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Key Words

cysteine, iron sulfur, oxidative stress, redox, signaling

Abstract

Glutathione, a tripeptide with the sequence γ-Glu-Cys-Gly, exists either in a reduced form with a free thiol group or in an oxidized form with a disulfide between two identical molecules. We describe here briefly the pathways involved in the synthesis, reduction, polymerization, and degradation of glutathione, as well as its distribution throughout the plant and its redox buffering capacities. The function of glutathione in xenobiotic and heavy metal detoxification, plant development, and plant-pathogen interactions is also briefly discussed. Several lines of evidence indicate that glutathione and glutaredoxins (GRXs) are implicated in the response to oxidative stress through the regeneration of enzymes involved in peroxide and methionine sulfoxide reduction. Finally, emerging functions for plant GRXs and glutathione concern the regulation of protein activity via glutathionylation and the capacity of some GRXs to bind iron sulfur centers and for some of them to transfer FeS clusters into apoproteins.

Contents

Glutathione: the major nonprotein cellular thiol

GSH: reduced glutathione

GSSG: oxidized glutathione

Glutathionylation: the binding of a glutathione to a thiol group to protect the protein from irreversible inactivation or to regulate protein activity

Glutaredoxin (GRX): small-molecular-weight thiol oxidoreductase

INTRODUCTION

Glutathione is a tripeptide with the sequence γ-L-glutamyl-L-cysteinyl-glycine that plays an important role in a wide range of organisms. Glutathione is nearly ubiquitous, although it is absent in some organisms that use different thiol cofactors such as coenzyme A, mycothiol, or ergothioneine (58). Glutathione can exist either in the reduced state (GSH) or in an oxidized state (GSSG), in which two glutathione molecules are linked via a disulfide bond. In plants, glutathione is necessary not only for maintaining the redox balance, but also for xenobiotic detoxification, heavy metal detoxification, and more generally for cell signaling (**Figure 1**). Because GSH and its derivatives are involved in numerous developmental processes and because it is a major player in the cell redox chemistry, it has been studied extensively over the past decades. Recent reviews have described the synthesis and degradation of this compound, its role in xenobiotic elimination, its role in cell signaling in interaction with other redox systems such as ascorbate or peroxides, and also its role in signaling pathways induced by jasmonic acid and other plant hormones (25, 65, 73, 77, 78). We give a brief overview of these aspects but describe more extensively the emerging importance of glutathionylation reactions in plants and the recent exciting developments concerning glutaredoxins (GRXs), the catalysts of these deglutathionylation/glutathionylation reactions that are also involved in stress response and in iron sulfur assembly reactions.

GLUTATHIONE AND HOMOLOGS IN PLANTS

Structure, Chemical Properties, Synthesis, Reduction, Polymerization, and Degradation

The redox buffering properties and the intracellular concentrations of GSH are conditioned by its chemical properties, its mode of synthesis, and reduction. Likewise, the formation of GSH polymers (phytochelatins), and the degradation of GSH are reactions that control the intracellular GSH pool and GSH/GSSG ratios. These various reactions are discussed in the following sections.

Structure of the oxidized and reduced forms. The linear structure of GSH reveals an anomaly in the organization of this compound. Unlike in traditional proteins or peptides the glutamate residue is linked to the neighboring cysteine via its side chain carboxylate, resulting in a pseudopeptide bond. In its oxidized form, GSSG, the two chains of glutathione are linked via a disulfide bond with elimination of two protons and two electrons.

It is a gross simplification to represent glutathione, either oxidized or reduced, as a linear molecule. In reality GSH or GSSG adopts

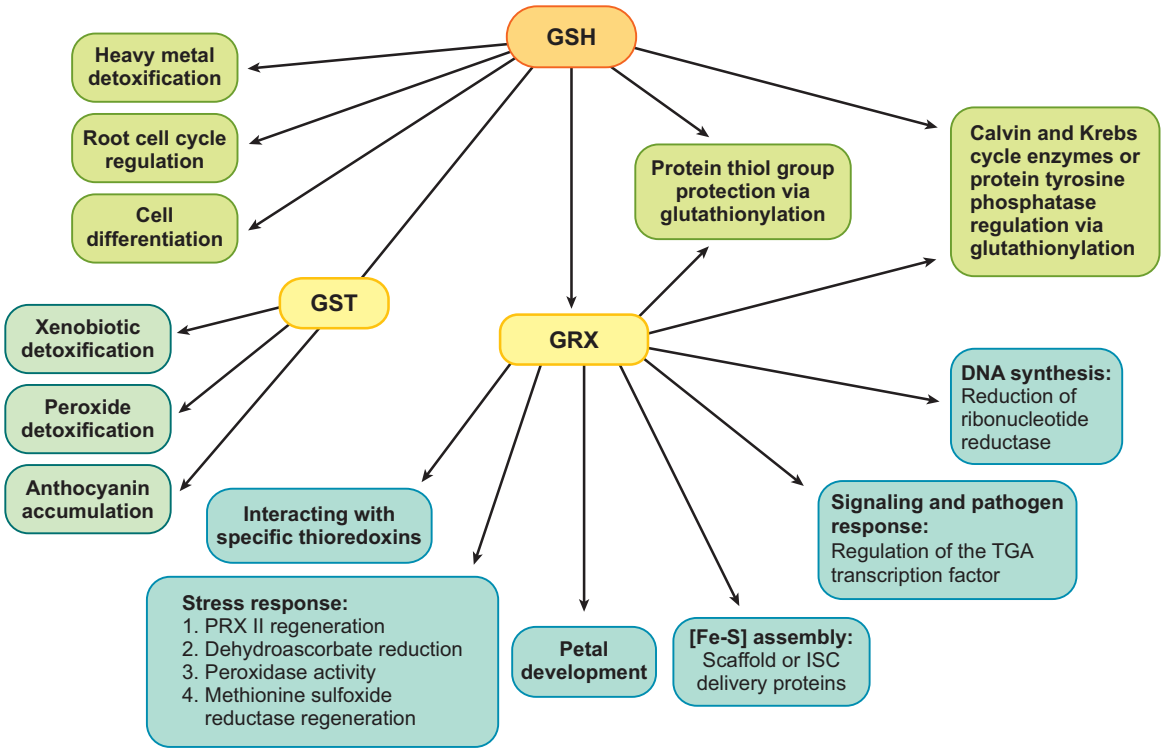

Figure 1

Reduced glutathione (GSH)-, glutathione-*S*-transferase (GST)-, and glutaredoxin (GRX)-linked processes. Abbreviations: ISC, iron sulfur cluster; PRX, peroxiredoxin.

three-dimensional structures that are far from linear. Although several protein structures contain bound glutathione, data regarding the structure of this compound in solution are scarce. The crystal structure of GSH was solved a long time ago and later refined (32), but surprisingly only recently have Klochkov and coworkers (54) succeeded in solving the structure of GSH by NMR and residual dipolar coupling analysis. To solve this structure, the authors aligned the molecules in a lyotropic crystalline medium. This certainly indicates that glutathione is a flexible molecule with few structural constraints, presumably because of its small size. The examination of several protein structures containing bound GSSG (1GRA, 2GRT, and 1YKC) also indicates that even in its more complex oxidized form, glutathione is flexible, it can adopt many conformations, and thus the glutathione binding sites present in enzymes that utilize this compound are varied. As a result, the analysis of the primary sequences of glutathione-dependent enzymes does not reveal any similarity in the glutathione binding sites.

Glutathione and its variants. There are homologs of glutathione in some plants and especially legumes; the most frequently observed is homoglutathione (hGSH) in legumes. hGSH is similar to glutathione, but the glycine residue is replaced by a β-alanine. Its synthesis requires the participation of a specific hGSH synthetase (62). In soybean, for example, hGSH is preferred to GSH for the glutathione-*S*-transferase (GST)-linked detoxification of the xenobiotic acifluorfen (100). Hydroxymethylglutathione is another alternative compound to GSH in which the glycine residue is replaced by a serine; this

TRX: thioredoxin

compound is found in graminae, including rice, wheat, and barley (82). A vacuolar carboxypeptidase of the Y type may be involved in the synthesis of hydroxymethylglutathione (82).

Redox potential and cysteine pKa values. The E_m value of the couple GSH/GSSG is –240 mV at pH 7.0 (65). This value is actually close to the value determined for many thioredoxins (TRXs) (from –280 to –330 mV), which constitute alternative reducing systems in plants and elsewhere. However, as GSSG dissociates upon reduction into two separate GSH units, its influence on the cellular redox status depends on both the total glutathione concentration and the GSH/GSSG ratio (65, 73). The unusual chemical nature of the pseudopeptide bond between γ-Glu and Cys influences only marginally the pKa value of the cysteine residue, which is critical for redox activity. The pKa value for this cysteine has been determined experimentally to be ~8.6–9 compared to the value of 8.3 for free cysteine (50).

Synthesis, reduction, and degradation. Being a pseudopeptide, glutathione is produced not via classical protein synthesis, but rather through the operation of two successive enzymes: γ-glutamyl-cysteine ligase or γ-glutamyl-cysteine synthase (γ-GCL or GSH1) and glutathione synthetase (GS or GSH2). The first of these enzymes catalyzes the formation in the chloroplast of the pseudopeptide bond between Glu and Cys in the presence of ATP, leading to γ-Glu-Cys (also known as γ-EC). Plant γ-GCL are monomeric enzymes of ~450 amino acids in the processed form, whereas the mammalian proteins are composed of a large catalytic subunit associated with a small regulatory subunit (46). Plant γ-GCL contain one or two disulfide bonds and are inhibited by reduction. The reduction of one of the disulfides, conserved in all plant species, governs the dimer-to-monomer transition of the enzyme, whereas the reduction of the second disulfide, present only in some plant species including Brassicaceae, shields access to the active site, thereby decreasing catalytic efficiency (41). The significance of this redox effect, its existence in different species, and the nature of its in vivo effector (TRX system or GSH/GRX system) are still unclear. The second enzyme of the synthesis pathway, glutathione synthetase, catalyzes the ATP-dependent formation of glutathione from γ-EC and glycine (45). This second step likely takes place both in the chloroplast and in the cytosol, but some variations in this distribution may occur between plants (107). The *Arabidopsis thaliana* glutathione synthetase is composed of 480 amino acids, with a glycine-rich loop covering the active site of the enzyme, but no redox regulation has been reported (108).

Although in most physiological conditions intracellular glutathione is essentially in the reduced form, it can be converted to the oxidized form, GSSG, in an oxidizing environment. The regeneration of GSH is performed by glutathione reductase, a NADPH-dependent dimeric flavoprotein that contains one FAD and one disulfide per subunit. The three-dimensional structure of human glutathione reductase reveals that the FAD is facing the disulfide, and thus an intramolecular electron flow from NADPH to FAD and then to the disulfide is facilitated (52). After reduction of the catalytic disulfide of glutathione reductase, a classical dithiol disulfide exchange reaction takes place between the reductase and GSSG, and two GSH molecules are released. There are no structural data available concerning plant glutathione reductases to date, but the high sequence similarities to other reductases from different biological origin suggest that they should be structurally similar. Glutathione reductase exhibits some similarity to other flavin-containing disulfide oxidoreductases such as TRX reductase and lipoamide dehydrogenase, but generally these other enzymes cannot reduce the same substrates. However, in *Drosophila melanogaster*, which lacks a functional glutathione

reductase, GSSG is reduced by a TRX reductase in the presence of TRX, indicating some overlap between the two major reducing systems (51).

Glutathione is a cellular redox buffer, but it can also be considered as a reservoir of cysteine for the cell. If cysteine is needed, it is then necessary to degrade the glutathione molecule. This is achieved via the successive operation of two different enzymes, γ-glutamyl transferase (γ-GT) and dipeptidase (99). The degradation process conforms to the following equations, where AA stands for any amino acid:

$$\gamma\text{Glu} - \text{Cys} - \text{Gly} + \text{AA} \rightarrow \gamma\text{Glu} - \text{AA} + \text{Cys} - \text{Gly}. \quad 1.$$

$$\text{Cys} - \text{Gly} + 2\text{H}_2\text{O} \rightarrow \text{Cys} + \text{Gly}. \quad 2.$$

Several γ-GT in plants transfer the γ-Glu residue to a variety of amino acids with the release of the dipeptide Cys-Gly (Equation 1), which in turn is cleaved by a dipeptidase to release the required cysteine (Equation 2). L-Glu is regenerated from γ-Glu-AA via the action of γ-glutamyl cyclotransferase and 5-oxoprolinase. γ-GT can also degrade xenobiotic-conjugated glutathione derivatives. Two forms of the enzyme have been detected in many plants; one enzyme is located outside the plasma membrane facing the cell wall and the other enzyme is soluble (61, 74). The soluble enzymes are heterodimers (subunits of 21 and 42 kDa) originating from a single precursor that is cleaved during biogenesis, whereas the bound γ-GTs are either heterodimers or monomers of 61 kDa. In plants the activity of γ-GT is believed to be linked to secondary metabolism and fruit ripening (99).

Distribution in Planta and Tissular and Subcellular Localization

Glutathione is present in many tissues and subcellular compartments, with concentrations in the millimolar range. In chloroplasts, the concentration of glutathione is estimated to be between 1 and 4.5 mM (78). Labeling with monochlorobimane and confocal microscopy were used to quantify cytoplasmic glutathione in *Arabidopsis* cells, with estimates of its concentration in the 3-mM range (66). Similar labeling in the main cell types of poplar gave values ranging between 0.2 and 0.3 mM in both photosynthetic and nonphotosynthetic cells (35). Using this same technology, Meyer & Fricker (64) estimated the rates of in vivo synthesis of glutathione in *Arabidopsis* cell culture suspensions. An alternative method for the detection of glutathione is the use of specific antibodies, although in general they also recognize the dipeptide γ-glutamylcysteine (40). Recently, a redox-sensitive GFP (roGFP2) has been engineered to estimate in vivo the intracellular redox potential (34), and it will constitute an important tool in the future to estimate the glutathione redox state in different tissues and subcellular compartments. Experiments performed with roGFP2 in *A. thaliana* demonstrated that the redox potential in the cytosol of non-stressed cells is –320 mV (63). In addition, in vitro characterization of this roGFP2 showed that it monitors the redox potential of the cellular glutathione buffer via GRX-linked reactions but not through the TRX-dependent pathways (63).

Glutathione and glutathione reductase have been detected in mitochondria; in addition, GSH and ascorbate are present in peroxisomes, suggesting that these organelles contain a full complement of these antioxidant systems (47). Glutathione is also present in the apoplast and endoplasmic reticulum. It remains unanswered how glutathione is transported between organelles or compartments that do not possess the ability to synthesize it. Some plasma membrane-localized transporters transport glutathione and especially import GSSG from the apoplast to the cytosol (44, 114), but to date, experimental evidence for a glutathione transport mechanism from the cytosol to organelles or vice versa is scarce. Selective antibodies versus glutamate, cysteine, and glycine together with

PRX: peroxiredoxin

gold labeling have been used to detect the glutathione precursors in *Cucurbita pepo* roots by using transmission electron microscopy, leading to the conclusion that glutathione degradation occurs in the vacuole or the tonoplast but not at the plasmalemma and/or apoplast (113).

Physiological Roles

GSH has physiological roles in reactive oxygen species detoxification, heavy metal detoxification, xenobiotic conjugation, and also in a variety of cellular and tissular functions. These roles are discussed in the following sections.

Redox buffer and reactive oxygen species detoxification. Glutathione is the most abundant low-molecular-weight thiol in cells and it constitutes a redox buffer that keeps the intracellular environment reduced. Glutathione plays a major role in the detoxification of reactive oxygen species (ROS). GSH can be oxidized to GSSG by some ROS, such as H_2O_2; GSH can react with nitric oxide to form the GSNO derivative, but it also participates in the glutathione/ascorbate cycle, in which GSH allows regeneration of reduced ascorbate, the other major antioxidant in plant cells. GSH also provides electrons to diverse peroxidases. Indeed, although plant glutathione peroxidases are reduced by TRX rather than by glutathione and should thus be renamed thioredoxin-peroxidases (37, 75), other peroxidases of the peroxiredoxin (PRX) family use glutathione alone or with GRX (23, 27, 91). Other enzymes, such as some GSTs or GRXs, also possess a glutathione-dependent peroxidase activity (14, 55).

Phytochelatins and heavy metal detoxification. One of the most documented systems for eliminating heavy metals comprises metallothioneins, small-molecular-weight proteins containing a high density of cysteine residues. Similar to other organisms, plants contain multiple metallothioneins, but they also possess an alternative system of detoxification, phytochelatins (PCs) (12). These compounds are characterized by a general structure of the type $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, but in some plant species the terminal Gly is sometimes replaced by an Ala, Gln, Glu, or Ser, leading to the formation of isophytochelatins (21). In *A. thaliana*, GSH is the in vivo precursor to PC synthesis, which also occurs independently of the protein synthesis machinery. PCs are synthesized through PC synthase. This enzyme is a ~50-kDa polypeptide that catalyzes the net transfer of a γ-EC unit of GSH to another GSH molecule or a PC molecule, mediating its extension from the C to N terminus. Toxic metals such as nickel, cadmium, mercury, or arsenate are efficiently detoxified via glutathione and PCs owing to the high affinity of these polymers for heavy metals (12, 42). Nickel hyperaccumulators produce PC constitutively and increase glutathione synthesis, leading to a better tolerance for this metal (26). The glutathione S-conjugates can be sequestered in the vacuole via γ-glutamyl transpeptidase (33).

Glutathione conjugates. When plant cells need to eliminate xenobiotics, they first conjugate them to glutathione to target the molecules that need to be removed. The enzymes that perform the ligation of glutathione to external molecules are called GSTs and they are found in nearly all living organisms. There are several classes in the plant GST superfamily, including the ϕ (phi), τ (tau), θ (theta), ζ (zeta), λ (lambda), and dehydroascorbate reductase (DHAR) classes; the ϕ and τ classes are specific to plants (19). The glutathione conjugates are then transferred to the vacuole by ATP-dependent GS-X pumps and degraded.

Other physiological functions. Glutathione is involved in many diverse biological processes in plants such as the G1/S transition of the cell cycle during postembryonic root development (105), tracheary cell differentiation (36), anthocyanin accumulation

(110), programmed cell death, and pathogen resistance (16, 24, 71). Most of these suggested functions are based on the analysis of plants containing low levels of glutathione, such as *Arabidopsis* γ-GCL mutants *rax1–1* (4), *cad2–1* (13), and *rml1* (105) or wild-type plants treated with buthionine sulfoximine, a specific inhibitor of γ-GCL. However, such low levels of glutathione are likely to affect the function of GRXs by impairing their reduction and to alter the regulation of protein activities by precluding, for example, glutathionylation. Therefore, the requirement of GSH in all these processes may be linked to the existence of proteins that are regulated by GRX and/or by glutathionylation.

GLUTATHIONYLATION

Glutathionylation/ Deglutathionylation Reactions

In addition to its well-established roles described above, glutathione is involved in a posttranslational modification called glutathionylation. This reversible modification consists of the formation of a mixed disulfide bond between a cysteine residue and glutathione (**Figure 2**). Theoretically, glutathionylation can occur spontaneously by different reaction mechanisms (68). The most widely accepted mechanisms occur in the presence of GSSG or in the presence of GSH and oxidants. Thus, in the cell, protein

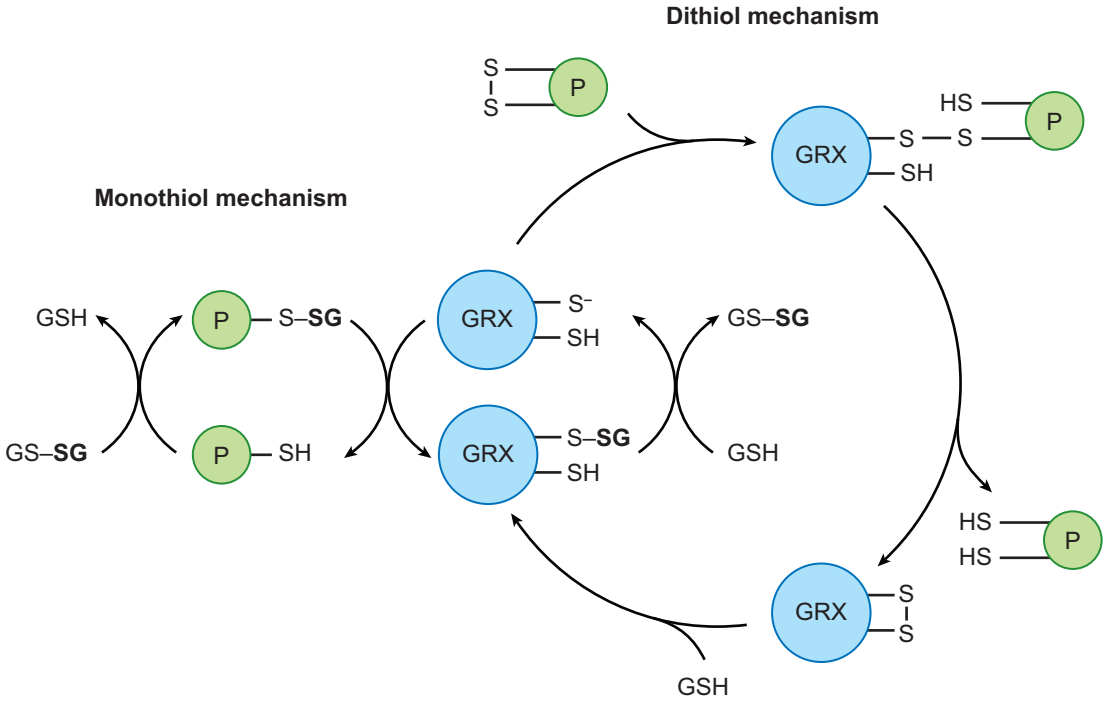

Figure 2

Monothiol and dithiol glutaredoxin (GRX)-dependent catalytic mechanisms. GRX can reduce both disulfide bridges or protein-glutathione adducts with the help of two glutathione molecules; these reactions have different mechanisms. In the dithiol mechanism, two thiol groups of GRXs are required and form an intramolecular disulfide bond between the two active-site cysteines that is subsequently reduced by reduced glutathione (GSH). In the monothiol mechanism used for deglutathionylation reactions, only one active-site cysteine is generally needed to reduce the mixed disulfide between the target protein (P) and glutathione. The glutathionylated GRX form is regenerated by another glutathione molecule. Abbreviation: GSSG, oxidized glutathione.

glutathionylation is favored under conditions of enhanced ROS production. The mechanism prevailing in vivo remains unknown. However, in vitro studies have suggested that GSH and ROS promote glutathionylation much more efficiently than GSSG alone (15, 67, 112). Starke and coworkers (98) reported that a human GRX could catalyze glutathionylation in the presence of glutathionethiyl radicals. In contrast, the reverse reaction, deglutathionylation, is likely catalyzed by GRX. Other disulfide oxidoreductases, such as TRX or protein disulfide isomerases (PDI), are poorly efficient in catalyzing deglutathionylation compared with CPYC-GRX (49, 81, 83; M. Zaffagnini & S.D. Lemaire, manuscript in preparation). The reaction is based on a monothiol mechanism because it only requires the most N-terminal cysteine of the active site of CPYC-GRX (**Figure 2**) and an external GSH for the GRX regeneration (8). Nevertheless, CGFS-GRXs from yeast or *Chlamydomonas reinhardtii*, which contain a disulfide, can also catalyze deglutathionylation, likely through a dithiol mechanism but without external GSH (102; M. Zaffagnini & S.D. Lemaire, manuscript in preparation) (**Figure 3**).

Glutathionylation Targets

Most studies on glutathionylation have been performed in mammals. Proteomic studies, based mainly on the use of ^{35}S-labeling or biotinylated glutathione, have allowed the identification of approximately 150 targets of glutathionylation involved in diverse processes such as glycolysis, signal transduction, protein degradation, intracellular trafficking, and protein folding. Glutathionylation can protect protein thiols from irreversible inactivation but can also alter, either positively or negatively, the activity of many proteins. Mammalian carbonic anhydrase III is one

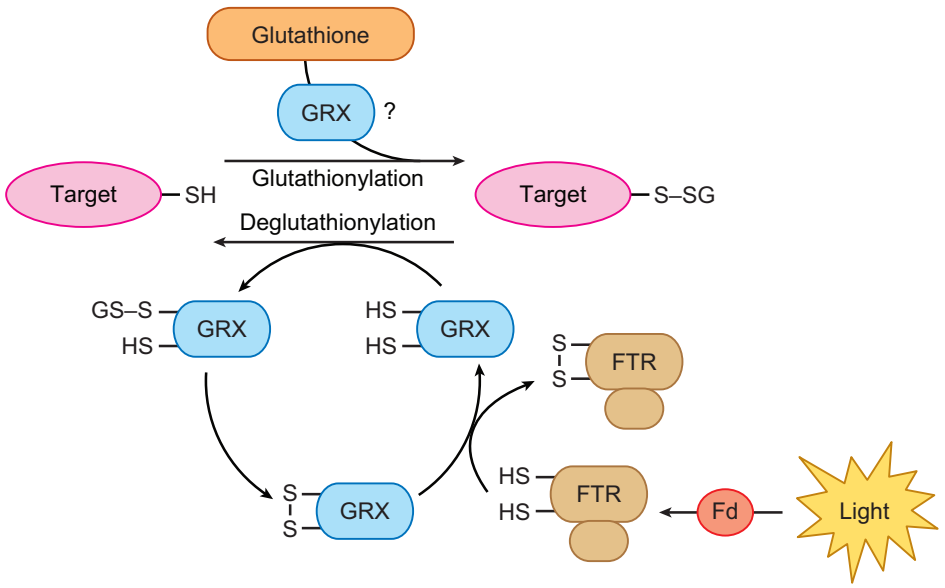

Figure 3

CGFS-GRX-dependent mechanism for deglutathionylation reactions. This mechanism, demonstrated for *Chlamydomonas reinhardtii* GRX3, a homolog of GRXS14, differs from those described in **Figure 2** in that the glutathionylated catalytic cysteine of GRX is attacked by an extra active-site C terminus cysteine. The resulting disulfide is then reduced by FTR in chloroplasts. Abbreviations: GRX, glutaredoxin; FTR, ferredoxin-thioredoxin reductase; Fd, ferredoxin.

of the first enzymes for which regulation by glutathionylation has been demonstrated (9).

The studies on glutathionylation in mammals, yeast, and bacteria have been reviewed recently (30, 42, 68, 96). In contrast, little is known so far about glutathionylation in plants. We focus on the most recent studies that suggest that glutathionylation is also a mechanism of regulation and redox signaling in plants. The first report of a plant protein undergoing glutathionylation concerned *A. thaliana* GSTs that possess a DHAR activity and/or a glutathione-dependent thioltransferase activity (17). These enzymes, which contain a catalytically essential cysteine, are glutathionylated in vitro in the presence of GSSG with a concomitant loss of enzymatic activity. This form would constitute an intermediary step of the catalytic mechanism, allowing glutathione-dependent reduction of dehydroascorbic acid (DHA). The second study, based on the in vivo use of biotinylated glutathione with *A. thaliana* cell suspensions, reported the glutathionylation of two enzymes: cytosolic triose-phosphate isomerase, a glycolytic enzyme, and fructose-1,6-bisphosphate (FBP) aldolase, a Calvin cycle enzyme (43). In vitro, the activity of recombinant cytosolic triose-phosphate isomerase is inhibited by GSSG. More recently, the inactivation of a soybean protein tyrosine phosphatase (PTP) by glutathionylation was also reported (18). Although a similar inactivation of several animal PTPs has been described (5, 104), in the case of soybean PTP, glutathionylation was suggested to involve a different mechanism implicating the catalytic cysteine and two additional cysteines (18).

In human cells, one of the three extra active-site cysteines of TRX was also identified as a glutathionylation target in vivo under oxidative stress (10). Plant TRXs also undergo glutathionylation in vitro. The glutathionylation of mitochondrial TRX h2 from poplar leads to an increase in the redox potential of the active-site disulfide and is thus likely to affect the activity of the protein (28). Among the four types of TRX (f, m, x, and y) present in chloroplasts, the f-type TRXs can undergo glutathionylation (67). These f-type TRXs are reduced in the light by way of photoreduced ferredoxin and ferredoxin-thioredoxin reductase (FTR) and are involved mainly in the light-dependent regulation of carbon metabolism enzymes, including several Calvin cycle enzymes (7, 57). Glutathionylation of *Arabidopsis* TRX f strongly decreases its reduction by FTR and thus its ability to activate target enzymes in the light (67). Cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme inactivated by glutathionylation (69). In higher-plant chloroplasts, two types of GAPDH, A_2B_2 and A_4, participate in the Calvin cycle. *Arabidopsis* A_4-GAPDH is inactivated by glutathionylation in vitro, whereas A_2B_2-GAPDH is not (112). However, the activity of A_2B_2-GAPDH is regulated by TRX f, whereas A_4-GAPDH is not. Consequently, under conditions leading to protein glutathionylation in chloroplasts, the activity of both types of GAPDH is likely to be decreased. More generally, the results obtained about the glutathionylation of TRX f, GAPDH, and FBP aldolase suggest that this posttranslational modification could constitute a new mechanism of regulation of Calvin cycle enzymes under oxidative stress (57, 67).

Using biotinylated glutathione and dark-grown *Arabidopsis* cell suspensions, Dixon and coworkers (20) identified 10 glutathionylated proteins in vivo (sucrose synthase, tubulin α and β, acetyl-CoA carboxylase, actin, cytosolic GAPDH, transducin, and Hsc70–1) and 71 glutathionylated proteins in vitro. However, these techniques did not distinguish glutathionylated proteins from those interacting with glutathionylated proteins. Using denaturing treatments, Dixon and colleagues have shown that among the targets identified in vitro, only 22 are likely to be glutathionylated, but unfortunately the identity of these 22 proteins was not revealed. In vitro analyses on methionine synthase and alcohol dehydrogenase revealed that a protein present in *Arabidopsis*

FTR: ferredoxin-thioredoxin reductase

extracts was required to trigger glutathionylation of these enzymes (20). The first in vivo proteomic study on photosynthesizing cells was recently performed in *Chlamydomonas* using ^{35}S-radiolabeling and allowed the identification of more than 20 glutathionylated proteins, most of which are located in the chloroplast (L. Michelet, S.D. Lemaire & P. Decottignies, manuscript in preparation). These proteins are involved in diverse processes such as photosynthesis, chloroplast translation, amino acid and ATP metabolism, protein folding, acetate metabolism, and oxidative stress. Many enzymes related to stress were identified, such as chloroplast chaperones or PRXs. Two new Calvin cycle enzymes, distinct from those known to undergo glutathionylation, were also identified. Glutathionylation was confirmed in vitro for three of these targets: the chloroplastic enzymes HSP70B and 2-Cys PRX and also isocitrate lyase, an enzyme involved in acetate metabolism.

GLUTAREDOXINS

The Glutaredoxin Family

Approximately 30 different GRX isoforms have been identified in higher plants, whereas only 6 are found in the eukaryotic green alga *C. reinhardtii* and 3 in the cyanobacterium *Synechocystis* sp. (56, 87, 90). In land plants, including mosses, GRX isoforms can be classified into three distinct subgroups. All these GRXs share several conserved motives, a conserved three-dimensional structure, and they possess a cysteine or a serine in the fourth position of the active-site motif (CxxC or CxxS) (90). The first class, or CPYC-type, which contains GRXs with C[P/G/S][Y/F][C/S] motifs, is homologous to the classical dithiol GRXs such as *Escherichia coli* GRX1 and GRX3, yeast GRX1 and GRX2, and mammalian GRX1 and GRX2. The second class, or CGFS-type, has a strictly conserved CGFS active-site sequence and includes GRXs homologous to yeast GRX3, GRX4, and GRX5 and to *E. coli* GRX4 (38). Plants have generally four different members in this group (GRXS14 to GRXS17). GRXS14 and GRXS15 are small proteins (approximately 170 amino acids) with a single repeat of the GRX module, GRXS16 is larger (around 290 amino acids) with the GRX module linked to an N-terminal extension, and GRXS17 possesses a TRX-like module in the N-terminal part followed by two to three GRX domains depending on the organisms. The third class, or CC-type, which regroups proteins with CC[M/L][C/S/G/A/I] active sites, is apparently restricted to land plants, because it is absent in the genomes of lower photosynthetic organisms such as *Chlamydomonas* or *Synechocystis* and also in bacteria and mammals. The only functional data reported for CC-type GRX isoforms concern their involvement in petal development (111) and pathogen responses through jasmonic acid/salicylic acid signaling (76), two processes specific to higher plants.

Subcellular Localization

Predictions of subcellular localizations suggest that most CC-type GRXs are cytosolic. It is also the case for several GRXs containing a CPYC active site, although in plants two or three of these GRXs (GRXC2, GRXC3, or GRXC4) are predicted to be secreted and might correspond to GRXs highly abundant in the phloem sap (101). In addition, two *Arabidopsis* GRXs (GRXC5 and GRXS12) belonging to the CPYC-type but containing an altered active site (WCSYC or WCSYS, respectively) are predicted to be targeted to chloroplasts. In contrast, CGFS-type GRXs are predicted to be localized in the cytosol/nucleus (GRXS17), the mitochondria (GRXS15), or the chloroplast (GRXS14 and GRXS16). The chloroplastic localization of GRXS14 and GRXS16 and the mitochondrial localization of GRXS15 have been confirmed experimentally (11; S. Bandyopadhyay, F. Gama, M. Molina-Navarro, J. M. Gualberto, R. Claxton, S. G. Naik, B. H. Huynh,

E. Herrero, J. P. Jacquot, M. K. Johnson, N. Rouhier, manuscript in revision).

Structural and Biochemical Properties

GRXs use two different catalytic mechanisms that involve one or two conserved cysteines to reduce a mixed disulfide between a protein and glutathione or a disulfide bridge. One catalytic dithiol mechanism is similar to that employed by TRXs, i.e., the N-terminal cysteine forms a transient disulfide with the oxidized target protein and the second resolving cysteine is required to reduce this disulfide and generate the reduced target protein. In the monothiol mechanism, a cysteine of a target protein reacts with glutathione and the first N-terminal active-site cysteine of GRX reduces this mixed disulfide. Another glutathione molecule is then needed to regenerate the reduced GRX (**Figure 2**). In addition, a third mechanism used by some CGFS-GRXs at least is depicted in **Figure 3**. Following the deglutathionylation reaction, an intramolecular disulfide bridge is formed between the catalytic cysteine and an extra active-site cysteine. The regeneration of this oxidized GRX is dependent on FTR but not on glutathione (M. Zaffagnini & S.D. Lemaire, manuscript in preparation).

The high number of GRX genes in plants compared with other organisms and the low conservation of the primary sequences, especially near the active site, suggest that some of the GRXs should possess different structural and biochemical properties. GRXs are generally considered to be disulfide oxidoreductases of the TRX family that are reduced by glutathione and have a redox potential ranging from –170 to –230 mV. These general features are likely conserved in all CPYC-type GRXs, which possess DHAR and disulfide reductase activities but can also catalyze deglutathionylation (88; M. Zaffagnini & S.D. Lemaire, manuscript in preparation). Site-directed mutagenesis has shown that these dithiol GRXs can use a monothiol mechanism with some substrates such as hydroxylethyl disulfide, dehydroascorbate, or PRX IIB (88, 89). However, the recent biochemical characterization of *Chlamydomonas* GRX3, a chloroplastic CGFS-GRX homologous to GRXS14, revealed unique properties. This GRX3 exhibits a lower midpoint redox potential, closer to that of TRXs, and is efficiently reduced by FTR in the light but not by glutathione. It has no disulfide reductase or DHAR activities but catalyzes deglutathionylation efficiently (M. Zaffagnini & S.D. Lemaire, manuscript in preparation). This finding suggests that the numerous GRX isoforms present in plants may indeed exhibit different biochemical properties that will have to be explored.

To date, only two plant GRX structures, poplar GRXC1 and GRXC4, have been solved (22, 79). The previously solved GRX structures of other organisms indicated a monomeric organization and a typical TRX fold. From NMR data, it appears that GRXC4, which displays a CPYC active site, has a typical TRX fold, but it is in equilibrium between a monomeric and a dimeric form; the auto-association surface comprises both the active site and the GSH binding site (**Figure 4**). In the dimer, this creates a small free pocket that could accommodate the presence of a prosthetic group such as an iron sulfur center were it not prevented by the side chain of the Pro residue of the active site (79). This observation was later confirmed by the description of a dimeric poplar GRXC1 (active site CGYC) bridging an iron sulfur cluster (ISC) around the active site (**Figure 4**) (22).

ISC: iron sulfur cluster

Functions of Glutaredoxins

The structural and redox properties of plant glutaredoxins govern their biochemical reactivities. We describe in the following section the present knowledge concerning the physiological functions of glutaredoxins in photosynthetic organisms.

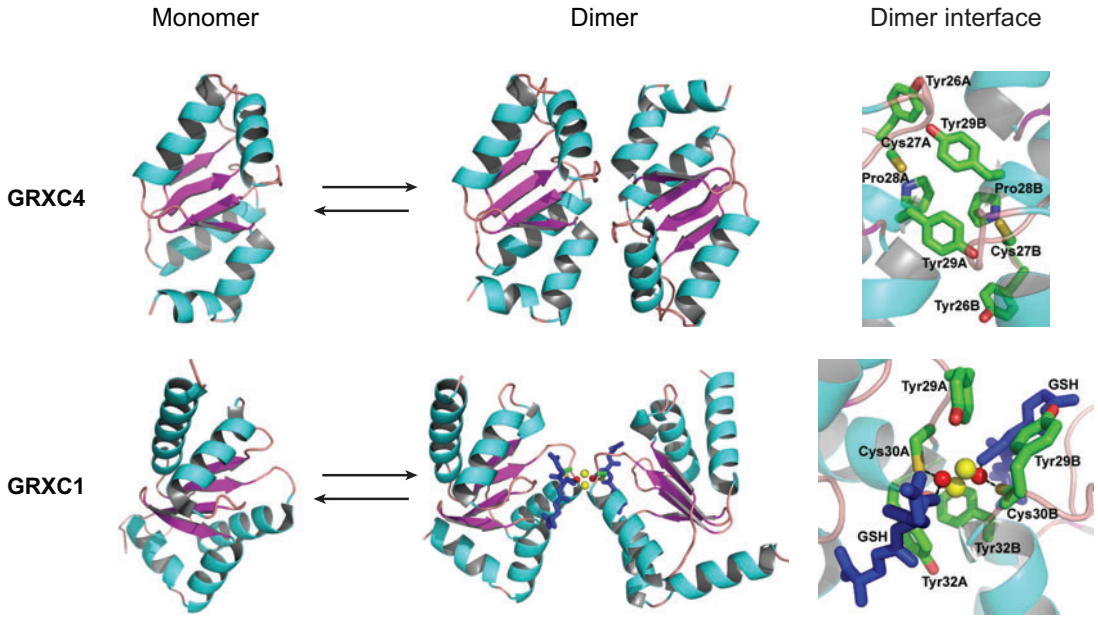

Figure 4

Monomer-to-dimer transition of poplar glutaredoxin homologs GRXC1 and GRXC4. The two proteins oscillate between a monomeric and a dimeric state. In GRXC4, the monomers are arranged in a head-to-tail orientation (79), whereas in GRXC1 (Accession numbers 1Z7R, 2E7P) they are in a mirror conformation and the two subunits are bridged by a [2Fe-2S] cluster (22, 93). In GRXC4, the side chain of the active-site proline residue (replaced by a glycine in GRXC1) likely prevents the incorporation of an iron sulfur cluster (ISC). The iron atoms are colored red, the heterosulfur atoms are colored yellow, and the ligand glutathione molecules are colored dark blue. The figure was drawn with PyMOL.

Role in oxidative stress response. To date, the most documented functions of GSH and GRXs in plants are their involvement in oxidative stress responses. GSH is crucial, especially in the chloroplast and in the cytosol, serving as an electron donor to DHAR for the maintenance of a reduced pool of ascorbate, which itself is used by ascorbate peroxidase for the removal of H_2O_2. GRXs are implicated in many different ways, for example, by directly reducing peroxides (55) or dehydroascorbate (88; M. Zaffagnini & S.D. Lemaire, manuscript in preparation), or by reducing PRXs, a group of thiol-dependent peroxidases (23, 27, 91). Peroxiredoxins are thiol containing enzymes that have the capacity to reduce not only hydrogen peroxide but also more complex alkyl hydroperoxides. The interaction between GRXC4 and PRX IIB has been well described. Mutagenesis studies on the cysteines of both partners indicate that the sulfenic acid formed on the peroxidatic cysteine of PRX IIB after the reduction of the peroxide can likely be attacked either by GRX or by GSH, forming a glutathionylated PRX IIB, the latter efficiently reduced by GRXC4 but not by glutathione alone (89). The second hypothesis has been inferred from NMR studies on PRX IIB, which showed that this protein oscillates between a dimeric reduced form and a monomeric glutathionylated form (80). These type II PRXs are the only glutathione- or GRX-dependent peroxidases; other PRXs and GPXs, contrary to their initial denomination based on their homology to mammalian glutathione peroxidases, use TRXs but not glutathione for their regeneration (37, 75, 92). Thus, the glutathione-dependent peroxidase activities observed in many studies on plant extracts are probably due to peroxidase

activities linked either to the direct peroxide reduction by GSH or to GRXs, GSTs, or some specific PRXs.

Another aspect concerning the roles of GRXs in oxidative stress response is the capacity of some CPYC-GRXs to replace TRXs as reductants to methionine sulfoxide reductases, a group of enzymes that reduce methionine sulfoxide back to methionine (106). Methionine is one of the most oxidation-sensitive amino acids, but this oxidation does not necessary lead to the inactivation of the protein. Random oxidation of methionine could constitute a sink for ROS and thus constitute a process of detoxification (97).

The *Arabidopsis* knockout mutant of chloroplastic GRXS14 presents defects in early seedling growth under oxidative stress and increased protein carbonylation in the chloroplast (11). Although its precise mechanism of action is not known, this GRX is involved in the stress response. One hypothesis might be that part of the iron sulfur assembly machinery is defective in this mutant (see below), liberating free iron, which could lead to an oxidative stress situation. A GRXC2 from the extremophile *Deschampsia antarctica*, identified as a gene involved in cold acclimation, is likely also involved in the response to an osmotic stress (31).

Roles in iron sulfur cluster assembly. Yeast mutant cells in which the *grx5* gene has been deleted (*a*) are impaired in mitochondrial ISC biogenesis and thus in respiratory growth, (*b*) accumulate free iron, and (*c*) are more sensitive to oxidative stress (86). Some prokaryotic and eukaryotic monothiol CGFS-GRXs are effective functional substitutes for yeast GRX5 (70). Although the specific role of yeast GRX5 in ISC biogenesis remains to be elucidated, studies of knockout mutants suggest that it likely facilitates the transfer of clusters preassembled on the Iscu1p scaffold protein onto acceptor proteins (72). Another hypothesis formulated from in silico analysis suggests that GRX5 may rather serve in the initial assembly of ISC into scaffold proteins (2). The recent discovery that GRX5 is also required for vertebrate heme synthesis raises the possibility that cluster-bound GRX5 plays a direct role in regulating heme biosynthesis in mammals by facilitating the assembly of a [4Fe-4S] cluster on iron regulatory protein 1 or by activating ferrochelatase through the insertion of the catalytically essential [2Fe-2S] cluster (109). Moreover, some *E. coli* GRXs increase ISC incorporation into the oxygen sensor fumarate nitrate reductase regulator, a protein that requires a [4Fe-4S] cluster for its function, presumably by reducing disulfides involving the ligand cysteines and formed in the apoprotein (1).

In other respects, poplar GRXC1 (CGYC active site) and human GRX2 (CSYC active site) are holodimers in which the subunit-bridging [2Fe-2S] cluster is ligated by one active-site cysteine of each monomer and the cysteines of two glutathione molecules (22, 48, 93) (**Figure 4**). The ISC was proposed to function as a redox sensor for the activation of GRX2 during conditions of oxidative stress. Nevertheless, whereas human GRX2 is localized both in the cytosol and mitochondria, poplar GRXC1 is essentially a cytosolic protein. Although ISC assembly machineries are located in the organelles, some iron sulfur (Fe-S) proteins (e.g., aconitase) are present in the cytosol and in the nucleus (103). A system involving the mitochondrial ISC assembly machinery, a few other proteins, and glutathione is required in *Saccharomyces cerevisiae* both for the export of ISC from mitochondria and for the assembly of cytosolic ISC-containing proteins, but many components that contribute to this process have not been identified (60). Initial cluster transfer experiments between holo-GRXC1 and a chloroplastic apo-ferredoxin were not successful, suggesting either that this GRX does not efficiently transfer ISC or that it has specific cytosolic partners not yet identified (S. Bandyopadhyay and M.K. Johnson, unpublished results).

An additional argument for the involvement of GRX in general and of GRXs with a

CGFS active site in ISC biogenesis/assembly derives from mutagenesis studies on poplar GRXC1 that indicate that incorporation of an ISC is likely to be a general feature of plant GRXs possessing a glycine or a small amino acid adjacent to the catalytic cysteine. Especially, the GRX with natural CGFS active sites (such as yeast GRX5) might incorporate an ISC as the mutation of the GRXC1 active site (CGYC) into CGFS still allows the incorporation of an ISC (93). Preliminary work with plant CGFS-GRXs indicates that two chloroplastic GRXs (GRXS14 and GRXS16) out of four CGFS-GRXs stably incorporate a [2Fe-2S] cluster during an anaerobic purification and that the fastest transfer observed to date occurs between GRXS14 and *Synechocystis* apo-ferredoxin (S. Bandyopadhyay, F. Gama, M. Molina-Navarro, J. M. Gualberto, R. Claxton, S. G. Naik, B. H. Huynh, E. Herrero, J.P. Jacquot, M. K. Johnson, N. Rouhier, manuscript in revision). These results strongly suggest that these proteins would function as scaffold proteins for de novo synthesis and transfer of Fe-S clusters, as Fe-S cluster delivery proteins for mediating the transfer of Fe-S clusters from Isc or sulfur mobilization (Suf) scaffold proteins to acceptor proteins, or as a regulator of the Suf machinery by interacting with SufE1 (**Figure 5**).

To date, although Fe-S proteins are required for many essential processes for life, such as photosynthesis, respiration, and nitrogen and sulfur assimilation, the different pathways involved in iron sulfur assembly and biogenesis in plants are poorly characterized (3). However, some proteins (cysteine desulfurase and scaffold proteins) homologous to those of bacterial, yeast, and mammalian assembly machineries have been characterized both in mitochondria and chloroplasts (4), and the finding that GRX may be involved in iron sulfur assembly is a new and exciting area of research.

Other functions. In addition to their role in oxidative stress responses, in ISC assembly, and for the two CC-type GRXs characterized so far in petal development and in pathogen response, GRXs might be involved in several other processes and metabolic pathways, in particular through the regulation of proteins by deglutathionylation/glutathionylation. GRX affinity columns allowed the identification of 94 putative targets in *A. thaliana*, *Solanum tuberosum*, *Pisum sativum*, and *Populus trichocarpa* × *deltoides* and 42 in *Synechocystis* (59, 94). There is an apparent overlap between the numerous putative targets of TRX and GRX identified by proteomics and the proteins that undergo glutathionylation (68). Although dual regulation by TRX/GRX and glutathionylation is possible for some of the targets, and despite the low specificity of TRX and GRX affinity columns, some targets may be specifically redox regulated by TRX or GRX or glutathionylation. Bacterial or mammalian GRXs have also been detected primarily through their ability to sustain the activity of ribonucleotide reductase and 3′-phosphoadenosine-5′-phosphosulfate (PAPS) reductase (39), and similar activities have been reported for poplar GRXC4, the most studied protein with the canonical CPYC active site (95). In addition, in plants the bacterial PAPS reductase is replaced by an adenosine-5′-phosphosulfate reductase, which contains a GRX module in the C terminus of the reductase domain (6).

GRX/TRX cross talk. Whereas GRXs are normally reduced by NADPH, glutathione reductase, and GSH, some GRXs can be alternatively reduced by TRX reductase, such as *Chlamydomonas* GRX3 (M. Zaffagnini & S.D. Lemaire, manuscript in preparation); some TRXs are reduced by the GSH/GRX system rather than by TRX reductases (29). The glutathionylation of some plant TRXs represents another area of interaction between TRX and GRX. In addition, the analysis of an *Arabidopsis* mutant deficient in mitochondrial and cytosolic TRX reductase indicated that some cytosolic TRXs are still partially reduced

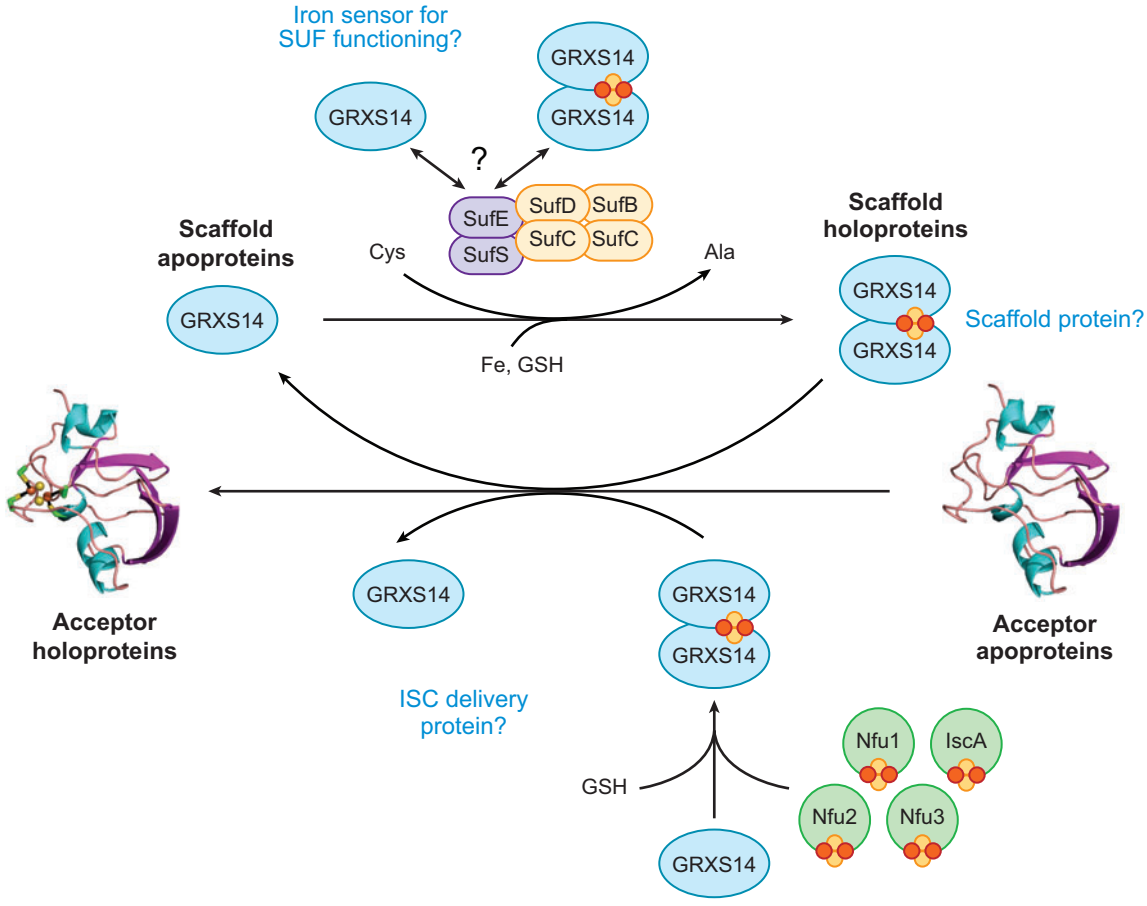

Figure 5

Proposed roles for chloroplastic CGFS-glutaredoxins (GRXs) in iron sulfur assembly. In addition to other known chloroplastic scaffold proteins (IscA, Nfu1, Nfu2, and Nfu3), GRXS14 and others could function as scaffold proteins for de novo synthesis and transfer of Fe-S clusters, as Fe-S cluster delivery proteins for mediating the transfer of Fe-S clusters from Isc or Suf scaffold proteins to acceptor proteins, or as a regulator of the Suf machinery by interacting with SufE1 via the BolA domain. In ISC, the iron atoms are colored red and the heterosulfur atoms are colored yellow. The acceptor protein represented here is a spinach ferredoxin (Accession number 1OFF). Abbreviations: ISC, iron sulfur cluster; GSH, reduced glutathione; Suf, sulfur mobilization.

through an unknown glutathione-dependent pathway that might involve some GRXs (84).

FUTURE DEVELOPMENTS

Several questions concerning the roles of GSHs and GRXs in plants remain to be answered. The importance of the cross talk between TRXs, GRXs, and glutathionylation reactions needs to be addressed in the future, especially because the number of redox-regulated or glutathionylated proteins in plants is likely to increase with the development of proteomic studies. The factors involved in glutathionylation regulation, the role of GRXs, and the functional importance of glutathionylation will also have to be addressed. The emergence of posttranslational regulation of TRXs and GRXs by glutathionylation or nitrosylation could also

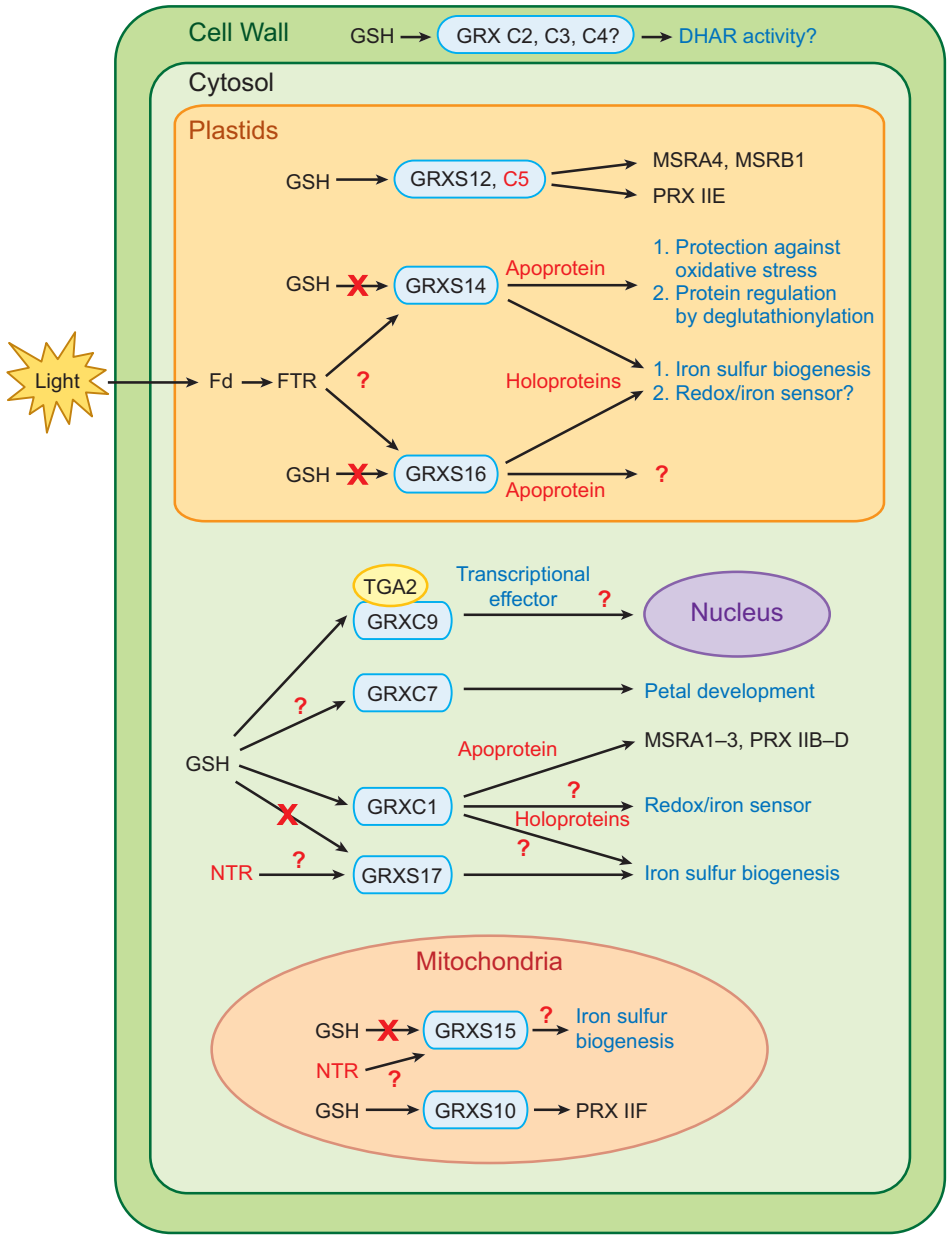

Figure 6

Putative localization and function of glutaredoxins (GRXs) in plant cells. Compartments, such as the vacuole, endoplasmic reticulum, or peroxisomes, for which the presence of GRX has not been established have been omitted. The phloem is also omitted in this scheme, although a GRXC2 from *Ricinus communis* was identified in this compartment (101). GRXC1, GRXC2, GRXC3, GRXC4, GRXC5, and GRXS12 belong to the first GRX subgroup; GRXS14, GRXS15, GRXS16, and GRXS17 belong to the second subgroup, and GRXC7, GRXC9, and GRXS10 belong to the third subgroup. Abbreviations: DHAR, dehydroascorbate reductase; MSR, methionine sulfoxide reductase; NTR, NADPH thioredoxin reductase; PRX, peroxiredoxin; GSH, reduced glutathione; FTR, ferredoxin-thioredoxin reductase; Fd, Ferredoxin; TGA2, TGA2 transcription factor.

constitute regulatory mechanisms of these reductases. In this respect, S-nitrosoglutathione (GSNO) is an important messenger molecule in animal cells and its role is still obscure in plants. The role of GSH in cell signaling is established but whether GRX also plays a role needs to be evaluated. Especially, the recent advances showing that phosphatases, kinases, and transcription factors can be redox regulated, sometimes by glutathionylation reactions, suggest important roles for GRXs in cell signaling (53, 76, 85).

Another point of interest will be to study systematically the reducing systems needed for each class of GRX. Indeed, the conventional CPYC-GRXs probably use GSH, but nothing is known in particular about the mode of reduction of the CC group, and only preliminary studies suggest that oxidized CGFS-GRXs are regenerated via TRX reductase. A possible involvement of TRXs for the reduction of the CGFS proteins is also an open question.

Overall, the functions of all the plant GRX isoforms are far from established. The present knowledge is summarized in **Figure 6**. The results obtained from organisms with fewer GRX genes, which can be easily deleted or disrupted, can serve as a working model for those organisms that have a more complex GRX organization. The high number of GRX genes present in land plants certainly suggests that they should be involved in a broad spectrum of processes. The question of the specificity or redundancy within the GRX family remains unanswered, and the study of GRX knockout lines and possibly of multiple knockouts should shed some light on this domain.

SUMMARY POINTS

1. Glutathione is a major redox buffer in the cell.
2. Glutathione and its derivatives are involved in the detoxification of xenobiotics and heavy metals.
3. Photosynthetic organisms contain a broad multigenic glutaredoxin (GRX) family.
4. GRXs and glutathione are involved in deglutathionylation/glutathionylation reactions.
5. Some GRXs bind an ISC (iron sulfur center); glutathione and iron-sulfur-containing GRXs are involved in iron sulfur assembly.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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