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REVIEW

Thioredoxins, glutaredoxins, and glutathionylation: new crosstalks to explore

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Abstract Oxidants are widely considered as toxic molecules that cells have to scavenge and detoxify efficiently and continuously. However, emerging evidence suggests that these oxidants can play an important role in redox signaling, mainly through a set of reversible post-translational modifications of thiol residues on proteins. The most studied redox system in photosynthetic organisms is the thioredoxin (TRX) system, involved in the regulation of a growing number of target proteins via thiol/disulfide exchanges. In addition, recent studies suggest that glutaredoxins (GRX) could also play an important role in redox signaling especially by regulating protein glutathionylation, a post-translational modification whose importance begins to be recognized in mammals while much less is known in photosynthetic organisms. This review focuses on oxidants and redox signaling with particular emphasis on recent developments in the study of functions, regulation mechanisms and targets of TRX, GRX and glutathionylation. This review will also present the complex emerging interplay between these three components of redox-signaling networks.

Keywords Arabidopsis · Chlamydomonas · Glutaredoxin · Glutathione · Glutathionylation · Oxidants · Photosynthetic organism · Redox signaling · Thioredoxin

Abbreviations

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRX	Glutaredoxin
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
MSR	Methionine sulfoxide reductase
$NO^{\cdot}$	Nitric oxide radical
NTR	NADPH TRX reductase
PCD	Programmed cell death
PKC	Protein kinase C
PRX	Peroxiredoxin
PTP	Protein tyrosine phosphatase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOH	Sulfenic acid
SO_2H	Sulfinic acid
SO_3H	Sulfonic acid
TPI	Triose phosphate isomerase
TRX	Thioredoxin

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Introduction

Life in an oxygen rich environment implicates the production of oxidants. As a consequence of their aerobic life, photosynthetic organisms as well as

non-photosynthetic organisms are exposed to these oxidants that can cause wide-ranging damage to several cell components, including DNA, lipid membranes, sugars, and proteins. Various oxidants can be generated under oxidative stress as well as during the course of normal metabolism. These oxidants can be classified in two main categories: reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), and peroxide radicals, and reactive nitrogen species (RNS), such as nitric oxide radicals ($NO^{\cdot}$) and peroxynitrite radicals ($ONOO^{\cdot-}$).

In order to prevent oxidant damage, plant cells have developed effective defense mechanisms composed of a set of antioxidant molecules and enzymes. The major non-enzymatic antioxidants in plants are glutathione, ascorbate, and tocopherols. Different enzymes are also involved in ROS scavenging including superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase, and peroxiredoxins (PRX) (Apel and Hirt 2004).

However, if oxidants have been traditionally considered as toxic molecules with deleterious effects for cells, recent studies suggest that these reactive species could be produced by cells on purpose and be involved in signaling processes, especially through modifications of the oxidation state of protein residues. The exposure of proteins to oxidants can lead to the carbonylation of arginine, lysine, proline, and threonine residues, the oxidation of methionine residues into methionine sulfoxides, and nitration of tyrosines (Stadtman 1993). With regard to cysteines, different oxidation states can exist (Fig. 1). Cysteine thiols can be oxidized into sulfenic (SOH), sulfinic (SO_2H), or sulfonic (SO_3H) acids. Oxidation of cysteines can also lead to the formation of intra- or interprotein disulfide bonds and to S-thiolation, which consists in the formation of mixed disulfide bridges between the free thiol of a protein and a low molecular weight thiol such as cysteine (S-cysteinylation), cysteamine (S-cysteaminylation), or glutathione (S-glutathionylation). In addition to these oxidations, cysteines can undergo nitrosylation in the presence of RNS. However, these modifications by oxidation are not all involved in redox regulation mechanisms. One of the main criteria allowing to consider these post-translational modifications as regulatory mechanisms and not only as oxidative damages is their reversibility. For example, carbonylation is generally considered as an oxidative damage since this modification is irreversible and leads to degradation of the oxidized protein by the proteasome (Stadtman 1993; Dunlop et al. 2002; Grune et al. 2003). Cysteine oxidations into sulfinic and sulfonic acids are also generally considered as irreversible oxidations. However, recent studies have shown that in the particular case of 2-cys peroxiredoxins, the sulfinic form of the catalytic cysteine could be reduced to the sulfenic acid form by sulfiredoxin with the help of thioredoxin (TRX) (Biteau et al. 2003; Woo et al. 2005). Reversible modifications are those involving disulfide bonds (intraprotein, interprotein, and S-thiolation) but also methionine sulfoxides, sulfenic acids and nitrotyrosines. The reduction of oxidized residues is controlled by two major protein families: TRX and glutaredoxins (GRX). Consequently, these ''redoxins'' play a major role in redox signaling. Methionine sulfoxide reductases (MSR) are involved in the reduction of methionine sulfoxides using TRX as electron donors (for a review see Rouhier et al. in this special issue). Studies suggest that tyrosine nitration could also be reversed enzymatically, but the identity of the enzymes involved remains to be determined (Kamisaki et al. 1998; Irie et al. 2003). Nitrosylation of cysteines could also be reversed by TRX (Ravi et al. 2004). Sulfenic acids can be directly reduced by reduced glutathione (GSH) or lipoic acid or can be stabilized within the protein environment and recycled via disulfide bond intermediates by TRX and GRX. Intra- and interprotein disulfide bridges can be reduced by TRX but GRX could also play some role. In addition, GRX are likely to play a major role in the regulation of S-thiolation notably by reduction of glutathionylated cysteines to the thiol form.

Through these reversible modifications, oxidants can modulate the activity of a range of proteins and play a role of messenger within the cell. This review will focus on redox regulation in photosynthetic organisms with particular emphasis on recent developments in the study of TRX, GRX and glutathionylation. The emerging complex interplay between these three components of redox-signaling networks will also be discussed.

Thioredoxins

Thioredoxins are small redox proteins of ca. 12 kDa found in all free-living organisms. These proteins are involved in thiol-disulfide exchange reactions by way of their conserved active-site W-C-G(P)-P-C. Their redox potential is generally comprised between – 270 and – 300 mV (Åslund et al. 1997; Hirasawa et al. 1999; Collin et al. 2003, 2004). TRX also share a common three-dimensional structure motif, ''the thioredoxin fold'', which consists of four α-helices surrounding a β-sheet composed of five strands (Martin 1995). Different aspects of the TRX system in both photosynthetic

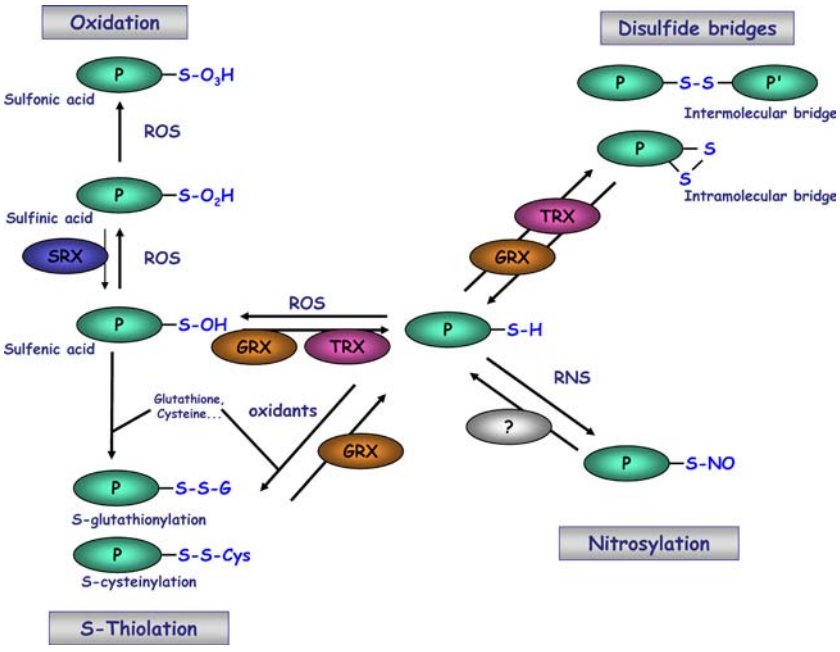

Fig. 1 The different oxidation states of cysteine residues. In the presence of oxidants, free and accessible thiols on proteins can undergo several modifications, reversible or not. Thioredoxins and glutaredoxins can regulate the formation of intra- or intermolecular disulfide bridges between two cysteines. Cysteines can also be oxidized into sulfenic acids (SOH) by reactive oxygen species (ROS), which can be reduced by thioredoxins and glutaredoxins. Further oxidation of these sulfenic acids can lead to the formation of sulfinic acids (SO_2H), which can be reduced back in some cases by sulfiredoxins (SRX), or to irreversible sulfonic acids (SO_3H). The presence of both oxidants and low molecular weight thiols such as glutathione or cysteine can allow S-thiolation of proteic cysteines via different mechanisms. These modifications are, respectively, called S-glutathionylation and S-cysteinylation, depending on the free thiol involved. S-thiolation can be reversed by glutaredoxins as well as thioredoxins, even if the latter is much less efficient. In addition to these modifications, cysteines can also undergo nitrosylation in the presence of reactive nitrogen species (RNS), a reversible modification that could be reversed by thioredoxins

and non-photosynthetic organisms have been discussed in several recent reviews (Holmgren 2000; Schürmann and Jacquot 2000; Buchanan et al. 2002; Jacquot et al. 2002; Vlamis-Gardikas and Holmgren 2002; Lemaire et al. 2003b; Lemaire and Miginiac-Maslow, 2004; Gelhaye et al. 2005; Buchanan and Balmer 2005; Holmgren et al. 2005). TRX was first identified as a hydrogen donor to ribonucleotide reductase in *Escherichia coli* (Laurent et al. 1964). Non-photosynthetic eukaryotes contain a limited number of TRX with usually one or two isoforms in the cytosol and one in mitochondria, reduced by an NADPH dependent TRX reductase (NTR) present in these compartments. These TRX have been implicated in defense mechanisms against oxidative stress by reducing methionine sulfoxides via MSR and by participating in ROS detoxification via PRX. TRX also participate in sulfate assimilation by reducing PAPS reductase and can control the DNA-binding activity of several transcription factors (Vlamis-Gardikas and Holmgren 2002).

In photosynthetic organisms, two types of TRX were initially identified in the late 70s: TRX f and m (reviewed in Schürmann and Jacquot 2000; Jacquot et al. 2002). These TRX, located in the chloroplast, are reduced in the light by photoreduced ferredoxin and ferredoxin thioredoxin reductase (FTR). Subsequently, they are able to reduce regulatory disulfides on their target enzymes. The first targets identified for these TRX were key enzymes of the carbon metabolism such as Calvin cycle enzymes (fructose-1,6-bisphosphatase, phosphoribulokinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), NADP-malate dehydrogenase, Rubisco activase, or ATP-synthase γ-subunit. All these enzymes are weakly active or inactive in the dark and activated by TRX under illumination. Biochemical studies suggest that f-type TRX could specifically regulate the activation of carbon assimilation enzymes. A third type of TRX was discovered a decade later: the h type TRX (h for heterotrophic), located in the cytosol (reviewed in Gelhaye et al. 2004a) and sometimes in mitochondria (Gelhaye et al. 2004b). These TRX h, reduced by the NADPH/NTR system, are involved in the mobilization of seed reserves during germination (Kobrehel et al. 1992; Besse et al. 1996; Cho et al. 1999), in self-incompatibility mechanisms (Bower et al. 1996; Cabrillac et al. 2001), and also in

ROS detoxification as electron donors to MSR (Gelhaye et al. 2003; see Rouhier et al. in this issue) and PRX (Dietz 2003; Rouhier and Jacquot 2005). More recently, the sequencing of plant genomes revealed the existence of multiple isoforms and led to the discovery of mitochondrial TRX o (Laloi et al. 2001) and of two new types of chloroplastic thioredoxins named x (Mestres-Ortega and Meyer 1999) and y (Lemaire et al. 2003a). These new chloroplastic TRX seem to be involved in defense mechanisms against oxidative stress since these proteins are efficient substrates for chloroplastic 2-cys PRX and PRX Q (Collin et al. 2003, 2004; Rouhier et al. 2004a; Dietz 2003; Rouhier and Jacquot 2005). These TRX might also be efficient electron donors for chloroplastic MSR (Vieira dos Santos et al. 2005).

In addition to the identification of new TRX types, the completion of the genomes of the higher plant *Arabidopsis thaliana* and of the green alga *Chlamydomonas reinhardtii* revealed an unsuspected multiplicity of sequences (Lemaire et al. 2003b). These genomic data revealed that TRX constitute a multigenic family in photosynthetic eukaryotes with six different TRX types (h, o, f, m, x and y). Chlamydomonas was found to contain eight different TRX, namely 2 TRX h, 1 TRX o, 2 TRX f, 1 TRX m, 1 TRX x, and 1 TRX y, whereas 19 TRX were identified in Arabidopsis, which displays much more isoforms for each TRX type with 8 TRX h, 2 TRX o, 2 TRX f, 4 TRX m, 1 TRX x and 2 TRX y (Fig. 2). It should be noted that these organisms also contain atypical TRX and a number of TRX-like proteins, which were excluded from the analysis presented here (Meyer et al. 2005; Meyer et al. in this special issue). This multiplicity of TRX genes raised questions about their specificity and redundancy. Moreover, with the identification of such a multiplicity, we came to the point where there were more isoforms of TRX identified in Arabidopsis than known TRX targets. Indeed, classical biochemical approaches had only allowed identification of 16 TRX targets in higher plants at that time (Buchanan and Balmer 2005). This prompted several groups to develop strategies aimed at the identification of new TRX targets. Some of these studies were based on the capacity of monocysteinic thioredoxin mutants with only the N-terminus cysteine in their active-site (the second active-site cysteine being replaced by site-directed mutagenesis by an alanine or a serine) to form stable mixed disulfides with target proteins. Such a mutated TRX can be immobilized on a column which is loaded with protein extracts. After washing, proteins bound to the TRX affinity column can be eluted using reducing agents such as dithiothreitol and subsequently identified by proteomics. Approximately 130 putative TRX target proteins have been identified using such approaches (Motohashi et al. 2001; Goyer et al. 2002; Lindahl and Florencio 2003; Balmer et al. 2003, 2004a; Yamazaki et al. 2004; Lemaire et al. 2004). A similar strategy was employed to trap *E. coli* putative targets (Kumar et al. 2004). Columns with immobilized wild-type TRX were also used in order to identify proteins interacting electrostatically with TRX (Balmer et al. 2004b). Techniques using thiol-specific fluorescent probes, such as monobromobimane or Cy5 maleimide, have been developed in order to visualize target proteins after in vitro reduction by TRX. This fluorescent labeling of reduced proteins allowed the identification of ca. 70 putative targets (Yano et al. 2001; Marx et al. 2003; Wong et al. 2003, 2004; Maeda et al. 2004). Another approach consisted in thiol-labeling with radioactive alkylating agents such as iodoacetate (^{14}C IAA) or iodoacetamide (^{14}C IAM). In this technique, free thiols of a protein extract are derivatized with cold IAA or IAM prior to in vitro reduction treatment using a NTR/TRX system. This approach is thus restricted to TRX efficiently reduced by NTR, i.e., extra-chloroplastic TRX. The reduced thiols are then labeled with ^{14}C IAA or ^{14}C IAM and identified by proteomics. This method allowed to identify 45 putative targets from Arabidopsis total leaf extracts (Marchand et al. 2004). The two-hybrid technique in yeast is a classical approach that allows identification of partner proteins by testing protein–protein interactions in vivo. Monocysteinic TRX have been used as baits in this kind of approach but in commercial strains, dedicated to such experiments, very few targets have been identified (Verdoucq et al. 1999; Vignols et al. 2005). It has been suggested that the mixed disulfide between the monocysteinic TRX and its target could be disrupted by endogenous TRX. To overcome this, a new yeast strain has been recently developed, knocked out in the two genes coding for cytosolic TRX in yeast (Vignols et al. 2005). This strain could thus be used in future studies to explore TRX-interacting proteins in vivo. These different approaches have led to the identification of nearly 300 targets, implicated in many distinct cell processes such as sulfur and nitrogen metabolism, fatty acids biosynthesis, glycolysis, Calvin cycle, oxidative stress defense mechanisms, amino-acids biosynthesis, glyoxylate cycle, or translation (Table 1, supplemental data). Most of the previously established TRX targets could be recovered by these proteomic approaches. This indicates that the methods employed were efficient to identify TRX targets since very few established TRX targets were missed, such as the glucan water dikinase (Mikkelsen et al. 2005). Among the numerous new putative targets, only a few

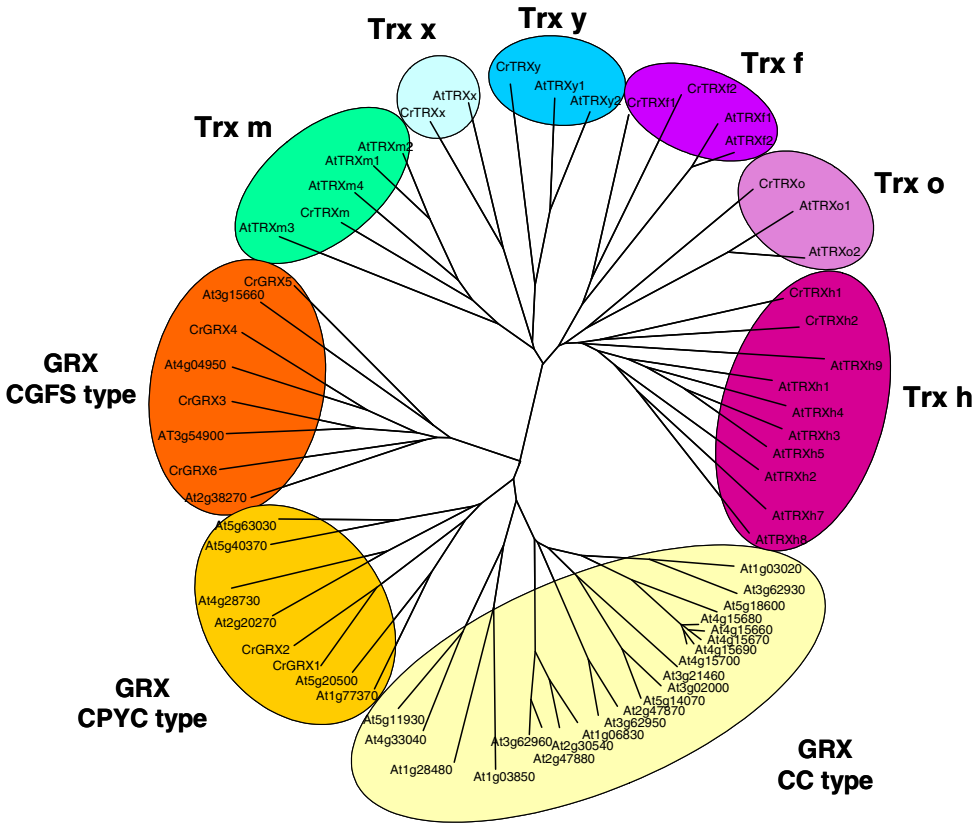

Fig. 2 The thioredoxin and glutaredoxin families in Arabidopsis and Chlamydomonas. The unrooted phylogenetic tree was constructed with the Clustal X program. Gaps were excluded. Accession numbers: *Chlamydomonas reinhardtii* (Cr) TRX h1, P80028; h2, AAO20258; o, AAO20259; m, P23400; f1, AAO20261; f2, AV622215; x, AAO20260; y, AAO20257; Glutaredoxin proteinID codes at http://www.genome.jgi-psf.org/Chlre3/ GRX1: 195746, GRX2: 195611, GRX3: 194929, GRX4: 195766, GRX5: 195767, GRX6: 195615. For *Arabidopsis thaliana* (At) the names indicated correspond to AGI codes for GRX. Accession numbers for TRX are as follows: h1, Z14084; h2, Z35475; h3, Z35474; h4, Z35473; h5, Z35476; h7, AAD39316; h8, AAG52561; h9, AAG51342; o1, AAC12840; o2, AF396650; m1, O48737; m2, Q9SEU8; m3, Q9SEU7; m4, Q9SEU6; f1, Q9XFH8; f2, Q9XFH9; x, AAF15952; y1, AAF04439 corrected for intron splicing; y2, AAM91085 corrected with EST AY128276

have been biochemically confirmed to be regulated by TRX. These targets include higher plants cyclophilins (Motohashi et al. 2003; Gopalan et al. 2004; Buchanan and Luan 2005), cyanobacterial phosphoglucomutase (Lindahl and Florencio 2003) and catalase, isocitrate lyase and 3-isopropylmalate dehydrogenase from Chlamydomonas (Lemaire et al. 2004). Most of the other proteins identified as possible targets still need to be validated in biochemical experiments.

Despite the use of different TRX isoforms known to exhibit distinct preferences for TRX targets, no specificity has been observed with monocysteinic TRX columns. This drawback could be due to the mutation of one active-site cysteine, which could induce a slight change in the micro-environment of the active-site. The high concentration of protein bound on the column can also favor the formation of heterodisulfides regardless of enzyme preference. The different methods consisting in an in vitro reduction by NTR/TRX systems followed with free thiol labeling are not specific either, due to the presence of endogenous TRX systems in the protein extracts. Several studies have shown, using complementation of a yeast TRX mutant strain by plant TRX genes, that Arabidopsis cytosolic and chloroplastic TRX isoforms exhibit distinct specificities in vivo (Mouaheb et al. 1998; Bréhélin et al. 2000; Issakidis-Bourguet et al. 2001). Thus, the recently developed yeast two-hybrid strain deficient for cytosolic thioredoxins (Vignols et al. 2005) appears as a promising tool to allow in vivo analysis of TRX-target specificity.

Glutaredoxins

Glutaredoxins are small redox proteins of the TRX superfamily using GSH as electron donor. They were

initially described as alternative hydrogen donors to ribonucleotide reductase in an *E. coli* TRX mutant (Holmgren 1976). Compared to TRX, GRX exhibit a similar overall 3D structure, but have a more positive redox potential comprised between – 190 and – 230 mV (Åslund et al. 1997). For detailed information on GRX, we refer the reader to different recent reviews focused on this subject (Holmgren 2000; Lemaire 2004; Rouhier et al. 2004b; Fernandes and Holmgren 2004; Shelton et al. 2005; Holmgren et al. 2005; Herrero et al. in this special issue). GRX seem to play a major role in oxidative stress responses. Indeed, genes coding for GRX are induced by oxidants in bacteria and yeast (Prieto-Alamo et al. 2000; Grant 2001). Moreover, GRX have been reported to exhibit dehydroascorbate reductase (Park and Levine 1996; Washburn and Wells 1999), glutathione reductase (Collinson et al. 2002) and glutathione-S-transferase (Collinson and Grant 2003) activities. These proteins are also involved in redox signaling by regulating transcription factors related to oxidative stress in mammals: NF-1, PEBP2, NF-κB and AP-1 (Bandyopadhyay et al. 1998; Zheng et al. 1998; Nakamura et al. 1999; Hirota et al. 2000). These functions are partially redundant with those of TRX. GRX are also able to catalyze protein deglutathionylation by reduction of GSH-mixed disulfides, while TRX are much less efficient (Jung and Thomas 1996; Nulton-Persson et al. 2003). In non-photosynthetic organisms, three major types of GRX have been identified. Classical GRX harbor a CPYC active-site sequence (GRX1 and GRX3 in *E. coli*, GRX1 and GRX2 in *S. cerevisiae*). A second group, with a CGFS active-site, corresponds to yeast GRX3, GRX4 and GRX5 and *E. coli* GRX4. Finally, *E. coli* GRX2 is an atypical GRX corresponding to a GST containing the GRX CPYC active-site (Xia et al. 2001). GRX can reduce disulfide bridges using a monothiol or dithiol mechanism (Vlamis-Gardikas and Holmgren 2002). Recent studies have suggested that GRX could also be involved in iron–sulfur cluster assembly. Indeed, a yeast mutant deficient in GRX5, a mitochondrial CGFS-type GRX, is unable to assemble iron–sulfur clusters (Rodriguez-Manzaneque et al. 2002; Tamarit et al. 2003). This function appears to be conserved in other organisms (Wingert et al. 2005; Molina-Navarro et al. 2006). Moreover, some GRX were shown to form a complex with iron–sulfur clusters (Lillig et al. 2005; Feng et al. 2006).

In photosynthetic organisms, studies have focused on TRX while very little is known about GRX even though cDNA sequences have been available for a long time (Minakuchi et al. 1994; Szederkenyi et al. 1997). Recent genomic analyses (Lemaire 2004; Rouhier et al. 2004b) have identified three different types of GRX: the CPYC and CGFS groups already described in *E. coli* and yeast; and an additional type called CC, composed of GRX with a CCXC or CCXS active-site (Fig. 2). This latter subgroup seems to be specific of higher plants. Chlamydomonas contains six GRX (2 CPYC-type GRX and 4 CGFS-type GRX) while Arabidopsis contains at least 31 GRX (6 CPYC-type GRX, 4 CGFS-type GRX, and 21 CC-type GRX). Therefore, the situation is different from that observed for the TRX family. Indeed, the number of isoforms of TRX and GRX is higher in Arabidopsis (19 TRX, 31 GRX) than in Chlamydomonas (8 TRX, 6 GRX). However, in the case of TRX, all types (h, o, m, f, x, and y) are present in Chlamydomonas with fewer isoforms for each type. In contrary, in the case of GRX, the difference in the number of isoforms is mainly due to the absence of the CC-type in Chlamydomonas.

Glutaredoxins were shown to be abundant in the phloem sap, like TRX and PRX (Ishiwatari et al. 1995; Balachandran et al. 1997, Szederkenyi et al. 1997; Rouhier et al. 2001). To date, the only biochemical data have been obtained on poplar CPYC-type GRX and have revealed that a GRX was able to reduce a type II PRX, involving GRX in oxidative stress responses (Rouhier et al. 2001, 2002). A CC type glutaredoxin, called ROXY1 has also been shown to be involved in petal development in *A. thaliana* (Xing et al. 2005). However, the targets of GRX remain largely unknown. A first approach has been led recently to identify glutaredoxin targets in photosynthetic organisms, using affinity columns made with a mutated GRX from poplar (a classical GRX with an active-site sequence mutated into CPYS), a strategy similar to those used to identify TRX targets. This approach allowed identification of 94 putative GRX targets from different higher plant tissues (Rouhier et al. 2005). However, most targets identified in this study had already been retained on monocysteinic TRX columns, setting again the problem of the specificity of targets identified using such approaches (Table 1, supplemental data). Clearly, additional work is required to clarify the respective specific functions and/or redundancy of TRX and GRX as well as the interplay between the two systems. Further studies will also be necessary to validate putative GRX targets biochemically and to identify new targets using additional approaches and other types of GRX.

Redox signaling

Redox-signaling mechanisms mediated by dithiol/disulfide exchanges under the control of TRX have

been extensively studied. It is the best characterized mechanism of redox signaling in photosynthetic organisms. Apart from the particular case of light dependent TRX signaling in the chloroplast, the concepts of redox signaling and redox regulation have evolved, during the last 30 years, from the concept of oxidative stress tending to consider ROS as ''bad'' and antioxidants as ''good'' (Ghezzi et al. 2005). Many studies have established that ROS and antioxidants could function as intracellular messengers being produced by cells on purpose under normal growth conditions and not only in stress conditions. Reversible oxidations of cysteine residues appear to play a major role in ROS mediated signaling under the control of TRX, GRX, and related proteins. In non-photosynthetic organisms, transcription factors such as *E. coli* OxyR or yeast Yap1 play an important role in stress signaling. They are regulated by oxidants through oxidation of cysteine residues into sulfenic acids leading to the formation of disulfide bonds (reviewed in Poole et al. 2004). These transcription factors can be later regenerated in their reduced form by TRX and GRX. Recently, it has been suggested that the oxidation of 2-cys PRX catalytic-cysteine into sulfinic acid and its reduction by sulfiredoxins could play a major role in H_2O_2 intracellular messenger function (Wood et al. 2003; Rhee et al. 2005a, 2005b). Moreover, in mammals, the programmed cell death (PCD) or apoptosis is induced by ROS production in mitochondria, under the control of the mitochondrial TRX (Tanaka et al. 2002).

In photosynthetic organisms, several redox-signaling mechanisms have been described. During plant pathogen reactions, ROS are intensively produced. This increase in ROS production is due to an enhanced enzymatic activity of plasma-membrane-bound NADPH-oxidase, cell wall-bound peroxidases and amine oxidases in the apoplast, concomitant with a decrease in catalase and ascorbate peroxidase activities. This oxidative burst activates PCD, which allows to limit the spread of disease from the infection point (Apel and Hirt 2004). The OXI1 kinase seems to participate in redox signaling during oxidative burst (Rentel et al. 2004). ROS produced by NADPH oxidases also play a role in abscissic acid and auxin signaling mechanisms (reviewed in Laloi et al. 2004). Consequently, ROS are involved in stomatal closure (Kwak et al. 2003), root elongation (Foreman et al. 2003) and germination (Torres et al. 2002), processes known to be under the control of abscissic acid. A role for H_2O_2 in auxin signaling and gravitropism in maize roots has also been reported (Joo et al. 2001). ROS produced in plant cells can influence the expression of several genes (Neill et al. 2002), but the mechanisms of signal sensing and signal transduction remain unknown. In Arabidopsis, a signaling cascade involving Mitogen Activated Protein Kinases and Protein Tyrosine Phosphatase (PTP) seems to control indirectly the activation of transcription factors in response to H_2O_2 (reviewed in Apel and Hirt 2004; Laloi et al. 2004). A more direct activation of transcription factors, similar to OxyR or Yap1 activation has recently been shown in plants in the systemic acquired resistance process. NPR1 and TGA1 factors are redox-regulated by reduction of disulfide bonds in response to salicylic acid (Mou et al. 2003; Després et al. 2003). This regulation seems to be linked with the glutathione pool, suggesting that GRX could participate in the reduction of these transcription factors.

As in animals, the glutathione pool constitutes a major redox buffer in photosynthetic organisms. However, in contrast to most animal cells, plant cells also synthesize abundant amounts of ascorbate (vitamin C) and tocopherols (vitamin E), which constitute additional redox buffers. The balance between the different redox buffers and oxidants, called redox homeostasis, determines ROS signaling duration and specificity (Foyer and Noctor 2005). In photosynthetic cells in the light, the chloroplast is the main site of oxidants production (Foyer et al. 1997). Several environmental parameters, such as light and temperature, can affect the efficiency of photosynthetic electron transfer and change the redox state of the chloroplast. High light, for instance, can lead to enhanced ROS production (Foyer and Noctor 2003). The chloroplast can thus participate in the sensing of environmental conditions and redox signals derived from the chloroplast can allow adaptation to stress conditions. While it has been well established that the redox state of the plastoquinone pool can control the expression of several nuclear genes (Pfannschmidt 2003; Fey et al. 2005; Beck 2005), the signaling cascade allowing transduction of the signal from the chloroplast to the nucleus remains to be identified. Chloroplasts, and especially H_2O_2 molecules produced as byproducts of photosynthesis and photorespiration, seem to play a key role in PCD mechanisms (for a review, see Foyer and Noctor 2005). Recent studies have also shown that cell death induced by singlet oxygen produced in chloroplasts is not a result of the oxidative damage caused by this extremely reactive ROS but is due to the execution of a genetic program of PCD via the EXECUTER1 pathway (Wagner et al. 2004).

Reactive oxygen species have been involved in various cell processes but the underlying pathways as well as the sensors of these signals remain poorly understood. These mechanisms of redox signaling are likely to involve regulations by oxidation of cysteine residues. In photosynthetic organisms, much attention has been paid to regulation by dithiol/disulfide exchanges controlled by TRX while the function of GRX remains largely unknown. Similarly, many studies have been focused on the importance and function of glutathione in plants, but very little is known on glutathionylation while the importance of this post-translational modification and its role in redox signaling begins to appear in mammals.

Glutathionylation and signaling

S-thiolation consists in the formation of a mixed disulfide between a free thiol on a protein and a low molecular weight thiol (Gilbert 1984, 1990, 1995). Since glutathione represents the major non-proteic thiol in both photosynthetic and non-photosynthetic cells, the main S-thiolation occurring in cells is S-glutathionylation. This post-translational modification of growing importance in mammals remains much less documented in plants. After an introduction on the function of glutathione, this section will detail the mechanisms, functions and targets of glutathionylation in non-photosynthetic and photosynthetic organisms.

Glutathione

Glutathione is a tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) of 307 Da that can exist in a reduced (GSH), or oxidized (GSSG) form. Oxidized glutathione is continuously regenerated into GSH by glutathione reductase using NADPH as a reductant. As the main free soluble thiol of low molecular weight, glutathione is classically considered to constitute a redox buffer that maintains the intracellular environment in a reduced state. In photosynthetic organisms, the chloroplastic concentration of glutathione is estimated between 1 and 4.5 mM in the stroma, GSSG representing ca. 10% of this pool (Noctor and Foyer 1998). Glutathione has multiple functions in plants (reviewed in Ogawa 2005; Meyer and Hell 2005; Foyer and Noctor 2005). This tripeptide plays a major role in oxidant detoxification either by direct reaction with ROS and RNS, via the ascorbate-glutathione cycle, or as an electron donor for antioxidant enzymes such as some GST and GRX exhibiting glutathione peroxidase activities. Glutathione also participates in xenobiotic detoxification with glutathione-S-transferases (Edwards and Dixon 2005) and heavy metals detoxification, as a substrate for phytochelatin biosynthesis (Cobbett and Golsbrough 2002). Glutathione seems to play a role in various other cell processes such as the cell cycle G1-to-S phase transition (Vernoux et al. 2000), cell differentiation (Henmi et al. 2001, 2005), flowering (Ogawa et al. 2001, 2004), anthocyanins accumulation (Xiang et al. 2001), in PCD and in resistance against pathogens (Mou et al. 2003; Després et al. 2003; Foyer and Noctor 2005). In addition to all these functions, recent studies have suggested that glutathione could also play a major role in redox signaling through modification of cysteine residues. Indeed, glutathione can regulate the oxidation state of cysteines either directly by protein glutathionylation or indirectly, via GRX. In mammals, it has been shown that glutathionylation could modulate the activity, the localization or the stability of a growing number of proteins. Compared to the abundant literature on mammals, data obtained in photosynthetic organisms are rather scarce.

Glutathionylation as a redox regulation mechanism

If glutathionylation as a redox regulation mechanism has only recently emerged, this modification has been discovered a long time ago. It has been known for many years that in liver, under normal conditions, 1% of the glutathione molecules are bound to proteins (Brigelius et al. 1982, 1983) and that under stress conditions, these mixed disulfides can represent up to 20–50% of the glutathione pool (Gilbert 1984). Various studies have allowed the identification of different proteins susceptible to undergo glutathionylation since then. This post-translational modification can constitute an antioxidant mechanism that evolved to protect cysteine residues from irreversible oxidation to sulfinic or sulfonic acids (Fig. 1). Moreover, it has been clearly established that glutathionylation can modulate either positively or negatively the activity of numerous enzymes and play an important role in redox signaling. Protein glutathionylation has been recently the subject of several reviews detailing the role of this modification in mammals (Fratelli et al. 2004; Ghezzi 2005a, b; Ghezzi et al. 2005; Hurd et al. 2005; O'Brian and Chu 2005; Shackelford et al. 2005).

Mechanisms of glutathionylation

Protein glutathionylation is a dynamic process. Theoretically, several mechanisms can lead to protein

glutathionylation but the precise mechanisms occurring in vivo are still far from being well understood.

The reaction can occur through a thiol/disulfide exchange in the presence of GSSG:

$$PSH + GSSG \rightarrow PSSG + GSH \quad (1)$$

Glutathionylation can also occur through direct oxidation:

$$GSH + PSH \rightarrow PSSG + 2H^+ \quad (2)$$

However, a direct oxidation of two thiols in the presence of oxygen for instance is unlikely to occur because this reaction would implicate a ternary collision between the two thiols and the oxidant. This kind of reaction can, however, be catalyzed by oxidants such as diamide (Kosower and Kosower 1987):

$$\begin{aligned}(CH_3)_2NCON{=}NCON(CH_3)_2 + RS^- + H^+ &\rightarrow (CH_3)_2NCON(SR)NHCON(CH_3)_2\\ (CH_3)_2NCON(SR)NHCON(CH_3)_2 + R'S^- + H^+ &\rightarrow RSSR' + (CH_3)_2NCONHNHCON(CH_3)_2\end{aligned} \quad (3)$$

where R and R′ can be either glutathione (G) or a protein (P).

Another possible mechanism requires prior oxidation of one of the two thiols involved in the reaction. Protein thiols oxidized into sulfenic acids can then react with a molecule of glutathione and generate a disulfide:

$$PSOH + GSH \rightarrow PSSG + H_2O \quad (4)$$

It has been shown that human protein tyrosine phosphatase 1B can undergo glutathionylation via this mechanism (Barrett et al. 1999b).

Glutathionylation can also occur if the thiol on the glutathione molecule is oxidized into a sulfenic acid:

$$GSOH + PSH \rightarrow PSSG + H_2O \quad (5)$$

The oxidation of protein thiols (6) or of glutathione thiol (7) by the hydroxyl radical ($HO^{\cdot}$), can lead to the formation of thiyl radicals:

$$PSH + HO^{\cdot} \rightarrow PS^{\cdot} + H_2O \quad (6)$$

$$GSH + HO^{\cdot} \rightarrow GS^{\cdot} + H_2O \quad (7)$$

Thiyl radical from a protein, reaction (8), or from glutathione, reaction (9), can lead to a radical mixed disulfide. This radical mixed disulfide will then transfer an electron to oxygen to form the superoxide anion $O_2^{\cdot-}$ leaving a mixed disulfide:

$$PS^{\cdot} + GSH \underset{H^+}{\rightleftarrows} PS^{\cdot}\text{-}SG \underset{O_2\,O_2^{\cdot-}}{\rightleftarrows} PSSG \quad (8)$$

$$GS^{\cdot} + PSH \underset{H^+}{\rightleftarrows} GS^{\cdot}\text{-}SP \underset{O_2\,O_2^{\cdot-}}{\rightleftarrows} PSSG \quad (9)$$

It has been suggested that a GRX could catalyze reaction (9) (Starke et al. 2003).

Glutathionylation can also be induced by nitric oxide (NO). Nitrosylated thiols can react with glutathione to form mixed disulfides:

$$PSNO + GSH \rightarrow PSSG + HNO \quad (10)$$

NO exposure can also lead to glutathione nitrosylation. This S-nitrosylated glutathione (GSNO) can then react with a protein thiol and lead to glutathionylation of the protein:

$$GSNO + PSH \rightarrow PSSG + HNO \quad (11)$$

Some oxidized forms of glutathione, such as GS–O–SG can also favor the formation of GSH-protein mixed disulfides (Huang and Huang 2002).

All these mechanisms are possible, but the one prevailing in vivo remains unknown. Although glutathionylation by thiol/disulfide exchange (reaction (1)) with oxidized glutathione is widely considered as the major reaction leading to mixed disulfide formation, this possibility is questioned by several studies. First, this mechanism implies that protein glutathionylation is directly linked to the redox state (GSH/GSSG) of the glutathione pool. However, in vivo GSSG concentration is very low compared to GSH concentration and the GSSG concentration required for efficient protein glutathionylation in vitro is usually higher than the physiological concentration. Moreover, during the oxidative burst in human neutrophils, a massive increase in protein glutathionylation is observed without any increase in GSSG concentration (Chai et al. 1994). Interestingly, a glutathionylated protein, the human actin, does not seem to undergo this modification by thiol/disulfide exchange, in vitro as well as in vivo (Dalle-Donne et al.

2003, 2005). Moreover, it has been suggested that a proteic factor could be required for the glutathionylation of some proteins in vitro (Dixon et al. 2005b).

Glutathionylation is a reversible modification. It has been shown that GRX can catalyze the deglutathionylation of proteins by reducing the mixed disulfide between a protein and the molecule of glutathione with a far higher efficiency than TRX or protein disulfide isomerases (Jung and Thomas 1996; Nulton-Persson et al. 2003).

Glutathionylation targets in non-photosynthetic organisms

Many studies on the function of protein glutathionylation have been performed in non-photosynthetic organisms, especially in mammals. These studies have allowed to identify many targets of glutathionylation thus implicating this modification in the regulation of various cell processes. Glutathionylation can protect vulnerable protein thiols from irreversible oxidation. This modification can also regulate a specific protein activity as in the case of the bifunctional carbonic anhydrase III, which exhibits also a phosphatase activity tightly regulated by the glutathionylation of two cysteine residues (Koester et al. 1981; Thomas et al. 1995; Cabiscol and Levine 1996). The activity of the HIV protease 1 and of the HTLV-I protease is also regulated by glutathionylation of two cysteine residues (Davis et al. 1996, 1997, 2003).

The cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can undergo glutathionylation. In addition to the central role of this protein in glycolysis and energy production, GAPDH has been recently involved in many other functions including regulation of apoptosis, DNA repair or nuclear RNA export (reviewed in Sirover 1999, 2005). The enzyme presents a very reactive active-site cysteine that is presumably the target of glutathionylation, leading to inhibition of the protein activity (Ravichandran et al. 1994; Schuppe-Koistinen et al. 1994; Grant et al. 1999; Mohr et al. 1999). In yeast, a H_2O_2 treatment leads to glutathionylation of GAPDH as well as of two other glycolytic enzymes: enolase and alcohol dehydrogenase (Shenton and Grant 2003). Many other glycolytic enzymes (aldolase, phosphoglycerate kinase, pyruvate kinase and triose phosphate isomerase) can also undergo glutathionylation in human cells (Fratelli et al. 2003). These data suggest that glutathionylation could coordinate cellular metabolism in response to oxidative stress by modulating glycolysis.

Glutathionylation also seems to regulate several enzymes involved in signaling pathways such as enzymes of the protein kinase C (PKC) family: PKCε, which stimulates cell growth, is inactivated by this modification whereas PKCδ, which play an important role in DNA damage-initiated apoptotic pathways, seem to be activated by S-thiolation (Ward et al. 2002; Chu et al. 2003). PKCα can also be inactivated by glutathionylation (Ward et al. 2000). Several transcription factors can be regulated by glutathionylation such as the c-Jun subunit of the AP-1 complex (Klatt et al. 1999a; b), the p50 subunit of the NF-κB factor (Pineda-Molina et al. 2001, 2002), or NF-1 (Nuclear Factor 1) (Bandyopadhyay et al. 1998). Glutathionylation of these transcription factors results in an inhibition of DNA binding.

Cytoskeletal arrangements and intracellular trafficking can also be regulated by glutathionylation. Indeed, this modification can regulate actin polymerization (Wang et al. 2001, 2003; Dalle-Donne et al. 2003, 2005). Moreover, annexin A2, which interacts with actin, is also glutathionylated (Caplan et al. 2004), as well as tubulin (Landino et al. 2004).

Several enzymes that have a cysteine in their active-site, like GAPDH, are inhibited by S-thiolation, such as α-ketoglutarate dehydrogenase (αKGDH) (Nulton-Persson et al. 2003), creatine kinase (Reddy et al. 2000) or cAMP Dependent Protein Kinase (CDPK) (Humphries et al. 2002). Protein tyrosine phosphatase 1B (PTP 1B), which is involved in the regulation of αKGDH activity, is also inhibited (Barrett et al. 1999a, b).

However, protein glutathionylation has not always an inhibitory effect. H-ras, for instance, a G protein involved in many different transduction pathways, is activated by glutathionylation (Adachi et al. 2004a). The calcium pump SERCA, implicated in muscle relaxation, apoptosis and proliferation, is also activated by glutathionylation (Adachi et al. 2004b) whereas this kind of regulation inhibits the chloride channel CFTR (Wang et al. 2005). The regulation of several chaperones of the HSP family has also been described. The multimeric aggregate size of HSP27 seems to be regulated by glutathionylation (Eaton et al. 2002a) and the chaperonin activity of HSP70 is activated by this modification (Hoppe et al. 2004).

Glutathionylation in non-photosynthetic organisms has been also studied by proteomic approaches (Klatt et al. 2000; Eaton et al. 2002b; Fratelli et al. 2002, 2003; Lind et al. 2002; Shenton and Grant 2003). Most of the techniques used were based on ^{35}S radioactive labeling of the glutathione pools. Alternative methods using biotinylated glutathione, anti-glutathione antibodies or affinity columns with oxidized or reduced glutathione as well as immobilized glutathione ana-

logs, have been also developed (for review, see Fratelli et al. 2004). More than 120 proteins have been identified as putative glutathionylation targets in mammals (Table 1, supplemental data). However, in most cases, the functional effect of this modification remains unknown.

Glutathionylation targets in photosynthetic organisms

Very few data are available about glutathionylation in photosynthetic organisms. To our knowledge, the first study reporting that a plant protein could undergo glutathionylation has been published by Dixon and co-workers (2002). These authors identified GST proteins from Arabidopsis with dehydroascorbate reductase and/or GSH-dependent thiol transferase activity. These GST have a catalytic cysteine that is essential for their activity. This cysteine can undergo glutathionylation in vitro in the presence of GSSG, leading to inhibition of enzymatic activity. However, the authors suggested that the mixed disulfide formed between the catalytic cysteine and glutathione could be an intermediary step in the reaction mechanism of glutathione-dependent dehydroascorbate reduction.

In 2003, a second publication reported a larger scale approach to identify glutathionylated proteins in Arabidopsis cell suspensions using biotinylated-glutathione labeling (Ito et al. 2003). In this study, 20 glutathionylated proteins have been visualized, two of which have been identified: chloroplastic fructose-1,6-bisphosphate aldolase, a Calvin cycle enzyme, and cytosolic triose phosphate isomerase (TPI), a glycolytic enzyme. Additional experiments revealed that recombinant TPI could be inactivated by GSSG in vitro.

The inactivation of a soybean PTP by S-thiolation has also been recently reported (Dixon et al. 2005a). However, this regulation seems to involve a mechanism different from the regulation of human PTP, which is also inactivated by glutathionylation (Barrett et al. 1999a).

A mitochondrial TRX from poplar has been reported to undergo glutathionylation in vitro (Gelhaye et al. 2004a). The increase of the redox potential of the active-site disulfide upon glutathionylation suggests that the activity of this TRX towards still unidentified targets could be affected. Casagrande and co-workers (2002) have reported specific glutathionylation of human TRX on an extra cysteine, distinct from those of the active-site. Moreover, S-thiolation appears to affect TRX activity, probably by decreasing the efficiency of TRX reduction by NTR. These findings prompted us to investigate the glutathionylation of all chloroplastic TRX from Arabidopsis and Chlamydomonas (Michelet et al. 2005). This study revealed that f-type thioredoxins are the only chloroplastic TRX able to undergo glutathionylation in vitro. The target residue is an extra cysteine, distinct from the two active-site cysteines, which is strictly conserved in all f-type TRX. Moreover, the reconstitution of the thylakoid dependent reduction system allowed to demonstrate that glutathionylation of the protein impairs its reduction in the light, resulting in impaired activation of targets. Since f-type TRX appear to be specifically involved in the regulation of carbon assimilation enzymes by light, their glutathionylation could constitute a new mechanism of regulation of photosynthetic metabolism under oxidative stress (Michelet et al. 2005).

In addition to studies focused on a particular type of enzymes, a proteomic approach has been recently led on Arabidopsis cell suspensions in order to identify a large number of proteins undergoing glutathionylation in vivo (Dixon et al. 2005b). However, technical difficulties only allowed the identification of a limited number of proteins: sucrose synthase, tubulins α and β, Acetyl-CoA Carboxylase, actin, cytosolic GAPDH, transducin, and Hsc70–1. Due to these problems, the authors have then studied in vitro glutathionylation of proteins in the presence of biotinylated-GSSG. Proteic extracts from Arabidopsis cells grown in the dark were used in this study because of the problems encountered with foliar extracts. A total of 73 proteins have been identified with among them, methionine synthase and alcohol dehydrogenase. Interestingly, the in vitro thiolation of the two latter proteins was found to require unidentified proteic factors present in Arabidopsis cell extracts. This is the first large-scale approach led in a photosynthetic organism to identify glutathionylated proteins, pinpointing the many technical difficulties inherent to this kind of studies. Other studies in more physiological conditions will be needed to identify other proteins susceptible to undergo glutathionylation with particular emphasis on chloroplastic targets since chloroplast is the major site of oxidant production under light. Regulation by glutathionylation thus could play an important role in this compartment.

TRX, GRX, and glutathione: new crosstalks to explore

Emerging evidence suggests that TRX, GRX and glutathione/glutathionylation could constitute a complex

Table 2 Putative or established targets of a dual redox regulation by "redoxins" (thioredoxins and or glutaredoxins) and by glutathionylation

	Thioredoxin targets		Glutaredoxin targets		Glutathionylated proteins	
	Photosynthetic organims	Non-photosynthetic organisms	Photosynthetic organims	Non-photosynthetic organisms	Photosynthetic organims	Non-photosynthetic organisms
14-3-3 protein			x^{63}			$2x^{67}$
Acetyl-CoA carboxylase	$x^{26,39,53,77}$				x^{56}	
Aconitase	$x^{40,48,51}$	x^{46}	x^{63}		x^{56}	x^{5}
Actin	$x^{48,51}$				x^{56}	$x^{5,20,28,30,36,42,54,66,67}$
Adenylate kinase	$x^{40,48}$					x^{66}
Alcohol dehydrogenase	$x^{40,49,52}$				x^{56}	$x^{35,57}$
Aldehyde dehydrogenase	$x^{40,51}$		x^{63}			x^{35}
Aldose reductase	x^{49}					$x^{13,23,25,67}$
AP1 (c-Jun subunit)		$x^{6,8,76}$		x^{8}		$x^{10,11,57}$
Argininosuccinate synthetase	$x^{32,48}$				x^{56}	
Carbonic anhydrase	$2x^{26,39,50}$		x^{63}			$x^{3,4,15,57,86}$
Cyclophilin (peptidylprolyl cis–trans isomerase)	$2x^{17,44,49,50,51,75}$	$2x^{46}$	x^{63}			$x^{30,42,66,70}$
Cytochrome c oxidase	$2x^{40}$					$2x^{30,66}$
Enolase	$2x^{26,37,48,49,51}$	x^{46}	x^{63}		x^{56}	$x^{35,66,67}$
Fructose-1,6-bisphosphate aldolase	$2x^{37,48,50,51,52}$	x^{46}	x^{63}		$x^{31,56}$	$x^{35,66,67}$
Globulin	$4x^{37,51}$				x^{56}	
Glutaredoxin	x^{40}					x^{57}
Glutathione S-transferase	x^{50}				$6x^{21,56}$	$x^{16,27,29,42}$
Glyceraldehyde 3-P dehydrogenase (NAD)	$4x^{37,49,51}$	x^{46}	x^{63}		x^{56}	$x^{1,2,5,9,12,34,35,57}$
Glycine decarboxylase	$3x^{40}$		$2x^{63}$		x^{56}	
HSP60 Heat shock/Chaperonin protein 60 kDa	$2x^{32,40,48,51,52}$		$3x^{63}$		x^{56}	$x^{66,67}$
HSP70 Heat shock/Chaperonin protein 70 kDa	$3x^{26,39,40,48,51,52}$		$3x^{63}$			$x^{30,35,42,45,66,67}$
HSP90 Heat shock/Chaperonin protein 90 kDa		x^{46}				x^{67}
Inorganic pyrophosphatase	$2x^{48}$					x^{67}
Isocitrate dehydrogenase	$x^{40,50}$	x^{46}	x^{63}			x^{38}
Malate dehydrogenase (NAD)	$x^{37,49,50,51,52}$		x^{63}			x^{160}
Malic enzyme	x^{40}		x^{63}		$2x^{56}$	
Methionine synthase	$x^{51,52}$		x^{63}		x^{56}	
NF-κB (p50 subunit)		^{4,8,68}		x^{8}		$x^{18,57}$
Nuclear factor-1				x^{7}		x^{7}
Nucleoside diphosphate kinase	$x^{40,49}$	x^{46}	x^{63}			$x^{5,67}$
OxyR				x^{55}		x^{60}
1-Cys Peroxiredoxin	$x^{32,33,37,51}$					$x^{61,67}$
2-Cys Peroxiredoxin	$2x^{17,26,48,49,51,78,79}$		x^{63}		x^{56}	$x^{62,66,67}$
Peroxiredoxin type II	$x^{48,50,52,73,74}$		$3x^{63,73,74}$			$x^{30,42,67}$
3′-adenylylsulfate (PAPS) reductase		$x^{71,72}$		$x^{71,72}$		x^{72}
Phosphoglycerate kinase	x^{39}		x^{63}			x^{66}
Protein disulfide isomerase	$x^{14,19,37,48,50,51,58}$					$3x^{66,67}$
Pyruvate kinase		x^{46}				$x^{67,69}$
Ran	x^{48}					x^{30}
Reversibly glycosylated polypeptide	$2x^{37,50,51}$		x^{63}		$2x^{56}$	

Table 2 continued

	Thioredoxin targets		Glutaredoxin targets		Glutathionylated proteins	
	Photosynthetic organims	Non-photosynthetic organisms	Photosynthetic organims	Non-photosynthetic organisms	Photosynthetic organims	Non-photosynthetic organisms
Ribosomal protein S5	x[39]					x[35]
40S ribosomal protein S12	x[51]					x[66]
Rubisco-binding protein	2x[26,39]				x[56]	
Sulfate adenylyltransferase	x[32]				x[56]	
Superoxide dismutase (Cu–Zn)	x[49,51]					x[34,57]
Triosephosphate isomerase	2x[26,37,49,50,51]	x[46]	x[63]		x[31]	x[5,35,66]
Tubulin	2x[51]				2x[56]	2x[42,47,67]
Ubiquitin conjugating enzyme	x[51]					x[30,64,66]
UDP-glucose Ppase	x[51]				x[56]	

For each protein, the type of regulation (TRX, GRX, or glutathionylation) and the type of organism (photosynthetic, non-photosynthetic) is indicated with an ''x'' in the corresponding column. When several isoforms of the protein have been identified, the 'x' is preceded by the number of isoforms reported. Superscript numbers indicate the corresponding references as follows: **1**: Ravichandran et al. (1994), **2**: Schuppe-Koistinen et al. (1994), **3**: Thomas et al. (1995), **4**: Cabiscol and Levine (1996), **5**: Eaton et al. (2002b), **6**: Schenk et al. (1994), **7**: Bandhyopadhyay et al. (1998), **8**: Hirota et al. (2000), **9**: Grant et al. (1999), **10**: Klatt et al. (1999a), **11**: Klatt et al. (1999b), **12**: Mohr et al. (1999), **13**: Cappiello et al. (1996), **14**: Trebitsh et al. (2000), **15**: Kim and Levine (2005), **16**: Dafre et al. (1996), **17**: Motohashi et al. (2001), **18**: Pineda-Molina et al. (2001), **19**: Trebitsh et al. (2001), **20**: Wang et al. (2001), **21**: Dixon et al. (2002), **22**: Eaton et al. (2002a), **23**: Cappiello et al. (1994), **24**: Pineda-Molina and Lamas (2002), **25**: Cappiello et al. (2001), **26**: Balmer et al. (2003), **27**: Sies et al. 1998, **28**: Dalle-Donne et al. (2003), **29**: Celli et al. (2003), **30**: Fratelli et al. (2003), **31**: Ito et al. (2003), **32**: Lindahl and Florencio (2003), **33**: Marx et al. (2003), **34**: Tao and English 2004, **35**: Shenton and Grant (2003), **36**: Wang et al. (2003), **37**: Wong et al. (2003), **38**: Kil and Park 2005, **39**: Balmer et al. (2004a), **40**: Balmer et al. (2004b), **41**: Caplan et al. (2004), **42**: Fratelli et al. (2004), **43**: Gelhaye et al. (2004a); **44**: Gopalan et al.(2004), **45**: Hoppe et al. (2004); **46**: Kumar et al. (2004), **47**: Landino et al. (2004), **48**: Lemaire et al. (2004), **49**: Maeda et al. (2004), **50**: Marchand et al. (2004), **51**: Wong et al. (2004), **52**: Yamazaki et al. (2004), **53**: Hunter and Ohlrogge 1998, **54**: Dalle-Donne et al. (2005); **55**: Zheng et al. (1998), **56**: Dixon et al. (2005b), **57**: Klatt et al. (2000), **58**: Levitan et al. (2005), **59**: Maeda et al. (2005), **60**: Kim et al. (2002), **61**: Manevich et al (2004), **62**: Sullivan et al. (2000), **63**: Rouhier et al. (2005), **64**: Jahngen-Hodge et al. (1997), **65**: Florencio et al. (1993), **66**: Fratelli et al. (2002), **67**: Lind et al. (2002), **68**: Hirota et al. 1999, **69**: Mannervick and Axelsson (1980), **70**: Ghezzi et al. (2006), **71**: Lillig et al. (1999), **72**: Lillig et al. (2003), **73**: Rouhier et al. (2001), **74**: Rouhier et al. (2002), **75**: Motohashi et al. (2003), **76**: Hirota et al. (1997), **77**: Sasaki et al. (1997), **78**: Goyer et al. (2002), **79**: König et al. (2002), **80**: Gelhaye et al. (2003), **81**: Casagrande et al. (2002), **82**: Adachi et al. (2004a), **83**: Reddy et al. (2000), **84**: Ward et al. (2000), **85**: Humphries et al. (2002), **86**: Ji et al. (1999), **160**: Eaton and Shattock (2002)

network involved in redox signaling with multiple crosstalks between these three components. As previously mentioned, most of the 94 putative plant targets bound to a GRX column were previously identified as possible TRX targets underlining the connections and/or redundancy between these two redox regulators.

Studies on redox-regulated proteins in non-photosynthetic organisms have focused essentially on the identification of glutathionylated proteins while the diversity of TRX targets remains to be examined by proteomics approaches. In contrary, work on photosynthetic organisms has allowed identification of many putative TRX and GRX targets while much less is known on glutathionylated proteins. However, it can be noticed that several proteins identified as glutathionylatable in mammals and in Arabidopsis exhibit a direct or indirect link with TRX and/or GRX. In order to get a complete overview of these possible interactions, we have collected the list of proteins identified as established or putative targets of TRX or GRX but also those undergoing glutathionylation (Table 1, supplemental data). Analysis of these data revealed that many proteins were identified as being both glutathionylated and putative targets of TRX and/or GRX (Table 2). Among the glutathionylated proteins identified in non-photosynthetic organisms, many homologous proteins have been identified as putative TRX targets on monocysteinic TRX columns in plants such as actin, adenylate kinase, enolase, NAD dependent malate dehydrogenase, HSP 60, HSP 70, Ran, TPI, and fructose-1,6-bisphosphate aldolase. For some of these proteins a double regulation by reduction of a disulfide bond under the control of TRX/GRX on one side and by glutathionylation of a cysteine on the other side is possible. For example, the transcription factors c-Jun and NF-κ B are TRX targets and are glutathionylated. TRX are also known to be efficient electron donors to PRX and human PRX I, IV, V, and VI have been identified as proteins susceptible to undergo glutathionylation. Similarly, cyclophilin A is glutathionylated in human cells (Ghezzi et al. 2006) while several chloroplastic cyclophilins have been shown to be redox-regulated by TRX (Motohashi et al. 2003; Gopalan et al. 2004; Buchanan and Luan 2005). Nevertheless, if GRX are far more efficient than TRX in reducing GSH/protein mixed disulfides, TRX are also able to catalyze this reaction (Jung and Thomas 1996). It is thus possible that some proteins bind to monocysteinic TRX columns because of their glutathionylation. This would suggest that some of the proteins identified using these approaches could not only be TRX and GRX targets but also S-thiolation targets.

In addition, several studies have demonstrated the existence of more direct interactions between TRX, GRX, and glutathione/glutathionylation. Human TRX has been shown to undergo glutathionylation in human cells (Casagrande et al. 2002). In photosynthetic organisms, chloroplastic f-type thioredoxins (Michelet et al. 2005) as well as a mitochondrial TRX from poplar (Gelhaye et al. 2004a) have also been shown to undergo this modification. Interestingly, glutathionylation of these TRX is susceptible to affect their activity toward their targets and thereby could lead to metabolic changes. Another direct link was observed in plants when a thioredoxin from poplar was found to be reduced by GRX and GSH instead of NADPH-thioredoxin reductase (Gelhaye et al. 2003) and two glutathione peroxidases were shown to be TRX-dependent (Herbette et al. 2002). Direct crosstalks have also been observed in non-photosynthetic organisms: *Saccharomyces cerevisiae* GRX5 (CGFS type) could be more efficiently reduced by TRX than by GSH (Tamarit et al. 2003) and *E. coli* Grx4, a CGFS-type GRX, has been reported to act as a substrate for *E. coli* thioredoxin reductase after GSSG oxidation (Fernandes et al. 2005). In addition, in some organisms such as drosophila that have apparently no glutathione reductase to regenerate GSH, the TRX system is involved in GSSG reduction (Kanzok et al. 2001). It has also been recently shown that a protein, called TGR for Thioredoxin/Glutathione Reductase, composed of a thioredoxin reductase and a GRX domain, was able to reduce both TRX and GSSG, and that it was able to use GSH as an electron donor to the GRX domain (Sun et al. 2001, 2005). Another link between TRX and GRX systems has been evidenced with human mitochondrial GRX2. This GRX harbors a CSYC active-site and lacks one conserved non-active-site cysteine residue present in human GRX1. Due to the absence of this cysteine, human GRX2 is less prone to inactivation by oxidants and GSSG. Moreover, this GRX can be reactivated directly by TRX reductase as well as GSH, suggesting that human GRX2 could operate in an oxidative cell environment (Johansson et al. 2004).

Additional studies are thus required to clarify the respective specific functions and redundancy of TRX, GRX and glutathione/glutathionylation as well as the interplay between these three key components of the cellular redox-signaling network. Unraveling the role and importance of this crosstalk will probably constitute one of the main challenges of future studies on redox signaling.

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