate and cysteine can be released by only very few enzymes and thus protects GSH from catabolism by intracellular aminopeptidases. Lack of efficient degradation capabilities for the γ-peptide bond also contributes to hyperaccumulation of γ-EC in *gsh2* knockout mutants (Pasternak et al., 2008). The only enzymes known to be capable of cleaving the γ-peptide bond are the GGTs (see above), which are located in either the apoplast or the vacuole (Grzam et al., 2007, Martin et al., 2007; Ohkama-Ohtsu et al., 2007a; Ohkama-Ohtsu et al., 2007b). Similar to glutathione *S*-conjugates, GSSG might be exported to the vacuole for degradation. Such sequestration has been discussed as an overspill valve for extremely high GSSG concentrations in the cytosol under conditions of extreme stress (Foyer et al., 2001). The only cellular compartment for which high concentrations of GSSG have been shown is the ER (Hwang et al., 1992) and it can be assumed that with each vesicle transported to the plasma membrane or the vacuole some glutathione is lost. In both cases this glutathione would become accessible to degradation by GGTs. In analogy to animals other enzyme activities of the γ-glutamyl cycle have been described. These enzymes include carboxypeptidases, Cys-Gly dipeptidases, γ-glutamyl cyclotransferase and 5-oxo-prolinase (Martin, 2003 and refs. cited therein). However, so far none of these enzymes have been characterized at the molecular level.

IV. Cellular Functions of Glutathione in Plants

A. Detoxification of Xenobiotics and Endogenous Compounds

Plants are generally taking up many toxic compounds from their natural environment with little indiscrimination (Coleman et al., 1997). There are, however, efficient detoxifying systems in place to avoid long-term damage. The most important pathway for detoxification of electrophilic compounds is based on conjugation of these compounds to glutathione through reactions catalyzed by glutathione *S*-transferases (GSTs). The Arabidopsis nuclear genome contains 54 genes with high homology to GSTs (Edwards and Dixon, 2005). Most plant GSTs are predicted to be present in the cytosol, but there are also reports of microsomal GSTs, nuclear- or apoplast-localized enzymes and of gene products bearing plastid-targeting signal peptides (Frova, 2003 and refs. cited therein). The GSTs are a group of homo- or heterodimeric enzymes and due to multiple heterodimer formation a large number of different combinations is possible, which might contribute to the broad range of different substrates for conjugation to glutathione (Edwards and Dixon, 2005). After conjugation the glutathione-moiety acts as an efficient tag marking the conjugate for vacuolar sequestration. This sequestration is achieved by multidrug-resistance associated proteins (MRPs), a subfamily of ATP-binding cassette proteins (Rea et al., 1998; Martinoia et al., 2000; Rea, 2007). A number of vacuolar ABC-transporters have been shown to transport glutathione *S*-conjugates in vitro (Martinoia et al., 1993; Li et al., 1995), but even multiple knockouts of ABC-transporters did not significantly affect vacuolar sequestration in vivo (A.J. Meyer and M.D. Fricker, unpublished results). The latter observation is supported by the identification of at least 10 MRPs on the tonoplast (Jaquinod et al., 2007). In vitro ABC-transporters on the tonoplast membrane have also been shown to transport GSSG with Km values of 73 to 400 μM (Foyer et al., 2001). It has been discussed whether vacuolar sequestration of GSSG excessively formed under conditions of oxidative stress might contribute to maintenance of a reduced cytosol (Foyer et al., 2001), but this hypothesis has not been tested experimentally in vivo.

After their formation glutathione *S*-conjugates are further processed. Using MCB as a fluorescent probe for GSH it was shown that vacuolar sequestration of glutathione *S*-bimane adducts is very fast and completed within 30 min (Fricker et al., 2000; Meyer and Fricker, 2000; Meyer et al., 2001; Meyer and Fricker, 2002). Using the in vivo labeling of GSH with MCB in combination with HPLC analysis it was recently shown that degradation of glutathione *S*-conjugates in Arabidopsis is much slower than vacuolar sequestration (Grzam et al., 2006; Ohkama-Ohtsu et al., 2007b). Vacuolar degradation of

conjugates in Arabidopsis is initiated by a rate-limiting γ-glutamyl transpeptidase cleaving the γ-amide bond and releasing glutamate (GGT4, At4g29210; Grzam et al., 2007; Ohkama-Ohtsu et al., 2007b). The remaining cysteinylgylcine conjugate is quickly undergoing further degradation to cysteine conjugates (Grzam et al., 2006, 2007). The latter reaction might be catalyzed by a vacuolar dipeptidase, but no enzymatic activity has been ascribed yet. Despite very fast vacuolar sequestration of glutathione *S*-conjugates the cytosolic enzyme phytochelatin synthase (PCS) has been suggested to play an important role in catabolism of glutathione *S*-conjugates by cleaving the glycine residue in the initial degradation step (Beck et al., 2003; Blum et al., 2007). The minor activity of PCS towards glutathione *S*-conjugates and the concomitant release of γ-EC-conjugates is, however, much slower than sequestration of the glutathione *S*-conjugates even in the presence of PCS-activating heavy metals (Grzam et al., 2006).

Besides exogenous toxic compounds normal metabolism also generates a range of toxic by-products, which need to be detoxified. One example for endogenous toxic compounds that are detoxified via conjugation with GSH is methylglyoxal. Glycolysis leads to formation of highly toxic methylglyoxal through non-enzymatic β-elimination of the phosphate group of triose phosphates. Methylglyoxal is reacting non-enzymatically with GSH to form a hemithioacetal, which is then further degraded by the enzymes glyoxalase I and II (Singla-Pareek et al., 2003). During this reaction GSH is released again. Similarly, GSH is required as a cofactor in isomerization reactions catalyzed by zeta- and phi-isoforms of GSTs (Edwards et al., 2000; Edwards and Dixon, 2005). Such an isomerase activity has been exploited to bioactivate a thiadiazolidine herbicide through GST-mediated isomerization with a GSH conjugate as an intermediate (Edwards et al., 2000).

B. Detoxification of Heavy Metals via Phytochelatins: the Glutathione-Based First Line of Defense

Higher plants (but also *Schizosaccharomyces pombe* and *Caenorhabditis elegans*; Rea et al., 2004) may detoxify heavy metals via enzymatic biosynthesis of metal-binding thiolpeptides, the so-called phytochelatins (Clemens et al., 2002; Tong et al., 2004; Clemens, 2006). The enzyme PCS is associated with the papain superfamily of cysteine proteases (Rea, 2006; Romanyuk et al., 2006). It operates as a γ-glutamylcysteine dipeptidyl transferase, transferring a γ-glutamylcysteine unit from one GSH molecule to another to form PC_2, which may be further extended by repeated transfer of additional γ-glutamylcysteine units. Depending on the species and tissue, phytochelatins may assume different lengths, but in general PC_{2-4} are the most abundant ones. The reaction mechanism of the PCS enzyme has attracted much interest, in particular its activation by heavy metal ions. Initially, PCS was thought to be directly activated by binding of heavy metal ions to Cys residues located in its highly conserved N-terminal half which has been shown to provide the fold for core catalysis. However, closer inspection later revealed that PCS catalyzes a bisubstrate transpeptidation reaction in which both free GSH and its corresponding metal thiolate are co-substrates (Vatamaniuk et al., 2000; Rea et al., 2004). During catalysis, the PCS enzyme forms a covalent γ-glutamylcysteine acyl intermediate. In fact, transient acylation of PCS occurs at two sites, however, with different ligand requirements (Vatamaniuk et al., 2004). Recently, the existence of prokaryotic PCS homologs has been discovered. These enzymes are only half the size of eukaryotic PCS proteins, and contain only the N-terminal catalytic domain (Tsuji et al., 2004; Vivares et al., 2005). For the enzyme from *Nostoc* sp., *Ns*PCS, an enzymatic function has been demonstrated; however, this enzyme shows both GSH hydrolase and PCS activities, being more peptidase- than transpeptidase in its mode of action (Tsuji et al., 2004; Vivares et al., 2005; Rea, 2006). The protein structure of the *Ns*PCS enzyme has recently been resolved (Vivares et al., 2005).

PCS expression appears to be constitutive in roots and shoots of higher plants, but may be further up-regulated in response to heavy metal exposure at the transcript and protein level, respectively (Lee et al., 2002; Heiss et al., 2003). In plants, PCS activation provides the first line of defense against toxic levels of heavy metal ions, positioning its precursor glutathione in the center of heavy metal tolerance. In fact, the first

Arabidopsis mutations showing increased Cd sensitivity could be assigned to defects in PCS (*cad1-1*; Howden et al., 1995b) and GSH1 (*cad2-1*; Howden et al., 1995a; Cobbett et al., 1998), respectively (see above). When PCS activity is up-regulated in response to Cd exposure, phytochelatins (expressed as GSH equivalents) may accumulate 10-fold the tissues GSH concentration (Schäfer et al., 1998; Haag-Kerwer et al., 1999; Heiss et al., 1999; Heiss et al., 2003 and refs. cited therein), causing a transient decrease of GSH content (Ducruix et al., 2006; Herbette et al., 2006; Nocito et al., 2006). In response to the strong metabolic sink for GSH generated during the rapid accumulation of phytochelatins, the biosynthesis of GSH and cysteine and the entire sulfate assimilation pathway all become activated (Schäfer et al., 1998; Heiss et al., 1999). This activation is based on a coordinate transcriptional up-regulation (Schäfer et al., 1998; Xiang and Oliver, 1998; Heiss et al., 1999; Xiang et al., 2001; Herbette et al., 2006; Nocito et al., 2006), and extends to the transcriptional activation of high affinity sulfate transporters (Nocito et al., 2002; Nocito et al., 2006). During early biosynthesis of PCs, γ-glutamylcysteine content may, at least transiently, increase while cysteine levels remain unaffected (Ducruix et al., 2006; Nocito et al., 2006), pointing to a limitation of glycine and/or GSH2 activity. As feeding glycine to Cd-exposed Arabidopsis suspension-culture cells prevented the accumulation of γ-glutamylcysteine, it is likely that glycine may turn into a limiting factor when GSH synthesis is upgraded during PC synthesis; however, this may largely depend on the tissue and be less relevant in leaves with active photorespiration. This interpretation is supported by earlier studies on poplar transformed with γ-glutamylcysteine synthetase from *E. coli* (Noctor et al., 1999), where γ-glutamylcysteine was shown to accumulate in leaves only in the dark phase.

The observation of a (at least transiently) reduced GSH content as early as 2 h after onset of Cd exposure indicates that the cellular redox poise will be significantly affected (Herbette et al., 2006; Nocito et al., 2006). In their recent study on Cd-exposed maize seedlings, Nocito et al. (2006) reported a threefold decrease of reduced GSH 3 h after Cd exposure, with the content of γ-glutamylcysteine remaining almost unaffected. Such a shift in the glutathione-based cellular redox potential is expected to act as a strong signal, and indeed, genes involved in the response to reactive oxygen species were among the Cd-responsive genes in Arabidopsis (Suzuki et al., 2001) and *B. juncea* (Minglin et al., 2005). Recently, Gillet et al. (2006) have shown in a proteomic approach that in the unicellular photosynthetic algae *Chlamydomonas reinhardtii* several proteins related to oxidative stress response are indeed up-regulated in response to Cd exposure, and that most of the Cd-sensitive proteins were also regulated via thioredoxin (TRX) and/or GRX.

Based on the concept of phytoremediation, several attempts have been made to upgrade the potential of plants to accumulate heavy metals (Pilon-Smits, 2005 and refs. cited therein). Since detoxification of heavy metals via the GSH-based formation of PCs is pivotal to both heavy metal tolerance and accumulation, genes of the cysteine biosynthesis pathway, genes encoding GSH biosynthesis (GSH1 and GSH2), and PCS, have all been overexpressed in plants, individually or in combination (Zhu et al., 1999a,b; Xiang et al., 2001; Dominguez-Solis et al., 2004; Bittsanszky et al., 2005; Pomponi et al., 2006; Wawrzynski et al., 2006). While, as expected, an increased Cd tolerance and accumulation have been achieved in several plant species, including tobacco, poplar and *B. juncea*, the overall potential of engineering PC biosynthesis, directly or indirectly, appears to be rather moderate. In particular, the phytoremediation-relevant root-shoot transfer of heavy metals proved to remain a major bottleneck. While for Arabidopsis, transfer of PCs and Cd from root to shoot and vice versa has been demonstrated (Gong et al., 2003; Chen et al., 2006), the overexpression of PCS from Arabidopsis in tobacco enhanced only Cd tolerance but not Cd transfer from root to shoot (Pomponi et al., 2006). Whether at the whole plant level, redistribution of the PC precursor GSH via modulation of its long-distance transport plays a role in the potential of roots (or shoots) to mount their PC defense line is not known, however, the observed changes in expression of a putative GSH transporter in *B. juncea* at both transcript and protein level is indicative of such an adaptation (Bogs et al., 2003). To what extent the GSH-degrading γ-glutamyl transpeptidases are

involved in long-distance transport of GSH (i.e. by catalyzing phloem loading and/or unloading of GSH or GSSG) remains to be conclusively shown (Ohkama-Ohtsu et al., 2007a); if their role in GSH transport can be confirmed it would be interesting to study their expression in response to Cd exposure.

In summary, GSH plays its pivotal role in cellular detoxification of heavy metals most likely in two ways, i.e. being the precursor for PC biosynthesis, and acting as a cellular redox sensor due to the transient but significant decrease of reduced GSH in response to heavy metal exposure.

C. GSH as a Reductant in Normal Metabolism

GSH is now well established as an important factor in reversible protein modification catalyzed by GRXs (Fernandes and Holmgren, 2004; Shelton et al., 2005). The original discovery of GRXs as important mediators of GSH-derived electrons, however, was related to a metabolic process. The GRX system was first discovered in thioredoxin deficient *E. coli*, which were still able to reduce ribonucleotides (Holmgren, 1976). In this case electrons are transferred from NADPH to glutathione catalyzed by GR and finally to the target protein ribonucleotide reductase in a reaction catalyzed by GRXs. Besides a large number of diverse GRXs plants also contain GRX-motifs fused to other proteins. The best described example is the APS reductase which contains a GRX-like domain at its C-terminus and uses GSH as hydrogen donor (Bick et al., 1998).

While the main function of plant GSTs is the conjugation of xenobiotics (see section IV.A.) some subgroups of this highly diverse gene family have other catalytic functions that do not lead to formation of glutathione *S*-conjugates. Several isoforms belonging to the tau-, phi- and theta-classes of GSTs have been shown to exhibit glutathione-dependent peroxidase activity with reductive activity towards organic hydroperoxides. In these reactions GSH is oxidized to the respective sulfenic acid, which spontaneously reacts with a second GSH molecule to form GSSG (Edwards et al., 2000).

The most prominent use of GSH as an electron donor in normal metabolism, however, is the GAC in which electrons are transferred from GSH to dehydroascorbate (DHA) to regenerate ascorbate. The enzyme responsible for this electron transfer is dehydroascorbate reductase (DHAR), which has also been shown to belong to the GST superfamily (Dixon et al., 2002). These enzymes, in contrast to most other GSTs, are monomeric. The active site serine residue in this case is replaced by a single cysteine, a catalytically essential residue that has been proposed to form mixed disulfides with GSH during the catalytic cycle (Dixon et al., 2002). Arabidopsis contains four different DHARs which are predicted to be targeted to the cytosol, the mitochondria and chloroplasts. The presence of DHAR in both mitochondria and chloroplasts has been confirmed by proteomic studies (Chew et al., 2003b).

D. The Glutathione–Ascorbate Cycle (GAC): Removing Reactive Oxygen Species (ROS)

Depending on their in situ concentration and on the metabolic context, ROS may act as cellular stressors, or, alternatively, as primary signals which initiate specific developmental or defense programs, either directly or via affecting the redox state of cellular antioxidant molecules (Foyer and Noctor, 2005 and refs. cited therein). Consequently, plants have developed mechanisms to induce, to increase or to quench ROS accumulation. Thus, under pathogen attack, plasma membrane bound NADPH oxidase is activated and initiates the "oxidative burst" in the apoplast, which in turn plays a major role in orchestrating the cellular defense reaction, culminating in programmed cell death (hypersensitive response) (Vanacker et al., 2000; Dangl and Jones, 2001; Ameisen, 2002; Mou et al., 2003). Also, it was recently shown that NADPH oxidase-mediated formation of ROS in the apoplast provides an important signal for developmental processes like root hair formation (Foreman et al., 2003). Conversely, to cope with excessive and unregulated accumulation of stress-induced ROS in different compartments, e.g. chloroplasts, mitochondria, peroxisomes and the cell wall space, a set of small antioxidant molecules provides a regulated network present in different cellular compartments (Foyer and Noctor, 2005; Kanwischer et al., 2005). Included are glutathione, ascorbic acid, α-tocopherol and several secondary plant products

with antioxidant activities (e.g. flavonoids). While α-tocopherol is a membrane-soluble, lipophilic antioxidant, glutathione and ascorbic acid are soluble antioxidants present in up to millimolar concentrations in most cellular compartments. Functionally, the latter are linked in the GAC (Fig. 2). In principle, electrons from NADPH are used to detoxify ROS (namely H_2O_2) via a sequence of enzymatic steps, which involve the sequential oxidation/reduction of glutathione and ascorbic acid, reflecting the redox potentials of the named antioxidants. The GAC has been suggested to operate in the cytosol, chloroplasts, mitochondria and peroxisomes (Chew et al., 2003b; Kuzniak and Sklodowska, 2005a; Leterrier et al., 2005). While for chloroplasts and mitochondria, GAC function is certainly required for ROS detoxification in these organelles, its operation in peroxisomes might also be required to regenerate NADP for metabolic function (del Rio et al., 2002; Kuzniak and Sklodowska, 2005b). Interestingly, for GR and several other GAC enzymes dual targeting to chloroplasts and mitochondria has been demonstrated (Creissen et al., 1995; Chew et al., 2003b). As the biosynthesis of glutathione and ascorbic acid are strictly compartmentalized (Smirnoff et al., 2001; Wachter et al., 2005), transport systems must exist to shuttle these antioxidants between different compartments. As yet, little is known about the involved transporters and their mechanism for antioxidant exchange between different compartments. While in the apoplast, ascorbic acid appears to provide the only redox buffer (Horemans et al., 2000; Sanmartin et al., 2003; Foyer and Noctor, 2005; Pignocchi et al., 2006), in other organelles glutathione and ascorbic acid always operate together in the GAC. The role of the central vacuole in cellular redox poise has not attracted much attention. However, clearly ROS, including H_2O_2, may equilibrate with the vacuolar compartment which often makes up 90% of the cellular volume. Several secondary plant products, including flavonoids, which exhibit antioxidant activity, are localized in the vacuole and may possibly act as redox buffer. Recently, Bienert et al. (2007) could show that H_2O_2 is transported via certain types of aquaporins (Arabidopsis TIP1;1 and TIP1;2), providing a route for regulated movement of H_2O_2 across the tonoplast membrane. Interestingly, Arabidopsis lines mutated in TIP1;1 exhibit a strong growth phenotype (cell and plant death) with a signature reminiscent of oxidative stress (Ma et al., 2004).

The above considerations assign a central role to the antioxidant pair glutathione–ascorbate for

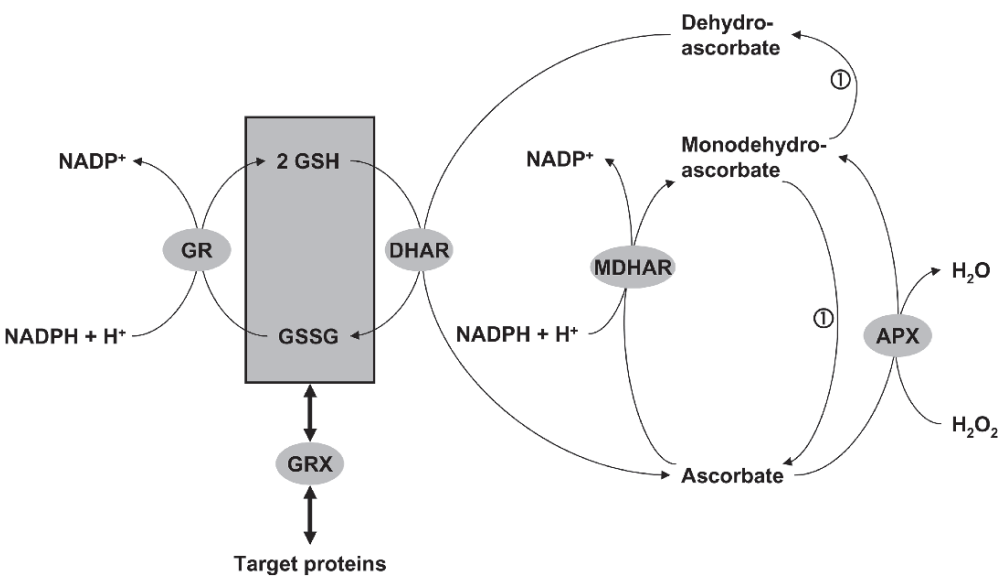

Fig. 2. The glutathione–ascorbate cycle. GR: glutathione reductase; DHAR: dehydroascorbate reductase; MDHAR: monodehydroascorbate reductase; APX: ascorbate peroxidase; GRX: glutaredoxin; ①: spontaneous disproportionation of monodehydroascorbate. The ascorbate part of the cycle is not shown stoichiometrically.

maintaining cellular redox poise. In particular, regulation and compartmentation of glutathione and ascorbate biosynthesis, degradation, and the exchange of glutathione and ascorbate between different compartments via specific transporters all impact on GAC function by affecting the pool sizes of both antioxidants. As both compounds are also largely, but not exclusively synthesized in the leaves, at the whole plant level source-sink relationships are superimposed on the local distributions. Thus, ascorbate levels in the root are more than 10-fold lower than in photosynthetic tissues (C. Kiefer and T. Rausch, unpublished). Within GAC itself, individual components of this complex enzyme network, including multiple isoforms for SOD, APX, MDHAR, DHAR, and GR for different cellular compartment, have all be shown to be regulated in their expression and/or activity in response to increased ROS exposure. Mutant analysis and attempts to engineer the expression of single enzymes of GAC, or of enzymes involved in the biosynthesis of one of the major cellular antioxidants have revealed substantial "cross talk" between GAC components. Prominent examples are the GSH1 mutant *rax1* in Arabidopsis (Ball et al., 2004), the effect of ectopic overexpression of DHAR (Chen et al., 2003), the tocopherol cyclase mutant *vte1*, and the low ascorbate *vtc1* mutant (Kanwischer et al., 2005). In all these cases, changing the level of one antioxidant significantly affected the concentration of other antioxidants. In the *rax1* mutant (Ball et al., 2004), a significantly decreased affinity of GSH1 for its substrate cysteine results in a lowered GSH content (Hothorn et al., 2006), causing a constitutive up-regulation of a specific APX isoform. In the *vte1* mutant, increased contents of GSH and AA were observed, whereas in the *vtc1* mutant the content of α-tocopherol was enhanced (Kanwischer et al., 2005). Interestingly, the ectopic expression of a wheat DHAR in tobacco and in maize caused not only an increase of total AA content, but also led to an up-regulation of total GSH content, and in both cases the reduced forms were even more affected (Chen et al., 2003). The molecular mechanisms of these various types of "cross-talk" between the different biosynthetic pathways and/or redox states of individual antioxidants are still a matter of speculation. Obviously, a comprehensive and dynamic analysis of all enzymes (including isoforms) and antioxidant metabolites at compartmental and high temporal resolution, respectively, would be required to develop a model for the observed interdependencies. While microarray analysis allows to simultaneously follow the expression of all contributing enzyme isoforms, at least at the transcript level, the recent demonstration of post-transcriptional and post-translational controls (Hothorn et al., 2006; Jez et al., 2006) as well as the dynamic changes of subcellular compartmentation preclude any simply predictions based on transcriptomics alone.

Finally, another important aspect of GAC-mediated redox control in different cellular compartments is the continuous requirement for NADPH to reduce GSSG via GR. At present, no experimental studies or model calculations are available that would address the metabolic costs of GAC operation in different tissues and under different stress conditions. While in light-exposed leaves, the required NADPH is continuously regenerated via photosynthetic electron transport, the situation is different in the dark and in heterotrophic tissues. Here, GAC operation is most likely fueled by NADPH originating from the oxidative pentose phosphate (OPP) cycle. It would be interesting to determine whether oxidative stress results in significant changes of OPP metabolites and what the metabolic costs are in terms of glucose oxidized as compared to cellular respiration.

E. Glutathione-Dependent Redox Signaling

The most important function of glutathione is its role in redox buffering of the intracellular milieu and, related to this, sensing and transmission of deviations form the steady state redox poise. Evolution of GSH is thought to be related to the evolution of oxygenic photosynthesis and it is assumed that the two step biosynthesis pathway has evolved in forward direction (see section II.A). Due to rapid autocatalytic oxidation of cysteine in the presence of transition metals and concomitant formation of hydroxyl radicals through a Fenton reaction cysteine does not meet the requirements of a suitable redox sensor (Meyer and Hell, 2005). Formation of the distinct γ-amide bond between glutamate and cysteine leads to blocking of the α-amino group of cysteine and an α-amino acid

like domain at the glutamate residue. This feature already greatly reduces metal-catalyzed thiol oxidation of the cysteine residue, which can be even further reduced by adding the glycine residue to the C-terminus of cysteine (Fahey and Sundquist, 1991). Despite these important features in terms of interactions with transition metals, the redox properties of GSH and its precursors are highly similar. The standard reduction potentials range from −216 mV for the cysteine/cystine couple and −240 mV for the GSH/GSSG couple (pH 7.0, 25 °C) (Schafer and Buettner, 2001; Jones, 2002). The reduction potential of thiols is largely dependent on the degree of protonation of the thiol group and thus for quantitative comparisons an adjustment of −5.9 mV per 0.1 increase in pH needs to be considered for quantitative calculations. More important, however, is the fact that two GSH molecules form a single GSSG molecule during oxidation. Thus, the concentration of GSH enters the Nernst equation as a squared term resulting in dependence of the reduction potential on both, the total concentration of GSH equivalents ([GSH] + 2[GSSG]) and the degree of oxidation of the glutathione pool (Equ. 1). The GSH concentration in the cytosol is considered to be in the low millimolar range (Meyer et al., 2001) and thus 10–50 times higher than the cysteine concentration. With the assumption of a similar degree of oxidation the reduction potential of glutathione would be about 200 mV more negative than that of cysteine. Replacement of the glycine residue by other amino acids like serine or glutamate does not significantly change the redox properties of the thiol group (Krezel and Bal, 2003).

$$E_{hc} = -240\ \text{mV} - (59.1/2)\ \text{mV} * \log([\text{GSH}]^2/[\text{GSSG}]) \quad (1)$$

The reduction state of the glutathione pool is frequently described as > 90% (Noctor, 2006). Assuming a total glutathione concentration of 1 mM and a degree of oxidation of only 1% at pH 7.0 would, according to Equ. 1, result in a reduction potential of −219 mV. The recently developed roGFP enables direct redox measurements in living cells (Dooley et al., 2004; Hanson et al., 2004; Jiang et al., 2006). This sensor has a midpoint reduction potential of −280 mM at pH 7.0 (Hanson et al., 2004) and equilibrates specifically with the cellular glutathione redox buffer (Meyer et al., 2007). Imaging of roGFP expressed in the cytosol of Arabidopsis and tobacco showed that the sensor is almost completely reduced. This observation strongly suggests that the actual reduction potential of the cellular glutathione redox buffer is far more negative than formerly assumed on the basis of biochemical analysis of cell extracts. With total glutathione concentrations in the low millimolar range GSSG would thus be present in only sub-micromolar concentrations.

The reduction potential of glutathione can equilibrate with other redox buffers. Unless enzymatically catalyzed such an equilibration would be far too slow to have any physiological significance. A particular family of disulfide-oxidoreductases, the GRXs, are capable of reversibly transferring electrons between glutathione and thiol groups on target proteins (Fig. 3). It is now apparent that GRXs are involved in a large number of different cellular processes and that GRXs play a crucial role in response to oxidative stress in all organisms (Fernandes and Holmgren, 2004; Shelton et al., 2005; Xing et al., 2006). The ability of GRXs to rapidly equilibrate the glutathione reduction potential with the reduction potential of protein thiols can be exploited for signaling purposes. Analogous to *O*-phosphorylation reversible post-translational modification of specific thiols can also efficiently regulate protein functions. Depending on the protein structure this redox-dependent regulation can occur in different forms. Single cysteine residues might be glutathionylated and in other cases formation of disulfide bridges between two cysteines might occur. Based on these two possibilities target proteins for redox-dependent modification have been classified in Type I- and Type II-nanoswitches (Schafer and Buettner, 2001; Meyer and Hell, 2005).

The Arabidopsis genome encodes at least 31 GRXs (Lemaire, 2004; Xing et al., 2006), which are predicted to be present in all subcellular compartments apart the vacuole. Based on the cysteine motif of the active site plant GRXs cluster in three distinct groups, denominated CPYC-, CGFS- and CC-type (Xing et al., 2006). The CPYC group is structurally equivalent to the classical dithiol GRXs from other organisms, while the CC-type GRXs are specific for plants and most prominent in higher plants (Xing et al., 2006). The high number of different GRXs implicates a large number of diverse target proteins.

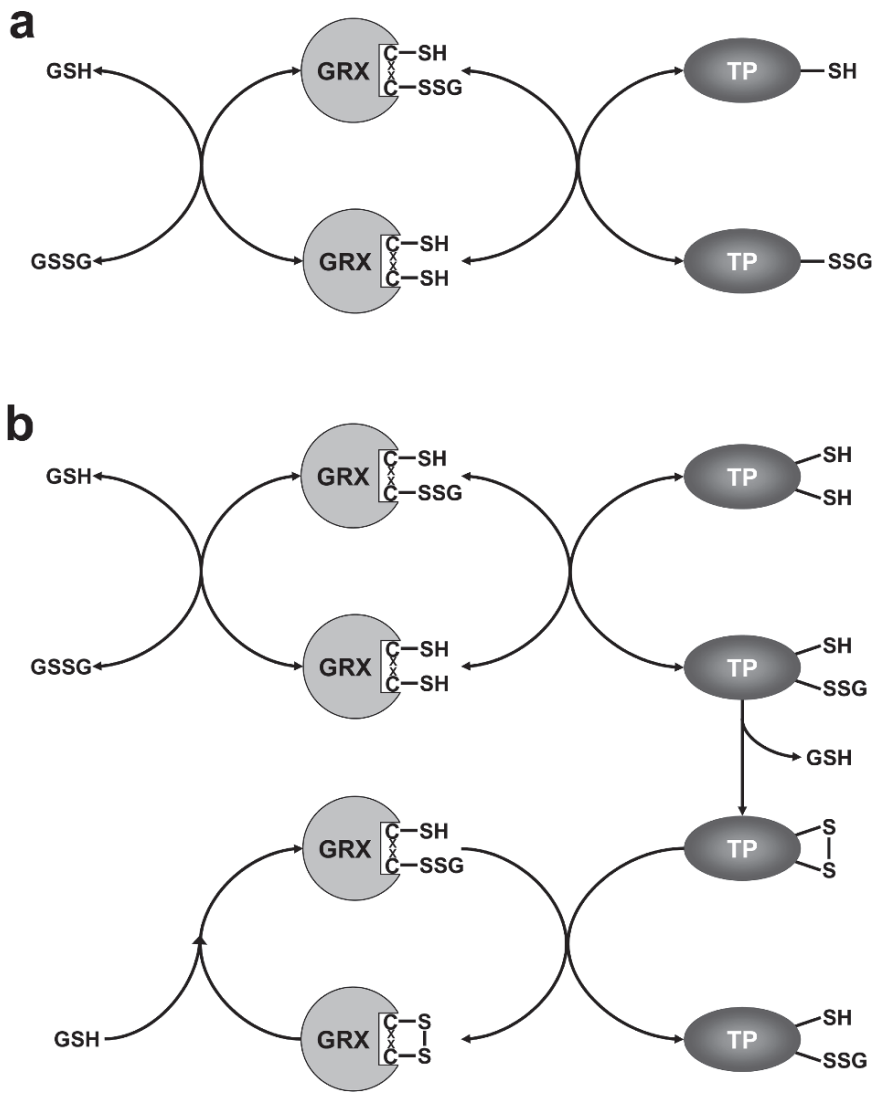

Fig. 3. Mechanism for reversible interaction of glutaredoxins with target proteins. (a) Glutathionylation of a type I target protein (TP). (b) Oxidation and reduction of a type II target protein. Cartoons shown for the oxidation reactions (top) are for a dithiol GRX, but reactions are exactly the same for monothiol GRXs, because the catalytic reaction leading to oxidation of a target protein involves only the N-terminal cysteine of the GRX motif. The glutathionylated intermediate of the target protein is instable and a second nearby cysteine can substitute the glutathione moiety thus forming an intramolecular disulfide bridge. In contrast to the oxidation reaction, the reduction of target protein disulfides by GRX might involve both active site cysteines (bottom).

Because the catalytic mechanism involves only the N-terminal cysteine and because GRXs only transfer GSH rather than forming mixed disulfides with target proteins (Peltoniemi et al., 2006), most approaches for trapping of mixed disulfides between GRXs and their targets similar to successful approaches for TRXs have failed. The only exception is a report by Rouhier et al. (2005) in which 94 putative targets of poplar GRX C4 were trapped after mutating the second cysteine from the active site. Applying a different approach based on labeling of proteins with radioactive GSH and mass-spectrometry, different studies have recently shown that plant cells, like other eukaryotic cells, contain several proteins that undergo *S*-glutathionylation (Ito et al., 2003; Dixon et al., 2005; Michelet et al., 2005). *S*-glutathionylation can protect protein thiols from irreversible oxidation by ROS (Ghezzi, 2005). This protective mechanism is

important for retaining protein structure during desiccation of resurrection plants (Kranner et al., 2002). In addition, reversible protein *S*-glutathionylation as well as GRX-dependent formation of disulfide bridges are means for regulation of signal transduction and are currently emerging as novel mechanisms involved in cellular regulation. To be effective as a regulatory mechanism a number of different criteria need to be met: First, the function of the modified protein must change. Second, the redox-dependent change must occur within intact cells in response to a physiological stimulus. Third, redox-dependent changes must occur at relatively low degree of oxidation of the cellular glutathione pool, i.e. very low concentrations of GSSG. Fourth, there must be rapid and efficient mechanisms for specific protein modifications in place. And finally, there must be rapid and efficient mechanisms for reversing the redox-dependent change. While the first criterion, change of function, is fulfilled by the described glutathionylated proteins TRXf, aldolase and triose-phosphate isomerase (Ito et al., 2003; Michelet et al., 2005), all other criteria remain largely unresolved, mainly because few target proteins have been established so far.

The best evidence for full reversibility of GRX-dependent post-translational protein modification results from expression of redox-sensitive fluorescent proteins (rxYFP, (Østergaard et al., 2001); roGFP (Hanson et al., 2004; Jiang et al., 2006). These proteins act as artificial targets for GRXs (Østergaard et al., 2004; Meyer et al., 2007) and redox-dependent changes in fluorescence can directly be observed in living cells. Changes of fluorescence occur in response to the cytosolic glutathione levels and different stimuli affecting the glutathione pool. Due to its redox-potential of −280 mV roGFP is extremely sensitive to minute changes of GSSG indicating that the cytosolic glutathione pool in non-stressed cells is almost completely reduced with only submicromolar concentrations of GSSG (Meyer et al., 2007). Kinetic analysis of fluorescence showed that the redox-dependent alteration of roGFP in living cells occurs much faster than in vitro indicating the involvement of interacting proteins, which are likely to be GRXs. GRXs at the same time guarantee the full reversibility of the reaction.

In the light of the very small number of described glutathionylated proteins the major challenge for the future thus is the identification of target proteins interacting with GRXs and exploring the specificity in these interactions. In this context it will also be necessary to study the exact localization of the entire set of GRXs at subcellular level and the expression during development and under stress conditions. Identification of target proteins will likely highlight the potential for cross-talk between different signal transduction pathways, which has been shown for the cross-talk between plastidic GRX and TRX systems already (Michelet et al., 2005). Due to the emerging complexity of interacting signaling pathways the entire signaling network cannot be described solely by biochemical approaches. Instead, quantitative descriptions of glutathione-dependent signaling and its integration into the cellular signaling system, will also rely on the use of computer-based modeling tools.

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