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COMPREHENSIVE INVITED REVIEW

Thioredoxin and Glutaredoxin Systems in Plants: Molecular Mechanisms, Crosstalks, and Functional Significance

Yves Meyer, Christophe Belin, Valérie Delorme-Hinoux, Jean-Philippe Reichheld, and Christophe Riondet

Abstract

Thioredoxins (Trx) and glutaredoxins (Grx) constitute families of thiol oxidoreductases. Our knowledge of Trx and Grx in plants has dramatically increased during the last decade. The release of the *Arabidopsis* genome sequence revealed an unexpectedly high number of Trx and Grx genes. The availability of several genomes of vascular and nonvascular plants allowed the establishment of a clear classification of the genes and the chronology of their appearance during plant evolution. Proteomic approaches have been developed that identified the putative Trx and Grx target proteins which are implicated in all aspects of plant growth, including basal metabolism, iron/sulfur cluster formation, development, adaptation to the environment, and stress responses. Analyses of the biochemical characteristics of specific Trx and Grx point to a strong specificity toward some target enzymes, particularly within plastidial Trx and Grx. In apparent contradiction with this specificity, genetic approaches show an absence of phenotype for most available Trx and Grx mutants, suggesting that redundancies also exist between Trx and Grx members. Despite this, the isolation of mutants inactivated in multiple genes and several genetic screens allowed the demonstration of the involvement of Trx and Grx in pathogen response, phytohormone pathways, and at several control points of plant development. Cytosolic Trxs are reduced by NADPH-thioredoxin reductase (NTR), while the reduction of Grx depends on reduced glutathione (GSH). Interestingly, recent development integrating biochemical analysis, proteomic data, and genetics have revealed an extensive crosstalk between the cytosolic NTR/Trx and GSH/Grx systems. This crosstalk, which occurs at multiple levels, reveals the high plasticity of the redox systems in plants. *Antioxid. Redox Signal.* 00, 000–000.

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I. Introduction

Thioredoxins (Trx) were identified several decades ago in *Escherichia coli* as efficient disulfide reducers, allowing the transfer of redox power to ribonucleotide reductase (RNR). RNR is responsible for the reduction of NDP to dNDP, which is a key step that is involved in the synthesis of dNTP and, consequently, in DNA synthesis (117). *E. coli* Trx were shown to depend on an NADPH Trx reductase (NTR), a flavin containing homodimer, for their reduction (155). Subsequently, glutaredoxins (Grx) were discovered for their ability to perform the same RNR reduction (90). Grx are reduced by reduced glutathione (GSH), releasing oxidized glutathione (GSSG), which is itself reduced by glutathione reductase (GR), a flavoprotein related to NTR (Fig. 1). Trx and Grx, also called redoxins, are also implicated in antioxidant defence by furnishing reducing power to thiol peroxidases (PRX), methionine sulfoxide reductases (MSR), and arsenate reductases. They are also mandatory for the reduction of sulfate to sulfide, which is dependent on phosphoadenylyl sulphate (PAPS) reductase, another reductase that obtains its reducing power through a pair of cysteines. Most Trx and Grx have in common a redox center, CxxC, composed of two cysteines that swap from the reduced dithiol form to the oxidized disulfide form. They present a similar three-dimensional folding, with the redox center on the surface of the redoxin, but their complete amino-acid sequences cannot be aligned, and they probably have no common ancestor. Further work has confirmed that Trx and Grx are present in most organisms and are reduced in similar ways in most bacteria and eukaryotes [for reviews, see Refs. (66, 148, 233)].

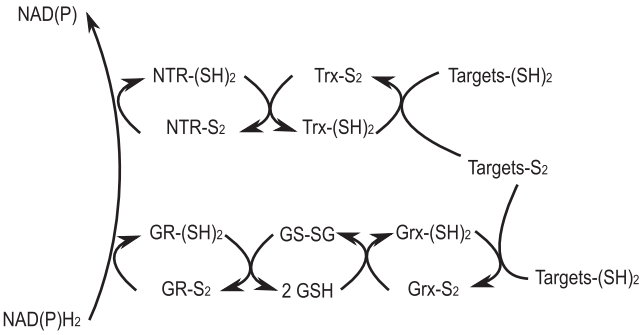

FIG. 1. Thioredoxin (Trx) and glutaredoxin (Grx)-dependent reduction pathways in *Escherichia coli*. *E. coli* Trxs are reduced by homodimeric flavoproteins Trx reductases. Trx reductases obtain their reducing power from either NADPH or NADH and are, therefore, called NAD(P)H-dependent Trx reductases (NTR). Grx are generally reduced by reduced glutathione (GSH). Subsequently, the oxidized glutathione (GSSG) molecules are reduced by NTR-related homodimeric flavoproteins, glutathione reductases (GR). Similar to NTR, GR obtain their reducing power from the pool of NADPH or NADH. As discussed in the text, Trx and Grx are able to reduce different types of oxidized cysteine residues and have a large spectrum of target peptides. Some of these targets are common between both types of oxidoreductases, while others are specific to each class. Therefore, Trx and Grx pathways are partially redundant, because they share common targets and the primary reducer NAD(P)H. Similar pathways reduce Trx and Grx in the cytosol and the mitochondria of plants and animals.

Sequencing programs revealed that Trx and Grx are encoded by several genes in most organisms, including bacteria. In contrast, NTR and GR are encoded by a small number of genes in both prokaryotes and eukaryotes. In *E. coli*, genetic approaches have confirmed the importance of Trx and Grx in DNA synthesis, antioxidant defence, and sulfate assimilation. The phenotypes of *trx* and *grx* mutants show complex and partial redundancies between the different Trx and Grx members.

In plants, Trx were first identified as regulators of photosynthesis, a rather different function from that previously identified in *E. coli*. This discovery stemmed from the observation that two chloroplastic enzymes involved in carbon metabolism, fructose-1,6-bisphosphatase (FBPase) and NADP-dependent malate dehydrogenase (NADP-MDH), are inactive when extracted from the plants maintained in the darkness, but are fully active when isolated from the plants maintained in the light. Further work established that these enzymes are activated by chloroplastic Trx, which are only reduced in the light by a ferredoxin (Fdx)-dependent Trx reductase (FTR). This system is, thus, completely unrelated to the NTR (202). Significantly, the Trx-dependent reduction of FBPase and NADP-MDH does not act on catalytic disulfides, but rather regulates the disulfides involved in protein conformation switches. Moreover, thiol regulation is also modulated by the redox potential of target proteins and by metabolic control systems (*e.g.*, the presence of Fru-1,6-BP is needed for FBPase thiol activation) (181). Thus, these factors add another level of complexity to the thiol regulation. The concept of Trx and Grx acting through conformational modifications has now been extended to all organisms. It was later shown that plants also have a cytosolic (189) and a mitochondrial system (113), in which Trx are reduced by NADPH-dependent NTR (96). Moreover, plants also contain cytosolic, mitochondrial, and

chloroplastic GR/GSH/Grx systems (194). One characteristic of plants is the very large number of Trx and Grx types, with most being encoded by several genes and all being encoded by the nuclear genome. This high complexity is supported by the fact that both Trx and Grx are involved in a large number of metabolic pathways. Moreover, a growing body of evidence suggests that these two systems support specific functions in terms of biochemical characteristics. Interestingly, recent work supported by genetic evidence has demonstrated that several types of crosstalk occur between Trx and Grx systems, pointing to the high plasticity of these redox systems in plants.

II. Peptidyl Cysteine Oxidation and Reduction by Trx and Grx

Trx and Grx are able to reduce different types of oxidized thiols and generally use distinct reduction mechanisms. Peptidyl cysteine in the reduced state (thiol) can be oxidized, leading to a disulfide bridge between two peptidyl cysteines that are present either in same or in two different peptides. Alternatively, a disulfide bridge can be formed between a peptidyl cysteine and the cysteine of a glutathione (glutathionylation). A thiol can also be oxidized by oxygen to a sulfenic acid or by S-nitrosylation, leading to the formation of S-nitrosothiols residues (RSNOs), a post-translational modification that is involved in signal transduction and nitric oxide (NO) transport.

A. Reduction of disulfide bridges and sulfenic acids

Trx are able to reduce disulfide bridges between two cysteines present in peptides by using a so-called dithiol mechanism. The reduction process always begins with the first cysteine of the redox site, which is called the catalytic cysteine, releasing a thiol on the target protein and forming a disulfide bond with the second target cysteine. In a second step, the second cysteine (resolving cysteine) of the Trx reduces the intermediate complex, releasing the reduced target and the oxidised Trx (Fig. 2A). Dithiol Grx (Grx harboring another cysteine, either the second active site cysteine or a C-terminal resolving cysteine) is also able to perform a dithiol mechanism. However, this mechanism is probably less frequent than the monothiol mechanism (see next).

Some cysteines are oxidized in the form of a sulfenic acid, without forming a disulfide bond. Although Trx-dependent disulfide bridge reduction activities have been extensively described, their capacity to reduce sulfenic acid residues has not yet been described. Nevertheless, in *E. coli*, DsbG and DsbC, two Trx-related proteins, have been shown to control the global sulfenic acid content of the periplasm and to protect single cysteine residues from oxidation (59).

B. S-glutathionylation/deglutathionylation and isomerization

Some peptidyl cysteines undergo glutathionylation, which can occur spontaneously by different mechanisms, mostly under conditions of enhanced reactive oxygen species (ROS) production (152, 194, 253). Human Grx was reported to catalyze glutathionylation (209). Moreover, in all organisms, deglutathionylation is performed by redoxins. In *Saccharomyces cerevisiae*, Trx appear to play a major role in deglutathionylation (83). However in all other organisms including

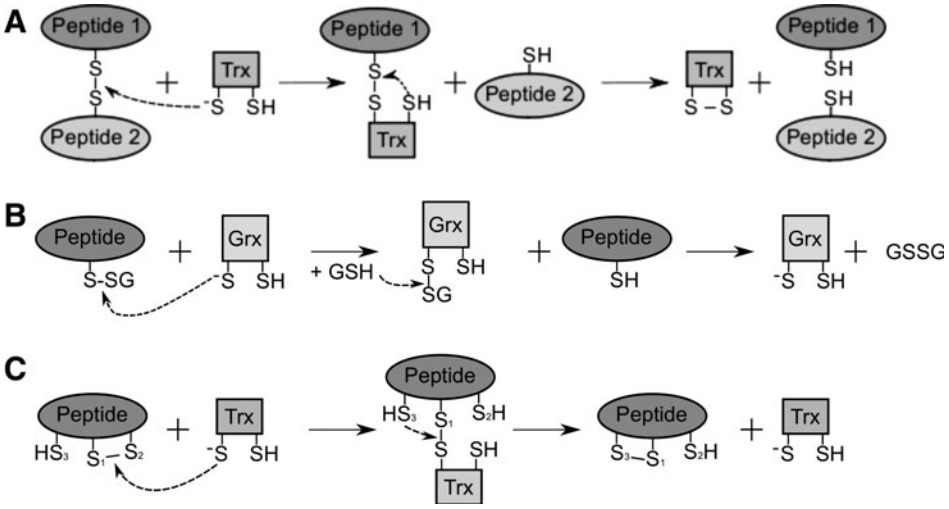

FIG. 2. Trx and Grx reduction mechanisms. (A) Reduction of a disulfide bridge between two peptidyl cysteines by Trx. Both the catalytic and the resolving cysteines of the Trx are needed to complete the reaction. The catalytic cysteine is deprotonated (S^-), acts as a nucleophile, and attacks the disulfide bridge. A mixed disulfide bridge is formed between the catalytic cysteine of the redoxin and a cysteine of the target peptide. This disulfide bridge is attacked by the resolving cysteine of the redoxin (SH), which forms a disulfide with the catalytic thiol, and the reduced form of the target peptide is released. **(B)** Deglutathionylation of a target protein by Grx. Here, only the catalytic cysteine and GSH are necessary. Deglutathionylation is generally considered to be performed by mono or dicysteinic Grx. The catalytic thiol of Grx attacks the disulfide of the glutathionylated peptide, releases the reduced peptide, and becomes glutathionylated. Another molecule of GSH reduces the glutathionylated thiol of Grx. **(C)** Isomerization of a target protein by Trx. Only the catalytic cysteine is necessary. Isomerization can be performed by mono or dicysteinic Trx, as well as by protein disulfide isomerases (not reviewed in this article). As for previous reactions, the catalytic cysteine attacks the disulfide bridge (S_1-S_2) and forms a mixed disulfide. The third thiol of the peptide (HS_3) serves as a resolving thiol and reduces the mixed disulfide, releasing the reduced Trx and forming a disulfide with S_1.

plants, this particular disulfide bridge is preferentially reduced by Grx, which itself becomes glutathionylated. The glutathionylated Grx is reduced by a GSH molecule releasing GSSG. In this process, the resolving cysteine of the redox site of Grx is not implicated (Fig. 2B). As described later, an alternative dithiol mechanism may exist for some Grx such as yeast Grx5 (216) or Chlamydomonas Grx3 (252). These different mechanisms have been described in several reviews (60, 152, 194, 254).

Some target proteins present cysteines in addition to those implicated in a disulfide bridge. In some cases, one of these can act as the resolving cysteine, releasing the already oxidized target protein but in which the disulfide bridge is isomerized (Fig. 2C). In fact, some organisms, including plants, encode the Grx and Trx variants exhibiting a CxxS redox site (named monocysteinic) that are able to deglutathionylate and isomerize target proteins but are unable to reduce a disulfide bridge formed between two peptidyl cysteines (205).

C. S-nitrosylation/denitrosylation

S-Nitrosylation/denitrosylation plays a major function in cell signaling (203, 208). A mammalian Trx was proposed to promote transnitrosylation through mechanisms involving nonactive site thiols (85, 153). More recently, two mechanisms were proposed for the denitrosylation of peptidyl S-nitrosothiols by Trx (16). Both implicate Trx as a reducer and differ only in the intermediate step in which Trx forms a complex with the protein or is transiently nitrosylated on its catalytic cysteine. In both cases, the catalytic and resolving cysteines are necessary to complete the reaction (Fig. 3). Trx have been shown to be active in the denitrosylation of mammalian caspase 3 (15). In mammalian cells, additional Trx transnitrosylation and denitrosylation target proteins have been isolated using the isotope coded affinity tag (ICAT) method in conjunction with the biotin-switch technique (17, 245). It remains to be determined whether dicysteinic Grx also plays a similar role. Current knowledge about the roles of Trx in the regulation of cellular processes by S-nitrosylation has been recently reviewed by Sengupta and Holmgren (203). The functions of Trx in the control of nitrosylation/denitrosylation have been studied in mammals but have not yet been investigated in plants.

III. Trx-Dependent Reduction System in Plants

A. Trx reduction

As previously described, in plants, the reduction of organellar and cytosolic Trx is performed by distinct pathways. Plastidial Trx are reduced in plastids by an FTR. FTR is only present in plant plastids and cyanobacteria. It contains a [4Fe-4S] cluster that plays a central role in the Trx-reduction process (45). *Arabidopsis thaliana* is composed of a catalytic peptide that is encoded by a unique gene which is associated with a variable peptide encoded by two genes. The protein is rather unstable after extraction from plants, and a recombinant protein is produced only from a cyanobacterium (56) as well as from spinach (76). This allowed a fine analysis of the structure and the reaction mechanism (56, 248). In the chloroplast under illumination, Fdx is directly reduced by the electron flux from photosystem 1. The major function of Fdx in the chloroplast during photosynthesis is to transfer the electron flux to the Fdx:NADP reductase (FNR), which, in turn, reduces NADP to NADPH, while a minor part of the redox flux allows FTR reduction (Fig. 4). In contrast, Fdx reduction in nongreen plastids depends on the oxidative pentose phosphate (OPP) pathway, which allows the NADPH used by the FNR to reduce Fdx (Fig. 5). Fdxs are encoded by five genes in *Arabidopsis* (86), and it is believed that the most abundantly expressed gene in the leaf encodes the Fdx which is responsible for photosynthetic NADP reduction and Trx reduction in the chloroplast. The inactivation of this gene perturbs photosynthesis and growth but is not lethal (234). In order to evaluate the function of the FTR/Trx system in plastids, the phenotype of a mutant of the variable subunit of the FTR was analyzed. It was found to be significantly more sensitive to oxidative stress (high light or paraquat) and has a decreased activity in NADP-MDH (104). Most probably, this limited phenotype is due to compensation by the second gene encoding the variable subunit of the FTR. Inactivation by virus-induced gene silencing of the catalytic subunit of FTR in tomato does not affect plant size and morphology, at least in the early stage of development. Nevertheless, several defence proteins are overexpressed, and necrotic lesions appear on the leaves. H_2O_2 accumulates, and plants become resistant to several bacterial pathogens. Thus, the FTR-silenced tomato presents a typical hypersensitive response (131). The simplest explanation is that, in the absence of reduction of several

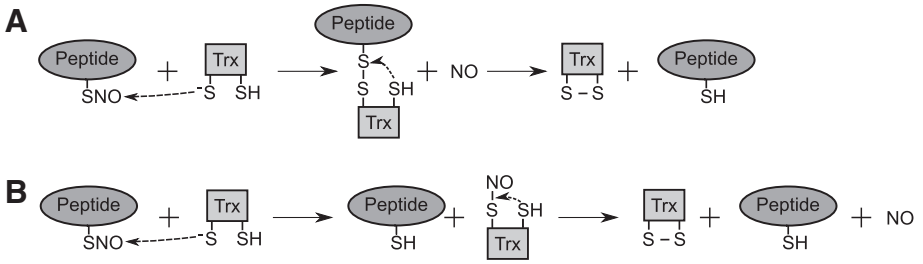

FIG. 3. Denitrosylation of nitrosylated target protein by Trx. Two alternative reaction mechanisms of S-nitrosothiol (SNO)-peptide reduction have been proposed. Both the catalytic and the resolving cysteines of the Trx are needed to complete the reactions. In the literature, denitrosylation is considered to be performed by Trx, and Grx may probably act in a similar manner. **(A)** The first step is the nucleophilic attack of the SNO by the catalytic cysteine, releasing nitric oxide (NO) and forming a mixed disulfide intermediate that is reduced by the resolving thiol of Trx. **(B)** In the alternative reaction, the first step is the same, but the NO is transferred to the catalytic thiol and the reduced peptide is released. The SNO bound is then reduced by the resolving cysteine, releasing NO.

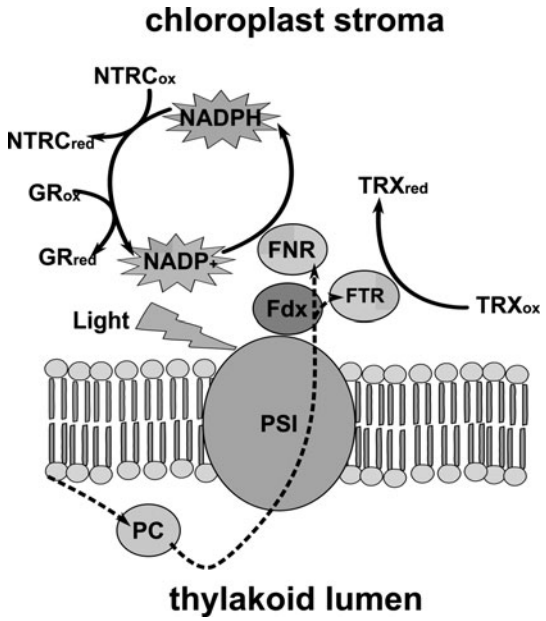

FIG. 4. Photosynthetic reduction of chloroplastic Trx in the light. Ferredoxin (Fdx) is reduced by the plastidial photosynthetic electron flux (*dashed line*). Fdx delivers electrons primarily to Fdx:NADP reductase (FNR) for the reduction of NADP to NADPH, while probably a smaller amount of electrons is needed by the Fdx:Trx reductase (FTR) to keep the Trx pool reduced. NADPH is also necessary for the reduction of NTRC and GR. PSI, photosystem 1; PC, plastocyanin.

plastidial Trx, H_2O_2 accumulates as a by-product of photosynthesis. This strongly suggests that the antioxidant activity of plastidial Trx (through peroxiredoxin [Prx] and glutathione peroxidase [Gpx] reduction) is particularly important in spite of the presence of other antioxidant systems, including NTRC, which are FTR independent (see next). In contrast, at least in this experiment, the lack of Trx-regulated day/night metabolism seems to have a minor impact on plant development.

The reduction of cytosolic (96) and mitochondrial (113) Trx is performed by NTR, which has a high similarity to the *E. coli* NTR. The active protein is a homodimer that is associated with a flavin which is responsible for the yellow color of the protein. The structure has now been established (55). In *Arabidopsis*, two genes (*NTRA* and *NTRB*) encode both a cytosolic protein and a mitochondrial protein, each gene expressing a short and a long mRNA (182). As discussed later, both proteins are dispensable for plant development (183). Most recombinant cytosolic Trx tested so far are efficiently reduced by recombinant NTR (Table 1). In addition, several plastidial Trx can also be reduced by NTR *in vitro*.

B. Trx and putative target proteins

Trx of vascular plants are now classified in 15 subgroups on the basis of sequence similarity (Fig. 6) with several members in each subgroup (79, 147). This includes the typical Trxs (MW about 10–12 kDa), which have a canonical WCG/PPC active site and are generally reduced by Trx reductases. Moreover, there are many "Trx-like" proteins with atypical active sites, multiple Trx domains, or proteins in which Trx motifs are associated with other domains (Table 1). Some of the latter proteins are not well characterized in terms of biochemical activities and reduction systems. Members of the 15

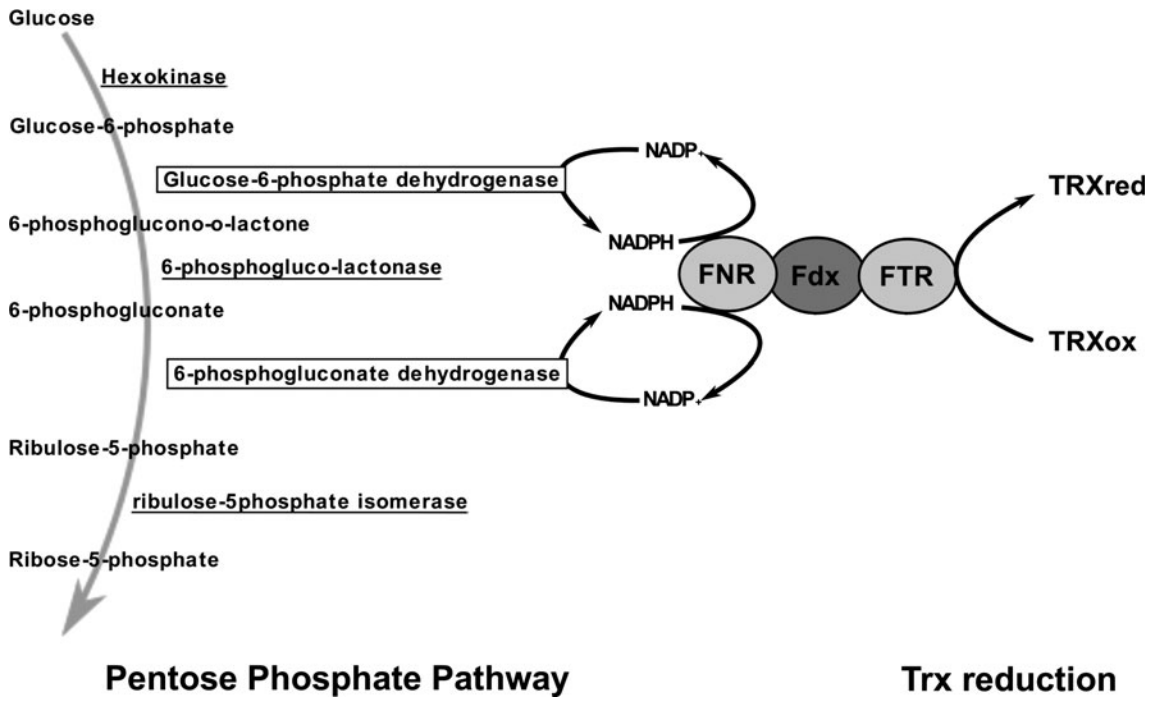

FIG. 5. Deactivation of enzymes of the pentose phosphate pathway by Trx. Trx reduction in nongreen plastids: The oxidative pentose pathway produces NADPH at the expense of glucose. NADPH reduces FNR, which, in turn, reduces Fdx (in the reverse way of the chloroplast pathway). Then, Fdx reduces FTR and Trx.

Table 1. Thioredoxin in *Arabidopsis*

Short name	*AGI number*	*Localization*	*Protein domains*	*Redox site*	*Production in* Escherichia coli	*Reducer*
h1	At3g51030	Cytosol (189)	Type hI 1 Trx domain	WCGPC	Soluble	NTR (189)
h3	At5g42980	Cytosol (189)		WCPPC	Soluble	NTR (189) GSH/Grx C1, 2 (183)
h4	At1g19730	Cytosol (189)		WCPPC	Soluble	NTR (189)
h5	At1g45145	Cytosol (189)		WCPPC	Soluble	NTR (189)
h2	At5g39950	Cytosol (189) Mitochondrial (143)	Type hII 1 Trx domain	WCGPC	Soluble	NTR (189) GSH/Grx C1, 2
h7	At1g59730	Cytosol putative		WCGPC	?	?
h8	At1g69880	Cytosol putative		WCGPC	Insoluble (personal data)	?
h9	At3g08710	Cytosol membrane (143)	Type hIII 1 Trx domain	WCGPC	Soluble	GSH/Grx (77, 106, 143)
h10	At3g56420	Cytosol		WCVPC	?	?
CxxS1	At1g11530	Cytosol (205)		WCIPS	Insoluble solubilizable	GSH (205)
CxxS2	At2g40790	Cytosol (205)		WCLPS	Insoluble solubilizable	GSH (205)
TDX	At3g17880	Cytosol (230) Nuclear putative	3 TPR motifs 1 Trx domain	WCGPC	Soluble (230)	NTR (230)
o1	At2g35010	Mito (48, 113)	1 Trx domain	WCGPC	Soluble (113)	NTR (113)
o2	At1g31020	?			Soluble (113)	NTR (113)
Picot AtGrxS17	At4g04950	Cytosol	1 Trx domain 3 Grx domains	WCDAS RCGFS KCGFS KCGFS	Soluble (personal data)	?
Clot	At5g42850	Cytosol putative	1 Trx domain	WCPDC	?	?
NRX1	At1g60420	Cytosol nuclear putative	Nucleoredoxins 3-2Trx domains	WCGPC SYRCP WCPPC	Insoluble (personal data)	?
NRX2	At4g31240	Cytosol nuclear putative		WCRPC WCPPF	Insoluble (personal data)	?
f1	At3g17880	Chloro (47)	Subgroup f 1 Trx domain	WCGPC	Soluble	FTR
f2	At3g17880	Chloro (47)			Soluble	FTR
m1	At1g03680	Chloro (47)	Subgroup m 1 Trx domain	WCGPC	Soluble	FTR
m2	At4g03520	Chloro (48)			Soluble	FTR

(*continued*)

Table 1. (Continued)

Short name	*AGI number*	*Localization*	*Protein domains*	*Redox site*	*Production in* Escherichia coli	*Reducer*
m3	At2g15570	Chloro (47)			Soluble (47)	FTR
m4	At3g15360	Chloro (47)			?	FTR
x	At1g50320	Chloro (47)	Subgroup x 1 Trx domain	WCGPC	Soluble (47)	FTR
y1	At1g76760	Chloro (47)	Subgroup y 1 Trx domain	WCGPC	Soluble (47)	FTR
y2	At1g43560	Chloro (48)			Soluble (48)	FTR
lilium1	At1g08570	Chloro (57)	Subgroup lilium 1 Trx domain	GCGGC	Soluble (57)	?
lilium2	At4g29670	Chloro		WCASC	Soluble (57)	?
lilium3	At5g61440	Chloro		GCGGC	?	?
lilium4	At2g33270	Chloro		SCGGC	?	?
lilium5	At4g26160	Chloro (31, 57)		WCGGC	Soluble (57)	?
lilium6	At1g07700	Chloro		ACGSC		?
CDSP32	At1g7608	Chloro (184)	1 degenerated Trx domain 1 Trx domain	KSDKS HCGPC	Soluble (28)	FTR
HCF164	At4g37200	Chloro transmembrane stroma thylacoid	1 Trx domain	WCEVC	Soluble	Trx m (157)
TARWCGPC	At1g52990	Cytosol putative	No ortholog outside *Arabidopsis*	WCGPC	?	?
WCRKC1	At5g06690	Chloro (31)	WCRKC 1 Trx domain	WCRKC	?	?
WCRKC2	At5g04260	Chloro (31)	1 Trx domain	WCRKC	?	?
z	At3g06730	Cytosol (188) Chloro (1, 201)		WCGPC	Soluble (44)	FTR (44)
APR1	At4g04610	Chloro (137)	aps reductase 1 PapsR domain 1 Trx domain	WCPFC	Soluble (84, 206)	GSH (21)
APR2	At1g62180	Chloro			Soluble (206)	
APR3	At4g21990	Chloro			Soluble (206)	
NTRC	At2g41680	Chloro (204)	1 NTR domain 1 Trx domain	ACAIC TCGPC	Soluble (204)	NADPH (204, 207)
WCGVC	At3g53220	Cytosol putative	1 Trx domain	WCGVC	?	?

FTR, ferredoxin:thioredoxin reductase; GSH, reduced glutathione; NTR, NADPH thioredoxin reductase; Trx, thioredoxin; APR, APS reductase; ?, unknown.

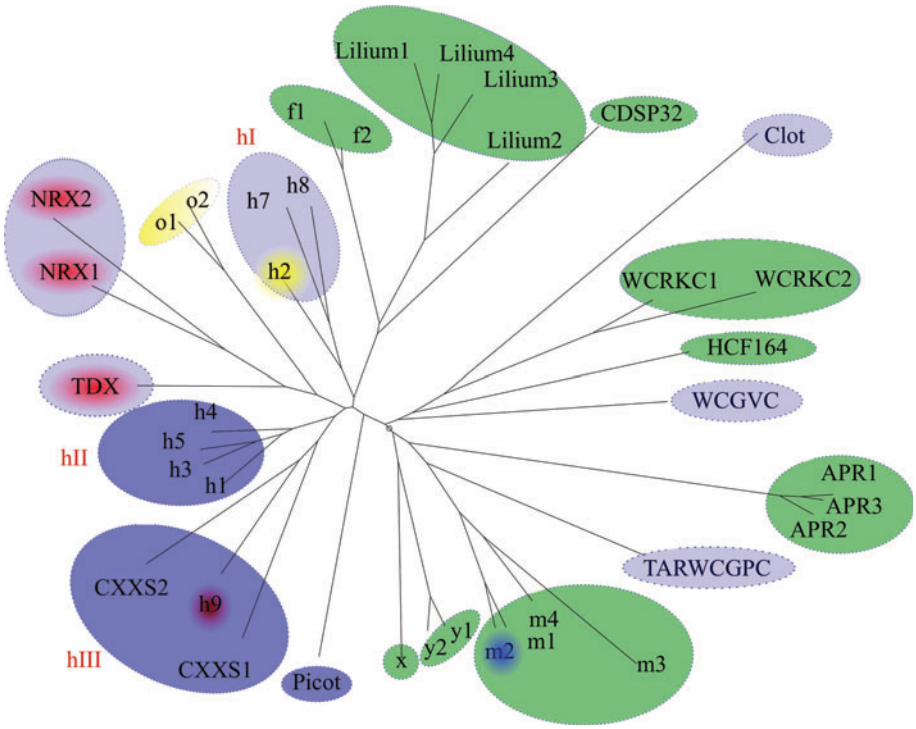

FIG. 6. Phylogenic tree of *Arabidopsis thaliana* Trx. Members of the different subgroups of Trx are clustered, and the cellular localization is represented by different background colors. The green color corresponds to the chloroplast, blue to the cytosol, yellow to the mitochondrion, red to the nucleus, and maroon to the membrane. A degraded color circle indicates a double localization. A faded color circle represents a putative localization. The AGI numbers corresponding to each protein are presented in Table 1. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

subgroups are found in all genomes of vascular plants that have been sequenced so far, and some are present in bryophytes, lycophytes, eukaryotic algae, and even in cyanobacteria (42). Table 1 shows the different subgroups of Trx, the members of these different subgroups in *Arabidopsis*, and the subcellular localization, if this has been demonstrated. In most cases, all members in a subgroup have the same subcellular localization. Most Trx have been easily produced in *E. coli*, allowing a fine analysis of the reaction mechanism, but some of them are only produced either at low levels or in an insoluble form.

1. Plastidial Trx. Most Trx are plastidial: Both the Trxm and Trxf were discovered early in plant Trx research (30, 94, 95, 100, 241), while the CDSP32 (28), x (47, 144), y (48, 120), z (176, 188), WCRKC (31), and Lilium (57) types were only discovered either by an analysis of genome sequences or during genetic screens (Fig. 6).

a. Metabolic and antioxidant functions. The involvement of chloroplastic Trx in the regulation of the day/night metabolisms has been the subject of intensive research during the four last decades. In the light, photosynthesis builds up a proton gradient that is used by the ATP synthase for ADP phosphorylation and which provides the electrons necessary for NADP reduction (Fig. 4). The NADPH and ATP that are produced by the photosynthetic electron transport are used for CO_2 assimilation by the Calvin–Benson cycle (Fig. 7A). The photosynthetic electron flux also reduces Trx, which, in turn, reduces and activates several enzymes of the Calvin–Benson cycle (Figs. 4 and 7A). Since the early discovery of the light-dependent regulation of chloroplast enzymes by Trx (240), especially enzymes of the Calvin–Benson cycle, a large number of studies have allowed not only a deciphering of the molecular mechanisms underlying the regulation of four Calvin–Benson cycle enzymes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), FBPase, PRK, and SBPase, but also the regulation of other enzymes such as NADP-MDH, ATP synthase, G6PDH, ACCase, ADP-glucose pyrophosphorylase (AGPase), Rubisco activase, several types of Prx, Gpx, TR-BAMY, MsrB2, GWD, and CP12 (see references 43, 121, 136, 197, and 202 for further details). The specificity of reduction by different plastidial isoforms has been reported for many of these enzymes (Table 2). Interestingly, the Trxm and Trxf are devoted to the regulation of the target proteins that are involved in the photosynthesis-related chloroplast metabolism. The best-studied enzymes are those of the Calvin–Benson cycle (with the exception of SBPase) and NADP-MDH. For these enzymes, Trxf is generally a more efficient reducer than Trxm. FBPase and B4-B8-GAPDH also exhibit a strict dependency on Trxf-dependent regulation. All other plastidial Trx are inefficient for their reductive activation (43, 121, 136, 197, 202). The molecular and structural bases of the Trx-dependent regulation of these enzymes have been investigated in great detail. However, many enzymes involved in antioxidant systems are preferentially reduced by Trxx, Trxy, Trxz, CDSP32, and NTRC (42, 47, 48, 165, 175, 229). Therefore, carbon metabolism enzymes are strictly regulated by FTR-dependent Trx (Trxf and Trxm), enabling a finely tuned day/light regulation of carbon metabolism. However, antioxidant enzymes have a broader range of reducers: Some of them are dependent on light, whereas others are dependent on NADPH (*i.e.*, NTRC, see next), allowing ROS detoxification also during the night (175). Interestingly, several antioxidant enzymes have been shown to be reduced by chloroplastic Grx, suggesting overlapping functions between Trx and Grx in antioxidant responses (Table 2) (see next). Thus, there are clear specificities between chloroplastic Trx for metabolic and antioxidant proteins in the chloroplast. These specificities are only partially related to the value of the midpoint redox

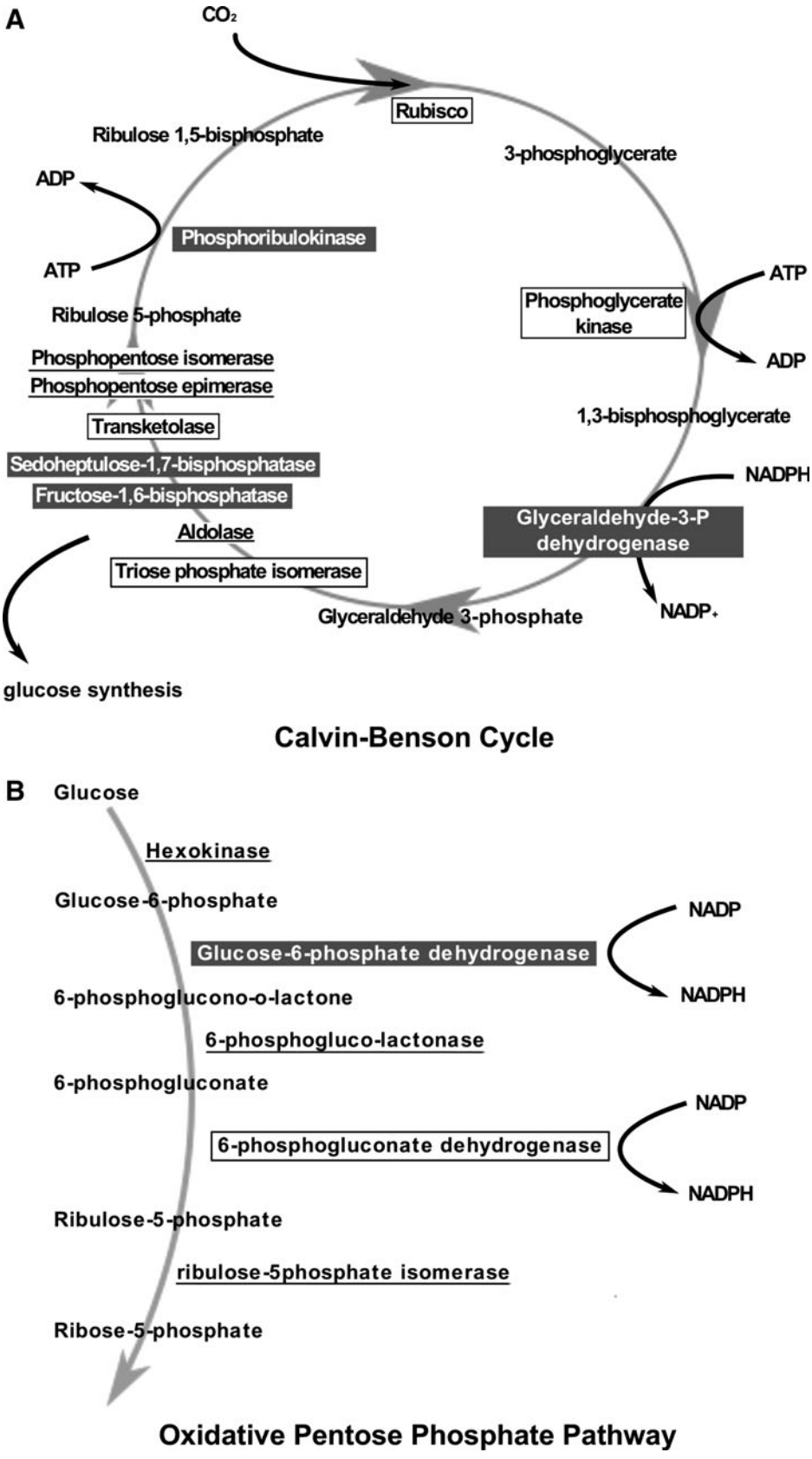

FIG. 7. Regulation of carbon metabolism by chloroplastic Trx. (A) Calvin–Benson cycle **(B)** A part of the oxidative pentose phosphate (OPP) pathway. In the light, Trx activates sugar synthesis through the Calvin–Benson cycle and inactivates the OPP pathway. The Calvin–Benson cycle enzymes that are activated by Trx reduction are boxed in gray. Other putative Trx target enzymes that are identified by proteomic approaches are boxed in white. The pentose phosphate pathway enzyme that is inactivated by Trx reduction is boxed in gray. In the night, Trxs are no longer reduced; the Calvin–Benson cycle proteins and NADP-dependent malate dehydrogenase (NADP-MDH) are oxidized; and carbon assimilation stops. The OPP pathway is activated, allowing sugar catabolism that is necessary for the basal metabolism of the chloroplast.

Table 2. Preferential Reduction of Several Target Proteins by *Arabidopsis* chloroplastic Thioredoxin and Grx *In Vitro*

	Artificial substrate			*Plastidial protein*																	
Target	*DHA*	*HEDS*	*Insulin*	*CP12 complex*	*PRK*	*TR-BAMY*	*Rubisco activase*	*G6PDH*	*GAPDH A4*	*GAPDH B4*	*GAPDH B8*	*NADP-MDH*	*FBPase*	*Gpx*	*Prx Q*	*2-cys Prx*	*PrxII-E*	*FNL*	*MSRA*	*MSRB1*	*MSRB2*	*AGPase*
Trxf1			+	+ + +	+ + +	+ +	+ +	+ + +	−	+ + +	+ + +	+ + +	+ + +							−	+ +	+ +
Trxf2			+									+ +	+ + +									
Trxm1			+	+	+	+	−	+ + +	−	−	−	+ +	−							−	+ + +	
Trxm2			+	+	+				−	−	−	+ +	−							−	+ +	+ +
Trxm3			+					+ +		+		−	−							−	−	
Trxm4			+					+ + +				+ +	−							−	+ +	
Trxx			+					−	−			−	−	+	+ +	+ +				−	−	
Trxy1			+			+		+ / −	−			−	−	+ +	+ +	+ +				−	+ +	
Trxy2			+									−	−	+ +						−	+ + +	
Trxz			+									−		+ +	+ +	−	+ +	+ +	+ +	−	−	
CDSP32			−												+ +	+ +				+ +	−	
CDSP32 C−ter			+												+ +	+ +				+ + +	−	
NTRC									−							+ +				−	−	+ +
GrxS12	+ +	+ +							+ +							+ +	+ +			+ +	−	
GrxC5	+ +	+ +															+ +			+ +		

The signs + or − indicate the activity levels, while the blank cells correspond to untested combinations. + / − means that the enzyme is efficient for activation of the target protein but inefficient for inhibition of the protein (167).

Most original references can be found in (121), those for GAPDH, CP12, PRK, and G6PDH in (136, 251, 252) and (167), those for MSR in (219, 229), AGPase in (150), Trx z in (1, 44, 201), and GrxS12 and GrxC5 in (53, 218).

DHA, dihydroascorbate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gpx, glutathione peroxidase; HEDS, hydroxyethyl disulfide; MSR, methionine sulfoxide reductase; NADP-MDH, NADP-dependent malate dehydrogenase; Prx, peroxiredoxin; FBPase, fructose-1,6-bisphosphatase; FNL, fructokinase-like; AGPase, ADP-glucose pyrophosphorylase.

potential of the distinct Trx/target couples, that is, Trx f reduce target proteins that have a broad range of redox potential (92, 167). Other parameters such as conformational characteristics and fine-tuning regulations by metabolic ratios (ATP/ADP, Mg^{2+} ion, pH gradients, and NADPH/NADP) are also likely involved (61).

The oxidative pentose phosphate (OPP) pathway and the Calvin–Benson cycle play antagonistic functions with regard to carbohydrate metabolism. These two pathways share some metabolites and enzymes that might lead to futile cycles. To avoid such a waste of energy during the day, Trxf and Trxm reduce glucose-6-phosphate dehydrogenase (G6PDH), a key enzyme of the OPP pathway that regulates the carbohydrate mobilization (Fig. 7B). The reduction of G6PDH leads to inactivation of the enzyme and, therefore, inhibition of the OPP cycle. However, during the night, Trxm and Trxf are no longer reduced (Fig. 7B). Subsequently, sugar synthesis stops, while the OPP pathway is reactivated by the Trx-dependent oxidation of the G6PDH, allowing some sugar catabolism and the production of ATP and NADPH that are necessary for the basal metabolism of the chloroplast (167, 200, 237) (Fig. 7B).

b. New functions of chloroplastic Trx. In addition to these classical biochemical studies, several genetic screens and proteomics approaches have revealed new functions for some plastidial Trx. It has been recently shown that Trxm2, a prototype of chloroplastic Trx, is partially retained in the cytosol and is involved in the heterodimerization of two G6PDH isoforms (G6PDH1 and G6PDH4). Subsequently, this dimer is addressed to the peroxisome, allowing an efficient OPP pathway to be performed in the peroxisome (146).

Another surprising role for a plastidial Trx concerns the implication of Trxm3 in cell-to-cell trafficking. Trxm3 was previously shown to be atypical. In contrast to the three other members of the Trxm subgroup, it is unable to reduce most plastidial target proteins. The only reported exception is a low activity in G6PDH1 (167). While AtTrxm1, m2, and m4 efficiently complement the Trx-mediated resistance to H_2O_2 in a *S. cerevisiae* mutant exhibiting Trx inactivated genes, Trxm3 increases the sensitivity to the oxidants (93). A screen aiming at identifying the proteins implicated in cell-to-cell trafficking was conducted using an *Arabidopsis* line, which expresses a GFP under the control of the Sucrose -H^+ Symporter 2 (SUC2) promoter, allowing gene expression only in the phloem. GFP transfer into the neighboring tissues takes place through plasmodesmata. After EMS mutagenesis, 1000 F2 lines were screened for the presence of GFP limited to the phloem in the root, that is, for lines with "GFP Arrested Trafficking" (*gat*). *gat1* was severely affected; it was unable to maintain root growth and was lethal at a later stage. The targeted gene encodes AtTrxm3. A fine analysis of the mutant showed the presence of callose in the plasmodesmata as well as the accumulation of ROS. The phenotype, including callose deposition, can be mimicked either by application of exogenous H_2O_2 or by decreasing the cellular GSH content (18, 19). The reduced starch content in *gat1* root columnella cells and the partial rescue of the mutant phenotype by sucrose suggest that Trxm3 is implicated in carbon partitioning between source and sink tissues and that its inactivation induces a strong oxidative stress situation. Interestingly, a redox-dependent plasma membrane targeting and dimerization of the sucrose transporters (SUT1) has also been reported, suggesting that carbohydrate partitioning is tightly redox regulated in plants (109). Nevertheless, the link between a plastidial Trx and its potential function in plasmodesmata is not obvious. As observed in Trxm2, precursors of chloroplastic Trx can be targeted to other subcellular compartments (146). Therefore, the cellular localization of Trxm3 in relation to its function in plasmodesmata will need further investigation. It is difficult to define AtTrxm3 orthologs in other plants due to the high homology with other Trxm. Complementation of the *gat1* mutant with other Trxm should reveal whether several Trxm share the same function in other plants. Further studies on gene expression and/or the biochemical properties of the protein could help gain more insight on the characteristics of AtTrxm3 that sustain its functions.

The subcellular localization of Trx reveals other surprises. Trxz was first described as being a Cf-9-interacting Trx (CITRX) in tomato, a cytosolic Trx interacting with the Cf-9 resistance protein and negatively regulating resistance to *Cladosporium fulvum* (188). It was then shown that Trxz is an adaptor between Cf-9 and the protein kinase ACIK1 and that it acts independently of its redox activity (168). More recently, independent articles have demonstrated that Trxz is mostly a plastidial protein in *A. thaliana* (143, 176) (under the name Trxp) (1, 201). In tomato, the CITRX has the size of the Trxz chloroplastic precursor and is probably partly retained in the cytosol by its interaction with Cf-9 and ACIK1. The inactivation of Trxz in *Arabidopsis* and *Nicotiana benthamiana* profoundly affects chloroplast development (1, 143). Interestingly, *trxz* mutants are affected in transcription of the chloroplast genome, particularly the genes transcribed by the nuclear-encoded plastid RNA polymerase (NEP), while transcription of the nuclear genome is unaffected. Trxz was found to be associated with NEP, suggesting that Trxz is implicated in the transcription of the plastidial genome. Trxz was found to interact with several other proteins, including FNR and two proteins with similarities to fructokinase-like (FNL) proteins. The catalytic cysteine of the Trxz redox site is necessary for the FNL/Trxz interaction (201).

Recent data suggest the implication of plastidial Trx in the machinery, allowing protein import into the chloroplast. Three inner plastid envelope proteins were isolated on Trx affinity columns. These proteins belong to a superfamily of Rieske iron-sulfur proteins that are involved in protein translocation and chlorophyll metabolism. They include the translocon protein TIC55, the precursor NADPH:protochlorophyllide oxidoreductase translocon protein PTC52, which operates as protochlorophyllide a-oxygenase, and the lethal leaf spot protein LLS1, which is identical with a pheophorbide a oxygenase (12). Furthermore, preprotein import into chloroplasts was shown to be regulated by redox state (211). For recent reviews, see Refs. (7, 108).

c. Atypical chloroplastic Trx. Plastids also contain larger proteins with a Trx domain that is associated with other functional domains. Three genes encode APS reductase, an enzyme performing the reduction of sulfate to sulfite (137, 206). The sulfate assimilation pathway in vascular plants differs from that of *E. coli* in being dependent on APS rather than on PAPS. Furthermore, *E. coli* PAPS reductase and plant APS reductase (APR) are structurally different. While *E. coli* PAPS reductase is a single domain protein that is reduced by a Trx or, alternatively, a Grx in vascular plants, APS reductase

(APR) is composed of two domains: The APS reductase domain at the N-terminus is associated with a Trx domain that provides the reduction power for APS reduction. The association of the two domains allows sulfate reduction independently of additional Trx or Grx. In spite of showing homology to Trx, plant APS reductases are reduced by GSH, and are, thus, functionally similar to a Grx (21).

NTRC is another gene encoding a chloroplastic two-domain protein with orthologs in plants and most cyanobacteria (204). NTRC is a Trx reductase that is combined to a Trx domain. The NTR domain in the N-terminal region directly reduces a C-terminal Trx domain at the expense of NADPH. NTRC preferentially reduces Prxs (175), allowing H_2O_2 and hydroperoxide reduction when Trx are inactive in chloroplasts during the night or in nongreen plastids. NTRC inactivation reduces plant growth and photosynthesis, particularly during short days (175). NTRC is present in both green and nongreen plastids. This protein is also able to activate the AGPase, a central enzyme of starch synthesis (163). In *Arabidopsis*, an *ntrc* KO mutant shows decreased activation of AGPase and lower starch accumulation in leaves and roots during both the day and the night. In addition, the activation of the root AGPase in response to sucrose feeding is strongly reduced in the mutant. Thus, NTRC regulates starch synthesis in response to light and sucrose. In heterotrophic tissues, NTRC acts as a sensor of light and adjusts starch synthesis accordingly (151) (Fig. 8).

Most studies concerning plastidial Trx have been focused on their chloroplastic function, but several Trx proteins such as Trxy1 (48), Trxf (58), NTRC, and APR are expressed in nongreen tissues, including roots and seeds, while their implication in seed germination has also been reported for cereals (127).

Most Trx proteins in plastids are located in the stroma. In contrast, HCF164 exhibiting the unique redox center WCEVC is a transmembrane protein bridging the thylakoid lumen with the stroma (157, 171). The inactivation of the *Hcf164* gene induces a high chlorophyll fluorescence phenotype and severe deficiency in the accumulation of cytochrome b(6)f complex subunits (124). In *Chlamydomonas reinhardtii*, it was further suggested that the CCS5 protein (similar to HCF164) is involved in the assembly of two plastid *c*-type cytochromes (*f* and c_6) (72). In *A. thaliana*, HCF164 has also been proposed to transfer reducing equivalents to several proteins in the thylakoid lumen. Among them are the cytochrome *f* and Rieske FeS proteins as well as the PSI-N subunit of photosystem I (157). HCF164 itself is reduced by a stromal Trxm (157), and a CcdA protein may act as a mediator in thylakoid membranes

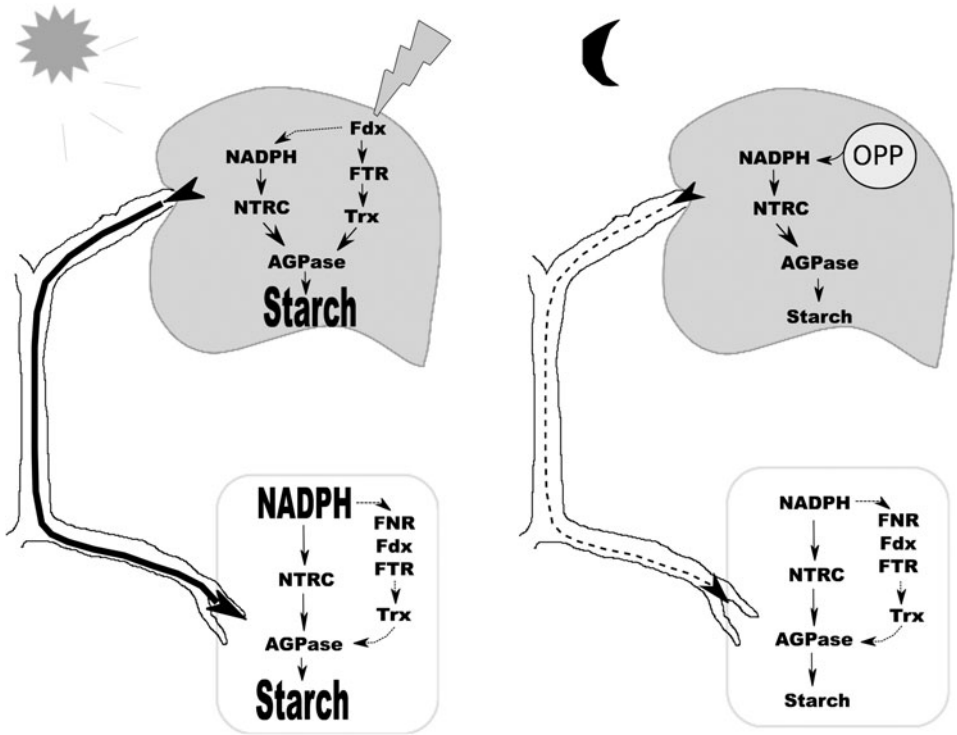

FIG. 8. NTRC regulates starch metabolism in green and nongreen tissues. Potential function of the Fdx/Trx system and NTRC in linking photosynthesis and sucrose to starch synthesis. NTRC serves as an alternate system for transferring reducing power to ADP-glucose pyrophosphorylase (AGPase) in leaves, thus enhancing storage starch synthesis. In the light (*left scheme*), NTRC is mainly linked to photoreduced Fdx *via* FNR (*dashed arrow*) and complements the FTR/Trx system in activating AGPase. In the dark (right scheme), NTRC is primarily linked to sugar oxidation *via* the initial reactions of the OPP and regulates AGPase in this way, independently of the Fdx/Trx system. Fdx/Trx and NTRC mechanisms also link light and chloroplasts in leaves to starch synthesis in root amyloplasts. When the light is on (*left*), the Fdx reduced by the photosynthetic electron transport chain serves as a source of reducing power for FTR and Trx, thereby enhancing photosynthetic CO_2 assimilation and sucrose synthesis. The increase in leaf sucrose in the light leads to enhanced long-distance transport of sucrose (*solid arrow*) and the activation of AGPase in roots *via* NTRC and, to a lesser extent, the FNR/Fdx/FTR/Trx system. In the dark (*right*), the decrease in leaf sucrose accumulation leads to decreased sucrose trafficking (*dashed arrow*) and lower starch synthesis in root amyloplasts.

by transferring reducing equivalents from the stromal to the lumenal side of the thylakoid membrane (158). The trans-thylakoid thiol-reducing pathway is implicated in the state transition, equilibrating the light excitation energy between photosystem I and photosystem II, likely by reducing protein kinase Stt7 (122). Nevertheless, it is not yet known how this redox regulation is performed.

2. Mitochondrial Trx. The mitochondrial Trx system is far simpler than the plastidial one. In *Arabidopsis*, only Trxo1 is specifically found in the mitochondria, while its paralog Trxo2 is expressed at a very low level, and its subcellular localization has not yet been established (113). In poplar, PtTrxh2 was shown to be present in mitochondria in spite of the absence of a typical mitochondrial transit peptide (78). Its ortholog, AtTrxh2, is present in both the cytosol and mitochondria (143). Little is known about the Trx function in mitochondria, although several putative target proteins have been identified by proteomics, including several Prxs, MSRs, and the membrane-bound alternative oxidase (4, 154). Some of these targets have been validated by *in vitro* enzymatic tests. PtTrxh2 is able to reduce alternative oxidase homodimers and to allow its activation in the presence of its effector pyruvate (78). The mitochondrial Trxo was also shown to serve as an efficient and specific electron donor to the pea Prx PsPrxIIF (11). No genetic data demonstrating the functions of mitochondrial Trx are available as yet. In *A. thaliana*, knockout mutants have been isolated for *trxo1* and *trxh2*, but no visible phenotype has yet been observed for either of these mutants (our unpublished data).

3. Cytosolic and nuclear Trx. In a broad sense, cytosolic Trx (*i.e.,* neither plastidial nor mitochondrial) are classified into the Trxh subgroup and several other subgroups. Some are represented by only one member in *Arabidopsis*, but they have orthologs in all vascular plants (Fig. 6). The original Trxh subgroup was split into three types when more sequence data became available. Little is yet known on the function of Trxh. Putative targets have been identified by proteomics (154, 249), including antioxidant proteins, but the specificity of Trxh has been tested in only a few cases as, for example, the reduction of NAD-MDH by Trxh1 (87) (Table 3).

Only a few genetic studies have assigned Trxh to specific functions. Among them, response to pathogens has been described in several reports. The *AtTrxh5* gene is responsive to the bacterial pathogen *Pseudomonas syringae* and is probably regulated by a WRKY transcription factor (114). Moreover, the conformation of the non-pathogenesis-related protein expressor 1 (NPR1), a master regulator of systemic acquired resistance (SAR), is regulated by the reversible oxidation of cysteine residues, switching NPR1 from a monomeric to a multimeric form (159). This redox regulation is performed through the opposing action of S-nitrosoglutathione (GSNO) and Trx (215). GSNO has been proposed to serve as a long-distance phloem mobile for SAR establishment (65). Here, both Trxh5 and Trxh3 are required for salicylate-induced NPR1 oligomer-to-monomer release during plant defense. In the *trxh3* and *trxh5* mutants, an NPR1-dependent SAR triggered after the local inoculation of an avirulent *P. syringae* ES4326/avrRpt2 strain was partially impaired. Moreover, the SAR was completely lost in the NTR mutant *ntra*, suggesting that additional Trx are involved in this response (215). In another report, a *trxh5* mutant was isolated in a genetic screen that was designed to identify resistance genes to victorin, a toxin of *Cochliobolus victoriae*. The toxicity is dependent on the dominant gene LOV1 encoding a coiled coil-nucleotide binding site-leucine rich repeat protein (CC-NBS-LRR). LOV1 confers specific sensitivity to victorin but not to several other toxins secreted by fungi. The victorin sensitivity of the *trxh5* mutant can be restored by complementation either by a wild-type Trxh5 or by a monocysteinic variant with the redox site WCPPS, but not by WSPPC or WSPPS, showing that Trxh5 acts through a monocysteinic mechanism. This mechanism might involve the highly conserved Cys507 residue in LOV1 (214).

The overexpression of AtTrxh3 has been shown to confer enhanced heat-shock tolerance in *Arabidopsis*, primarily through a chaperone function (173). Another phenotype was reported in Chlamydomonas, where deletion of Trxh1 resulted in an increased sensitivity to DNA-damaging agents, suggesting a possible role of Trxh1 in DNA repair that could not be complemented by Trxh2 (199). The last example, the phenotype associated with Trxh9 inactivation, will be described later, because this Trx presents a very specific reduction mechanism.

The tetratricoredoxin (TDX) is a two-domain protein with a cochaperone domain containing three tetratricopeptide motifs associated with a typical Trx in the C-terminal region. The TDX type is only found in vascular plants, but homologs of the cochaperone domain are present as free proteins in both animals [HIP cochaperone (89)] and plants (At4g22670 in *Arabidopsis*). AtTDX was shown to interact with Ssb2, an HSP70 chaperone, in a yeast two-hybrid test. The HIP domain of AtTDX interacts with the ATPase domain of the chaperone. The interaction between AtTDX and Ssb2 is released under oxidant conditions, but only if the Trx domain is present in association with a functional redox site (230). Furthermore, under heat shock, AtTDX shifts from a monomeric to an oligomeric state, and the activity shifts from a disulfide reductase activity toward a chaperone activity (118). The *attdx* mutant is viable and fertile under standard conditions.

The Trx-like Clot protein is encoded by a unique gene in *Arabidopsis* (42). It presents a high homology to the *Drosophila* Clot protein that is implicated in the synthesis of drosopterins, which are eye pigments (80). Clot and Sepia (encoding a dehydroascorbate reductase) are the two genes that are necessary for the reduction of dihydroneopterin triphosphate to drosopterins. The function of Clot in plants has not yet been analyzed.

Nucleoredoxins (Nrx) are composed of two or three Trx domains and contain putative nuclear localization motifs. Plant Nrx shows a limited homology with the mammalian Nrx, which is an important regulator of the Wnt/b-cathenin pathway (71). The ortholog of AtNrx1 in *Zea mays* was shown to have both cytosolic and nuclear localization (116). ZmNrx1 disulfide reduction activities have been shown *in vitro* (116), but its physiological reducer and targets have not yet been discovered. AtNrx1 was recently shown to play a major role in pollen tube growth in the pistil, but not in pollen tube growing *in vitro* (178), suggesting that this Nrx integrates signals from the maternal tissue and further guides the pollen tube to the ovule. In spite of having reduced pollen growth, *atnrx1* is male (and female) fertile.

Finally, Picot is a cytosolic protein with an N-terminal Trx domain presenting an atypical WCDAS motif and three Grx

Table 3. Preferential Reduction/Interaction of Several Target Proteins by *Arabidopsis* Cytosolic/Nuclear Thioredoxin and Grx *In Vitro*

	Artificial substrates			*Cytosolic/nuclear proteins*										
Target	*DHA*	*HEDS*	*Insulin*	*PrxII-B*	*cyt NADP-MDH*	*Scrambled RNAse*	*NPR1*	*PERIANTHIA*	*TGA1, 4–6*	*TGA2*	*TGA3, 7*	*TGA9*	*TGA10*	*HSP70*
h1			+ + +		+ + + (87)									
h2			+ + +	− (27)										
h3			+ + + (113, 205, 230)	− (27)		+ (205)	+ + + (215)							
h4			+ + +											
h5			+ + +				+ + + (215)							
CxxC1			+ (205)			+ + + (205)								
CxxC2			+ (205)			+ + + (205)								
TDX			+ + + (230)											+ + + (230)
C1	+ + (187)	+ + (187)	+ (187)	+ + (27, 187)										
C2	+ + (187)	+ + (187)	+ (187)	+ + (187)										
C3			+											
ROXY1								+ + + (126)	+ (126)	+ + (126)	+ + + (126)	+ + + (164)	+ + + (164)	
ROXY2												+ + + (164)	+ + + (164)	
ROXY18										+ + + (112)				
ROXY20							+ + + (166)			+ + + (166)				

The signs + or − indicate the activity levels, while the blank cells correspond to untested combinations. Data for which references are not indicated correspond to personal unpublished data. NPR1, nonpathogenesis related protein expressor 1; TDX, tetratricoredoxin.

domains, each with a K/RCGFS redox motif. Similar proteins previously designated as Picot (protein kinase C-interacting protein with a Trx homology domain) were described in animals (239). AtPicot will be presented in the Grx section.

IV. Grx-Dependent Reduction System in Plants

As previously described, Grx are generally reduced by GSH (Table 4). Glutathione is present in most cell compartments, where its levels and redox state are regulated at multiple levels (for recent reviews, see 70, 169).

A. Glutathione: synthesis, reduction, and compartmentation

Glutathione is synthesized in two steps that are catalyzed by gamma-glutamyl cysteine synthetase (GSH1) and glutathione synthetase (GSH2) (Fig. 9 and Table 5). In vascular plants, GSH1 is exclusively located in plastids, while GSH2 is dually targeted to plastids and cytosol (174, 235). As in other eukaryotes, GSH is required for plant development, as shown by the embryo lethality of *gsh1* knockout lines (32). In *Arabidopsis*, forward genetic screens have allowed the isolation of several mutants, which, after cloning and sequencing of the mutated genes, appear to be weak alleles of *GSH1*. The first mutant allele that was discovered was rootmeristemless 1 (*rml1*), which encodes an inefficient GSH1, allowing ~3% GSH synthesis in comparison to the wild-type (Fig. 10) (228). In the *rml1* mutant, homozygote seeds are formed with a normal embryo, but the root meristem fails to grow during germination. In contrast, the shoot meristem develops at least in the initial stage. Three other *gsh1* alleles are available: phytoalexin-deficient 2-1 (*pad2*-1) with about 16% GSH, cadmiumsensitive 2 (*cad2*) with about 20%–30% GSH, and regulator of APX2 1-1 (*rax1-1*) with 50% GSH. All these mutants present normal development under standard conditions and are fertile (Table 6) (2, 46, 91, 172). The *pad2-1* mutant was isolated on the basis of its sensitivity to several pathogens, possibly due to the inefficient production of phytoalexins and glucosinolates (172). *cad2* is hypersensitive to cadmium, possibly due to a limited synthesis of phytochelatins, which are GSH polymers (46, 91). *rax1-1* was isolated as a mutant constitutively overexpressing the *APX2* gene under photooxidative stress, suggesting that the level of glutathione interferes with the photooxidative stress signaling (2). In contrast to the *gsh1* KO mutant, a null mutant in *GSH2* shows normal embryogenesis, but the seeds do not germinate. The *gsh2* mutant accumulates high levels of the substrate of GSH2, gamma-GC, suggesting that this low-molecular-weight thiol only very partially compensates GSH in the early stage of plant development (174). Interestingly, Pasternak *et al.* (174) have shown by complementation of a *gsh2* mutant that the cytosolic GSH2 is sufficient for GSH synthesis, showing that gamma-GC is exported from the plastids to supply the cytosol with the precursor of GSH biosynthesis and that GSH is efficiently reimported into the plastids and mitochondria (Fig. 9). Several types of glutathione transporters have been described. Transport through the plasma membrane is performed by several homologs of oligopeptide transporters (24, 29, 177, 257). Tonoplast multidrug resistance-associated protein (MRP) transporters of the ATP-binding cassette family are able to transport GS-conjugates or GSSG from the cytosol to the vacuole (69, 138, 180). Recently, a genetic screen designed to isolate *Arabidopsis* suppressors of sensitivity to the glutathione biosynthesis inhibitor buthionine sulfoximine (BSO) allowed the isolation of three genes homologous to the *Plasmodium falciparum* chloroquine-resistance transporter. The corresponding proteins, called CLT1, 2, and 3, are suspected to be inner chloroplast envelope transporters that are involved in the chloroplast/cytosol transport of GSH and gamma-GC, and they regulate GSH levels in the cytosol (141). Recently, it has been demonstrated that the development of *gsh1* and *gsh2* embryos to a late stage is dependent on the level of GSH in the maternal plant, supporting the fact that glutathione is fed to the embryo by the maternal tissues (130).

Glutathione catabolism is known to play a major role in glutathione homeostasis. In *Arabidopsis*, it has been estimated to be at $30\,nmol.g^{-1}\ FW.h^{-1}$ (170), while the typical leaf glutathione contents are $300\,nmol.g^{-1}$ FW. Three major types of enzymes are involved in this regulation: carboxypeptidases, phytochelatin synthases, and gamma-glutamyl transpeptidases (GGT) (22, 23, 142, 210). Analyses of *Arabidopsis* mutants have described functions of the GGT1 and GGT2 in preventing oxidative stress by metabolizing extracellular GSSG and transporting glutathione into developing seeds (170).

By using independent techniques, consistent levels of total glutathione were found in almost all cellular compartments: plastids, mitochondria, peroxisomes, cytosol, nucleus, and apoplast (88, 98, 145, 170, 226, 255). Recent experiments using immunogold cytochemistry based on anti-glutathione antisera and transmission electron microscopy have been used to determine the relative concentrations of glutathione in different organelles of *A. thaliana*. Surprisingly, the highest level was found in mitochondria of both leaf and root cells that contained, respectively, sevenfold and fourfold higher glutathione levels than plastids, while the densities of glutathione labeling in the cytosol, nuclei, and peroxisomes were intermediate (255). How glutathione is imported into mitochondria or whether glutathione is synthesized in mitochondria is still unclear. A massive recruitment of glutathione into the nucleus was observed in proliferating *Arabidopsis* cells stained by the GST-dependent GSH-conjugating fluorescent agent 5-chloromethylfluorescein diacetate. This observation, showing a high abundance of GSH in the nucleus and severe depletion of the cytoplasmic GSH pool, suggests that extensive modifications of the whole-cell redox state occur during cell proliferation (232).

GSSG is reduced by GR. In plants, two genes encode dual-targeted GR, called GR1 and GR2. In *Arabidopsis*, GR1 encodes a cytosolic and a peroxisomal protein, and GR2 encodes a precursor targeted to both the chloroplast and the mitochondrion (Table 5) (41, 54, 103). GR2 inactivation is lethal at an early stage of embryo formation (225). It has not yet been established whether the embryo lethality of the *gr2* mutant is due to inactivation of the chloroplastic and/or the mitochondrial isoform of GR2. However, in the *gr2* mutant, the cytosolic GR1 is not able to compensate for the absence of organellar glutathione reduction. In contrast, the *gr1* mutant is aphenotypic under standard growth conditions in spite of producing 70% of the total GR activity compared with the wild type (139, 149). Despite accumulating high levels of GSSG, *gr1* is not hypersensitive to exogenously applied H_2O_2 (139). In *Arabidopsis*, the catalase 2 (*cat2*) mutant is inactivated in the major catalase and shows reduced growth in air. This

Table 4. Grx in *Arabidopsis*

Short name	*AGI identifier*	*Localization*	*Subgroup*	*Redox site*	*Nter sequence*	*Production in* E. coli	*Reducer*	*FeS cluster*
AtGrxC1	At5g63030	Cytosol (187)	Subgroup I Dicysteinic	YCGYC		(187, 193)	GSH (187)	Yes (187, 193)
AtGrxC2	At5g40370	Cytosol (187)		YCPYC		Soluble (187)	GSH (187)	no (193)
AtGrxC3	At1g77370	Cytosol		YCPYC		Soluble (193)	GSH	no (193)
AtGrxC4	At5g20500	Cytosol		YCPYC		Soluble (193)	GSH (191)	no (193)
AtGrxC5	At4g28730	Plastid (53)	Subgroup I WCSY[C/S]	WCSYC		Soluble (53)	GSH (53)	yes (53)
AtGrxS12	At2g20270	Plastid (53)		WCSYS		Soluble (193)	GSH (51)	no (51)
AtGrxS14	At3g54900	Plastid (8, 36)	Subgroup II CGFS	MCGFS		Soluble (125)	FTR putative	yes (8)
AtGrxS15	At3g15660	Mitochondria (8)		QCGFS		Soluble	?	no (8)
AtGrxS16	At2g38270	Plastidmitochondria (8)		QCGFS		Soluble	FTR putative	yes (8)
AtGrxS17Picot	At4g04950	Cyto (39)	Subgroup II 1 Trx	WCDAS RCGFS KCGFS		Soluble (personal data)	?	yes (8)
			3 Grx CGFS domains	KCGFS				
ROXY1	At3g02000	Cytosol/nuclear (126)	Subgroup III ROXY	TCCMC		One poplar ROXY member PopS7.2 is insoluble (52)	The redox activity of ROXY1 is established by complementation of the mutant. GSH is the most probable reducer.	?
ROXY2	At5g14070	Cytosolputative		TCCMC				
ROXY3	At3g21460			TCCMS				
ROXY4	At3g62950			SCCMC				
ROXY5	At2g47870			SCCMC				
ROXY6	At1g06830			SCCLS				
ROXY7	At2g30540			SCCMS				
ROXY8	At3g62960			SCCLC				
ROXY9	At2g47880			SCCLC				
ROXY10	At5g18600			SCCMS				
ROXY11	At4g15700			SCCMS				

(*continued*)

Table 4. (Continued)

Short name	*AGI identifier*	*Localization*	*Subgroup*	*Redox site*	*Nter sequence*	*Production in* E. coli	*Reducer*	*FeS cluster*
ROXY12	At4g15690			SCCMS				
ROXY13	At4g15680			SCCMS				
ROXY14	At4g15670			SCCMS				
ROXY15	At4g15660			SCCMS				
ROXY16	At1g03020			SCCMS				
ROXY17	At3g62930			SCCMS				
ROXY18	At1g03850	Cytosol (53)		GCCLG				
ROXY19	At1g28480	Cytosolputative		GCCMC				
ROXY20	At5g11930	Cytosol (193)		SCCMC				
ROXY21	At4g33040	Cytosolputative		SCCMC				
4CxxC1	At1g32760		Subgroup IV 4CxxC		MGGCVSSNLL	?	No redox activity established as yet for this family	?
4CxxC2 THRUMIN1	At1g64500	Membrane associated (237)			MGCTSSKQAK	An N-ter truncated variant is soluble (237)		
4CxxC3	At2g41330				MGSSVSKTAS	?		
4CxxC4	At3g28850				MGCASSKHRK			
4CxxC5	At3g57070				MGSSASKTAS			
4CxxC6	At4g10630				MGCVSSNLLN			
4CxxC7	At5g01420				MKSSRMKFAK			
4CxxC8	At5g03870				MGCVSSKLGK			
4CxxC9	At5g06470				MKGMKERLVK			
4CxxC10	At5g13810				MAGLE KNADF			
4CxxC11	At5g39865				MGCAS SKNRN			
4CxxC12	At5g58530				MWRPWRKSS			
4CxxC13	At3g11773				MMDMKERMV			
CPFC1	At4g10000	Plastid putative	Subgroup V GST-like CPF[C/A]	ACPFC		?	?	?
CPFC2	At5g16705	Plastid putative		DCPFC				
CPFC3	At5g42150	Mitochondria putative		ACPFC				
CPFA3	At5g02780	Cytosol putative		TCPFA				
CPFA4	At5g02790	Cytosol putative		VCPFA				
CPFA5	At3g55040	Cytosol putative		TCPFA				

?, unknown.

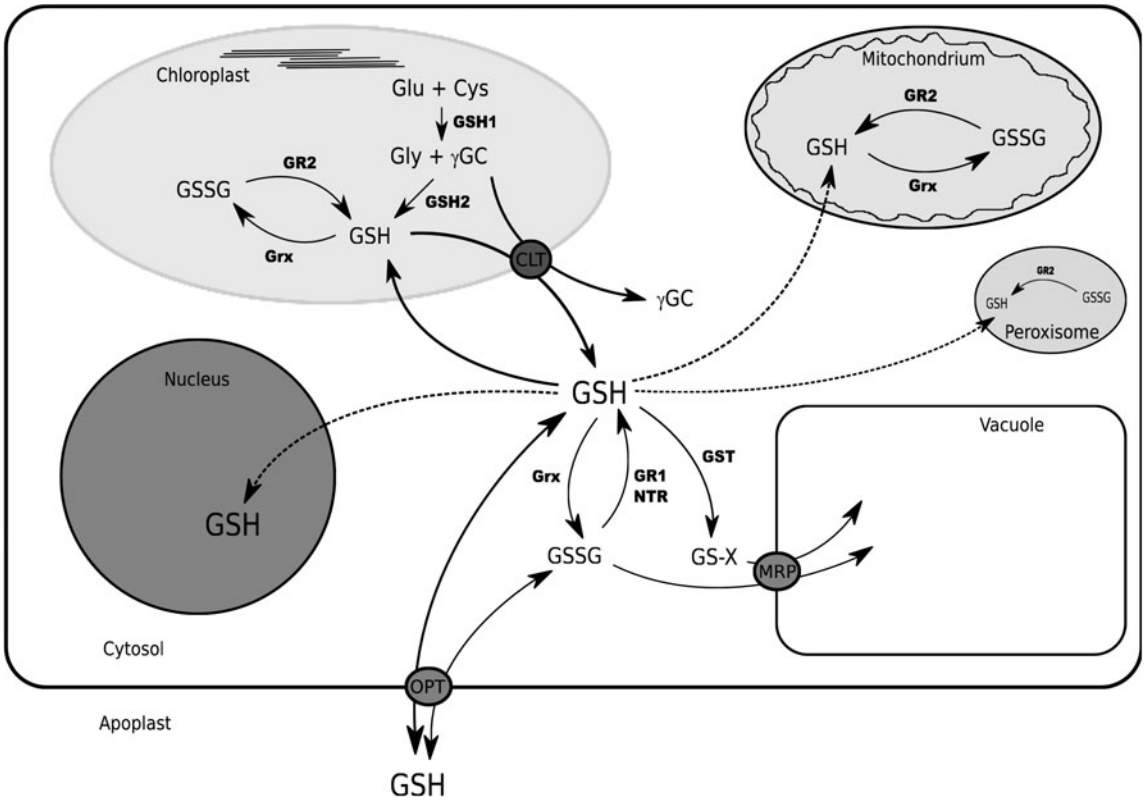

FIG. 9. Glutathione synthesis, reduction, and compartmentation in plant cells. Glutathione synthesis, reduction, and fluxes are represented by *blacks arrows*. Enzymes are represented by bold characters. When the glutathione fluxes are not clearly demonstrated, *broken lines* are used. Glu, glutamate; Gly, glycine; Cys, cysteine; γGC, gamma-glutamyl cysteine; GST, glutathione-S-transferase; CLT, chloroquine-like transporter; OPT, oligopeptide transporters; MRP, multidrug resistance-associated protein.

phenotype is due to the inability of the *cat2* mutant to scavenge the excess H_2O_2 produced in the peroxisomes during photorespiratory metabolism. This phenotype is rescued under high CO_2 conditions that inhibit photorespiratory metabolism (179). Interestingly, the double *gr1 cat2* mutant is highly affected under air conditions, showing that GR1 is implicated in oxidant defence when H_2O_2 accumulates endogenously (149). Moreover, the *gr1* mutation alters responses to pathogens and the expression of genes that are involved in defence hormone signaling, notably jasmonic acid-associated genes (149).

B. Grx and putative target proteins

The classification of vascular plant Grx was more difficult to establish than that of the Trx. Phylogenetic analyses based on similarity as well as characteristics of the redox centers define five subgroups (Fig. 11 and Table 4) (50). These include typical Grx (MW about 10–12 kDa) as well as proteins with multiple Grx domains or that are associated with other domains. In contrast to Trx, members of the five subgroups are not found in all species of the green lineage: subgroups III and IV are specific to vascular plants (147). Moreover, all members in a single subgroup do not have the same subcellular localization.

1. Subgroup I: C[P/G/S]Y[C/S] Grx. Six genes of this subgroup are encoded by the *Arabidopsis* genome. Four of them encode cytosolic Grx (C1, C2, C3, C4), while the other two members, C5 and S12, are plastidial. These isoforms have been well characterized biochemically in both *Arabidopsis* and poplar (51, 53, 187, 193, 253). Most of them have both disulfide reductase and deglutathionylation activities, and they are reduced by glutathione. Recent studies suggest a potential role for some of these Grx in redox sensing and redox signaling in the cytosol and chloroplasts (51, 53, 187, 253). A proteomic approach performed with poplar PtGrxC2 has identified 94 putative targets that are involved in many processes, including oxidative stress response, nitrogen, sulfur and carbon metabolisms, translation, and protein folding. Some of these proteins have been previously found to interact with Trx or to be glutathionylated in other organisms, but others could be more specific partners of Grx (192).

A very important point was the demonstration that PtGrxC1 (the ortholog of AtGrxC1 in poplar) is able to dimerize by incorporating an Fe-S cluster that is relatively stable under aerobic conditions (193). The holoprotein contains a subunit-bridging [2Fe-2S] cluster that is ligated by the catalytic cysteines of two Grxs and the cysteines of two glutathione molecules. This dimerized form is not only present in the recombinant protein, but has also been identified in plant protein extracts after chemical crosslinking during protein extraction (187). Nevertheless, it has not yet been demonstrated that this dimerized form incorporates a [2Fe-2S] cluster *in vivo*. AtGrxC1 is localized in the cytosol, and homologs having the same CGYC active site are found only in

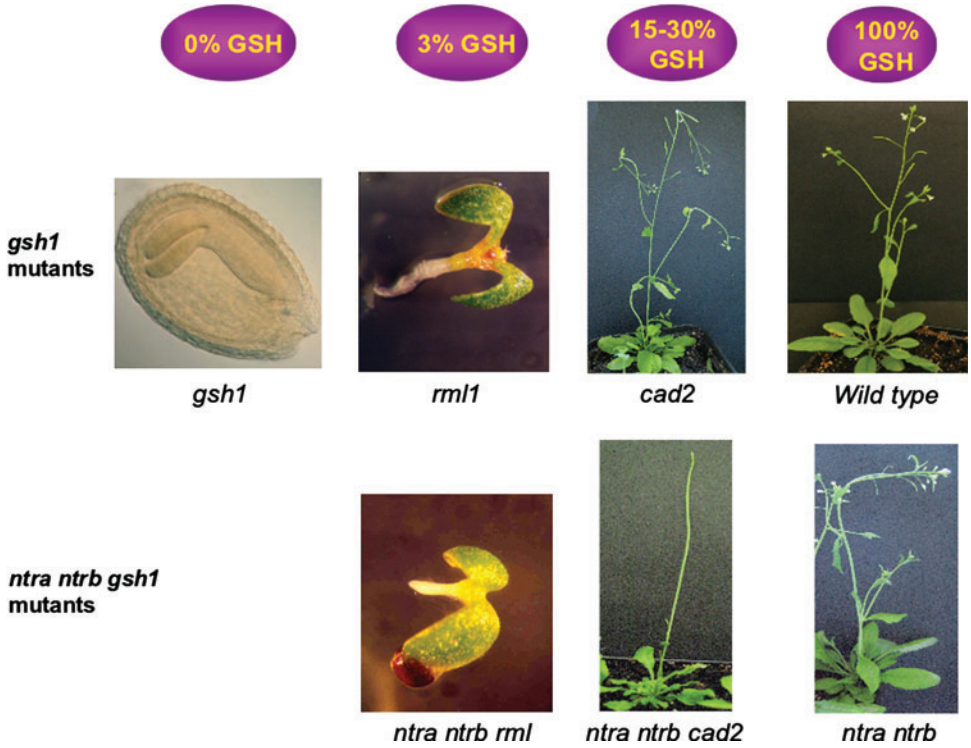

FIG. 10. Genetic crosstalk between glutathione and NTR pathways. The upper set of homozygous mutants represents the phenotypes of different conditional *gsh1* mutants. The percentages of total glutathione in the respective mutants as compared with wild-type plants have just been represented. The *gsh1* knockout mutant forms seeds with defective embryos. The rootmeristemless 1 (*rml1*) mutant, a severe allele of *GSH1*, germinates but is unable to maintain a functional root meristem. The cadmiumsensitive 2 (*cad2*) mutant, a mild allele of *GSH1*, has a normal development and is fertile. The lower set of mutants represents the phenotype of the same *gsh1* mutants in an *ntra ntrb* mutant background. *rml1 ntra ntrb* are unable to maintain both shoot and root meristems activities. *cad2 ntra ntrb* develops normally, but no flower is formed. The stem apex resembles a pin-like structure. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

dicot genomes (187). The sequence requirement to form a Fe-S bound dimer was analyzed by mutagenesis, showing the important role of a glycine following the first cysteine of the redox site (51, 53). Three other subgroup I Grx (C2, C3, and C4) have a proline in place of the glycine and are apparently unable to form FeS dimers, suggesting that the presence of the proline prevented Fe-S cluster assembly. This has been supported by mutagenesis studies that are conducted with both plant and human Grxs (20, 193). Nevertheless, the recently identified trypanosome Grx possessing a TCPYC active site is able to incorporate an Fe-S cluster (35). Moreover, *A. thaliana* GrxC5, presenting a WCSYC active site, was also shown to be able to form an Fe-S bound dimer, indicating that other structural determinants than the active-site amino acids are certainly involved in the formation of the Fe-S cluster (53). However, the GrxS12 isoform exhibiting a WCSYS active site is exclusively apomonomeric (51).

Both GrxC1 and GrxC5 are found as apo- and holoforms, but both proteins exhibit distinct characteristics. The apoform has disulfide and deglutathionylation capacities that are not found in the holoform. This is likely due to the fact that the catalytic Cys is involved in the Fe-S cluster (53, 193). Moreover, in GrxC1, the switch between the apo- and the holoform was shown to be dependent on redox conditions, suggesting that it could act as a redox sensor, responding to oxidative conditions (187), as suggested for human Grx (129) (Fig. 12). As described later, the ability of Grx to form a dimer with Fe-S clusters is not limited to GrxC1 and GrxC5.

In *Arabidopsis*, genetic studies have been performed on the *GrxC1* gene and its closest homolog *GrxC2* (187). The *atgrxc1* mutant is aphenotypic, but crossing the mutant with *atgrxc2*, which is itself also aphenotypic, does not allow isolation of homozygous double mutants. The female gametophytes *atgrxc1 atgrxc2* form viable seeds and plants when pollinated with wild-type pollen. Moreover, *atgrxc1 atgrxc2* pollen efficiently pollinates wild-type ovules, showing that the failure to obtain a double homozygote mutant is not due to gametophytic deficiencies but most probably to early lethality during embryo development (187). Since AtGrxC2 is unable to form [2Fe-2S] clusters, the lethality of the *atgrxc1 atgrxc2* double mutant is likely independent of the ability of AtGrxC1 to undergo [2Fe-2S] dimerization (187).

2. Subgroup II: CGFS Grx. The second subgroup of Grx is composed of four proteins in *Arabidopsis*, GrxS14, GrxS15, GrxS16, and GrxS17, all having the monocysteinic CGFS catalytic site. S14 and S16 are plastidial, S15 is mitochondrial, and S17 (Picot) is cytosolic (8, 38). While S14, S15, and S16 are monodomain Grx, S17 is a multidomain protein with a Trx-like domain and three CGFS Grx domains (Table 4). All

Table 5. Thioredoxin Reduction, Reduced Glutathione Synthesis, and Oxidized Glutathione Reduction in *Arabidopsis*

	AGI	*Localization*	*Activity*
NTRA	At2g17420	Cytosol	NADPH-dependent Trx reductase
NTRB	At4g35460	Cytosol	NADPH-dependent Trx reductase
FTR cat	At2g04700	Plast	Fdx-dependent Trx reductase catalytic subunit
FTR var1	At5g23440	Plast	Fdx-dependent Trx reductase variable subunit
FTR var2	At5g08410	Plast	Fdx-dependent Trx reductase variable subunit
GR1	At3g54660	Plast/mito	GR
GR2	At3g24170	Cytosol	GR
Gsh1	At4g23100	Plast/cytosol	Gamma-glutamylcysteine synthetase
Gsh2	At5g27380	Cytosol	Glutathione synthetase

Fdx, ferredoxin; GR, glutathione reductase.

members of this subgroup, except GrxS15, are also able to form Fe-S clusters. In poplar, PtGrxS14 and PtGrxS16 are able to transfer their iron cluster to an Fdx in an *in vitro* system (8, 9). Even though they have an Fe-S cluster, neither GrxC1 nor GrxC5 is able to perform the same transfer, suggesting that they may have a different substrate specificity (Fdx is a plastidial protein, while GrxC1 is cytosolic) or, alternatively, have a different function. The ability of several plant CGFS Grx to regulate Fe metabolism and iron cluster formation is further supported by their capacity to complement an *S. cerevisiae* mutant inactivated in its *Grx5* gene. The *grx5* mutant is unable to transfer an Fe-S cluster to several enzymes that are involved in the respiratory chain. Therefore, this mutant is no longer able to grow aerobically (38, 193). In contrast, PtGrxC1, AtGrxC1, and AtGrxC5 are unable to complement the *S. cerevisiae grx5* mutant, suggesting a different function from that of the CGFS subgroup. Therefore, in plants and other organisms, the reversible dimerization of several Grxs displaying an iron-sulfur cluster might allow them to act both as sensors of cellular iron and as scaffold proteins or carrier proteins of iron-sulfur clusters (Fig. 12) (53, 187, 195, 196).

Genetic analyses have revealed the potential roles of some CGFS Grx. The KO mutant of *atgrxs14* was shown to be hypersensitive to H_2O_2, suggesting a function of the GrxS14 in the chloroplastic antioxidant mechanism (37). Another study has demonstrated the implication of the cytosolic AtGrxS17 in auxin-dependent development. The *atgrxS17* KO mutant and some RNAi lines affecting AtGrxS17 were analyzed. At 22°C, the KO and RNAi plants are slightly shorter than the wild-type plants but develop well, and flowers are fertile. In contrast, at 28°C, the mutant does not form flowers, but instead, leaves are present near the meristem (39). Unfortunately, in this study, the structure of the meristem was not analyzed in detail. At 28°C, auxin transport (but not auxin level) is reduced in *atgrxS17*. The phenotype of the *atgrxS17* mutant is rescued at 22°C. For both GrxS14 and GrxS17, it is not known whether their characteristics allowing the formation of Fe-S clusters are related to their respective functions in chloroplastic antioxidant and auxin signaling mechanisms.

Recombinant Grx of most members of the first two subgroups have been produced in *E. coli*, and certain biochemical characteristics have been studied for several of them. Dicysteinic members show some dithiol disulfide reduction activity in insulin, a classical artificial substrate, in the presence of GSH (187). Nevertheless, this activity occurs with low efficiency compared with Trx (53, 74, 252). More consistently, monocysteinic Grx were shown to reduce several types of Prx, MSR, and various other target proteins (Tables 2 and 3).

3. Subgroup III: CCx[C/S/G] or ROXY Grx. Subgroup III, now named ROXY, is composed of 21 members in *Arabidopsis*, all putatively located in the cytosol and encoding proteins with a high similarity. The common characteristic of this subgroup is the presence of a redox site CCx[C/S/G]. ROXY genes are only present in land plant genomes, with two members in *Physcomitrella* and three in *Selaginella*. The gene number increases to 5 in *Pinus* and expands far more in flowering plants (258).

The biochemical characteristics of ROXY (Subgroup III) proteins have not yet been identified, because the proteins either cannot be produced or are insoluble in *E. coli* (166). Most members contain conserved residues which are necessary for glutathione binding, suggesting that they might be reduced by glutathione. Artificial variants suggest that the CCxC redox site may be compatible both with [2Fe-2S] dimerization and with a disulfide reductase activity as monomer (52).

Table 6. Available Gamma-Glutamyl cysteine Synthetase Alleles

Short name	*Long name*	*GSH content/wild-type content*	*Phenotype*
KO	Inactivated gene (32, 130)	0%	Embryo lethal
rml1	Root meristem less (228)	3%	No growth of the root meristem in seedlings
pad2-1	Phytoalexin-deficient2-1 (172)	16%	Hypersensitivity to *Pseudomonas syringae* and *Phytophthora brassicae*
cad2-1	Cadmium-sensitive 2-1 (46, 91)	20%–30%	Aphenotypic under standard conditions Hypersensitivity to Cd and Cu
rax1-1	Regulator of APX2 (2)	50%	Constitutive expression of APX2

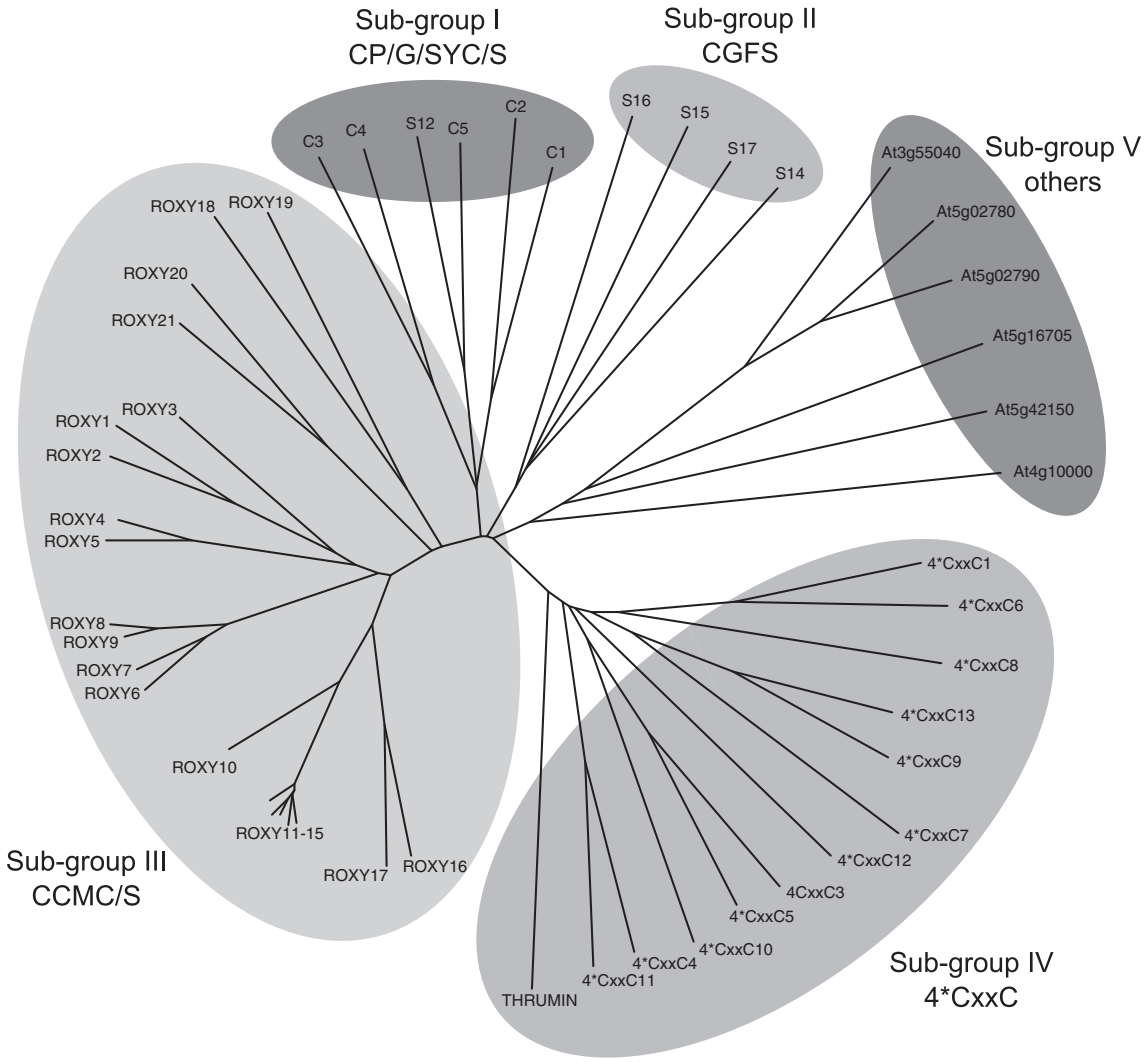

FIG. 11. Phylogenic tree of *A. thaliana* Grx. Members of the different subgroups of Grx were clustered in five different subgroups. The AGI numbers corresponding to each protein are presented in Table 4.

Moreover, we cannot exclude the possibility that the second cysteine of the C**C**x[C/S] site plays a redox function in the ROXY proteins. Such a mechanism has been recently suggested for the C-terminal extension of the *Drosophila* NTR (111). Understanding the function of ROXY members in development and defence in detail (see later) depends on our understanding of the enzymatic and structural characteristics of these proteins. Using another system for the production of the recombinant proteins may help to obtain soluble proteins (258).

Genetic evidence for the role of some ROXY members in *Arabidopsis* floral development originated from a genetic screen aiming at the identification of floral mutants having a variable and reduced number of petals. While wild-type *Arabidopsis* have flowers with four petals, mutant plants have two to four petals, with a mean value of 2.5. Cloning the mutated genes identified a Grx, which was named ROXY1. This gene encodes a Grx that belongs to the subgroup III in which the redox center is a CCMC. ROXY1 is theoretically able to reduce disulfide bridges through a monocysteinic or a dicysteinic mechanism (246). Complementing the *roxy1* mutant with a CCxS variant of ROXY1 restores the wild-type phenotype, suggesting that ROXY1 acts on the petal number through a monocysteinic mechanism, at least under the hypothesis that the redox activity of ROXY follows the same mechanism as other Grx. ROXY1 has a cytoplasmic/nuclear localization, but only the nuclear form is able to complement the ROXY1 phenotype (126). Since the ROXY family is composed of 21 genes in *Arabidopsis*, complementing the *roxy1* mutant with the coding sequence of several ROXY coding sequence under the control of the ROXY1 promoter restores the wild type in 7 cases out of the 12 ROXY members tested (126). This indicates that the expression pattern is particularly important for the function of the gene. ROXY1 is expressed early during flower formation in organ primordia, an expression pattern similar to PERIANTHIA (PAN, At1g68640), a bZIP transcription factor implicated in floral development and TGA2 (At5g06950), and another bZIP transcription factor expressed early during flower formation (126). A direct interaction between ROXY1 and PAN and different TGA transcription factors was shown in a yeast two-hybrid system and *in planta* in a tobacco system (126). A conserved C-terminal α-helical L**LL motif of ROXY possibly mediates the interaction

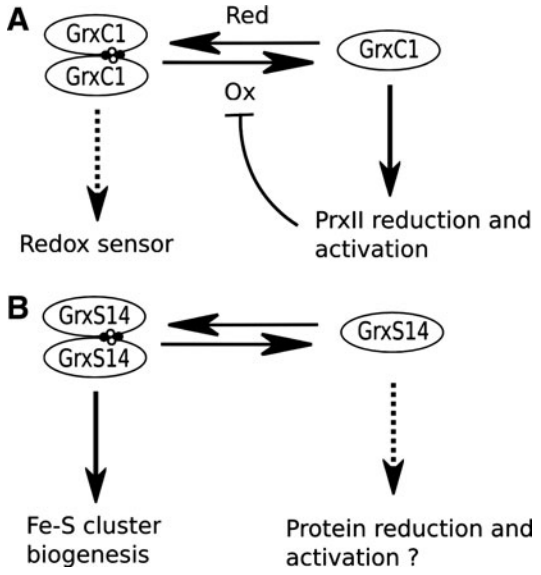

FIG. 12. Potential functions of subgroup I and II [2Fe-2S] cluster Grxs. (A) Cytosolic subgroup I GrxC1 likely oscillates between an apomonomer and a holodimer state binding an [2Fe-2S] cluster. This oscillation is dependent on redox constraints. An oxidative environment such as excess H_2O_2 or GSSG destabilizes the holodimer. However, reductive conditions such as DTT or GSH stabilize the holodimer. The apomonomer has disulfide bridge reduction capacities and activates PrxII, which may counterbalance oxidative conditions. The holodimer is unable to reduce disulfide bridges and may play distinct roles. **(B)** Chloroplastic subgroup II GrxS14 also likely oscillates between an apomonomer and a holodimer state. The apomonomer may exhibit deglutathionylation activity, but this activity has not yet been proved. The holodimer is able to transfer its [2Fe-2S] cluster to other proteins and has Fe-S cluster biogenesis capacities.

with TGA transcription factors (128). PAN has five cysteines, but only Cys340 is mandatory to complement the *pan* phenotype. TGA2 has no cysteine at the equivalent position but three other members of this bZIP family, At5g06839 (expressed in leaf whorl, flower, root, and seed), At5g10030 (TGA4), and At5g65210 (TGA1), described as a transcription factor and calmodulin-binding protein expressed in most tissues, have a cysteine at this position. They are putative target proteins for some members of the ROXY family, particularly those complementing the petal phenotype when expressed under the control of the ROXY1 promoter. The inactivation of ROXY2, the nearest homolog of ROXY1, is aphenotypic, but the double mutant *roxy1 roxy2* is impaired in anther formation and is male sterile. This demonstrates that ROXY1 and ROXY2 play a redundant function in another development (247). A phenotype similar to the *roxy1 roxy2* mutant is observed in the double mutant *tga9 tga10*. Consistently, both TGA9 and TGA10 interact with ROXY1 and ROXY2, suggesting that this interaction has a physiological impact on another development (164).

Members of the ROXY subgroup are also involved in the responses to pathogens. In *Arabidopsis*, an ectopic overexpression of the ROXY homolog leads to a higher susceptibility to the necrotrophic pathogen *Botrytis cinerea* (236). Moreover, ROXY19 is inducible by jasmonic acid, and its activity requires both the NPR1 and TGA transcription factors that are implicated in host/pathogen interactions (166). Interestingly, another member of the ROXY family, ROXY18, is required for plant infection by the necrotrophic fungus *B. cinerea*. In contrast to ROXY19, *ROXY18* gene expression is negatively controlled by jasmonic acid and TGA transcription factors but positively controlled by a *B. cinerea*-induced salicylic acid/NPR1 pathway (112). Both NPR1 and TGA factors exhibit cysteines that are prone to redox modifications and affect their activities (159, 190, 215). However, the mechanisms by which Grxs regulate TGA transcription factors remain to be determined. It has also been reported that a splicing variant of ROXY19 is involved in the protection against photooxidative stress in *Arabidopsis* (115).

4. Subgroup IV: 4CxxC Grx. The fourth Grx subgroup is composed of proteins with a Grx domain followed in the C-terminus by four CxxC repeats that may form a zinc finger (147, 165). We do not know much about the biochemical characteristics of the 4CxxC subgroup. Proteins with similar sequences are encoded by most eukaryotic genomes. No recombinant protein has been produced so far. A genetic screen that has been developed to select mutants which are unable to move their chloroplasts has identified *thrumin1*, a mutant which is inactivated in one 4CxxC Grx (238). In order to optimize light energy recovery, chloroplasts in mesophyll cells move toward light under low illumination but move away under strong illumination to avoid an overflow of the electron flux and production of ROS. Chloroplast movement is, in fact, induced by blue light *via* a photoreceptor and is transduced to the actin cytoskeleton at the level of the plasmalemma (212). THRUMIN1, similar to several 4CxxC Grx, presents an MGC N-terminal sequence, allowing myristoylation on the Gly residue and that is responsible for membrane anchoring. In addition, it has a Cys residue allowing palmitoylation. Thus, it is possible that THRUMIN1 is anchored on the plastidial membrane and interacts with the actin cytoskeleton to guide chloroplast movements. Nevertheless, it has not yet been established whether the chloroplast movements are controlled by redox changes.

The number of *Grx* genes within subgroups III and IV is not the same among different plant species, and it is, in most cases, not possible to define reliable orthologous relationships.

5. Subgroup V: CPF[C/S] Grx. The last subgroup is composed of six members that exhibit a dicysteinic CPFC or monocysteinic CFPS. Some members are located in the cytosol, mitochondria, and chloroplast. Biochemical evidence is lacking with regard to these members. Based on sequence homologies, four members have been classified within lambda-type glutathione S-transferases (GSTL), and close homologs of wheat have been shown to deglutathionylate flavonoids (62, 63).

V. Isolation of Trx and Grx Targets

Several strategies aiming at identifying Trx and Grx targets have been developed in the last decades. As just described, several targets have been identified by systematic activity tests under oxidizing or reducing conditions. More recently, proteomic approaches have been developed that systematically identify redox-regulated proteins. They were based on two techniques. As shown in Figure 13A, the first is

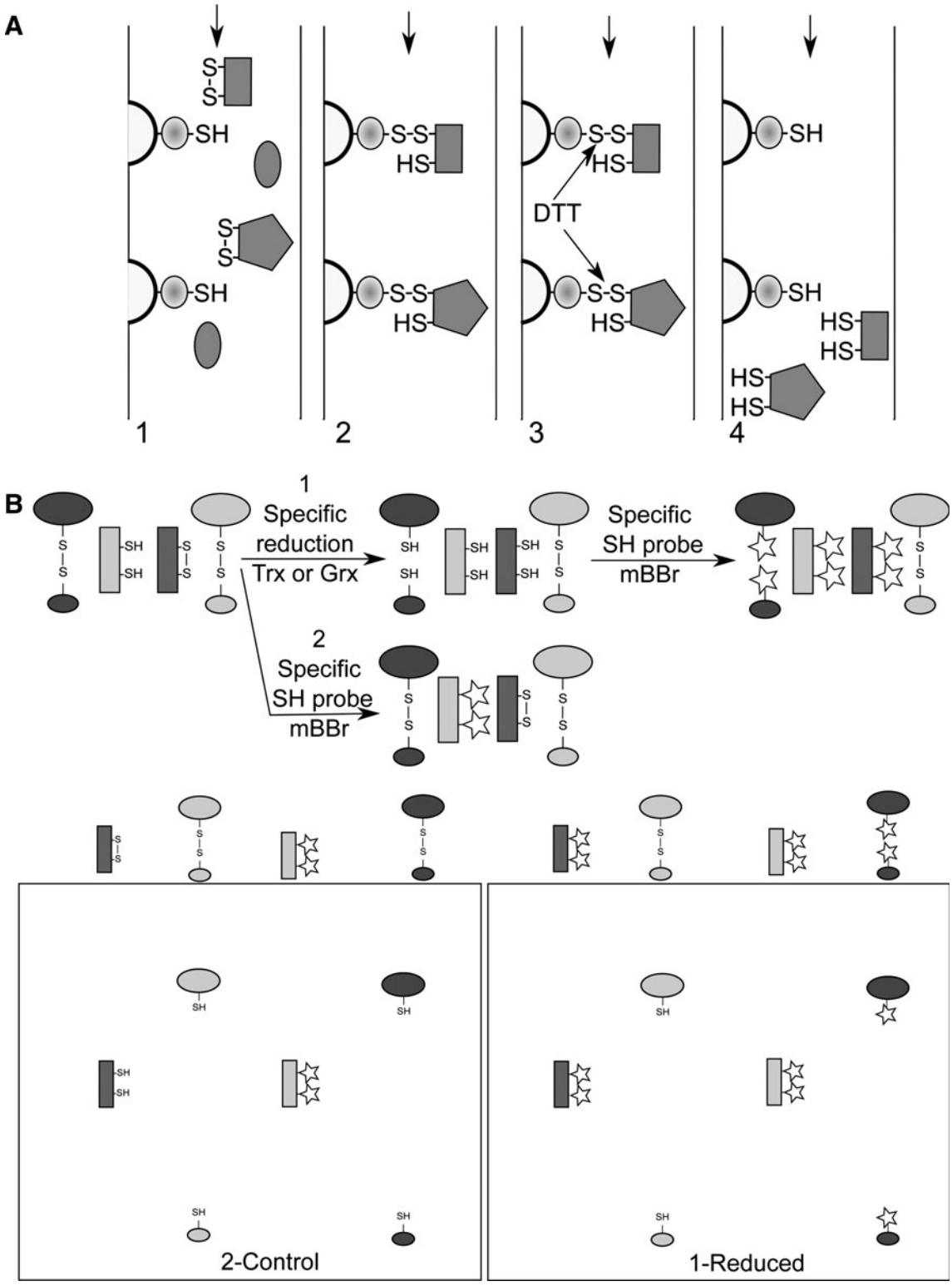

FIG. 13. Proteomic strategies that isolate Trx or Grx targets. (A) Isolation of Trx target proteins by affinity chromatography. Monocysteinic Trx or Grx variants (catalytic thiol) are grafted on column resin, and crude protein extracts are applied to the column [1]. Exposed disulfide bridges of Trx target proteins are attacked by the catalytic cysteine, allowing stable, mixed disulfide complex formation [2]. The column is washed to eliminate nonbound proteins, and the complex is then reduced by DTT [3], allowing recovery of the putative targets that are identified by mass spectrometry [4]. **(B)** Isolation of Trx or Grx target proteins by labeling and electrophoresis. A crude protein extract is treated by Trx or Grx for reducing the disulfide bridges [1]. Free thiols are then labeled with the fluorescent dye mBBr, and the proteins are separated by one- or two-dimensional electrophoresis, allowing identification by mass spectrometry. To detect unspecific thiols, the crude protein extract is directly labeled with mBBr [2]. Stars represent mBBr-labeled thiols.

based on the fact that an intermediate complex is formed between Trx and its target. It was first shown that a mutation of the second cysteine of the Trx redox center to serine stabilizes the complex (26). Expressing a mutated and tagged Trx in *S. cerevisiae* allowed the purification of a Trx/Prx complex (227). Evidence of Trx/Prx complexes has also been provided in plant extracts using coimmunoprecipitation (185). This was further extended to the purification of target proteins by affinity chromatography on columns with an immobilized Trx mutated in the second cysteine and allowed an identification of Trx targets in distinct cellular compartments (3, 28, 132, 154, 156) (Fig. 13A). The same technique was used to isolate targets of a cytosolic poplar GrxC4. Many of the identified proteins were already found to interact with Trx. Among them, the proteins involved in antioxidant pathways are potential common targets shared by Trx and Grx. More surprisingly, chloroplastic enzymes such as several Calvin–Benson cycle enzymes were also isolated. The physiological significance of a Grx-dependent regulation of these enzymes is questionable. Indeed, this would lead to a light-independent regulation of both the Calvin–Benson cycle and futile cycles. However, several targets have been shown to be glutathionylated, supporting the idea that they could be specifically deglutathionylated by Grx (192). By using the same strategy, a specific yeast two-hybrid strain has been designed that identifies Trx targets *in vivo* (231). Moreover, classical yeast two-hybrid strains have also been used to isolate relevant *in vivo* targets of plant Trx (1, 126, 146, 188, 230). The second approach consists of blocking free thiols of a plant extract, followed by a reduction of the disulfide bridges with Trx, and the labeling of the freshly released thiol with a fluorescent or radioactive thiol-specific probe (4, 6, 133–135, 140, 242, 243, 250) (Fig. 13B). Collectively, these proteomic approaches allowed the identification of numerous putative target proteins. Currently, ~500 potential targets have been identified in photosynthetic organisms. They take part in diverse processes, including metabolism, defence, and development, suggesting that the redox control acts as an integrator of all aspects of plant life. For exhaustive reviews, see (132, 154). For most of these targets, the type of reduction performed by Trx and Grx (disulfide reduction, isomerization, deglutathionylation, and denitrosylation) as well as the physiological relevance of these modifications remains to be determined.

Other redox proteomic strategies were designed to estimate differences in the oxidation of cysteine residues between two samples. These methods enabled the identification of thioltransferase target proteins *in vivo* (119, 123). These techniques combining mass spectrometric and ICAT have been used in several organisms to identify *in vivo* H_2O_2 oxidized target proteins and potential targets of Trx and glutathione pathways in corresponding gene mutants (119, 123) (see next). Adapting such techniques to plants would be of great interest in identifying physiologically target proteins of Trx and Grx *in vivo.*

VI. The Reduction Pathway of Cytosolic and Mitochondrial Trx, Grx, and GSH Revisited

Since the reduction of Trx, Grx, and GSSG is generally carried out by Trx reductase, GSH, and GR, respectively, several recent studies have reported that alternative reduction pathways are also occurring in plants and other organisms. This has important consequences for the regulation of the redox state of the different partners and for their downstream targets.

A. Trx reduction by Grx

In contrast to all other Trxh tested so far, the poplar Trxh4 presents an additional cysteine in position 4 and is not reduced by NTR but by the GSH/Grx system (77). Biochemical and X-ray crystallographic studies indicate that Trxh4 reduces target proteins using a classical mechanism involving its redox site, WCGPC. Cys58 performs the first nucleophilic attack, and the target protein is released after the attack of the resolving Cys61. In contrast to other Trxh, reduction involves Cys4, which reduces Cys61 and forms a disulfide bridge with Cys58, which is, in turn, attacked by a GSH molecule. This releases the reduced target and a glutathionylated Cys4 residue, which is then reduced by a Grx, finally liberating the fully reduced Trxh4 (Fig. 14) (106). A similar Trx with the MGxC N-terminal motif is present in flowering plants and conifers (seed plants) but not in *Physcomitrella* and *Selaginella*. Interestingly, it was demonstrated that the N-terminal Gly2 residue is modified by myristoylation in the *Arabidopsis* ortholog AtTrxh9, allowing membrane anchoring and palmitoylation that are responsible for cell-to-cell movement (143). Gly2 is required for membrane binding, while both Gly2 and Cys4 are needed for movement. Myristoylation is considered an irreversible modification, while palmitoylation is reversible. Thus, palmitoylation may interfere with the reduction of Trxh9 by the Grx system. The redox activity may be rescued after movement, when the palmitoylation is removed. In *Arabidopsis*, only AtTrxh9 encodes this type of protein. Trxh2, Trxh7, and Trxh8 are predicted to undergo myristoylation (http://ca.expasy.org/cgi-bin/myristoylator/myristoylator.pl), but only Trxh9 is predicted to undergo palmitoylation (http://csspalm.biocuckoo.org/online3.php). On the basis of the conservation of the sequence in all the seed plants analyzed so far, one should suspect a major role for this gene. The homozygous *trxh9* mutant is viable but seedling growth is severely impaired, particularly in agar in the absence of sucrose. On soil, plants are dwarf but, nevertheless, fertile (143). It is not yet known whether the myristoylation/palmitoylation is involved in the developmental phenotype of the mutant.

Although PopTrxh4 and likely AtTrxh9 are mainly reduced by Grx instead of NTR, other plant Trx are known to be optionally reduced by NTR or Grx. In *Arabidopsis*, cytosolic Trxh3 and Trxh2 are efficiently reduced by cytosolic GrxC1 and GrxC2 *in vitro* (Table 1) (183). The alternative reduction mechanism of Trxh3 and Trxh2 by Grx is still to be determined, but it is likely not the same as for PopTrxh4, as none of them exhibit an additional Cys4 at the N-terminal side. Indeed, several Trx have been reported to undergo glutathionylation, including human Trx (34), *Arabidopsis* Trxf1 (151), and poplar Trxh2 (78). Glutathionylation appears to decrease the activity of these Trx. This modification suggesting that Grx could regulate Trx activity by deglutathionylation will need further investigation.

Other types of Trx might be reduced by alternative reduction pathways. This may be particularly relevant for plastidial Trx. Although the chloroplastic carbon metabolism enzymes are strictly regulated by a light-dependent Trx regulation,

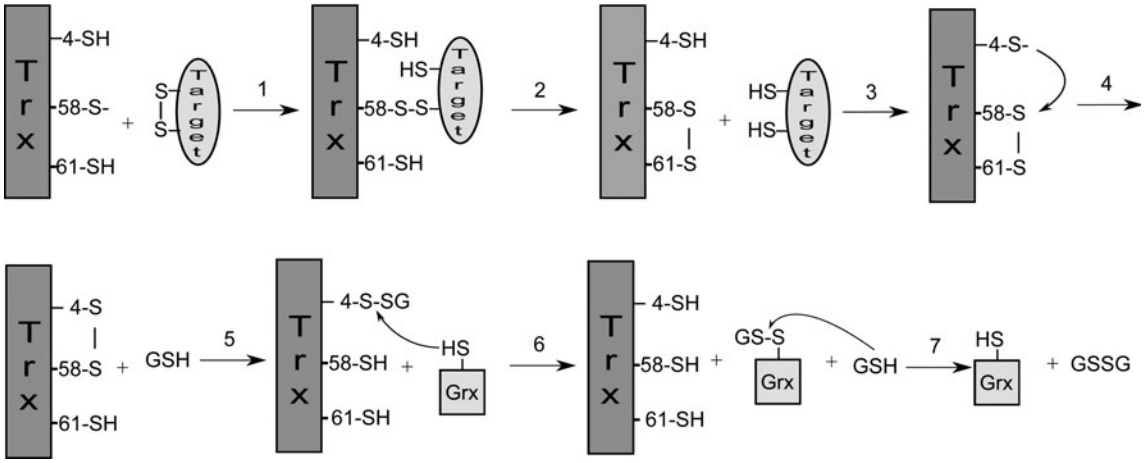

FIG. 14. Reduction mechanism of PopTrxh4 and AtTrxh9 by Grx and GSH. Here, three cysteine residues of Trx are necessary to perform the reduction mechanism: a catalytic Cys58 and two resolving Cys61 and Cys4. The reduction of the target peptide follows a classical mechanism [1, 2]. The Cys4 of PopTrxh4 attacks the disulfide bridge between Cys58 and Cys61 [3], releasing the reduced Cys61, and forms a disulfide bridge with Cys58 [4]. A GSH molecule attacks this bridge, releasing the reduced Cys58 and the glutathionylated Cys4 [5], which is deglutathionylated by a Grx [6]. The glutathionylated Grx is reduced by a second GSH molecule [7].

several plastidic Trx have also been found to be expressed in nonphotosynthetic organs (48, 58). For example, in the amyloplast, Trx is reduced by Fdx/FTR as in chloroplasts, but the Fdx is reduced by NADPH *via* FNR (Fig. 5) (5). Moreover, recent works showing the dual chloroplastic/cytosolic localization of the plastidial Trxm2 (146) may suggest that some chloroplastic Trx can be reduced by NTR. This statement has to be biochemically tested, and could support the convergent evolution of both the Trx reductases (NTR and FTR) present in plants (97). Trxf shows high homology to the cytosolic Trx and is supposed to be of eukaryotic origin (198), while most plastidial Trx are related to cyanobacterial Trx. The ability of FTR and NTR to reduce most Trx probably allowed the shift of the ancestor of Trxf from the cytosol to the plastid.

B. GSSG reduction by Trx

Alternative reduction of GSSG by Trx was originally shown in *P. falciparum* and *Drosophila melanogaster* (101, 102). In *Drosophila*, which is devoid of GR, the NTR/Trx system was shown to exhibit a higher redox potential than *E. coli* Trx, being compatible with the efficient reduction of GSSG (40). Alternative GSSG reduction by cytosolic Trx has also been found in *Arabidopsis* (Fig. 15). In this case, the cytosolic NTRA/Trxh3 acts as a backup system for GSSG reduction and exhibits a rate of GSSG reduction about 200-fold lower than GR1. As described later, the physiological function of this backup system was further supported by genetic evidence in mutants (139). Nevertheless, the biological significance of such a backup system will have to be further demonstrated in wild-type plants. Similarly, mutant analyses in *S. cerevisiae* have also described a GSSG alternative system, indicating that such backup reduction is conserved within several organisms (217). It was further shown by structural analyses that a specific Met35 residue in yeast Trx1 is important for GSSG reduction activity (10). Nevertheless, it seems that alternative residues are involved, as Met35 is not always conserved in the Trx involved in GSSG reduction.

C. Atypical types of reduction

Several types of alternative reduction have been described in a variety of organisms. The regeneration of an active reduced form of Grx is usually dependent on glutathione, but some specific members are also, or uniquely, regenerated by Trx reductases. *E. coli* Grx4 has a monothiol CGFS potential active site and, in the reduced form, contains three thiols. Treatments with GSSG result in glutathionylation and the formation of a disulfide bridge. The disulfide of Grx4 was shown to be a direct substrate for *E. coli* NTR, whereas the mixed disulfide was reduced by Grx1. Moreover, reduced Grx4 showed the potential to transfer electrons to oxidized *E. coli* Grx1 and Grx3 (67). Human mitochondrial and cytosolic isoforms of Grx2 contain the active site sequence CSYC and have a high affinity toward glutathionylated proteins. In

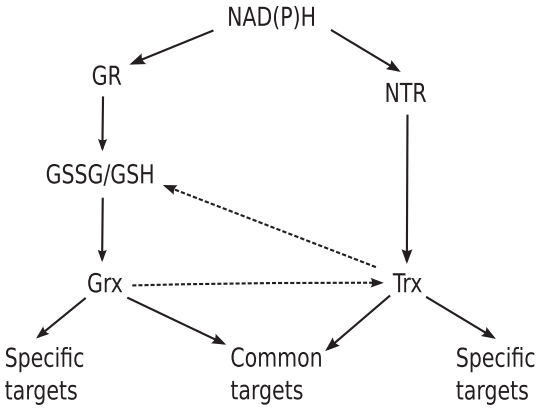

FIG. 15. Trx and Grx reduction in the cytosol. The classical reduction pathways NADPH/NTR/Trx on the right and NADPH/GR/GSH/Grx on the left of the figure are represented by *continuous arrows*, while the alternate pathways (Grx-dependent Trx reduction and Trx-dependent GSSG reduction) are indicated by *dashed lines*.

addition to glutathione, Grx2 is reduced by NTR, which efficiently reduces both the active site disulfide and the GSH-Grx intermediate formed in the reduction of glutathionylated substrates (99). Interestingly, *E. coli* Grx4 and human Grx2 exhibit CG/SYC/S active sites, which are also found in some plant Grx isoforms (Table 4). Whether these isoforms can be reduced by Trx reductases in plants is still to be determined. As discussed earlier, Grx exhibiting CG/SYC/S active sites have been shown to form an Fe-S cluster with GSH, implicating that this redox activity only occurs in the monomeric forms of Grx.

In *Chlamydomonas*, Grx3 forms an atypical intramolecular disulfide during its catalytic cycle between the first active site cysteine and a second cysteine located in the C-terminal part of the protein. This disulfide is further reduced by an FTR but not by glutathione. The more negative redox potential of Grx3 (−323 mV at pH 7.9) compared with other Grxs (−244 to −284 mV at pH 7.9) might explain its preference to receive electrons from the FTR, but this hypothesis has not yet been proved (252). Similarly, in *Arabidopsis*, chloroplastic subgroup II GrxS14 and GrxS16 have been proposed to be reduced by FTR (53). Moreover, *Chlamydomonas* homologs of GrxS15 and GrxS16 (Grx5 and Grx6) are also apparently not reduced by GSH (74).

As previously described, the reduction of vascular plant APR constitutes another type of atypical reduction pathway. While showing homology to Trx, APR is exclusively reduced by GSH, and is, thus, functionally similar to a Grx (21). Another type of atypical reduction pathway was found in budding yeast. Here, the mitochondrial 1-Cys Prx1 is reactivated by glutathionylation of the catalytic cysteine residue and a subsequent reduction by a Trx reductase (Trr2) coupled with GSH (82, 83).

VII. Genetic Evidence for Interactions Between Grx and Trx Pathways

Genetic studies constitute powerful approaches that decipher the functions of Grx and Trx pathways. In plants and other organisms, recent genetic evidence has also highlighted that crosstalks are occurring between Trx and Grx pathways.

A. Genetic crosstalk between Trx and Grx systems in yeast

In *S. cerevisiae*, the Trx system is composed of two cytosolic Trx (Trx1 and Trx2) and a mitochondrial Trx (Trx3). Cytosolic and mitochondrial Trxs are, respectively, reduced by cytosolic (Trr1) and mitochondrial (Trr2) NTR. Within the two dicysteinic Grx, Grx1 is cytosolic, whereas Grx2 is dually located in the cytosol and the mitochondria. Moreover, five monocysteinic Grx are found in *S. cerevisiae*.

Crosstalking between Trx and Grx systems has been extensively described in *E. coli*. In *E. coli*, simultaneous inactivation of the GSH and Trx pathways is unviable, but inactivation of either pathway is fully viable, which indicates that each pathway can fully operate the essential function of reducing disulphides in the absence of the other pathway [reviewed by Toledano *et al.* (221)]. Similarly, yeast strains that completely lack the cytosolic NADPH- Trx system (*trx1 trx2 trr1*), the mitochondrial system (*trr2* and *trx3*), or both cytosolic and mitochondrial systems (*trr1 trr2* and *trx1 trx2 trx3*) are also viable. However, strains that are simultaneously deleted for components of both systems (*trx1 trx2 grx1 grx2* and *glr1 trr1*) are not viable (64, 222–224). Nevertheless, several works indicate that, unlike *E. coli*, the yeast Trx and GSH/Grx pathways are indeed not redundant. Although inactivation of both Trx genes is not lethal, the *trx1 trx2* double mutant exhibits strong phenotypic perturbations. It is unable to assimilate sulfate and grows only in the presence of reduced sulfur. Moreover, the double mutant suffers from a perturbed cell cycle with a very long S phase and, as a compensation, a shortened G1 phase (160, 161). This phenotype is due to the lower ability of the double mutant to reduce RNR and, subsequently, to regenerate the pool of dNTP that is necessary for performing DNA synthesis. This also indicates that Grx are poor RNR reductants in yeast (33, 105). Le Moan *et al.* (123) revealed contrasted differences in the oxidized proteome of cells on inactivation of the Trx or GSH pathway, suggestive of very distinct thiol redox control functions and assigning an exclusive role for cytosolic Trx in H_2O_2 metabolism and a presumed thiol redox buffer function for glutathione. Interestingly, glutathione was also shown to be essential in yeast (81, 244) through its requirement in iron-sulfur clusters assembly, but it only serves as a Trx backup in cytosolic thiol-redox maintenance (110).

Crosstalking between the Trx and the GSH/Grx pathways has been studied by measuring the redox state of Trx and Grx in mutants that are lacking the GR and the Trx reductases (110, 223). In budding yeast, the cytosolic Trx reductase Trr1 is required for normal cell growth. However, the cytosolic/mitochondrial GR Glr1 is dispensable. An analysis of the redox state of Trx and Grx in the *glr1* mutant has revealed that Trxs are maintained in a reduced state independently of the glutathione system. In contrast, the redox state of Grxs is strongly correlated with the oxidation state of the GSSG/GSH redox couple. Trotter and Grant (223) have proposed that independent redox regulations of Trx enable cells to survive under conditions where the GSH/Grx system is oxidized. In contrast to the *glr1* mutant, the loss of Trr1 in the *trr1* mutant not only results in the oxidation of cytosolic Trxs but also shifts the redox state of Grx to a more reduced form. This latter observation likely accounts for the fact that Trx mutants contain elevated glutathione levels. *trx1 trx2* and *trr1* mutants show an increase in both GSSG and GSH levels, with the redox state of the GSSG/GSH being similar to that of the wild-type strain (223). Therefore, there is a link between the Trx system and the glutathione metabolism of the cell (75, 162). This may, therefore, account for the growth defect observed in the *trr1* mutant (223). Nevertheless, it is unclear whether Trxs are directly or indirectly involved in the reduction of glutathione (217).

In contrast to the cytosol, the inactivation of the mitochondrial Trr2 does not impact the redox state of the mitochondrial Trx3, indicating that an alternative reduction of Trx3 fully rescues the loss of Trr2. Similarly, an analysis of the redox state of Trx3 reveals that it is maintained in the reduced form in mutants lacking components of the cytoplasmic Trx system (*trx1 trx2* or *trr1*). However, the deletion of both Trr2 and Glr1 results in a partial oxidation of Trx3 during normal growth conditions, which is even more pronounced during oxidative stress or respiratory growth conditions. These data indicate that both Trr2 and Glr1 are required to maintain the redox state of the mitochondrial Trx3, but it is still unclear how this reduction occurs (224).

B. Genetic crosstalk reveals new functions of Trx and Grx systems in plant development

In order to identify the functions of cytosolic Trx, corresponding insertion lines were isolated from the available collections of *Arabidopsis*. First attempts at identifying phenotypes of several Trxh null homozygotes were unsuccessful. A multiple mutant inactivated in *h1*, *h2*, *h3*, and *h5* was constructed in our lab. It does not show any obvious growth defect under normal and several stress conditions. We suspected that redundancy with other Trx, Trx-like enzymes, or Grx was responsible for the absence of developmental or adaptive response to the inactivation of these Trx. An alternative approach targeted inactivation of the NTR. The rationale was to limit the reduction of all cytosolic and mitochondrial Trx by inactivating both NTR genes. Surprisingly, the double mutant *ntra ntrb* is still viable and fertile. It presents slightly reduced growth and accumulates more anthocyanins (13). It is not hypersensitive to exogenously applied H_2O_2 or other oxidants and is even more resistant to UV-B, probably due to the high anthocyanin level. This very limited phenotype is contrasted with the lethality of mouse embryos that are inactivated in their cytosolic or mitochondrial NTR genes (25, 49). A very important point that should be noted while understanding the viability of the NTR null mutant is the demonstration that Trxh3 is not completely oxidized in the *ntra ntrb* mutant, suggesting the existence of an alternative Trx reduction system in *Arabidopsis*. Two lines of evidence show that this system is dependent on GSH and Grx. First, *ntra ntrb* is hypersensitive to BSO, a specific inhibitor of the first enzyme of GSH synthesis. Second, this was confirmed by crossing the *ntra ntrb* mutant with the *rml1* mutant, a strong allele of the first enzyme in GSH synthesis containing only about 3% GSH, compared with the wild type. After germination, the root meristem of *rml1* is blocked, but the shoot meristem grows, at least in the early phase of plant development (Fig. 10) (228). In the triple mutant *ntra ntrb rml1*, both root and shoot apical meristems are blocked, and the cytosolic Trxh3 is fully oxidized. *In vitro*, recombinant Trxh3 cannot be reduced by GSH alone, but a GSH/Grx system can reduce it with a low but nevertheless significant efficiency. All these data are in favor of a GSH/Grx alternative reduction system for Trxh in *Arabidopsis* (183). Nevertheless, the fact that the *ntra ntrb* mutant exhibits important growth phenotypes suggests that this alternative pathway is not optimal *in vivo*. It is currently not known whether all cytosolic Trx can be reduced by the GSH/Grx system *in planta*. The reducers of several cytosolic and mitochondrial Trx have been identified, but for some other Trx, this has not yet been established (Table 1). Genetic studies are in favor of an alternative reduction of Trxh5 in *Arabidopsis*. An *ntra ntrb* mutant inactivated in both cytosolic and mitochondrial Trx reductases does not show the insensitivity to victorin of the single *trxh5* mutant, suggesting that Trxh5 can be efficiently reduced *in planta* in the absence of NTR (213). However, surprisingly, the single *ntra* mutant exhibits a complete loss of the SAR (215). This apparent discrepancy is likely due to the different reduction mechanisms of Trxh5 target proteins in the two types of pathogen responses. As previously described, in the victorin pathway, Trxh5 reduction is acting *via* a monocysteinic reaction (*i.e.*, glutathionylation) and is, therefore, likely reduced by deglutathionylation, potentially by a Grx (213). However, in the SAR, Trxh5 is reducing a disulfide bridge in NPR1, likely *via* a dicysteinic reduction mechanism that might not be reducible by Grx (215).

The absence of growth of the root apical meristem in the *ntra ntrb rml1* mutant was the first evidence for the implication of Trx in plant meristem development. As previously shown, the synthesis of gamma-glutamyl cysteine is the limiting step in GSH synthesis in *Arabidopsis*. Fortunately, in addition to the lethal KO and the very severe *rml1*, other milder mutant alleles of *gsh1* have been isolated in several genetic screens (Table 6). As discussed earlier, the *cad2* mutant accumulates ~20%–30% GSH in comparison to the wild type, is aphenotypic under standard growth conditions, but is hypersensitive to cadmium, most probably due to a low phytochelatin content (91). Crossing *ntra ntrb* with *cad2* allows the isolation of *ntra ntrb cad2* homozygotes. Their early development is similar to the *ntra ntrb* phenotype until the rosette to stem transition takes place. The plant subsequently presents a bushy phenotype lacking apical dominance, and the stems present a *pin1* phenotype, without flower formation. The *pin1* mutant is inactivated in a transporter that is responsible for polarized auxin transport (73). Similar to the *pin1* mutant, the triple *ntra ntrb cad2* mutant has limited ability to transport auxin. Intriguingly, its auxin transport capacity is similar to that of the *cad2* mutant, which is also impaired in auxin transport but does not present a *pin* phenotype. This suggests that glutathione plays a major role in auxin transport. This was further supported by the fact that perturbed glutathione levels affect the expression of PIN transporters (14, 107). Moreover, the reduced polarized transport is not, or at least not solely, responsible for the phenotype of the *ntra ntrb cad2* mutant. In contrast to the *pin1* mutant, which has a normal level of auxin in the apical meristem, the auxin level is reduced in the *ntra ntrb cad2* mutant (14). In this mutant, Trxh3 is partially reduced as in the *ntra ntrb* mutant, showing that, in spite of the glutathione level limited to 20%–30% of the wild type, it remains sufficient to maintain the alternative GSH/Grx reduction systems. Thus, the absence of flower formation in the triple mutant is most probably due to the simultaneous poor reduction of Trx and Grx. One challenge in future research will be to identify which Trx and Grx are implicated in the meristem defect and in auxin synthesis and transport. A good Grx candidate is the GrxS17, which has been shown to be involved in auxin signaling (39). Recent works also suggest a role of S-nitrosylation in the regulation of the auxin signaling (68, 220).

The "abnormal inflorescence meristem1" mutant shows some similarity to the *ntra ntrb cad2* mutant, including meristem anomalies and loss of apical dominance. It is impaired in a multifunctional protein (At4g29010) that is involved in the beta-oxidation of fatty acids and the remobilization of auxin from its storage inactive butyric acid conjugate (186). Studying whether perturbed beta-oxidation is responsible for the abnormal inflorescence meristem phenotype of the *ntra ntrb cad2* mutant could reveal the redox regulation of beta-oxidation.

As previously stated, the cytosolic *GR1* gene is dispensable in *Arabidopsis*, although it produces more than two thirds of the GR activity. No obvious phenotype is visible, even after H_2O_2 treatment. Crossing the *ntra ntrb* mutant with *gr1* allows the generation of homozygous plants for *ntra* and *ntrb* mutations but that are heterozygous for the *gr1* mutation. *ntra*

ntrb gr1 pollen is unfertile, while *ntra ntrb gr1* ovules form seeds when pollinated with the wild-type male gametophyte or that have at least one wild-type allele of the *NTRA*, *NTRB*, or *GR1* genes. This strongly suggests that in the *gr1* pollen, GSSG is reduced by NTRA or NTRB. As previously discussed, this is further supported by the ability of a recombinant NADPH/NTRA/Trxh3 system to reduce GSSG *in vitro*. Thus, in the cytosol of germinating pollen, GSSG is reduced either by GR1 or by an NTR/Trx pathway. Both the NTR and GSH systems are dependent on NADPH as a first redox source. Therefore, the interaction between the two pathways may allow the adaptation of metabolism and development to the availability of NADPH. The different pathways that are involved in the reduction of cytosolic Trx and Grx are summarized in Figure 15.

Another study has recently demonstrated the role of glutathione in pollen germination. Impaired glutathione synthesis in the *pad2-1* mutant or after BSO treatment inhibit pollen germination *in vitro*. Moreover, IAA addition rescues glutathione-deficient pollen, indicating that glutathione depletion and auxin metabolism are linked in pollen germination and early elongation of the pollen tube (256). Taken together, several genetic studies show that the Trx and GSH/Grx systems play an important role in controlling several plant development pathways, including pollen development, meristem growth, and auxin metabolism.

VIII. Conclusions

The discovery of Trx and Grx in plants and most knowledge on their functions were first established by biochemistry. Genome sequencing revealed the high number of Trx and Grx types and their conservation among vascular plants, suggesting conserved functions during evolution. Proteomic approaches have revealed that numerous proteins that are involved in almost all aspects of plant life are potentially regulated by disulfide reduction, and, consequently, that Trx and Grx regulation is an integrator between metabolism, development, and adaptation to the environment. Nevertheless, in most cases, the consequences of these regulations in terms of development, fitness, or adaptation to the changing environmental conditions have not yet been established *in planta*. This remains the case for the regulation of photosynthetic carbon metabolism. Although the redox regulation of more than 25 chloroplast proteins has been well described, we do not know what would be the consequence of a deregulation: probably a waste of energy, but would this only slightly reduce growth or be lethal? Another question is the specificity of each Trx and Grx for particular targets. Biochemical studies have demonstrated a high specificity of Trx for metabolic enzymes. This is particularly true for the photosynthetic-related metabolism in the chloroplast, for which a strict light-dependent regulation is required to avoid futile cycles and a waste of energy. Such enzymatic specificities are likely favoring the maintenance of Trx genes during land plant evolution. In contrast, some target proteins were shown to be common to several Trx and/or Grx, suggesting that overlapping functions either between Trx family members or between Trx and Grx superfamilies occurs. Although overlapping functions between Trx members is highly consistent because they generally share the same reduction mechanism, the biochemical characteristics of Trx and Grx are different. Trxs are good disulfide bridge reducers but poor catalysts of protein deglutathionylation, while Grxs catalyze this reaction very efficiently. In contrast to Trxs, Grxs are also able to assemble stable or labile iron-sulfur clusters. Therefore, from a biochemical point of view, overlapping between Trx and Grx more generally relies on crosstalking between both the systems. Obviously, genetic approaches are also necessary to address these questions *in vivo*. The availability of insertional mutants offered a good perspective and helped most labs secure the first results, but it became clear that most simple Grx or Trx mutants are aphenotypic under standard conditions. The *ntrc* insertion mutant is one of the rare cases in which the phenotype allowed the identification of a clear antioxidant function at the plant level. Fortunately, genetic screens performed by labs, which, in most cases, were not previously implicated in redox research, have added a lot of new data, even when the mutant had already been analyzed (*attrxh5* and victorin sensitivity). The *attrxm3* mutant had not been analyzed earlier, but it would have probably only been identified as an "early germination lethal phenotype," and the defect in cell-to-cell trafficking would have been missed. The ROXY analysis suggests that the TGA transcription factors that are implicated in plant development and defence are important targets of Grx and reinforce previous work. We are clearly only in the early phase of the genetic approach. The real challenge will be to perform the good crosses to overcome redundancy and develop phenotyping screens that identify balanced adaptation, even if the regulation has only an apparently limited effect on laboratory grown plants, but which may be of great significance in the natural environment.

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Abbreviations Used

γGC = gamma-glutamyl cysteine
AGPase = ADP-glucose pyrophosphorylase
APR = APS reductase
APS = adenosine-5′-phosphosulfate
At = *Arabidopsis thaliana*
BSO = buthionine sulfoximine
cad2 = *cadmiumsensitive 2*
cat2 = catalase 2
CITRX = Cf-9-interacting Trx
CLT = chloroquine-like transporter
Cys = cysteine
DHA = dihydroascorbate
FBPase = fructose-1,6-bisphosphatase
Fdx = ferredoxin
FNL = fructokinase-like
FNR = ferredoxin:NADP reductase
FTR = ferredoxin:thioredoxin reductase
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
gat = GFP arrested trafficking
GGT = gamma-glutamyl transpeptidase
Glu = glutamate
Gly = glycine
Gpx = glutathione peroxidase (in fact Trx dependent Prx in plants)
GR = glutathione reductase
Grx = glutaredoxin
GSH = reduced glutathione
GSNO = S-nitrosoglutathione
GSSG = oxidized glutathione
GST = glutathione-S-transferase
HEDS = hydroxyethyl disulfide
ICAT = *isotope coded affinity tag*
MRP = multidrug resistance-associated protein
MSR = methionine sulfoxide reductase
NADP-MDH = NADP-dependent malate dehydrogenase
NEP = *nuclear-encoded plastid* RNA *polymerase*
NPR1 = nonpathogenesis related protein expressor 1
Nrx = nucleoredoxins
NTR = NADPH thioredoxin reductase
OPP = oxidative pentose phosphate
OPT = oligopeptide transporter
pad2-1 = phytoalexin-deficient 2-1
PAPS = phosphoadenylyl sulfate
PRK = phosphoribulokinase
Prx = peroxiredoxin
rax1-1 = regulator of APX2 1-1
rml1 = rootmeristemless 1
RNR = ribonucleotide reductase
ROS = reactive oxygen species
SAR = systemic acquired resistance
SNO = S-nitrosothiol
TDX = tetratricoredoxin
TGA = subfamily of basic domain/leucine zipper (bZIP) transcriptional regulators
Trx = thioredoxin