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Poplar Peroxiredoxin Q. A Thioredoxin-Linked Chloroplast Antioxidant Functional in Pathogen Defense[1]

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Peroxiredoxins are ubiquitous thioredoxin- or glutaredoxin-dependent peroxidases, the function of which is to destroy peroxides. Peroxiredoxin Q, one of the four plant subtypes, is a homolog of the bacterial bacterioferritin comigratory proteins. We show here that the poplar (*Populus tremula* x *Populus tremuloides*) protein acts as a monomer with an intramolecular disulfide bridge between two conserved cysteines. A wide range of electron donors and substrates was tested. Unlike type II peroxiredoxin, peroxiredoxin Q cannot use the glutaredoxin or cyclophilin isoforms tested, but various cytosolic, chloroplastic, and mitochondrial thioredoxins are efficient electron donors with no marked specificities. The redox midpoint potential of the peroxiredoxin Q catalytic disulfide is -325 mV at pH 7.0, explaining why the wild-type protein is reduced by thioredoxin but not by glutaredoxin. Additional evidence that thioredoxin serves as a donor comes from the formation of heterodimers between peroxiredoxin Q and monocysteinic mutants of spinach (*Spinacia oleracea*) thioredoxin m. Peroxiredoxin Q can reduce various alkyl hydroperoxides, but with a better efficiency for cumene hydroperoxide than hydrogen peroxide and tertiary butyl hydroperoxide. The use of immunolocalization and of a green fluorescence protein fusion construct indicates that the transit sequence efficiently targets peroxiredoxin Q to the chloroplasts and especially to those of the guard cells. The expression of this protein and of type II peroxiredoxin is modified in response to an infection by two races of *Melampsora larici-populina*, the causative agent of the poplar rust. In the case of an hypersensitive response, the peroxiredoxin expression increased, whereas it decreased during a compatible interaction.

Organisms living under aerobic conditions have developed an array of antioxidative systems to protect the cells from oxidative damages caused by reactive oxygen and reactive nitrogen species. An emerging family of nonheme peroxidases, called peroxiredoxins (Prx), has been described in all kingdoms during the past years (Chae et al., 1994a, 1994b; Dietz et al., 2002). These ubiquitous proteins are able to reduce organic and inorganic hydroperoxides with electrons provided mainly by NADPH or NADH and different proteins such as thioredoxin (Trx), glutaredoxin (Grx), cyclophilin A, and subunit F of alkyl hydroperoxide reductase (Chae et al., 1994a; Niimura et al., 1995; Lee et al., 2001; Rouhier et al., 2002a). In addition to their documented activity as peroxidases, Prx are involved in resistance to reactive nitrogen

species (Bryk et al., 2000; Wong et al., 2002; Sakamoto et al., 2003). The Prx were first classified into two subclasses, the 1-Cys Prx and the 2-Cys Prx, according to the number of conserved cysteines. However, the increasing number of Prx found in all organisms required a more complex hierarchy. There are at least six Prx isoforms in mammals, five in yeast, and nine out of the 10 genes present in the genome of Arabidopsis are expressed (Park et al., 2000; Dietz et al., 2002; Fujii and Ikeda, 2002; Brehelin et al., 2003).

The plant sequences can be organized into four distinct subgroups (Dietz et al., 2002; Rouhier and Jacquot, 2002d; Dietz, 2003). The first one corresponds to the 1-Cys Prx. These are seed-restricted enzymes that are mainly localized in the nucleus of the embryo and the aleurone layer (Stacy et al., 1999). The second group, which comprises the dimeric 2-Cys Prx, is well characterized in terms of function and catalytic mechanism (Dietz et al., 2002; Goyer et al., 2002; Konig et al., 2002, 2003). These proteins, homologous to the mammalian Prx I to IV and bacterial AhpC, are chloroplastic enzymes. The third

class is a large group of monomeric type II Prx, with at least five expressed isoforms in Arabidopsis (Brehelin et al., 2003). Two of these are assumed to be cytosolic enzymes, the two other are probably chloroplastic and mitochondrial (Choi et al., 1999; Rouhier et al., 2001; Horling et al., 2002). Finally, the last class is Prx Q, an enzyme homologous to the bacterioferritin comigratory proteins (BCP). Until recently, this type of enzyme was thought to be present only in prokaryotes and lower eukaryotes, but an enzyme of *Sedum lineare* has been recently characterized (Kong et al., 2000). Apparently, this enzyme is a monomer with an intramolecular disulfide bridge between two Cys residues separated by only four amino acids (Kong et al., 2000). Nevertheless, mutagenesis studies on BCP indicate that this protein is functional with only the catalytic Cys (Jeong et al., 2000). These results are thus contradictory and deserve to be further investigated.

Moreover, the functions of BCP and Prx Q are not very well understood. BCP is involved in the resistance of *Escherichia coli* against various oxidants and are overexpressed in *Frankia sp.* during the formation of symbiosis with *Alnus glutinosa* (Jeong et al., 2000; Hammad et al., 2001). In Arabidopsis, it appears that transcripts of Prx Q are sensitive to redox changes. For example, its synthesis is promoted by high light and oxidants, and its disappearance is enhanced by reductants such as ascorbate (Horling et al., 2002, 2003). The physiological electron donor and the cellular and subcellular localization of this enzyme are not currently known even though Prx Q was shown to be retained from a chloroplastic stroma lysate on a column containing monocysteinic Trx m mutant in a recent proteomic study (Motohashi et al., 2001). This work focuses on the biochemical properties of poplar (*Populus tremula* x *Populus tremuloides*) Prx Q, with an emphasis on the catalytic mechanism and electron donor and substrate specificities. In addition, the cellular and subcellular localizations and the function of this enzyme were investigated. We found that Prx Q is definitely a chloroplastic enzyme as suggested by its N-terminal extension, and that it participates in the response to an infection of poplar by the rust fungus *Melampsora larici-populina*.

RESULTS

Sequence Analysis

The deduced amino acid sequence of the poplar enzyme studied here consists of 213 residues with a putative transit peptide of 64 residues, identified by analogy to the mature Prx Q of *S. lineare* (Kong et al., 2000). The recombinant mature form of poplar Prx Q used in this study contains 151 amino acids, including two residues (one Met and one Ala) that have been added to the N terminus immediately after the putative cleavage site of the precursor. Its calculated molecular mass and pI are 16,831 Da and 9.3, respectively.

An amino acid sequence comparison of three plant Prx Q and four BCP from cyanobacteria, bacteria, yeast, and archaebacteria is shown in Figure 1. All the plant sequences contain a N-terminal extension of 60 to 70 amino acids, which is proposed to be a chloroplastic target sequence by prediction software (http://www.cbs.dtu.dk/services/TargetP/ and http://
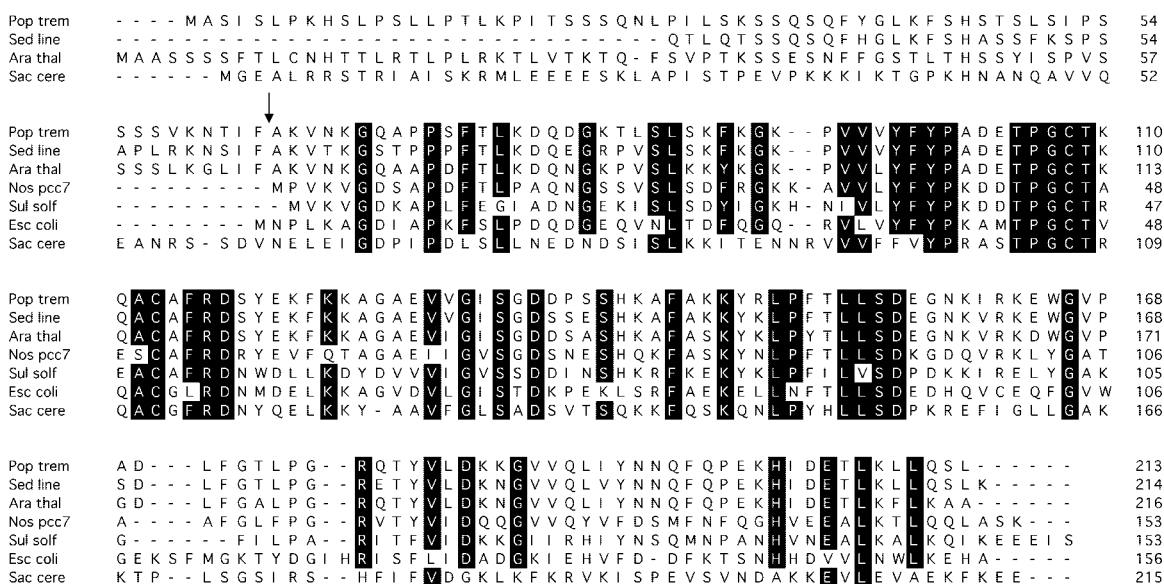
Figure 1. Alignment of poplar Prx Q with homologous proteins using ClustalW. Accession numbers are AY530803 for the poplar Prx Q (Pop trem), BAA90524 for *S. lineare* (Sed line), BAB01069 for Arabidopsis (Ara thal), NP_487223 for *Nostoc sp. PCC 7120* (Nos pcc7), NP_343463 for *Sulfolobus solfataricus* (Sul solf), P23480 for *E. coli* (Esc coli), and CAA86239 for *S. cerevisiae* (Sac cere). The arrow represents the site of truncation of the poplar sequence.

psort.nibb.ac.jp/form.html). The *Saccharomyces cerevisiae* enzyme also possesses a N-terminal extension, but the subcellular location of the yeast enzyme has not yet been identified. The sequences of the mature proteins, devoid of the transit peptides, are 149 to 156 amino acids long, and all of them contain the two putative active site cysteines in position 46 and 51 (poplar numbering of the shortened construction). Cys 46 is the residue that is homologous to the catalytic Cys present in all plant peroxiredoxins, whatever their subtype {consensus sequence P-(X)$_3$-T-[P/F]-(X)-C-[T/S/P]} and that is involved in catalysis via the transient formation of a sulfenic acid. The identity levels of the sequences of the mature plant proteins range from 84% to 88%. Comparisons between plant and nonplant enzymes show much lower identity levels, ranging from 35% to 55%.

Biochemical Characterization

Expression, Purification, and Mutagenesis

Using a combination of gel filtration and cation exchange chromatography, recombinant Prx Q WT, C46S, and C51S were purified to homogeneity (Fig. 2A). The overall yield was about 7 to 30 mg of homogenous protein L^{-1} of *E. coli* culture. The oxidized form of purified wild-type (WT) Prx Q is a monomeric enzyme as judged by its behavior during gel filtration (data not shown) and its migration in nonreducing 15% (w/v) SDS-PAGE (Fig. 2A, lane 4). The two monocysteinic mutants are able to form measurable amounts of homodimer, presumably via disulfide bonds involving the remaining Cys (Fig. 2A, lanes 5 and 6). Thiol titrations of the reduced enzymes confirmed the presence of two thiols M^{-1} enzyme for the reduced form of WT Prx Q, whereas no thiols could be detected in the oxidized state (data not shown). This observation suggests that there is an
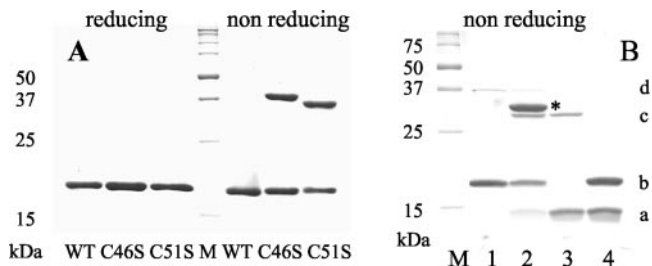

Figure 2. A, SDS PAGE (15%, w/v) analysis of the recombinant Prx Q proteins. Poplar Prx Q WT (lanes 1 and 4), Prx Q C46S (lanes 2 and 5), and Prx Q C51S (lanes 3 and 6) in reducing conditions (10 mM DTT [dithiothreitol], lanes 1–3) and in nonreducing conditions (lanes 4–6). B, Nonreducing SDS-PAGE showing the formation of heterodimers between Prx Q C51S and Trx m C40S. Lane 1, Prx Q C51S-TNB; lane 2, Prx Q C51S-TNB + Trx m C40S; lane 3, Trx m C40S; lane 4, Prx Q C51S-TNB + Trx m C40S + 10 mM DTT. The star represents the heterodimer between Prx Q C51S and Trx m C40S, a and c are respectively monomer and dimer of Trx m C40S, and b and d are monomer and dimer of Prx Q C51S, respectively.

Figure 3. Time course of H$_2$O$_2$ reduction mediated by the recombinant WT Prx Q and various Trx in the presence of DTT. □, Poplar Trx h2; ■, poplar Trx h1; △, poplar Trx h3; ○, spinach Trx m; ●, spinach Trx f; closed lozenges, *Chlamydomonas reinhardtii* Trx y; and closed triangles, poplar Grx, cyclophilin A, or controls: DTT alone, minus Trx, and minus Prx Q.

intramolecular disulfide in the monomer. Only one Cys was detected M^{-1} enzyme for the two mutants (data not shown).

Electron Donor and Substrate Specificity

Regardless of the conditions used, we could not observe DTT functioning as a direct electron donor to Prx Q using the ferrous oxidation in xylenol orange assay (FOX) detection method. Activity was only observed if Trx was added to promote catalysis (Fig. 3). To investigate whether the enzyme exhibits a preference for a given electron donor, the efficiency of several chloroplastic donors, including spinach (*Spinacia oleracea*) Trx m and f and the recently described *Chlamydomonas reinhardtii* Trx y, has been studied (Lemaire et al., 2003). In addition, one mitochondrial Trx (poplar Trx h2, E. Gelhaye, N. Rouhier, J. Gualberto, J. Gérard, M. Hirasawa, N. Navrot, P.I. Olsson, G. Wingsle, E. De Fay, D.B. Knaff, et al., unpublished data), two cytosolic Trx (poplar Trx h1 and h3), cyclophilin A, and one isoform of Grx have been also tested as electron donors. Figure 3 shows that unlike type II Prx, WT Prx Q cannot use Grx as an electron donor. Likewise, the poplar cyclophilin A used in this study was ineffective as a donor. On the other hand, all Trx tested were active with an apparent efficiency order h1>y>h3>[m, f, h2]. Experiments carried out with different Trx concentrations indicated that the reactions typically saturate at Trx concentrations between 5 and 10 μM. Experiments using NADPH reduction of poplar Trx h3, catalyzed by Arabidopsis NADPH thioredoxin reductase (NTR), provide a method for monitoring the Trx-dependent reduction of hydroperoxide by Prx Q spectrophotometrically. The kinetic parameters, de-

Table I. *Catalytic parameters of WT Prx Q versus various hydroperoxides*

These parameters were determined by following the NADPH oxidation in the presence of 1 μM WT Prx Q. The apparent K_m for the various hydroperoxides was measured at saturating concentrations of Trx h3. The K_m of Prx Q for Trx h3 is 1.5 μM.

	kcat	K_m	kcat/K_m	V_{max}
	s^{-1}	μM	$\times 10^3\ m^{-1}s^{-1}$	μmol NADPH ox $min^{-1}\ \mu mol^{-1}$
H_2O_2	2.93	367 ± 37.5	7.98	176 (10.46)[a]
tBOOH	0.896	375.2 ± 125.7	2.39	53.76 (3.19)[b]
CUOOH	2.35	98.8 ± 19.1	23.78	141.12 (8.38)[c]

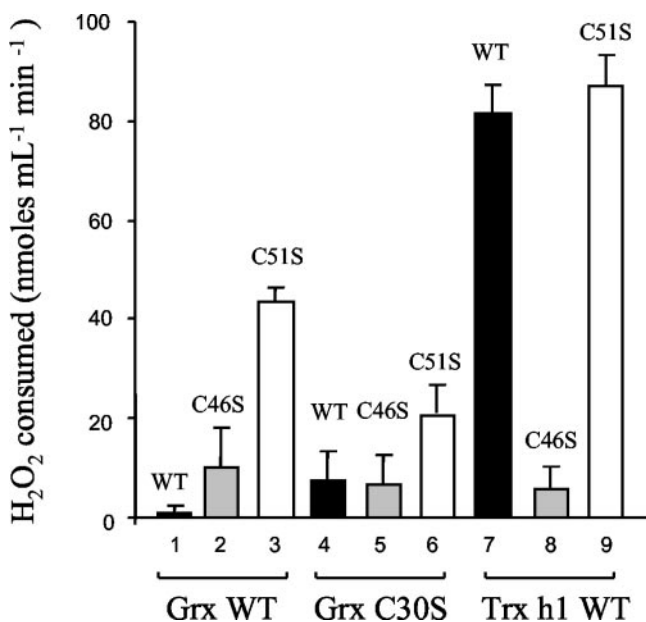[a] No. in parentheses is μmol NADPH ox $min^{-1}\ mg^{-1}$ protein.

termined in this fashion, are shown in Table I. Several hydroperoxides can be used as substrates by Prx Q in the presence of Trx h3. The reactions saturate at a Trx concentration of approximately 6 μM, and the K_m for Trx h3, the best electron donor is around 1.5 μM. In terms of catalytic efficiency (i.e. $k_{cat}\ K_m^{-1}$), the preferred substrate is cumene hydroperoxide (CUOOH) followed by H_2O_2 and t-BOOH, but only the reduction of H_2O_2 is physiologically relevant.

Characterization of the Active Site by Site-Directed Mutagenesis

Figure 4 shows the peroxidase activity of WT Prx Q, Prx Q C46S, or C51S with H_2O_2 as a substrate and in the presence of DTT and WT Grx, Grx C30S, or Trx

Figure 4. Trx- or Grx-dependent peroxidase activity of Prx Q WT, C51S, or C46S. The peroxidase activity was measured with 500 μM H_2O_2, 5 μM of WT Prx Q (columns 1, 4, and 7), Prx Q C46S (2, 5, and 8), or Prx Q C51S (3, 6, and 9), in the presence of 5 mM DTT with 50 μM of WT Grx (columns 1–3), Grx C30S (4–6), or Trx h1 WT (7–9). The activity was stopped 5 min after H_2O_2 addition and is expressed as a concentration of consumed H_2O_2 over 5 min. Each experiment was repeated three times. Background values of the reactions are in the order of 50 μM.

h1. As observed before, the WT Prx Q is only reduced by Trx h1 (Fig. 4, column 7) but not by Grx (Fig. 4, columns 1 and 4) even at high concentration (50 μM). The C46S mutant does not possess any biochemical activity, whatever the reductants (Trx or Grx) or the substrates (H_2O_2, tBOOH, or CUOOH) used (Fig. 4, columns 2, 5, and 8, and data not shown). On the other hand, whereas activity of C51S mutant is not detectable at low Trx concentration (approximately 5 μM), it can be partially rescued by increasing the Trx concentration to 50 μM (Fig. 4, column 9). Unexpectedly, this mutant possesses a peroxidase activity in the presence of WT Grx, but it is only weakly active with the mutant Grx C30S (Fig. 4, columns 3 and 6). These results were confirmed spectrophotometrically by following NADPH oxidation in the presence of the Trx or Grx systems (data not shown).

Complex Formation

The biochemical characteristics described above have been confirmed by establishing that heterodimers can be formed between the donors and Prx Q C51S. In agreement with the biochemical results, cyclophilin A was unable to form heterodimers with Prx Q (data not shown). On the other hand, heterodimers were present when mixing Prx C51S and the various monocysteinic Trx: Trx f C49S, Trx h1 C42S (data not shown), or Trx m C40S (Fig. 2B, lane 2). The dissociation of this complex by excess DTT proves that the heterodimer was linked by a covalent disulfide bridge (Fig. 2B, lane 4). The size of the heterodimer polypeptide (approximately 30 kD) is in good agreement with the addition of one Trx (13 kD) to one Prx Q (17 kD). Western-blot experiments with antibodies directed against poplar Trx h1 and Prx Q confirmed the nature of this additional band (data not shown). The observation that WT Grx does not form stable heterodimers with Prx Q C51S, although it is able to support the activity of this mutant, is consistent with the formation of a transient intermolecular disulfide bridge that is subsequently reduced by the second active site Cys of Grx (data not shown). Grx C30S does not form dimers with Prx Q C51S, although it makes excellent heterodimeric structures with type II Prx in agreement with its lower catalytic efficiency (Rouhier et al., 2002a).

Redox Potential

Figure 5 summarizes the results of redox titrations of the Prx Q disulfide over the pH range from pH 5.5 to 10.5. Each point represents the average of at least two determinations and the average deviations suggest that the experimental uncertainty in E_m is between 5 and 10 mV. Titrations at all pH values gave excellent fits to the Nernst Equation for a single two-electron redox couple. Although DTT is not a donor in the time scale used for the FOX assay, it is able to reduce the disulfide bridge of the enzyme upon longer periods of incubation. Titrations at three different total DTT concentrations in the redox buffers used (1, 1.75, and 2.5 mM) and at two different

redox equilibration times (2 and 3 h) gave identical titration curves (within the experimental uncertainties), as expected for titrations in which redox equilibrium has been established. The E_m value for the disulfide of Prx Q at pH 7.0, determined using the mBBr fluorescence method, is -325 ± 10 mV (Fig. 5A). A plot of E_m versus pH contains two linear regions (Fig. 5B). At pH values between 5.5 and 9.0, the slope is -59 mV pH unit^{-1}, the value expected for a process in which a two-electron reduction is accompanied by the uptake of two protons (Chivers et al., 1997). Between pH 9.0 and 10.5, the E_m versus pH slope is -29.5 mV pH unit^{-1}, indicating that the two-electron reduction is accompanied by the uptake of only a single proton over this pH range (Chivers et al., 1997). The intersection of these two straight-line segments corresponds to the pK_a of an acid/base couple with a protonation state that is linked to the redox state of Prx Q (Chivers et al., 1997). Although the simplest explanation for this data is that the more acidic active-site Cys of reduced Prx Q has a pK_a value close to 9.0, thermodynamic measurements alone cannot identify the actual amino acid(s) involved in this redox-coupled proton uptake.

## Expression and Localization of Prx Q

Localization of Prx Q

Several experiments aimed at investigating the cellular and subcellular localization of Prx Q have been undertaken. First, a transient expression experiment was carried out using a chimeric protein obtained by fusing the transit peptide of Prx Q to green fluorescent protein (GFP). After bombardment of tobacco (*Nicotiana tabacum*) leaf epidermis (which contain epidermal cells and underlying parenchymal photosynthetic cells), fluorescence was detected in the chloroplasts of the mesophyll cells (data not shown) and also in the chloroplasts of the guard cells of stomata (Fig. 6, A3). Control experiments showing the natural fluorescence of chlorophyll indicate that the GFP fluorescence coincides with the chloroplasts (Fig. 6, A2). These results indicate that the transit sequence at the N terminus of mature Prx Q efficiently targets the protein to chloroplasts and notably to those of the guard cells of stomata. In a second series of experiments, semi-thin sections of poplar leaves were incubated with purified Prx Q antibodies and the presence of the polypeptide detected by using anti-rabbit antibodies coupled to fluorescein isothiocyanate. As above with the GFP experiments, the secondary fluorescence was found in the chloroplasts of mesophyll cells (Fig. 6B). Immunocytochemistry experiments indicate that Prx Q is present in the stromal part of the chloroplasts and also is somehow associated with the thylakoid membranes in a distribution similar to the one observed for 2-Cys peroxiredoxin (Fig. 6C; Konig et al., 2002).

A

B
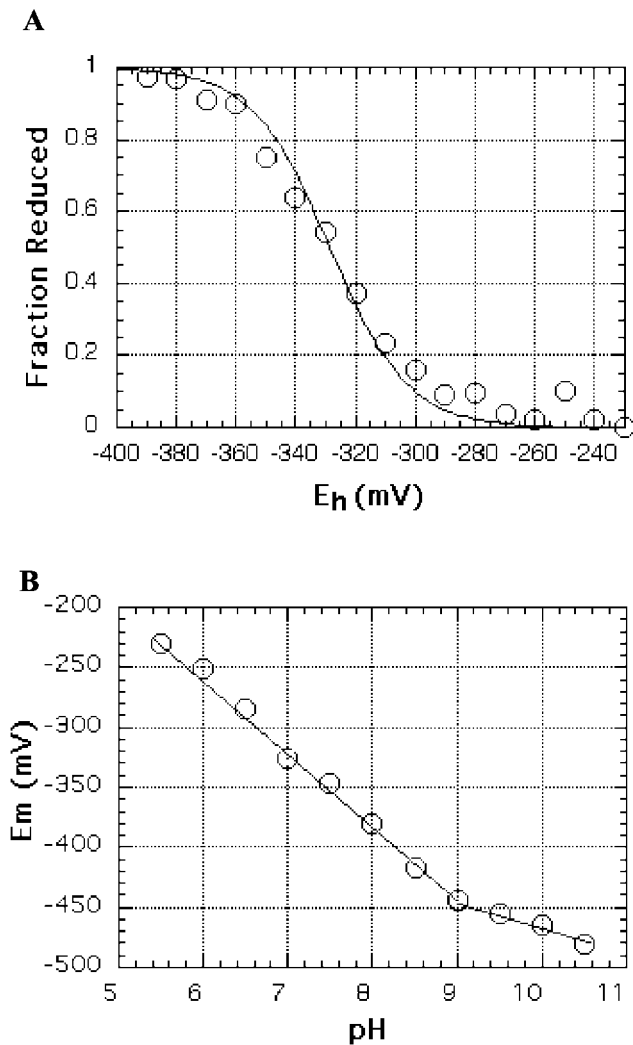
Figure 5. Oxidation-reduction properties of Prx Q. A, Oxidation-reduction titration of Prx Q at pH 7.0. Each reaction mixture contained 100 μg of Prx Q in 1.0 mL of 100 mM MOPS buffer (pH 7.0) containing DTT at a total concentration of 2.5 mM (E_h was adjusted by varying the ratio of reduced:oxidized DTT). Samples were equilibrated for 3.0 h before the addition of monobromobimane (mBBr). B, Effect of pH on the E_m value of Prx Q. Reaction conditions were as in A except for the pH buffers used (see "Materials and Methods").

Figure 6. A, Chloroplast localization of the fusion protein comprising the N-terminal sequence of Prx fused to GFP. 1, Image of stomata from *Nicotiana benthamiana* under visible light. Only the lower guard cell was transfected. 2, Autofluorescence of chlorophyll (red). 3, Fluorescence of the fusion protein (green). 4, Merged images. B, Immunofluorescence labeling of Prx Q on a poplar leaf transversal section. C, Immunogold labeling of Prx Q in a chloroplast of mesophyll cells from light grown poplar leaves.
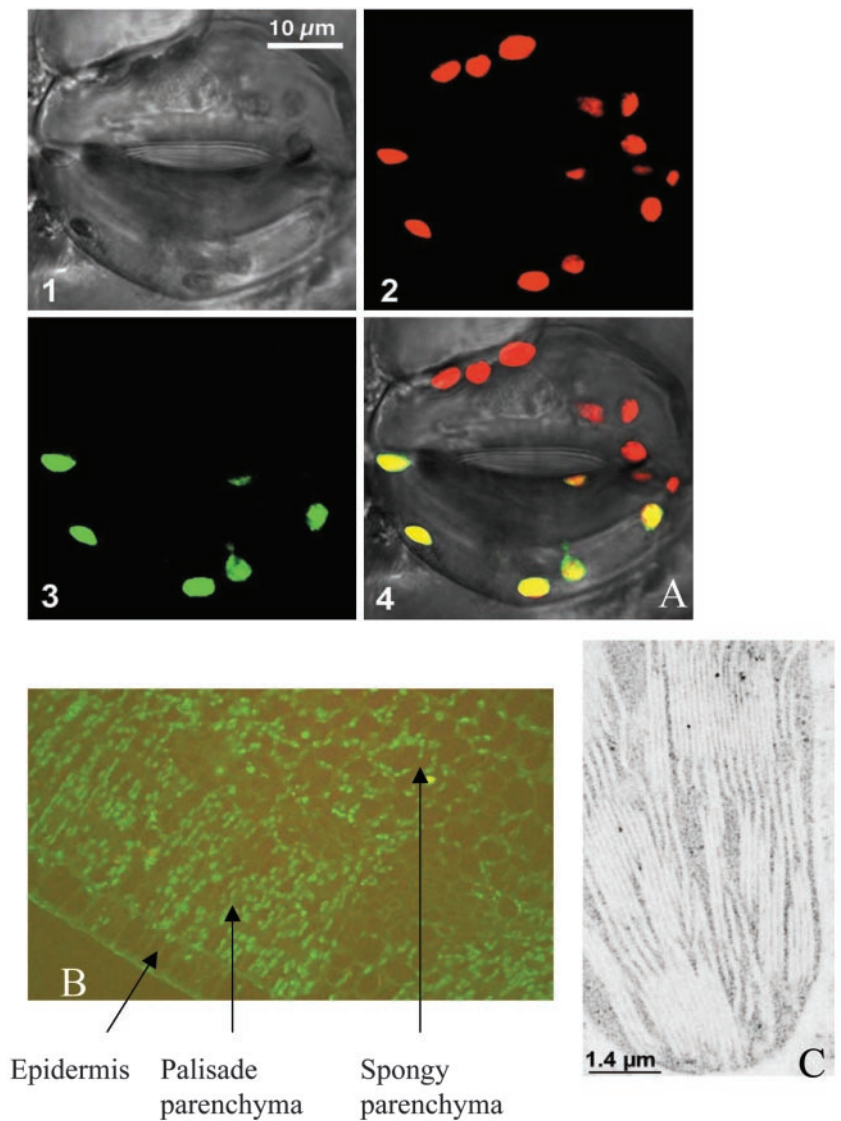
Expression of Prx Q in Untreated Plants

The expression of *prx Q* in plant organs was investigated by recording the number of expressed sequence tags (ESTs) encoding this protein among all the poplar ESTs present in the GenBank database. Only nine ESTs are present out of a total of approximately 125,000, which suggests that the gene is transcribed at very low levels in leaves (four hits), bark (three hits), flowers (one hit), and young tension xylem (one hit). The Prx Q content was also followed at the protein level in roots, stems, or leaves of healthy plants. Figure 7A shows that the Prx Q polypeptide is only present in detectable amounts in leaves, but not in stems or roots.

Prx Q Expression and the Pathogenic Response

Compatible or incompatible reactions result from the infection of poplar leaves by races of the rust fungus *M. larici-populina*. We have determined that the level of Prx Q and type II Prx polypeptide increases markedly with the time of infection in the case of a hypersensitive response (Fig. 7B) with a maximum at approximately 24 h. Conversely, in the compatible reaction, the Prx Q and type II Prx content decreased as infection proceeded (Fig. 7C). After 10 d of infection, the Prx Q content is approximately equivalent to the one before infection. In noninfected leaves (control), the relative amount of type II Prx remained nearly unchanged and the one of Prx Q is varied slightly presumably in the response to changes in light, as observed for *prx Q* transcripts in Arabidopsis.

DISCUSSION

Plant Prx, as their counterparts from other biological organisms, are currently subject to great interest
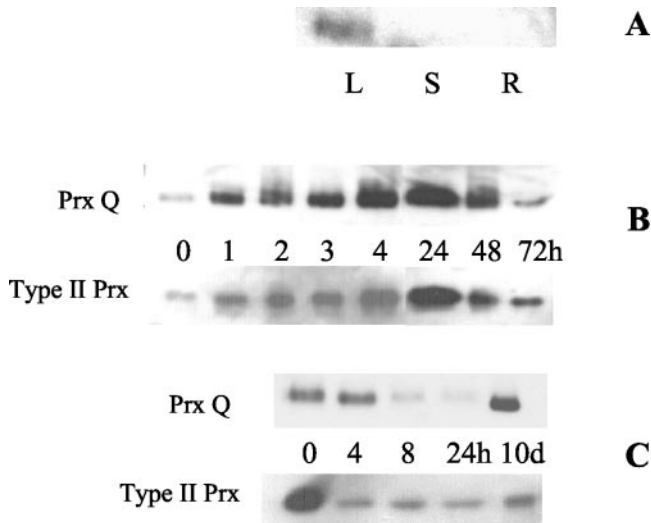
Figure 7. Expression pattern of poplar Prx Q and type II Prx in plant organs or in response to infection by *M. larici-populina.* A, Western blot showing the expression of Prx Q in leaves (L), stems (S), or roots (R) and the expression of Prx Q and type II Prx during an incompatible reaction between 0 and 72 h (B) or a compatible reaction between 0 and 24 h and 10 d after infection (C).

because of their documented or sometimes postulated protective role in oxidative or nitrosative stress. Two subtypes have been extensively studied, the chloroplastic dimeric 2-Cys Prx and the type II Prx. Less is known about the 1-Cys Prx and the Prx Q. Here, we present new data concerning the molecular mechanism, substrate and donor specificity, and in vivo function and localization of Prx Q in poplar, a woody species.
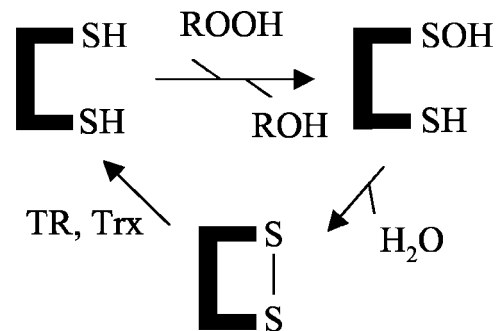
Catalytic Mechanism

Using recombinant technology and site-directed mutagenesis, we have identified the active site of the poplar enzyme as an intramolecular disulfide, which involves Cys 46 and Cys 51. Based on several lines of evidence, Cys 46 is proposed to be the catalytic Cys. First, it is in a conserved position with respect to all other Prx types where it has been shown to be the peroxidatic Cys. Second, the C46S mutant is inactive, whereas the C51S mutant is partially active at high electron donor concentration. We propose here that in the WT form of the enzyme, the regeneration of Cys 46 involves the transient formation of a disulfide with Cys 51, which is subsequently reduced by Trx but not Grx. In the C51S mutant, the sulfenic acid can be reduced directly by Trx or Grx. These data for the WT or the Prx Q C51S mutant are depicted in the reaction scheme presented in Figure 8. These observations are in agreement with results obtained for the BCP from *E. coli*, for which only the equivalent of Cys 46 is essential and the two other BCP cysteines are dispensable (Jeong et al., 2000). A complete alignment analysis of BCP is also consistent with this

observation. Whereas the first Cys is totally conserved, the second is not present in BCP from *Helicobacter pylori* or *Synechocystis sp.* (accession nos. AAD05701 and BAA16704, respectively). Although site-directed mutagenesis experiments carried out with the *S. lineare* enzyme have been interpreted in terms of two essential cysteines, the experiments using mutated *S. lineare* enzyme did not investigate the possibility of activity at high Trx concentrations (Kong et al., 2000).

Substrate Specificity

From the biochemical analysis of poplar Prx Q, we found that its catalytic efficiency for the reduction of H_2O_2 ($k_{cat} K_m^{-1}$ of 7.98×10^3 M^{-1} s^{-1}) is comparable with the value obtained for the *E. coli* BCP (2.45×10^3 M^{-1} s^{-1}) and to those of the 2-Cys Prx from Arabi-
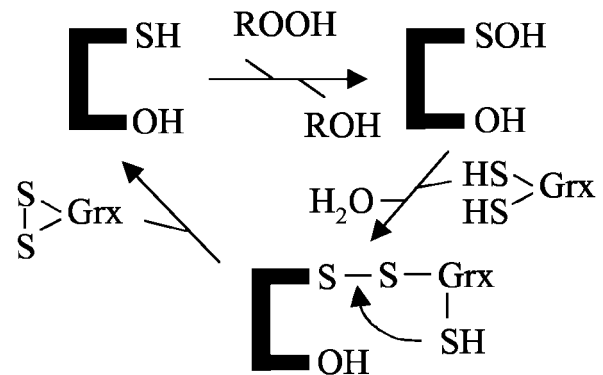
Figure 8. Reaction scheme of poplar Prx Q WT (A) and poplar Prx Q C51S or monocysteinic BCP from other organisms (B). In the case of Prx Q with two conserved cysteines (A), the catalytic Cys is transformed into a sulfenic acid intermediate that is reduced by the attack of the second Cys to form an intramolecular disulfide bridge. This disulfide is then reduced by Trx. In the case of BCP with only one conserved Cys or in the poplar Prx Q C51S mutant (B), the sulfenic acid is assumed to be reduced directly by bicysteinic Trx or Grx, forming a transient intermolecular disulfide bridge that is reduced by the second Cys of Trx or Grx, these electron donors being oxidized.

dopsis (36×10^3 M^{-1} s^{-1}; Jeong et al., 2000; Konig et al., 2002). In addition to reducing H_2O_2, Prx Q also catalyzes the reduction of nonphysiological peroxides, with a preference for CUOOH compared with t-BOOH. Given the important roles postulated for H_2O_2, especially in the chloroplast metabolism, these findings are likely to be of physiological importance.

Physiological Electron Donor

The chloroplast of photosynthetic organisms is a complex organelle that contains a multiplicity of proteins with possible similar function. In addition to many ascorbate or glutathione peroxidases, four Prx isoforms are also found in the chloroplasts of Arabidopsis: two dimeric 2-Cys Prx, one type II Prx, and one Prx Q (Dietz et al., 2002). On the other hand, a large number of Trx isoforms are likely to be located in this compartment: four Trx m, two Trx f, two Trx y, one Trx x, and one chloroplast drought-induced protein of 32 kD (CDP32), a thioredoxin-like protein that is able to reduce the 2-Cys Prx (Broin et al., 2002; Meyer et al., 2002). Earlier studies with the *S. linare* enzyme used the couple *E. coli* Trx/*E. coli* Trx reductase as an electron donor for the reaction, which is not a physiological reductant (Kong et al., 2000). We have carried out an extensive study of the electron donor specificity of Prx Q, testing a large number of chloroplastic and nonchloroplastic thioredoxins. Although all Trx tested were able to serve as electron donors, their efficiencies differed. The most efficient reductant is a cytosolic Trx, suggesting there is no marked specificity between the thioredoxins for the reduction of this enzyme (Fig. 3). On the other hand, two isoforms of the other documented donors, glutaredoxin and cyclophilin A, are totally inefficient with the WT Prx Q (Lee et al., 2001; Rouhier et al., 2002a). Nevertheless, other Grx or cyclophilin isoforms could possibly reduce the poplar WT Prx Q. This observation clearly separates Prx Q from poplar type II Prx, which can use Trx and Grx as electron donors (Rouhier et al., 2002a).

As has been observed for dimeric 2-Cys Prx, DTT cannot serve as a direct donor for Prx Q activity, in contrast to its ability to serve as a donor to type II Prx (Horling et al., 2003). As the redox potential of DTT is sufficient to reduce Prx Q (see below), this observation suggests an important role for the active site of Trx in the protein/protein recognition needed for the interaction with the catalytic disulfide of Prx Q. Although it will be necessary to obtain the three-dimensional structure of Prx Q to understand the detailed nature of these molecular contacts, some insight is provided by the recently resolved structure of a fusion protein from *Haemophilus influenzae* between Prx and Grx modules (Kim et al., 2003). The interactions between Prx and Grx derived from charged interactions between three Asp residues of Prx in position 148, 154, and 156 and Lys and Arg of

Grx, whereas the interactions between Prx and Trx are of hydrophobic nature. In poplar, the type II Prx, which was previously found to interact with Grx, possess two Glu residues equivalent to the Asp residues in position 148 and 156, partly explaining the interaction (Rouhier et al., 2002a; Kim et al., 2003). When comparing all of these proteins with poplar Prx Q, we can observe that Prx Q possesses two Glu residues (at positions 200 and 205) in positions equivalent to Asp148 and 154 of the Prx/Grx fusion that might be involved in similar electrostatic interactions. These observations could explain why Prx Q C51S is able to interact with Grx, but not why the disulfide bond of WT Prx Q is not reduced catalytically by Grx.

To assess whether a thermodynamic barrier exists for the reduction of Prx Q by Grx, we carried out an extensive series of oxidation-reduction titrations of Prx Q. The E_m value of -325 mV, measured for Prx Q at pH 7.0, explains why Grx, with an E_m value at pH 7.0 between -200 and -230 mV (Prinz et al., 1997) is such a poor donor in this reaction. The E_m value for poplar Prx Q is more negative than those of other peroxiredoxins, which range from -320 mV (2-Cys Prx B) to -288 mV (Prx II B; Konig et al., 2002) and reduction of the poplar enzyme by Trx would be slightly unfavorable. This situation is similar to those observed for reduction of the regulatory disulfide of chloroplast FBPase (E_m values of -305 and -315 mV have been reported for the spinach and pea [*Pisum sativum*] enzymes, respectively, whereas the E_m for pea and spinach Trx f is -290 mV) and the reduction of the C-terminal regulatory disulfide of sorghum NADP-malate dehydrogenase (E_m of -330 mV) by Trx m (E_m of -300 mV; Schurmann and Jacquot, 2000). Despite the slightly unfavorable ΔE_m values of 20 to 30 mV, reduction of these regulatory disulfides can occur efficiently, but the much greater ΔE_m of approximately 100 mV for reduction of WT Prx Q by Grx is probably too high for the reaction to proceed.

Expression and Localization of Prx Q

In uninfected and unstressed plants, Prx Q is only expressed in leaves, but not in roots or stems, and the level of expression is very low. The cellular and subcellular distribution of Prx Q in leaves has been investigated by immunofluorescence, immunogold labeling, or GFP fusion proteins. All techniques gave similar results, indicating that the protein is localized in the chloroplasts of mesophyll cells and also in those of the guard cells of stomata. This observation is in good agreement with the predictions made on the basis of the transit sequence analysis. As H_2O_2 is known to be a compound that affects stomatal closure, the intervention of Prx Q in this process is rather likely (Zhang et al., 2001). In addition, pathogen attacks of leaves very often involve the colonization of the fungus via the ostiole of the stomata (Laurans and Pilate, 1999; Mendgen and Hahn, 2002).

Peroxides and especially H_2O_2 are implicated in regulating disease resistance at various levels. H_2O_2 has a direct antimicrobial effect and it triggers the hypersensitive response in plants. In addition, it is a substrate for oxidative cross linking of cell wall material and a diffusible signal that induces the transcription of various resistance genes (Mellersh et al., 2002). Whatever the poplar/*M. larici-populina* interaction type (incompatible or compatible), the fungus is able to penetrate the stomatal cells (Laurans and Pilate, 1999). The future of the infection depends on the capacity of the plant to attack and destroy the pathogen. In an incompatible reaction, the plant does not develop any visible symptoms on the leaf. As in the case of animal cells, this involves an hypersensitive response that occurs via an oxidative burst with the production of massive amounts of H_2O_2 and nitric oxide in the infected tissues (Levine et al., 1994; Delledonne et al., 2001). Thus, it is likely that the high overexpression of Prx Q and type II Prx observed in those conditions (Fig. 7B) is related to the need to maintain the peroxide concentrations low outside the sites of infection and spare the uninfected cells. On the other hand, in a compatible reaction, the fungus takes over and lesions with urediniospores appear on the leaves after approximately 7 d in controlled conditions. In this second situation, the peroxide concentrations may be lower and the need for detoxification is presumably not as essential, resulting in a Prx Q or type II Prx content that decreases with time (Fig. 7C). The transcription of a *type II Prx* in pepper (*Capsicum annum*) leaves was shown to respond to H_2O_2 addition by an elevation of the mRNA levels (Do et al., 2003). In this study, pepper leaves have also been infected by bacteria, oomycetes, or fungi (*Xanthomonas campestris* pv. *Vesicatoria*, *Phytophtora capsici*, or *Colletotrichum gloeosporioides*) in compatible and incompatible reactions. In all cases, the levels of RNA transcripts varied considerably, and the nature of the variation depended on the nature of the pathogen and on the type of infection. The present study brings complementary information to those presented by Do et al. (Do et al., 2003). It shows that another peroxidase (Prx Q) exhibits a similar behavior, and, in addition, that the fluctuations are also observed at the protein level. Thus, it is likely that the over or underexpression of Prx Q and type II Prx recorded here is part of an extensive program that involves several peroxidases to control the pathogen infections.

MATERIALS AND METHODS

Materials

Poplar (*Populus tremula* x *Populus tremuloides*) Trx h1 WT and C42S, h2 WT, h3 WT and Grx WT or C30S were purified as described previously (Behm and Jacquot, 2000; Gelhaye et al., 2002; Rouhier et al., 2002c, 2002b; Gelhaye et al., 2003). Arabidopsis NTR and WT and mutant spinach (*Spinacia oleracea*) thioredoxin m and f were prepared as in Jacquot et al. (1994), Schurmann (1995), Capitani et al. (2000), and Balmer and Schürmann (2001).

The sequence of *C. reinhardtii* Trx y (CrTrx y) is described in Lemaire et al. (2003). The production and purification of poplar cyclophilin A will be described later.

Cloning of Poplar Prx Q and Site-Directed Mutagenesis

The open reading frame of poplar Prx Q in which the nucleotide sequence encoding the chloroplastic transit peptide was deleted was cloned by PCR from a leaf cDNA library from poplar into the expression plasmid pET-3d between the two restriction sites *Nco*I and *Bam*HI with the following oligonucleotides: forward oligonucleotide 5'CCCCC*CCATGG*CTAAGGTAAA-CAAAGG-3'; reverse oligonucleotide 5'-CCCCC*GGATCC*TCAAAGGC-TTTGAAGTAGTTT-3'. Both restriction sites are underlined. This construction was made possible by deleting the transit sequence until the Phe residue in position 63 by comparison with the mature Prx Q of *Sedum lineare*. Thus, the recombinant protein starts with the sequence MAKVN. The mutation of the two Cys residues at positions 46 and 51 to Ser was made by PCR-mediated site-directed mutagenesis with two complementary mutagenic primers. The mutagenic primers are as follows: C46S forward 5'-GATGAAACCCTGGG**A**GCACCAAACAGGCCTG-3'; C46S reverse 5'-CAGGCCTGTTTGGTGC**T**CCCAGGGGTTTCATC-3'; C51S forward 5'-GGGTGCACCAAACAGGCC**A**GTGCTTTTAGAGATTC-3'; C51S reverse 5'-GAATCTCTAAAAGCAC**T**GGCCTGTTTGGTGCACCC-3'. Mutagenic bases are in bold characters. The nature of each mutation was verified by DNA sequencing.

Expression and Purification of WT and Mutant Poplar Prx Q

Escherichia coli strain BL21(DE3) was cotransformed with the helper plasmid pSBET and each of the recombinant plasmids (Schenk et al., 1995). Cultures of 5 L of ampicillin- and kanamycin-resistant clones were induced with 100 μM isopropyl β-D-thiogalactoside for 4 h. The cells were then harvested by centrifugation at 4,400g and suspended in buffer A (30 mM Na-acetate, pH 5.5, 1 mM EDTA, and 200 mM NaCl) in the presence of 5 mM DTT. Bacteria were lysed by sonication for 5 min. The extract was then precipitated between 25% and 80% (w/v) of ammonium sulfate saturation. This fraction was successively subjected to gel filtration chromatography using ACA 44 equilibrated with buffer A. After dialysis against buffer A without NaCl, the sample was applied to a carboxymethyl cellulose column equilibrated with the same buffer. Proteins were eluted with a gradient of NaCl between 0 and 0.4 M and were then dialyzed and concentrated against buffer A without NaCl. The homogeneity of the proteins was assessed by 15% (w/v) SDS-PAGE.

Trx-Linked Peroxidase Activity

The reaction mixture (50 μL) contained 30 mM Tris-HCl, pH 7.0, 500 μM DTT, 1 μM Prx Q, and various concentrations of Trx or Grx ranging between 1 and 50 μM. The reaction was started by adding 500 μM H_2O_2. After 3 min, 5 μL was mixed with 495 μL of the FOX1 reagent (Wolff, 1994). The absorbance was then read at 560 nm after 1 h of incubation.

The peroxidase activity of Prx Q was also measured by following NADPH oxidation at 340 nm coupled to a thioredoxin-reducing system (Arabidopsis NTR/poplar Trx h3) that served as the electron donor and H_2O_2, t-BOOH, or CUOOH as an electron acceptor, following a procedure described previously for poplar type II Prx (Rouhier et al., 2002a). The only slight modification is that the reaction was made in 50 mM K^+/Na^+ phosphate buffer, pH 7.0. The determination of the catalytic parameters was effected with 1 μM Prx Q by varying the concentration of one substrate at saturating concentrations of the other substrate (6 μM Trx h3 or 200 μM hydroperoxide).

Formation of Heterodimers between Prx Q C51S and Trx m C40S or Trx f C49S

The formation of heterodimers between poplar Prx Q WT or C51S and spinach Trx m C40S or Trx f C49S was accomplished by derivatizing the various Prx Q with DTNB to form Prx Q-TNB as described for thioredoxin reductase in Wang et al. (1996) or by diamide treatment in the presence of

poplar donors (Trx h1 C42S, Grx C30S, and cyclophilin A) as in Rouhier et al. (2002a). For the DTNB treatment, Prx Q was reduced with 20 mM DTT, dialyzed, and was then reacted with a 50-fold excess of 5,5'-dithiobis nitrobenzoic acid (DTNB). The mixture was then dialyzed to eliminate this excess. Equimolar concentrations of Prx Q-TNB and reduced Trx or Grx monocysteinic mutants or WT cyclophilin A were used to create heterodimers in 30 mM Tris-HCl, pH 7.0, and 1 mM EDTA. The mixture was then subjected to SDS-PAGE under nonreducing conditions.

Thiol Titration

Five hundred micrograms of Prx Q WT, C46S, and C51S was reduced by incubation with 10 mM DTT for 30 min at room temperature, was precipitated with one volume of 20% (w/v) trichloroacetic acid (TCA), and was stored on ice for 30 min. The mixture was centrifuged for 10 min at 13,000g and was washed two times with 2% (w/v) TCA. The precipitate was resuspended in 30 mM Tris-HCl, pH 7.0, 1 mM EDTA, and 1% (w/v) SDS. The concentration was estimated by UV spectrophotometry ($\epsilon_M = 13,490$ M^{-1} cm^{-1}) before adding DTNB to a final concentration of 100 μM. After a 1-h incubation in the dark, the absorbance was read at 412 nm and the thiol content was estimated using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}.

Determination of the Redox Potential

Redox titrations were performed using mBBr as in Krimm et al. (1998). Prx Q at a concentration of 100 to 200 μg mL^{-1} (6–12 μM) was incubated in 500-μL aliquots at defined E_h values obtained by varying the relative amounts of oxidized and reduced DTT (at a total DTT concentration of 2.5 mM) for 3 h. An E_m value of –330 mV at pH 7.0 and a value of 9.2 for the pK_a of the more acidic thiol of reduced DTT were used for calculating E_h values (references listed in Krimm et al., 1998). The following buffers were used: MES, pH 6.0, MOPS, pH 6.5 to 7.5, Tricine, pH 8.0 to 8.5, and Bis/Tris-propane, pH above 8.5. After equilibrating the Prx Q sample at the defined E_h values with the DTT redox buffers, mBBr dissolved in acetonitrile was added to a final concentration of 10 mM. After a 20-min incubation with mBBr, the Prx Q was precipitated by addition of 500 μL of 20% (w/v) TCA. The resulting protein pellet obtained by centrifugation was washed and dissolved in 100 mM Tris-HCl buffer, pH 8.0, and 1% (w/v) SDS. The fluorescence of these samples was measured at 450 nm, excited by 380 nm light, using an spectrofluorimeter (Series 2 Luminsecence Spectrofluorimeter; Aminco-Bowman, Rochester, NY). Fitting of the data was done as previously described in Krimm et al. (1998).

Infection of the Leaves, Protein Extraction, Antibody Purification, and Western-Blot Analysis

Populus x *interamericana* "Beaupré" plants were grown for 12 weeks in a greenhouse from dormant cuttings in pots containing a sand-peat mixture. Fully expanded leaves were detached and spray inoculated on their abaxial surface with an urediniospore suspension in water-agar (0.1 g L^{-1}) adjusted to 5,000 urediniospores mL^{-1} or with water-agar (control). Two isolates of *M. larici-populina* were used: 98AG31 (pathotype 3-4-7) and 93ID6 (pathotype 3-4), virulent and avirulent, respectively, on Beaupré (Miot et al., 1999). The inoculated leaves were then incubated with the abaxial surface uppermost, floating on deionized water in petri dishes, at 20°C under artificial illumination, for various durations ranging from 1 h to 10 d. Four hundred milligrams of leaves was ground with a mortar and pestle on ice and then 4 mL of the extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2.5 mg mL^{-1} PVP, and 25 mM β-mercaptoethanol) was added. The mixture was centrifuged twice for 10 min at 14,000g and the supernatant was precipitated with 80% (w/v) acetone. Proteins were pelleted by centrifugation and washed with 80% (w/v) acetone. Proteins were resuspended in 125 mM Tris-HCl buffer (pH 6.8) containing 20% (w/v) glycerol, 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, and 0.05% (w/v) bromphenol blue, and their concentrations were determined using the Bradford reaction kit (Bio-Rad, Hercules, CA). Protein extraction of untreated leaves, stems, and roots was carried out as described in Rouhier et al. (2001). Polyclonal antibodies raised in rabbit against poplar Prx Q were purified by affinity chromatography on a Prx Q Sepharose column following the procedure described previously (Rouhier et al., 2001). Western blots were performed using polyvinylidene difluoride membranes (Millipore, Bedford, MA) and the Immune Star Goat Anti-Rabbit Detection kit (Bio-Rad).

Intracellular Localization via GFP Fusion

For in vivo intracellular localization, the first 195 bp of the poplar Prx Q cDNA, corresponding to the N-terminal presequence (65 amino acids), were cloned into the *Nco*I and *Bam*HI sites of pCK-GFP3A using the two following primers: 5'-CCCCCATGGCTTCCATTTCTCTC-3' and 5'-CCCCGGATCC-TTGGCAAAAATGGTATTCTT-3'.

The PCR-amplified fragment was fused to GFP at the *Bam*HI site, resulting in a chimeric protein where the transit sequence of Prx Q is present on the N terminus side of GFP and under the control of a double 35S promoter (Menand et al., 1998). *Nicotiana benthamiana* cells were transfected by bombardment of leaves with tungsten particles coated with plasmid DNA and images were obtained with a confocal microscope (LSM510; Zeiss, Jena, Germany).

Immunolocalization

Sections of 25 mm^2 were cut from expanded leaves of 2-month-old poplar plants and were fixed for 3 h at 4°C in 4% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The samples were subsequently rinsed in 0.1 M cacodylate, treated with 2% (w/v) OsO_4, dehydrated in increasing alcohol and propylene oxide, embedded in Durcupan (Fluka, Buchs, Switzerland), and finally polymerized at 56°C for 48 h.

For immunocytochemistry, ultrathin sections (70 nm) were placed onto 300-mesh nickel grids. Grids were floated for 15 min in a 50 mM Gly, 50 mM Tris-phosphate-buffered saline (TPBS) solution, transferred for 30 min on a blocking solution of 1% (w/v) bovine serum albumin (BSA) in TPBS/BSA, and then treated overnight at 4°C with purified rabbit Prx Q antibodies (diluted 100× in TPBS/BSA). This step was omitted for control samples. After washing, sections were incubated for 2 h in a solution of 50 mM Tris-HCl buffer containing 0.05% (w/v) polyethylene glycol with gold-labeled goat anti-rabbit immunoglobulin G (10 nm, dilution 1:20; Sigma, St. Louis). After rinsing, the sections were dried and stained with uranyl acetate and lead citrate. Observations were made using an electronic microscope (TEM 902; Zeiss).

For histochemical detection of Prx Q using fluorescence, 130-nm-thin sections were coated with poly-Lys (Sigma) on a glass slide. The resin was dissolved by incubation with ethanol saturated in NaOH for 20 min, washed with 100% (w/v) ethanol, and then washed with 100% (w/v) acetone. Slides were treated for 15 min with in TPBS solution containing 50 mM Gly, washed with TPBS, and finally treated with TPBS/BSA for 2.5 h before incubation with purified rabbit Prx Q antibodies overnight (diluted 100-fold). After rinsing with TPBS and TPBS/BSA, the slides were incubated for 2 h with secondary antibodies coupled to fluorescein isothiocyanate diluted 160-fold. Control experiments were performed by omitting primary antibodies or by testing preimmune serum. The slides were rinsed and mounted in TPBS containing 50% (w/v) glycerol. Sections were viewed on epifluorescence microscope (Optiphot II; Nikon, Melville, NY, coupled to a mercury lamp and B2A filters) and images were captured using a camera (D1; Nikon).

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