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Review

Glutaredoxins and thioredoxins in plants

Yves Meyer *, Wafi Siala, Talaat Bashandy, Christophe Riondet, Florence Vignols, Jean Philippe Reichheld

Université de Perpignan, Génome et dévelopement des plantes, CNRS-UP-IRD UMR 5096, Av P. Alduy, F 66860 Perpignan, Cedex, France

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Abstract

During the 70s and 80s two plant thioredoxin systems were identified. The chloroplastic system is composed of a ferredoxin-dependent thioredoxin, with two thioredoxin types (m and f) regulating the activity of enzymes implicated in photosynthetic carbon assimilation. In the cytosol of heterotrophic tissues, an NADP dependent thioredoxin reductase and a thioredoxin (h) were identified. The first plant glutaredoxin was only identified later, in 1994. Our view of plant thioredoxins and glutaredoxins was profoundly modified by the sequencing programs which revealed an unexpected number of genes encoding not only the previously identified disulfide reductases, but also numerous new types. At the same time it became clear that plant genomes encode chloroplastic, cytosolic and mitochondrial peroxiredoxins, suggesting a major role for redoxins in anti-oxidant defense. Efficient proteomics approaches were developed allowing the characterization of numerous thioredoxin target proteins. They are implicated in different aspects of plant life including development and adaptation to environmental changes and stresses. The most important challenge for the next years will probably be to identify *in planta* which redoxin reduces which target, a question which remains unsolved due to the low specificities of redoxins *in vitro* and the numerous redundancies which in most cases mask the phenotype of redoxin mutants.

Keywords: Thioredoxin; Glutaredoxin; Redox; *Arabidopsis*; Rice

The thioredoxins (Trx) and NADPH dependent thioredoxin reductases (NTR) were discovered 40 years ago as a system able to transfer reduction power to ribonucleotide reductase. In most organisms RNR is the unique way to produce deoxyribonucleotides and, consequently, allow cell division [1,2]. Later, glutaredoxins (Grx) were identified as alternative reductants of RNR. Grx are disulfide reductases which are reduced directly by glutathione [3]. Although the sequences of glutaredoxins show very limited similarities with thioredoxins, both types of disulfide reductases present similar folding and are now grouped in the thioredoxin superfamily (redoxins).

Plant thioredoxins were discovered in the 1970s as regulators of several chloroplastic enzymes related to photosynthesis and malate synthesis. Whereas water photolysis, the first step of photosynthesis, is intrinsically light dependent, carbon fixation performed in the Calvin cycle and malate synthesis are independent of light energy. Nevertheless, it was found that Fructose-1,6-bisphosphatase (FBPase), an enzyme of the Calvin cycle, is inactive when extracted from plants maintained in the dark but can be activated by a short illumination of the plant before extraction. A biochemical approach performed in B. Buchanan's lab demonstrated that the activation signal is ferredoxin-dependent, relayed by a protein that the authors identified as a thioredoxin (Trx f) and by a second fraction which was postulated to contain a ferredoxin-dependent thioredoxin reductase (FTR) [4]. The identification of the thioredoxin system in plant chloroplasts introduced a fairly new concept for thioredoxin functions: in contrast to RNR which is a reductase getting its reducing power from thioredoxin, FBPase is a phosphatase that does not need a reduction flux for activity. The thioredoxin induces a change of conformation in the FBPase, shifting the oxidized inactive FBPase to the reduced active conformation. Soon after the discovery of this process, it was shown that the activity of the chloroplastic NADPH-malate

* Corresponding author. Tel.: +33 4 68662225; fax: +33 4 68668499.
E-mail address: ymeyer@univ-perp.fr (Y. Meyer).

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dehydrogenase (MDH), which reduces oxaloacetate to malate using NADPH as reductant, is also activated by disulfide reduction in a ferredoxin-dependent process. That study revealed a second type of chloroplastic thioredoxin which was named Trx m. In contrast to Trx f, which efficiently activates FBPase or MDH, Trx m is only able to activate MDH and to deactivate glucose-6-phosphate dehydrogenase. Using the chloroplastic MDH as a substrate, a third type of plant thioredoxin was found in heterotrophic tissues (TRX h). This cytosolic thioredoxin is reduced by an NADPH dependent thioredoxin reductase.

Thus, in the 1970s it was clear that plants present three types of thioredoxins; the chloroplastic f and m, reduced by a ferredoxin-dependent thioredoxin reductase, and a cytosolic thioredoxin h reduced by an NADPH thioredoxin reductase. In the following years, considerable progress was made on the regulation of photosynthesis by the thioredoxin systems, including the discovery of new target proteins and fine analysis of the disulfide reduction due to the development of recombinant proteins. A plant glutaredoxin was identified from rice only later, in 1994 [5].

In the redoxin field, as in most fields of plant physiology, the sequencing programs have profoundly modified our knowledge and concepts. The release of the *Arabidopsis thaliana* genome in 2000 represents a milestone in our knowledge of the complexity of redoxins in plants. Soon after, proteomics forged our present view on redox regulation, which appears to be implicated in almost all functions necessary for plant development and adaptation to environmental changes including abiotic and biotic stresses.

1. Members of the thioredoxin superfamily and their reductants in plant genomes

As early as 1995, the *Arabidopsis* EST programs showed that each type of thioredoxin is encoded by multiple genes and new thioredoxin types were discovered. Due to their low amino-acid sequence similarity, blast queries and tree constructions were carried out independently for thioredoxins and glutaredoxins.

1.1. The thioredoxins encoded in the Arabidopsis genome

Searching the present *Arabidopsis* genome for thioredoxin genes reveals different families as previously shown [6]. In the present study we included Protein Disulfide Isomerases (PDI) (Fig. 1; Table 1). Typical PDIs are proteins constituted of two thioredoxin modules with a redox site, WCGHC, which is oxidizing. In addition, they exhibit a C-terminal KDEL motif which allows retention in the Endoplasmic Reticulum. Nevertheless, in addition to typical Trx with a WCGPC redox site and PDI, the genome of *Arabidopsis* encodes variants with non-canonical active sites or module numbers. For some members it is not obvious if they are Trx or PDI on the simple basis of the protein sequence. Thus, although this review will not include the rather limited knowledge on plant PDI, they are included in Fig.1 in order to obtain an exhaustive inventory of Trx homologues. Most members present in the lower part of the phylogenetic tree are thioredoxins with only one domain with a classical reducing WCGPC active site. Other Trxs present variant redox sites including two monocysteinic Trxs h. Some Trx show an N-terminal extension which was shown to be a transit peptide addressing the protein to the chloroplast (type m, f, x, y, HCF164, CDSP32) or the mitochondria (type o). Larger proteins show particular structures: Picot, CDSP32 and nucleoredoxins have 2 or 3 thioredoxin domains. TDX has an N-terminal Heat Shock Protein interacting (HIP) domain associated with a thioredoxin domain. In the upper part of the tree, a subgroup contains the PDI [7]. Some of these sequences harbor the classical PDI structure with two thioredoxin domains, each bearing an oxidant WCGHC redox site and a C-terminal KDEL domain allowing reticulum retention. A particularity of the *Arabidopsis* genome is the presence of 5 PDI pairs resulting from the last genome duplication which took place at the emergence of the *Brassicaceae*. While the two members of one pair possess a KDEL extension, in the three other pairs one member has a KDEL domain while the second member has lost this C-terminal extension. The last pair is constituted by two PDI homologues showing only one thioredoxin domain with atypical redox sites, WCYWC and WCYWS, and no KDEL terminal tag. In contrast to *Chlamydomonas* and several plasmodia which present a chloroplastic PDI, *Arabidopsis* does not seem to harbor such chloroplastic PDI. Loosely associated with the PDI, two flavin-dependent quescin sulfhydryl oxidases (QSOX) associate an oxidizing thioredoxin domain with an FAD containing ERV domain. These proteins are key elements of disulfide formation in the Endoplasmic Reticulum. The three adenosine 5′-phosphosulfate reductases (APR) are grouped with the PDI. These chloroplastic proteins implicated in sulfate assimilation present a domain responsible for adenosine 5′-phosphosulfate (APS) reduction and a thioredoxin domain related to PDI but with a reducing redox center, CPFC, similar to a glutaredoxin active site. In contrast to glutaredoxins which are reduced by glutathione, APS reductases are reduced by the ferredoxin-dependent thioredoxin reductase. Some loosely related proteins of unknown function were named APR Like 4–7 proteins in TAIR according to [7]. This appears very confusing because these proteins show significant structural similarities with the thioredoxin domain of APR but do not present APS reductase domains. Thus they are clearly not APR.

We indicate the subcellular localization of the *Arabidopsis* Trxs in Fig. 1 and Table 1. The plastidial localization of Trxs m, f, x, y, CDSP32 and NTRC and the mitochondrial localization of Trx o1 were established by cell fractionation or by expression of GFP Trx fusion proteins. It is generally suggested that thioredoxins h are cytosolic proteins due to the absence of a transit peptide, but this was proven experimentally only for *Arabidopsis* Trx h3 and Trx h5. In contrast Marcus et al. found evidence for the presence of castor bean Trx h in the mitochondria [8]. Similarly, the poplar Trx h2, which belongs to the subgroup h II is located in the mitochondria [9]. Thus the localization of the Trx h in *Arabidopsis* should be re-evaluated.

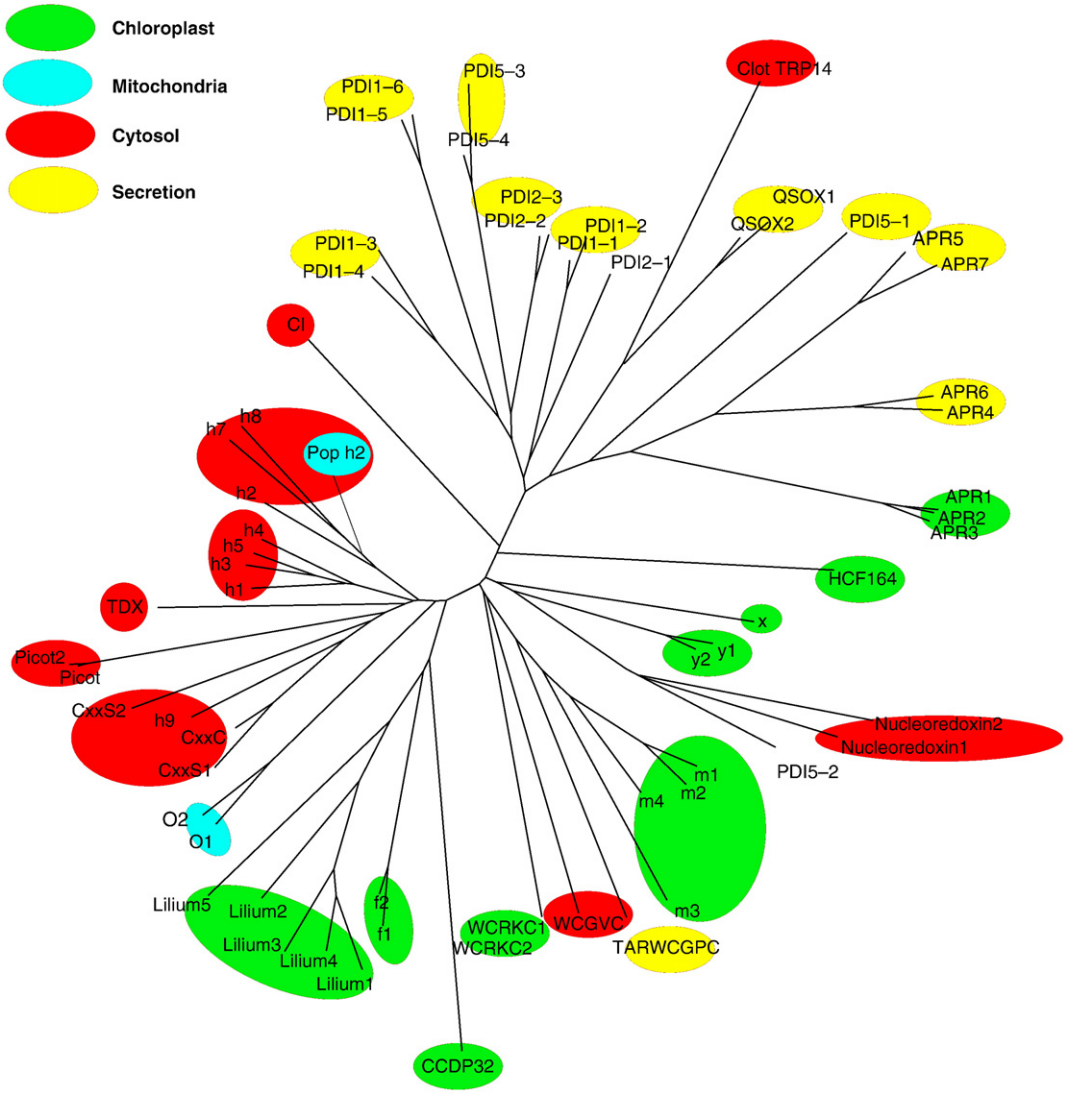

Fig. 1. The figure presents a phylogenic tree of the Trxs and PDIs of *Arabidopsis thaliana*. The AGI numbers corresponding to each proteins are presented in Table 1. We added in this tree the sequence Poplar thioredoxin popTrx h2 (GenPept AAL90749) which belongs to subgroup hII and was shown to be located in the mitochondria.

1.2. The glutaredoxins in Arabidopsis

Glutaredoxins constitute a rapidly growing family at least when the identification is based on sequence similarity. Only very recently, the genomic aspect was reviewed in several papers [6,8,10,11,12]. Briefly, about 50 *Arabidopsis* genes encode glutaredoxins or glutaredoxin-like proteins which can be subdivided into five groups, including a group with a CCxC or CCxS redox site uniquely present in higher plants. In most cases these Grx are identified only by sequence similarity. Only a few were studied for their biochemical properties.

1.3. The reductants of redoxins in Arabidopsis

The chloroplastic ferredoxin-dependent thioredoxin reductase was purified using affinity chromatography on ferredoxin-sepharose [13]. Since then, recombinant FTR protein has been produced and the structure identified. This allowed an understanding of how a ferredoxin, a single electron donor, reduces a disulfide bridge which needs the transfer of two electrons [14,15]. The ferredoxin thioredoxin reductases are heterodimeric proteins. In *Arabidopsis*, the catalytic subunit (FTRB) is encoded by one gene while the variable subunit is encoded by two genes (FTRA1, FTRA2) (Table 3).

The NADPH dependent thioredoxin reductases are encoded in *Arabidopsis* by two genes which were identified by the similarity of ESTs with *E. coli* NTR [16]. The structure of the *Arabidopsis* protein shows that the redox site is located in a groove and can probably be accessed only by small proteins with a disulfide on the surface [17]. Both NTR genes express a long and a short mRNA encoding a mitochondrial or a cytosolic NTR respectively [18]. Furthermore, a chloroplastic NTRC was recently identified in plants. In addition to the transit peptide, it is composed of two domains, an N-terminal

Table 1
Thioredoxins and PDI in *Arabidopsis*

Thioredoxin type	AGI ID	Common name	Subcellular localization in *Arabidopsis*
F	AT3G02730	f1	Chloroplast
	AT5G16400	f2	Chloroplast
M	AT1G03680	m1	Chloroplast
	AT4G03520	m2	Chloroplast
	AT2G15570	m3	Chloroplast
	AT3G15360	m4	Chloroplast
X	AT1G50320	X	Chloroplast
Y	AT1G76760	y1	Chloroplast
	AT1G43560	y2	Chloroplast
CDSP32	AT1G76080	CDSP32	Chloroplast
HCF164	AT4G37200	HCF164	Chloroplast
WCRKC	AT5G06690	WCRKC1	Chloroplast (homology)
	AT5G04260	WCRKC2	Chloroplast (homology)
Lilium	AT1G08570	Lilium1	Chloroplast (homology)
	AT4G29670	Lilium2	Chloroplast (homology)
	AT5G61440	Lilium3	Chloroplast (homology)
	AT2G33270	Lilium4	Chloroplast (homology)
	AT1G07700	Lilium5	Chloroplast (homology)
O	AT2G35010	o1	Mitochondria
	AT1G31020	o2	?
h subtype I	AT3G51030	h1	Cytosol
	AT5G42980	h3	Cytosol
	AT1G19730	h4	Cytosol
	AT1G45145	h5	Cytosol
h subtype II	AT5G39950	h2	Cytosol
	AT1G59730	h7	Cytosol (homology)
	AT1G69880	h8	Cytosol (homology)
h subtype III	AT2G40790	CxxS1	Cytosol (homology)
	AT3G08710	h9	Cytosol (homology)
	AT1G11530	CxxS2	Cytosol (homology)
	AT3G56420	CxxC2	Cytosol (homology)
WCGVC	AT3G53220	WCGVC	Cytosol (homology)
Cf-6 Interacting	AT3G06730	CI	?
Clot TRP14	AT5G42850	Clot	Cytosol (homology)
TDX	AT3G17880	TDX	Cytosol/nucleus
Nucleoredoxin	AT1G60420	Nucleor1	Nucleus (homology)
	AT4G31240	Nucleor2	Nucleus (homology)
Picot	AT4G04950	Picot1	Cytosol (homology)
	AT4G32580	Picot2	Cytosol (homology)
TARWCGPC	AT1G52990	TARWCGPC	Secretion (homology)
NTRC	AT2G41680	NTRC	Chloroplast
PDI-1-1	At1g21750		ER (homology)
PDI-1-2	At1g77510		ER (homology)
PDI-1-3	At3g54960		ER (homology)
PDI-1-4	At5g60640		ER (homology)
PDI-1-5	At1g52260		ER (homology)
PDI-1-6	At3g16110		ER (homology)
PDI-2-1	At2g47470		ER (homology)
PDI-2-2	At1g04980		ER (homology)
PDI-2-3	At2g32920		ER (homology)
PDI-5-1	At1g07960		ER (homology)
PDI-5-2	At1g35620		ER (homology)
PDI-5-3	At3g20560		ER (homology)
PDI-5-4	At4g27080		ER (homology)
QSOX1	At1g15020		ER (homology)
QSOX2	At2g01270		ER (homology)
APR1	At4g04610		Chloroplast
APR2	At1g62180		Chloroplast
APR3	At4g21990		Chloroplast
APRl4	At1g34780		Secretion (homology)
APRl5	At3g03860		Secretion (homology)
APRl6	At4g08930		Secretion (homology)
APRl7	At5g18120		Secretion (homology)

NTR domain associated with a C-terminal thioredoxin domain [19].

Because glutaredoxins are reduced by glutathione, they are indirectly dependent on the glutathione reductases (GR). In *Arabidopsis* as in pea, GRs are encoded by two genes. One encodes a protein which is dual targeted to the chloroplast and mitochondria [20] while the second gene encodes a cytosolic GR [21].

The amino-acid sequences of these redoxins and reductases in *Arabidopsis* are given in Supplementary material. The cysteines are highlighted for a rapid identification of the redox active site and putative glutathionylation sites.

1.4. Redoxins and their reductants in other plants

The recent release of the rice genome allowed comparison of redoxins in a dicot and a monocot. Rice encodes at least one member of each subfamily of Trxs, Grxs, FTRs, NTRs and GRs and all rice redoxins fall within the subfamilies defined using the *Arabidopsis* genome. This demonstrates that the subfamilies defined for *Arabidopsis* diverged before the appearance of mono and dicots and that the classification is reliable and applicable to all higher plants. It should be added that within most subfamilies the number of genes is different in *Arabidopsis* and rice and it is not possible to define clear orthologous relationships between *Arabidopsis* and rice genes. Some of these subfamilies are present in unicellular eukaryotic algae and in cyanophyceae. For example, NTRC homologues are present in several cyanophyceae, bacteria related to the ancestor of the chloroplast. Thus, although only recently identified in plants, from an evolutionary point of view NTRC is a very old protein. Nevertheless, thioredoxins h subtype III, TDX, o, and CCxC glutaredoxins are absent from *Chlamydomonas* and *Ostreococcus* [6]. It will be interesting to determine which subfamilies are present in multicellular mosses and ferns and if some of them appeared with the multicellular status of these organisms. The genome of *Physcomitrella patens* is now being sequenced and will provide important answers to this question. One first result is the presence of glutaredoxins with the CCxC redox site in *Physcomitrella* (http://www.cosmoss.org/cnt/).

2. Regulation of the chloroplastic metabolism by thioredoxins

2.1. The disulfide bridges of thioredoxin targets

Following the discovery of plant thioredoxins, the activation of FBPase by TRX f and MDH by TRX m was analyzed in detail. It appears that in both cases more than two cysteines of the proteins are involved in the night inactivation of these proteins. Mutation of pea FBPase C153S produces a constitutively active protein, while FBPases mutated either in C173S or C178S are still redox regulated. This suggests that a disulfide bridge, between C153 and C173 or C178, could inactivate the FBPase [22]. This conclusion is supported by the determination of the structure [23]. An even more complex situation exists for the homodimeric MDH in which five cysteines are implicated: the completely inactive enzyme presents a C-terminal disulfide

bridge which strongly affects the Km of the enzyme and an N-terminal bridge that closes the oxaloacetate binding site. In addition, an intermediate state exists with a low activity in which a cysteine of the C-terminal sequence forms a disulfide bridge with an internal cysteine of the companion homodimeric subunit. These data were obtained essentially by site directed mutagenesis [24] but have been confirmed by structural data [25,26]. Thus, it appears that the activation process of FBPase and MDH, the best investigated chloroplastic thioredoxin targets, is more complex than the simple reduction of a single disulfide bridge.

2.2. Function of the redoxin regulation in the chloroplast

Most enzymes involved in photosynthesis were tested for possible redox regulation. This demonstrated that, among the 8 enzymes of the Calvin cycle, 4 are activated by Trxs: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-1,6-bisphosphatase (FBPase), sedoheptulose-1,7-bisphosphatase (SBPase) and phosphoribulokinase (PRK) (a review in [4]). Other chloroplastic redox regulated proteins include Rubisco activase [27] acetyl-CoA carboxylase (ACCase) involved in fatty acid biosynthesis [28] and ADP-glucose pyrophosphorylase (AGPase) a rate limiting enzyme in starch synthesis [29]. All these enzymes are activated by reduction and are crucial for metabolic processes which use energy and redox potential derived from photosynthesis, suggesting that the thioredoxin activation adapts the activities of the chloroplast to the availability of light energy. The regulation of glucose-6-phosphate dehydrogenase (G6PDH), which is active in the oxidized state (in the dark) and inactivated by Trx reduction in the light, further supports this hypothesis: G6PDH is implicated in sugar catabolism necessary in the dark [30]. Thus, Trx regulation avoids the futile cycle in which catabolism and anabolism would take place at the same time. Alternatively, the closure of the active site of MDH by a disulfide bridge in the dark suggests a protection of the active site of some Trx-regulated enzymes in the oxidized environment during the night. In contrast to Trx reduction in the light, the mechanism of disulfide formation during the night is not understood. Oxidized thioredoxins are able to inactivate MDH *in vitro* (Miginiac-Maslow, personal communication), but more specific systems may act in the chloroplast. These dithiol disulfide regulations interact with other signal transduction pathways allowing an excellent adaptation of enzyme activity to changing physiological demands [31,32].

3. Thioredoxin and glutaredoxin in defense against oxidative stresses

3.1. Peroxiredoxins as anti-oxidants

After the discovery of thiol peroxidases in yeast and mammals, homologues were found in the *Arabidopsis* genome and in other plants. These enzymes are now named peroxiredoxins. They reduce H_2O_2 using a thiol and are subsequently reactivated by thioredoxins or glutaredoxins. The *Arabidopsis* peroxiredoxins are now exhaustively identified [33–35] and compose 5 subfamilies.

a) The 1-Cys peroxiredoxin is seed specific and locates to the cytosol and nucleus. It is not reducible *in vitro* by any glutathione, Grx or Trx tested so far, whereas ascorbate efficiently reduces this protein [36].
b) 2-Cys Prx are chloroplastic proteins which are reduced by most thioredoxins *in vitro* [33].
c) Glutathione peroxidases (Gpx) are named on the basis of their homology to corresponding mammal proteins but, in contrast to these, they are reduced exclusively by thioredoxins. Gpx members are found in different compartments including chloroplasts, mitochondria, and cytosol.
d) PrxII members are present in the cytosol, chloroplasts and mitochondria. The chloroplastic isoform is reducible by thioredoxins, while the cytosolic members of *Arabidopsis* are exclusively reduced by Grx [37]. This contrasts with the poplar homologues which are reducible by Grx or Prx [38].
e) Prx Q is a chloroplastic two cysteinic peroxiredoxin reduced by Trxs [34].

Members of the five Prx families are encoded in the rice and poplar genomes suggesting the presence of the five families in all higher plants. It is generally believed that the function of peroxiredoxins is to reduce H_2O_2. Although this is probably true for most Prx, in yeast and in mammals it was shown that some peroxiredoxins act as H_2O_2 sensors: in yeast when oxidized by H_2O_2, Gpx3 oxidizes the transcription factor YAP1 resulting in its activation. Subsequently, YAP1 induces its target genes which are mostly involved in anti-oxidant defense [39]. Other functions are suspected for some plant Prxs which bind to DNA and may preserve it from oxidative damage and possibly regulate gene expression.

3.2. Methionine sulfoxide reductases to repair damaged proteins

One consequence of the oxidative stresses is the generation of sulfoxided methionines, both free and in the form of peptidyl methionine sulfoxide (MetSO). The S enantiomer is reducible by Methionine sulfoxide reductase A (MsrA) and the R enantiomer by MsrB, two unrelated types of proteins. *Arabidopsis* encodes 5 MsrA and 9 MsrB genes. Based on the amino-acid sequences they are chloroplastic, secreted or cytosolic proteins. Some of them were shown to be reduced by thioredoxins or glutaredoxins [40–43].

4. Identification of putative thioredoxin and glutaredoxin targets by proteomics

After the release of the *Arabidopsis* genome sequence, we were faced with a strange situation in which the number of *Arabidopsis* thioredoxins was greater than the number of known targets proteins. This changed radically due to the development of methods allowing the identification of new targets by proteomic approaches.

4.1. Methods for the characterization of redox regulated proteins

Interactions between Trx and their target proteins are in most cases very unstable, but there are some exceptions.

a) Trx f undergoes a stable interaction with FBPase. Immobilization of thioredoxin f on agarose allowed Balmer et al. [44] to identify several redox regulated as well as none redox regulated proteins which interact electrostatically with Trx f.
b) Trx CI from tomato, involved in host pathogen interactions, was isolated by Tandem Affinity Purification with a tagged Cf-9 [45].
c) Two Trxs from *Brassica* implicated in pollen autoincompatibility were identified in a two-hybrid screen [46].

In fact, these cases are exceptions, the isolation of thioredoxin complexes and even two hybrid interactions between Trx and identified target proteins failed in most tested cases. This situation was overcome by the use of monocysteinic thioredoxins and glutaredoxins: it had been known for years that the reduction of a disulfide by a thioredoxin occurs in two steps [47]. First, the N-terminal thiol of the redox center of the reduced thioredoxin releases one electron to one cysteine of the disulfide bridge and forms a disulfide bridge with the second cysteine of the target. In the second reaction, the intermediate disulfide complex is reduced by the second cysteine of the Trx, releasing the reduced target protein and the oxidized Trx. Thus, using a Trx mutated in the second cysteine should allow stabilization of the Trx-target complexes. The feasibility of this approach was first demonstrated by expressing a mutated *Arabidopsis* Trx in yeast, which allowed the isolation of a complex with Ahp1, a protein which was shown to be a peroxiredoxin [48]. A far more efficient method was obtained by preparing affinity columns with mutated monocysteinic thioredoxins as bait. This method was used to isolate protein targets from whole plants or several subcellular fractions [49]. At the same time, another approach was developed: plant extracts were first alkylated in order to block all free thiols. Subsequently, the disulfide bridges were reduced by a thioredoxin treatment and the resulting free thiols were labeled by fluorescent or radioactive reactants [50,51]. After 2D electrophoresis, the labeled spots were identified by mass spectrometry.

4.2. Redox regulated proteins in almost all aspects of plant life

An impressive list of putative targets was established by both methods in several laboratories using thioredoxins or glutaredoxins as bait, most corresponding to plastidial proteins, but cytosolic, mitochondrial, nuclear and even peroxisomal targets were also identified (for review [52,53]). It is very likely that most of these proteins are subjected to disulfur dithiol regulation because almost all previously identified thioredoxin dependent proteins were re-isolated and all newly isolated proteins contain at least one cysteine, the minimal requirement to participate in a disulfide bridge. In addition, the protein set identified with both approaches using different thioredoxins or glutaredoxins on different plant extracts are widely overlapping. Furthermore, the list will probably be completed in the future when the methods have been adapted to the isolation of membrane proteins and to low abundance proteins. Currently, about 300 putative Trx/Grx targets have been identified. They are implicated in almost all pathways, including house keeping metabolism, photosynthesis, abiotic and biotic stress defense and development. This demonstrates an unexpected involvement of the dithiol disulfur regulation in almost all processes of plant life. One noticeable advance is the identification of thioredoxin target proteins in non-green plastids such as amyloplasts [54,55]. This is in good agreement with the expression of some plastidial Trx genes in non-green tissues.

5. Specific interactions between redoxins and their putative target proteins

The high number of thioredoxins and glutaredoxins in plants now fits well with the numerous targets. Nevertheless, the proteomic methods are almost as specific and do not allow the definition of an interaction between a given reductant and a particular protein. For example, chloroplastic targets have been identified with a cytosolic thioredoxin as bait, and almost the same targets were identified with thioredoxins or glutaredoxins.

5.1. Specificity of Trx in vitro

The question of the specificity of the reductant was asked early, when only a limited number of chloroplastic thioredoxins and target proteins had been identified. It is generally accepted that thioredoxin f is the *in vivo* reductant of FBPase because only recombinant thioredoxin f efficiently reduces the recombinant protein. Indeed, Trx f is able to interact electrostatically and stably with several redox regulated and redox independent proteins [44]. This property is not shared by other plant thioredoxins and may play a role in the specific reduction of FBPase by Trx f. In the case of MDH, Trx f and Trx m are almost as efficient reductants *in vitro*. Thus, the *in planta* reductant of MDH has not yet been identified. Recently, biochemical characteristics were re-evaluated using all types of chloroplastic *Arabidopsis* Trxs for their ability to reduce FBPase, MDH, several Prxs and MSRs *in vitro* [42,56–58]. The results are summarized in Table 2. Fairly clear differences appear between the different thioredoxin types suggesting the implication of Trx f, m1, m2, and m4 in photosynthetic carbon metabolism, while Trx x, y1, y2, CDSP32 and NTRc would be more efficient in anti-oxidant defense. An interesting point is the absence of activity of Trx m3 against the natural substrates, in spite of its activity on artificial substrates, showing that this member of the m group probably performs a function different from the other Trx m. The basis of the *in vitro* specificity was in part attributed to the redox potential of Trx x, y1 and y2 which are clearly less negative than the redox potential of isoforms f and m. This correlates with the less negative redox potential of 2-Cys Prx (−315 mV) compared to the C-terminal disulfide of MDH

Table 2
Glutaredoxins in *Arabidopsis*

Glutaredoxin type	AGI ID	Common name	Subcellular localization in *Arabidopsis*
CGFC	At3g28850		Mitochondria (homology)
CGFS	At2g38270		Chloroplast (homology)
	At3g55040	Picot	Mitochondria (homology)
	At4g04950		Cytosol
CxYC	At4g28730		ER (homology)
	At5g20500		ER (homology)
	At5g63030		Cytosol
	At5g40370		Cytosol
	At2g20270		Chloroplast (homology)
	At4g28730		Chloroplast (homology)
CCxC	At1G03020		Cytosol (homology)
CCxS	At1G03850		
	At1G06830		
	At1G28480		
	At2G30540		
	At2G47870		
	At2G47880		
	At3G02000		
	At3g21450-65		
	At3g62930		
	At3g62950		
	At3g62960		
	At4g15660		
	At4g15670		
	At4g15680		
	At4g15690		
	At4g15700		
	At4g33040		
	At5g11930		
	At5g14070		
	At5g18600		
4CxxC	At1g32760		Cytosol (homology)
	At1g64500		
	At2g41330		
	At3g11773		
	At3g28850		
	At3g57070		
	At4g10630		
	At5g01420		
	At5g03870		
	At5g06470		
	At5g13810		
	At5g39865		
	At5g58530		
CPFC	At4g10000		Chloroplast (homology)
CPFs	At5g02780		Cytosol (homology)
	At5g02790		Cytosol (homology)
	At5g16705		Chloroplast (homology)
	At5g42150	CAX inter	Mitochondria (homology)
	At3g55040		Cytosol (homology)

(−330 mV). Nevertheless, this single characteristic does not explain the absence of activity of Trx m3, and the relatively low activity of the more negative Trx on the anti-oxidant proteins.

5.2. Different expression pattern of the redoxin genes

Although the *in vitro* specificity appears relatively low, the expression pattern of each redoxin gene may be an important factor determining specific functions during the development or in response to environmental changes or stresses. This aspect is illustrated by the expression of Trx m3 in flower buds, while m1, m2 and m4 are expressed in leaves. As another example, y2 is expressed in leaves while y1 is expressed in roots [57]. The redox regulation of plastidial proteins in non-green tissues is now well established, as shown for an amylase [59] and by the involvement of Trx during seed formation and germination [60]. In non-photosynthetic plastids the redox flux depends on an NADPH ferredoxin reductase and transits through the Ftr as in the chloroplast. It has also been suggested that the chloroplastic Prx could be active during the night [61]. If they were reduced through an Ftr Trx mechanism, this would suppose a channeling to direct the reduction flux to the defense Trxs, but leaving the photosynthetic Trxs in the oxidized state. A direct reduction by NTRC is more likely and represent an obvious reason to maintain ferredoxin and NADPH dependent Prx reduction systems from the cyanophyceae to the higher plant chloroplast. Concerning cytosolic thioredoxins h1 to h5, different but overlapping expression patterns were shown using promoter reporter gene constructions, but only h5 is overexpressed after biotic and abiotic stresses [62]. Different oxidative stresses induce CDSP32 expression and accumulation in potato [63]. More details on the expression pattern of redoxins and their target proteins can be extracted at https://www.genevestigator.ethz.ch/ which compiles *Arabidopsis* affymetrics array data under different environmental situations, in different organs and in several mutants.

5.3. Other tools to reveal redoxin target interactions

Other tools were developed to gain more information on Trx specificity. Five Trx h were tested for their ability to complement the different aspects of the phenotype of an *S. cerevisiae* mutant in which the two cytosolic genes were inactivated. When expressed from a low copy number plasmid, all Trxs h restore a normal cell cycle but only Trx h2 restores sulfate assimilation and Trx h3 restores H_2O_2 tolerance [64]. These observations strongly suggested that although all *Arabidopsis* Trxs h are able to reduce RNR (restoration of the cell cycle), solely h2 reduces the PAPS reductase (sulfate assimilation) and solely h3 reduces peroxiredoxins. It should be noted that this specific complementation is less obvious if a high copy number vector is used. The same approach using chloroplastic Trxs revealed that m1, m2 and m4 acts as anti-oxidants in this system, while m3 expression increases the H_2O_2 sensitivity of the mutant [65], a further argument in favor of the unique characteristics of Trx m3. A two-hybrid yeast system was then developed to test Trx-target interactions. Standard two-hybrid systems are not useful with most thioredoxins. The first reason is the labile interaction between Trx and targets. But even using the monocysteinic Trx h3, which allows the stabilization of a disulfide bridge complex with the yeast Ahp1, no signal was obtained in a yeast two-hybrid test. Indeed, a Trx h3/Ahp1 complex was isolated by expressing Trx h3 in the mutant yeast with inactivated endogenous thioredoxins, but not when wild type yeast was used.

Table 3
Redoxins and glutathione reductases in *Arabidopsis*

Redoxin reductases	AGI ID	Common name	Subcellular localization in *Arabidopsis*
NADPH	AT2G17420	NTRA	Cytosol and mitochondria
Thioredoxin	AT4G35460	NTRB	Cytosol and mitochondria
Reductases	AT2G41680	NTRC	Chloroplast
Ferredoxin	At5g23440	FTRA1	Chloroplast
Thioredoxin	At5g08410	FTRA2	Chloroplast
Reductases	At2g04700	FTRB	Chloroplast
Glutathione	At3g54660	GR-ChloroMito	Chloroplast and mitochondria
Reductases	AT3G24170	GR-cyto	Cytosol

This strongly suggests that the yeast endogenous thioredoxins disrupt the complex and explains the failure to obtain a two-hybrid signal in commercial yeast. A new reporter yeast strain for two-hybrid test was constructed by inactivating both cytosolic thioredoxin genes. Using this strain it was possible to demonstrate directly the specific interaction of Trx h3 with two yeast peroxiredoxins and of Trx h2 with the PAPS reductase, confirming the data obtained by complementation [66]. We believe that this tool will allow a better discrimination of the specificity between thioredoxins and their targets. Moreover, it has additional potential for screening new targets and to perform by mutagenesis fine analysis of the sequences implicated in the interactions.

6. Glutaredoxins and iron–sulfur complexes

Our knowledge on the function of plant Grx is progressing only slowly. Some of these proteins have been expressed in *E. coli* and shown to be efficient reductants of Prxs and Msrs. A major discovery was recently made by demonstrating that glutaredoxin 1, a poplar cytosolic glutaredoxin, associates with a 2Fe-2S cluster [67]. The same property is shared by the *Arabidopsis* homologue. A similar result was obtained for a mammalian Grx and was interpreted as allowing the storage of this Grx in an inactive form. A far more exciting hypothesis can be proposed on the basis of the phenotype of an *S. cerevisiae* Grx5 knock out. Inactivation of this mitochondrial monocysteinic Grx leads to a drastic reduction in several proteins containing an FeS cluster suggesting that Grx5 is implicated in FeS assembly [68].

7. Genetic approaches to analyze the function of redoxins, their reductants and targets

7.1. Phenotypes resulting from redoxin or target inactivation

For a long time the plant redoxin field was developed exclusively by biochemists, but more recently genetic evidence on the function of plant redoxins has been published. To our knowledge, the first insight came from P. Rey's laboratory, who showed that the expression of antisense CDSP32 induces hypersensitivity of potatoes to oxidant stresses and 2-Cys Prx oxidation, thus indicating that CDSP32 participate in anti-oxidative defense [69]. The availability of insertional mutants from the Versailles, the Salk institute and the GABI-KAT collections suggested that analysis of the phenotype of *Arabidopsis* with inactivated redoxins would rapidly reveal the function of redoxin genes *in planta*. In fact, only limited success was obtained by this approach. An *ntrc* mutant was shown to be hypersensitive to oxidative stress, particularly under short day illumination or when maintaining the plants several days under darkness; showing that this gene plays an important defense role in the dark when the FTR dependent thioredoxins are most probably inactive [58]. A mitochondrial Prx II-F mutant presents reduced root growth under cadmium or salicylhydroxamic acid treatments [70]. A null mutant of the variable subunit of FTRA1 is hypersensitive to high light and paraquat stresses. This limited phenotype for the reduction system of all chloroplastic thioredoxins is most probably due to the presence of a second gene encoding the variable subunit FTRA2 [71]. Clearly, redundancies exist within the plant gene families. About 70% of all *Arabidopsis* genes are inactivated in at least one of the available collections of KO mutants. A survey at TAIR showed that a similar proportion of redoxin mutants are available. Thus, it is likely that numerous redoxin mutants have been analyzed, but not published, because no phenotypic difference was observed. In our lab Trx h1, h2, h3, h4, and h5 as well as several glutaredoxin mutants have been analyzed in the homozygous state under various environmental situations without detecting an obvious phenotype. Associating some of these mutations by crossing was no more successful. This is not a particularity of the redoxin families because it was noted early that the insertional mutants in the collection of Versailles present roughly 5 mutations in each plant but have a very low number of developmental defects [72]. This may be due to redundancies within gene families or to compensatory mechanisms by unrelated pathways. In spite of these difficulties, insertional mutants can reveal unexpected results as shown by the analysis of *ntra* and *ntrb* knock out mutants. Although the single mutants are asymptomatic, a double mutant was obtained which presents some defects including reduced size, wrinkled seeds and pollen with reduced fitness. Nevertheless, the most surprising result is that this mutant is viable and fertile although NTRA and NTRB were the only known reductants of cytosolic and mitochondrial thioredoxins [73]. Analyzing the reduction state of Trx h3 in the wild type shows a completely reduced protein while in the double mutant only half of the Trx protein is oxidized. This suggests an alternative reduction system for Trxs h which was identified as being glutathione dependent. Furthermore, crossing this mutant with root meristemless (*rml1*), a mutant of glutathione synthesis, reveals implication of Trxs in apical meristem growth: *rml1* plants have only 3% of the wild type level of glutathione and present a blocked root meristem but a fully active shoot meristem, at least in the early stages of germination. The triple mutant is blocked for both the root and the shoot meristems, showing that shoot meristem growth depends on Trxs which are reduced by the NTR pathway in *rml1* or by the glutathione pathway in *ntra ntrb* [74].

7.2. Redoxin genes identified in genetic screens

Three thioredoxin and one glutaredoxin mutants were isolated in genetic screens:

a) Trx HCF164 was isolated within a collection of the so-called high chlorophyll fluorescence (HCF) mutants and was shown to be deficient in cytochrome b6f. This thioredoxin is anchored to the thylakoid membrane at the luminal site. Although the genes encoding the b6f subunits are expressed, the proteins do not accumulate, suggesting that Trx HCF164 is implicated in cytochrome assembly [75–77]. It has been proposed that Trx HCF164 maintains the apocytochrome sulfhydryls reduced, allowing covalent ligation to the heme. Due to the thylakoid localization, a complex reduction scheme was proposed involving a transmembrane reductase [79] and a Trx m [80].
b) Trx CI was first isolated by tagged affinity as a protein interacting with the tomato Cf-9 resistance protein. As stated above, thioredoxin interactions are generally too weak to allow complex isolation. This suggests that Trx CI, like Trx f, presents a specificity which is not shared by most thioredoxins. It was later shown that virus induced gene silencing of Trx CI enhances the resistance of tomato to the pathogen *Cladosprium fulvum* [45]. An important point is that the redox activity of Trx CI is not required [78] for its negative regulation of Cf-9 but should rather act by its interacting properties as an adapter protein linking Cf-9 and ACIK1, a protein kinase. It is noticeable that Trx CI homologues exist in rice, tomato and *Arabidopsis* and that, in these evolutionarily distant species, all Trxs CI possess an active WCGPC redox site. Thus, in addition to interacting with Cf-9, Trxs CI are probably implicated in other processes in a redox way.
c) More recently, it was shown that Trx h5 is required for sensitivity to victorin, a toxin produced by *Cochliobolus victoriae* [79]. The first cysteine of the redox site of Trx h5 is the only cysteine necessary for sensitivity to victorin, suggesting that Trx h5 acts in victorin sensitivity by a monocysteinic, non-classical redox way. Trx h3 and Trx h5 originate from the duplication of a gene during the most recent genome duplication of the *Arabidopsis* ancestor shortly before the appearance of *Brassicaceae* [6]. Despite their high similarity, Trx h3 is not able to substitute for Trx h5 for victorin sensitivity. Trx h5 disruption abolishes sensitivity to victorin but not to other pathogen toxins like fumonisin B1 or coronatine [79].
d) Roxy1 mutants were isolated from the GABI-KAT collection for the presence of abnormal petals. All are inactivated in a glutaredoxin gene from the plant specific CCMC family [80]. The first cysteine of the redox site is necessary to revert the phenotype, showing that the protein acts through a monothiol redox pathway. The expression of Roxy is not detectable in leaves, but abundant in flowers and siliques. Surprisingly, the gene is also highly expressed in roots but no root phenotype is observed. Severe phenotypes were observed in the *roxy1 apetala* 1–10 double mutants.

8. Conclusions

On the basis of biochemical and proteomics data, the redoxin driven disulfide dithiol exchanges appear to play a major role in plant growth, development and response to environmental constraints. They act in two very different ways, either transferring reducing equivalent to some reductases like RNRs, Prxs and Msrs or by operating conformational changes of a large variety of other proteins. In addition, they are in direct relation with chaperones, as shown by their interaction with cyclophilins [81] and the structure of TDX, which associates an HSP interacting domain with a thioredoxin domain [82].

Their activity is dependent on the activity of their reductants, but it is now clear that some of them are subject to direct regulation, as was recently shown for glutathionylation which inactivates Trx f activity [83] and induces monomerization of a poplar Prx [84].

Unfortunately, the functional role of each member of the redoxin family *in planta* remains to be established because no phenotype can be associated with the inactivation of the gene. Even when a phenotype has been identified, as is the case for Trx CI and Trx h5, it is clear that it does not reveal the complete function and possibly not even the major function of these Trxs. For example, interaction of Trx CI with Cf-9 is redox independent and the sensitivity to victorin is mediated by Trx h5 through a monocysteinic reaction, although both are dithiol Trxs. The absence of phenotype results most probably from redundancy between members of the Trxs, and Grxs and between members of both families, as shown by genetic approaches in *E. coli* and *S. cerevisiae*. In plants, the high number of Trx and Grx genes precludes random association of mutations in all redoxin genes, as has been done in *E. coli* and yeast. Thus, additional data should be used to limit the number of crosses necessary to reveal a phenotype. The use of molecular markers, particularly the redox state of putative target proteins and the expression patterns in the mutants, could be an important approach to decipher the function of each gene. In addition, redox regulation is by far not limited to redoxins. Compensatory mechanism, for example in the response to oxidative stress, could be performed by dithiol disulfide independent mechanisms. Thus a survey of the genes and proteins implicated in redox homeostasis [85] as well as metabolic profiling [86] of the redoxin mutants should be considered.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2007.10.017.

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