Isolation and Characterization of a New Peroxiredoxin from Poplar Sieve Tubes That Uses Either Glutaredoxin or Thioredoxin as a Proton Donor[1]

Nicolas Rouhier, Eric Gelhaye, Pierre-Eric Sautiere, Annick Brun, Pascal Laurent, Denis Tagu, Joelle Gerard, Elisabeth de Faÿ, Yves Meyer, and Jean-Pierre Jacquot*

Unité Mixte de Recherche Interaction Arbres Microorganismes, Institut National de la Recherche Agronomique-Université Henri Poincaré Nancy I. Biochimie et Biologie Moléculaire Végétales, Université Henri Poincaré, 54506 Vandoeuvre cedex, France (N.R., E.G., A.B., P.L., D.T., J.G., E.d.F., J.-P.J.); Laboratoire d'Endocrinologie des Annélides Equipe Enseignement Supérieur Associé 97, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq cedex, France (P.-E.S.); and Laboratoire de Physiologie et de Biologie Moléculaire des Plantes, Unité Mixte de Recherche 5545, Université de Perpignan, 66025 Perpignan cedex, France (Y.M.)

A sequence coding for a peroxiredoxin (Prx) was isolated from a xylem/phloem cDNA library from *Populus trichocarpa* and subsequently inserted into an expression plasmid yielding the construction pET-Prx. The recombinant protein was produced in *Escherichia coli* cells and purified to homogeneity with a high yield. The poplar Prx is composed of 162 residues, a property that makes it the shortest plant Prx sequence isolated so far. It was shown that the protein is monomeric and possesses two conserved cysteines (Cys). The Prx degrades hydrogen peroxide and alkyl hydroperoxides in the presence of an exogenous proton donor that can be either thioredoxin or glutaredoxin (Grx). Based on this finding, we propose that the poplar protein represents a new type of Prx that differs from the so-called 2-Cys and 1-Cys Prx, a suggestion supported by the existence of natural fusion sequences constituted of a Prx motif coupled to a Grx motif. The protein was shown to be highly expressed in sieve tubes where thioredoxin *h* and Grx are also major proteins.

Reactive oxygen, sulfur, and nitrogen intermediates can cause serious damage to macromolecules such as proteins, lipids, or nucleic acids, eventually leading to pathological processes (Halliwell and Gutteridge, 1990; Beckman and Ames, 1997; Berlett and Stadtman, 1997). Among the antioxidant defense mechanisms developed by aerobic organisms, non-enzymatic reactions dependent on glutathione, ascorbate, or α-tocopherol play an important role. Alternatively, enzymatic detoxication reactions that involve catalases, superoxide dismutases, and ascorbate or glutathione peroxidases have been extensively studied and documented (Rhee et al., 1994).

A new family of antioxidative enzymes, called peroxiredoxins (Prx), recently has been characterized in all phyla (Rhee et al., 1999). These enzymes catalyze the reduction of either hydrogen peroxide (H_2O_2) or various alkyl hydroperoxides to water and the corresponding alcohol in the presence of a hydrogen donor, which is in turn converted to the oxidized form according to the equation:

$$ROOH + R'(SH)_2 \text{ — water} + H_2O + R'S\text{-}S$$
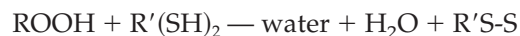
Prx associated with catalases or other peroxidases are believed to participate in signal transduction by regulating the intracellular concentration of H_2O_2, which in turn controls gene transcription and cell signaling through phosphorylation cascades (Jin et al., 1997).

Based on amino acid comparisons, Prx can be divided into two to three main groups: the so-called 2-Cys Prx (referred to as type A in this manuscript), the 1-Cys Prx (type B), and a new isotype that we will call type C. All these isoforms can be differentiated by the position of the Cys (Choi et al., 1999; Verdoucq et al., 1999; Seo et al., 2000). Prx differ from other peroxidases not only by their unique primary sequences but also by lacking prosthetic groups containing a metal ion, normally needed for the catalytic reaction to occur. Prx overcome this problem by using the conserved N terminus catalytic Cys that is converted into a sulfenic acid and regenerated via a proton donor. Site-directed mutagenesis has clearly identified this Cys residue (Cys 47 in yeast [*Saccharomyces cerevisiae*] thiol-specific antioxidant) as the catalytic residue (Chae et al., 1994a). In the case of 1-Cys Prx, the hydrogen donor remains essentially

unidentified, but could be Trx for the mammalian mitochondrial enzyme (Pedrajas et al., 2000). For the 2-Cys Prx, the donor is clearly thioredoxin (Trx) and the mechanism involves another conserved Cys of Prx (Kang et al., 1998). The hydrogen donor of the new isotype is also believed to be Trx (Chae et al., 1994b). The 2-Cys Prx exhibits a dimeric structure, with two identical subunits linked by a disulfide bridge (Choi et al., 1999). Biochemical evidence indicates that 1-Cys Prx are monomeric proteins with an intramolecular disulfide in the oxidized state (Kang et al., 1998). The molecular organization of type C Prx is still largely unknown. Nevertheless, it appears that all subunits are organized in a fold similar to Trx with a central pleated β-sheet surrounded by α-helices (Schroder and Pönting, 1998).

In mammals, Prx are associated with diverse cellular functions including apoptosis (Zhang et al., 1997; Kim et al., 2000), cell proliferation, and differentiation (Yamamoto et al., 1989; Prosperi et al., 1993). In the case of tumor necrosis factor-α-induced apoptosis, it was demonstrated recently that in the presence of reactive oxygen intermediates, human Trx peroxidase appears to be regulated by glutathione conjugation (Sullivan et al., 2000). This protein-glutathione mixed disulfide could then be reduced by oxidoreductases such as Trx, glutaredoxins (Grx) or protein disulfide isomerases. This regulatory mechanism of Cys oxidation reduction might prevent an irreversible oxidation of the concerned Cys into sulfonic or sulfinic acid.

As a general rule, many Prx sequences coexist in a single organism. For example, there are at least three Prx in *Escherichia coli*, five in yeast, and six isoforms characterized in mammalian cells (Zhou et al., 2000). One of the reasons explaining this multiplicity is the multiple subcellular localization of these proteins including the cytosol, peroxisomes, mitochondria, and possibly the nucleus (Stacy et al., 1999; Pedrajas et al., 2000; Zhou et al., 2000).

The systematic sequencing of Arabidopsis also indicates that there are many Prx genes in plants. It appears that the expression of some Prx genes is regulated both temporally and spatially, accounting for the high number of isoforms. In plants, the three types of Prx described above have been identified. The 1-Cys Prx, apparently encoded by a single gene, is expressed specifically in the aleurone layer and embryo of developing seeds of dicotyledonous and monocotyledonous plants (Haslekas et al., 1998; Stacy et al., 1999; Lewis et al., 2000). Its proposed function is to protect these tissues from reactive oxygen species produced during desiccation or as a by-product of respiration during imbibition of seeds. Moreover, there is indirect evidence suggesting that Prx are involved in maintenance of dormancy (Lewis et al., 2000). Apparently in plants, the 2-Cys Prx characterized so far are nuclear encoded, but localized in the chloroplasts (Baier and Dietz, 1997). The

chloroplastic 2-Cys Prx belong to a multigenic family and seem to be expressed in most plant tissues but the roots (Cheong et al., 1999). It is assumed to detoxify products of chloroplastic electron transport (Baier and Dietz, 1999). A recent report indicates that antisense plants suppressed for 2-Cys Prx compensate by overexpressing ascorbate peroxidase and dehydroascorbate reductase, suggesting that a subtle equilibrium exists between diverse antioxidant enzymes in chloroplasts (Baier et al., 2000).

Prx of the C type have been isolated recently from a flower bud cDNA library of *Brassica rapa* (Choi et al., 1999) and also from Arabidopsis (Verdoucq et al., 1999). In a very classic assay, the Prx from Chinese cabbage (*Brassica campestris* L. subsp. *pekinensis*) protects Gln synthetase from oxidation by radicals generated by the Fenton reaction. Both the Arabidopsis and *B. rapa* enzymes have been described to reduce H_2O_2 in the presence of NADPH, Trx reductase, and Trx. We describe here the isolation and characterization of a Prx of the type C from poplar (*Populus trichocarpa*) phloem and show that this protein is bifunctional, i.e. it can use either Trx or Grx as a hydrogen donor. This is discussed in the light of existing homologous prokaryotic sequences that contain a Grx motif fused to type C Prx. The protein was shown to be present in the plant and highly expressed in the sieve tubes of the phloem, which also contains high amounts of Trx and Grx.

RESULTS

Sequence Analysis and Cloning

Based on two overlapping expressed sequence tags (ESTs; AI163857 and AI162101) of respectively 458 and 553 nucleotides, a putative full-length sequence coding for a poplar Prx has been reconstituted. The corresponding cDNA sequence has been cloned by PCR and inserted into the expression plasmid pET-3d, yielding the construction pET-Prx. The open reading frame consists of 489 nucleotides coding for a polypeptide of 162 amino acids. Figure 1 shows an amino acid sequence comparison that includes plant Prx of the three types (A, B, and C). The sequence isolated in this study is strongly associated with the type C Prx from plants. It is clear that all type A plant Prx possess a N terminus extension of about 75 amino acids that presumably codes for a chloroplastic transit peptide as described in Baier and Dietz (1999). In general, Prx of the B and C type do not possess similar extensions and are thus probably not routed to mitochondria, chloroplasts, or to the vacuolar and extracellular compartments. A remarkable feature of the poplar sequence and all type C plant Prx is that they are all considerably shorter on the C-terminal end (type A and B show about 36 and 56 amino acids extensions, respectively). As a consequence, the type C sequences are among the shortest isolated for a Prx so far. The poplar Prx isolated in
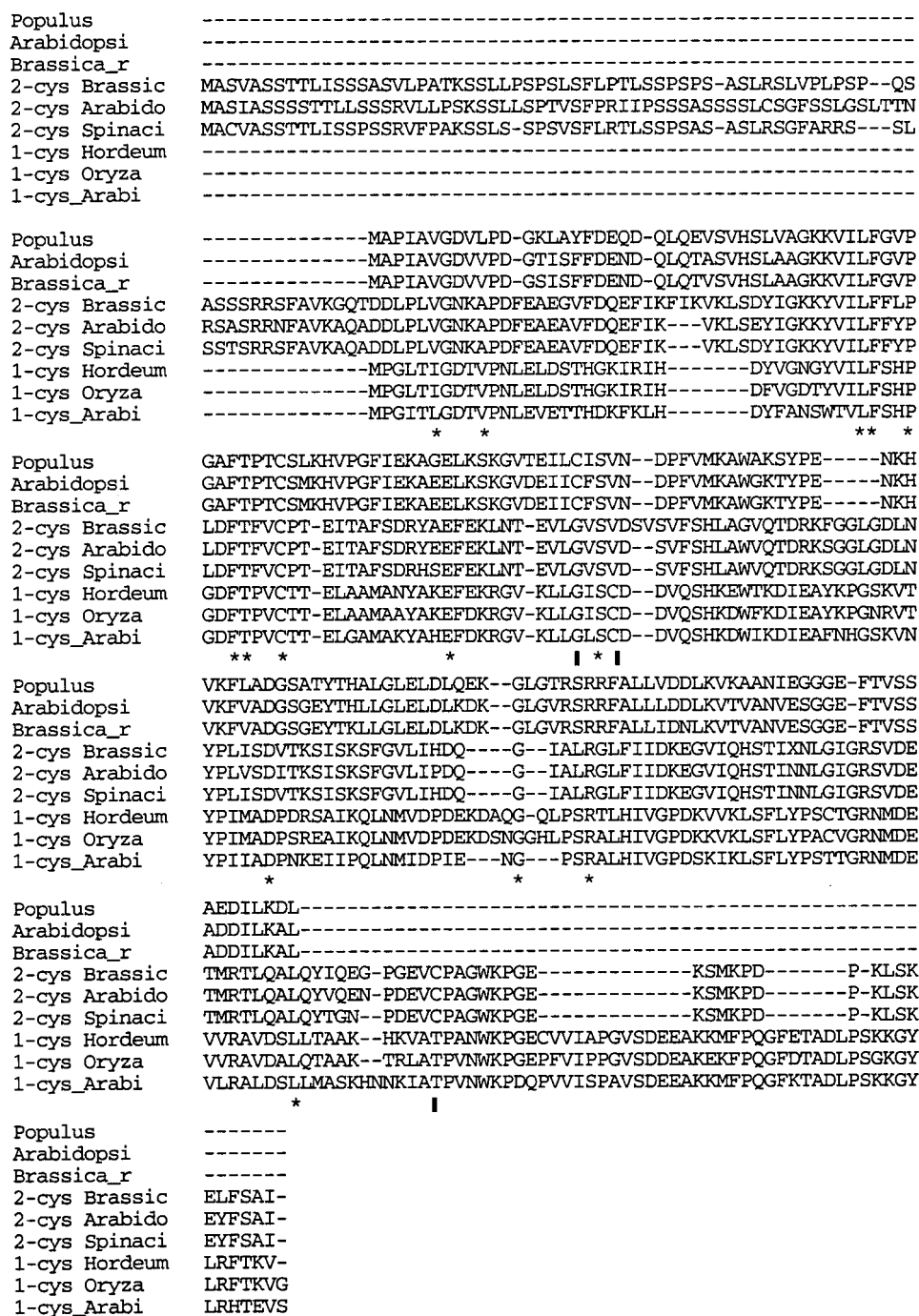
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Populus        ------------------------------------------------------------
Arabidopsi     ------------------------------------------------------------
Brassica_r     ------------------------------------------------------------
2-cys Brassic  MASVASSTTLISSSASVLPATKSSLLPSPSLSFLPTLSSPSPS-ASLRSLVPLPSP--QS
2-cys Arabido  MASIASSSSTTLLSSSRVLLPSKSSLLSPTVSFPRIIPSSSASSSSLCSGFSSLGSLTTN
2-cys Spinaci  MACVASSTTLISSPSSRVFPAKSSLS-SPSVSFLRTLSSPSAS-ASLRSGFARRS---SL
1-cys Hordeum  ------------------------------------------------------------
1-cys Oryza    ------------------------------------------------------------
1-cys_Arabi    ------------------------------------------------------------

Populus        --------------MAPIAVGDVLPD-GKLAYFDEQD-QLQEVSVHSLVAGKKVILFGVP
Arabidopsi     --------------MAPIAVGDVVPD-GTISFFDEND-QLQTASVHSLAAGKKVILFGVP
Brassica_r     --------------MAPIAVGDVVPD-GSISFFDEND-QLQTVSVHSLAAGKKVILFGVP
2-cys Brassic  ASSSRRSFAVKGQTDDLPLVGNKAPDFEAEGVFDQEFIKFIKVKLSDYIGKKYVILFFLP
2-cys Arabido  RSASRRNFAVKAQADDLPLVGNKAPDFEAEAVFDQEFIK---VKLSEYIGKKYVILFFYP
2-cys Spinaci  SSTSRRSFAVKAQADDLPLVGNKAPDFEAEAVFDQEFIK---VKLSDYIGKKYVILFFYP
1-cys Hordeum  --------------MPGLTIGDTVPNLELDSTHGKIRIH-------DYVGNGYVILFSHP
1-cys Oryza    --------------MPGLTIGDTVPNLELDSTHGKIRIH-------DFVGDTYVILFSHP
1-cys_Arabi    --------------MPGITLGDTVPNLEVETTHDKFKLH-------DYFANSWTVLFSHP
                             *      *                            **    *
Populus        GAFTPTCSLKHVPGFIEKAGELKSKGVTEILCISVN--DPFVMKAWAKSYPE-----NKH
Arabidopsi     GAFTPTCSMKHVPGFIEKAEELKSKGVDEIICFSVN--DPFVMKAWGKTYPE-----NKH
Brassica_r     GAFTPTCSMKHVPGFIEKAEELKSKGVDEIICFSVN--DPFVMKAWGKTYPE-----NKH
2-cys Brassic  LDFTFVCPT-EITAFSDRYAEFEKLNT-EVLGVSVDSVSVFSHLAGVQTDRKFGGLGDLN
2-cys Arabido  LDFTFVCPT-EITAFSDRYEEFEKLNT-EVLGVSVD--SVFSHLAWVQTDRKSGGLGDLN
2-cys Spinaci  LDFTFVCPT-EITAFSDRHSEFEKLNT-EVLGVSVD--SVFSHLAWVQTDRKSGGLGDLN
1-cys Hordeum  GDFTPVCTT-ELAAMANYAKEFEKRGV-KLLGISCD--DVQSHKEWTKDIEAYKPGSKVT
1-cys Oryza    GDFTPVCTT-ELAAMAAYAKEFDKRGV-KLLGISCD--DVQSHKDWFKDIEAYKPGNRVT
1-cys_Arabi    GDFTPVCTT-ELGAMAKYAHEFDKRGV-KLLGLSCD--DVQSHKDWIKDIEAFNHGSKVN
                **    *             *          ▮ * ▮
Populus        VKFLADGSATYTHALGLELDLQEK--GLGTRSRRFALLVDDLKVKAANIEGGGE-FTVSS
Arabidopsi     VKFVADGSGEYTHLLGLELDLKDK--GLGVRSRRFALLLDDLKVTVANVESGGE-FTVSS
Brassica_r     VKFVADGSGEYTKLLGLELDLKDK--GLGVRSRRFALLIDNLKVTVANVESGGE-FTVSS
2-cys Brassic  YPLISDVTKSISKSFGVLIHDQ----G--IALRGLFIIDKEGVIQHSTIXNLGIGRSVDE
2-cys Arabido  YPLVSDITKSISKSFGVLIPDQ----G--IALRGLFIIDKEGVIQHSTINNLGIGRSVDE
2-cys Spinaci  YPLISDVTKSISKSFGVLIHDQ----G--IALRGLFIIDKEGVIQHSTINNLGIGRSVDE
1-cys Hordeum  YPIMADPDRSAIKQLNMVDPDEKDAQG-QLPSRTLHIVGPDKVVKLSFLYPSCTGRNMDE
1-cys Oryza    YPIMADPSREAIKQLNMVDPDEKDSNGGHLPSRALHIVGPDKKVKLSFLYPACVGRNMDE
1-cys_Arabi    YPIIADPNKEIIPQLNMIDPIE---NG---PSRALHIVGPDSKIKLSFLYPSTTGRNMDE
                 *                       *       *
Populus        AEDILKDL----------------------------------------------------
Arabidopsi     ADDILKAL----------------------------------------------------
Brassica_r     ADDILKAL----------------------------------------------------
2-cys Brassic  TMRTLQALQYIQEG-PGEVCPAGWKPGE-------------KSMKPD-------P-KLSK
2-cys Arabido  TMRTLQALQYVQEN-PDEVCPAGWKPGE-------------KSMKPD-------P-KLSK
2-cys Spinaci  TMRTLQALQYTGN--PDEVCPAGWKPGE-------------KSMKPD-------P-KLSK
1-cys Hordeum  VVRAVDSLLTAAK--HKVATPANWKPGECVVIAPGVSDEEAKKMFPQGFETADLPSKKGY
1-cys Oryza    VVRAVDALQTAAK--TRLATPVNWKPGEPFVIPPGVSDDEAKEKFPQGFDTADLPSGKGY
1-cys_Arabi    VLRALDSLLMASKHNNKIATPVNWKPDQPVVISPAVSDEEAKKMFPQGFKTADLPSKKGY
                      *          ▮
Populus        -------
Arabidopsi     -------
Brassica_r     -------
2-cys Brassic  ELFSAI-
2-cys Arabido  EYFSAI-
2-cys Spinaci  EYFSAI-
1-cys Hordeum  LRFTKV-
1-cys Oryza    LRFTKVG
1-cys_Arabi    LRHTEVS
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Figure 1. Amino acid sequence alignment of plant Prx. The first three sequences belong to type C Prx, the three following sequences are 2-Cys Prx (type A), and the last three are 1-Cys Prx (type B). This alignment was performed using the Clustal W program, and the accession numbers for these sequences are: Arabidopsis, AAD28242; *B. rapa*, AAD33602; 2-Cys *B. rapa*, AF052202; 2-Cys Arabidopsis, BAB08951; 2-Cys *Spinacia oleracea*, O24364; 1-Cys *Hordeum vulgare*, CAA65387; 1-Cys *Oryza sativa*, BAA09947; and 1-Cys Arabidopsis, Y12089. Asterisk, Indicates strict homologies between all the sequences and conserved Cys within a Prx subtype.

this work displays strict identity of 80% to the Arabidopsis and *Brassica rapa*-type C Prx sequences. On the other hand, the identities are much lower compared with type A or B Prx (10%–15% and 13%–19%, respectively). Type A and B are similarly loosely related (21%–25% identity). Overall, plant Prx of the type A exhibit 77% to 85% identity, those of type B exhibit 68% to 84% identity, and those of type C exhibit 80% to 96% identity. At first, the absence of an N-terminal extension and of identified signals as Ser-

Lys-Leu for the peroxisomes suggest that the subcellular localization of the poplar Prx might be the cytosol, and the interrogation of the PSORT site (http://psort.nibb.ac.jp/form.html) supports this proposal. A very characteristic feature of all the Prx sequences is that one Cys residue (Cys 51 in the poplar enzyme) is strictly conserved with the surrounding consensus sequence: P[G/L][A/D]FT[P/F][T/V]C[S/P/T]. Site-directed mutagenesis has shown that this residue is the catalytic one in all Prx characterized so far (Chae et al., 1994a). The poplar sequence isolated in this work shows only one additional Cys (Cys 76), which is strictly conserved among type C Prx.

Expression of the Recombinant Protein, Purification, and Physical Characteristics

When the *E. coli* BL21 (DE3) strain was cotransformed by the plasmids pET-Prx and pSBET, a huge overexpression of the enzyme was observed, the recombinant Prx representing more than 50% of the total protein content (data not shown). This allowed us to purify the protein with a very high yield using two simple chromatographic steps consisting of an ACA 44 gel filtration column followed by a DEAE-Sephacel ion exchange. Nearly 40 mg protein was obtained per liter culture with a very high purity, based on a calculated molar extinction coefficient of 10,930 M^{-1} cm^{-1} (see Fig. 2). After staining with Coomassie Blue, the purified protein clearly shows a doublet band with an apparent molecular mass of about 18 kD, a value in close agreement with the predicted mass of the polypeptide (17,408 D). The origin of the polypeptide doublet is not fully understood, but it was observed when lysing whole bacterial cells that a similar doublet is already present, strongly suggesting that this property is not an artifact due to proteolysis during the protein purification process. Nevertheless, an N terminus amino acid analysis of the recombinant protein indicated a
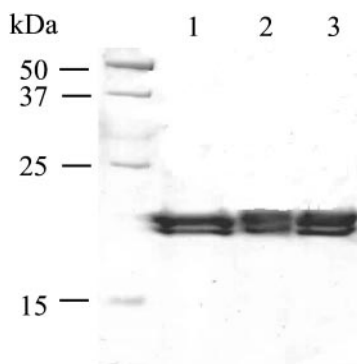
Figure 2. Size determination and purity of recombinant poplar Prx. The size marker used is Precision Protein Standards, unstained from Bio-Rad (Hercules, CA). Each lane contains about 3 μg of Prx. Lane 1, SDS and β-mercaptoethanol; lane 2, SDS alone; lane 3, SDS and dithiothreitol (DTT).
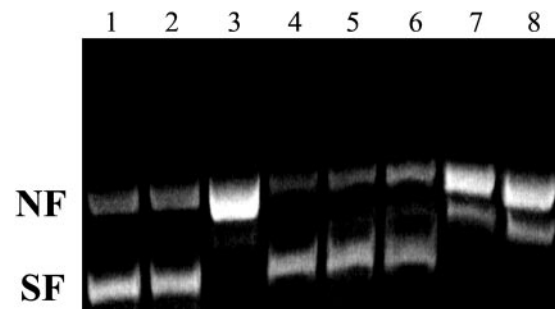
Figure 3. Prx-dependent inactivation of DNA cleavage by MCO. Plasmid pLBR19 was added in each reaction mixture after 40 min. Lane 1, pLBR19 alone; lane 2, DTT alone; lane 3, DTT + FeCl₃; lanes 4 through 6, DTT + FeCl₃ + various amounts of Prx (10, 20, and 40 μM); lane 7, DTT + FeCl₃ + bovine serum albumin (BSA; 20 μM); lane 8, ascorbate + FeCl₃. Nicked form (NF) and supercoiled form (SF) of pLBR19 are indicated on the left.

clipping of the N terminus with the superimposition of the sequences MAPIAV, APIAV, and PIAV, the MAPIAV sequence being more prevalent, accounting for more than 50% of the whole protein population. A similar doublet has been observed with various Prx and Kang et al. (1998) have shown that this doublet can be erased on type B Prx when the samples are treated with a strong reductant as DTT. However, Figure 2 shows that even when the samples are treated with a strong reductant (DTT instead of β-mercaptoethanol), at high temperature in the presence of SDS, a double component is always observed. Extraction of poplar Prx from the bacterial cells in the presence of 5 mM DTT and purification in the presence of 1 mM DTT also did not affect the SDS-PAGE pattern, suggesting that the oxidation of the highly conserved Cys (Cys 51) was not the cause of this behavior. Even when the protein was prepared in the absence of a reductant, it was found to be essentially in the reduced state. This was estimated by titrating the recombinant enzyme with 5,5',dithiobis-nitrobenzoic acid (DTNB). In the native state or in the denatured state (in the presence of 1% [w/v] SDS), 2 mol thiol groups were observed per mole enzyme, suggesting that the enzyme is fully reduced and that both thiols are accessible and not buried. The addition of concentrations of H_2O_2, ranging from 100 μM to 1 mM, resulted in the disappearance of 1.5 mol SH per mole enzyme. Thus, it is likely that the addition of the oxidizing substrate fully oxidizes the presumed catalytic Cys and partially Cys 76.

The oligomeric nature of Prx of the C type is not well documented in the literature so far. On the other hand, it has been proposed that Prx of the A type are dimers of identical subunits linked by a disulfide bridge. Treating the recombinant enzyme with SDS in the absence of a reductant (either β-mercaptoethanol or DTT) clearly indicates that such a hypothesis can be ruled out for the poplar enzyme because the protein always migrates as a monomer (data not shown). When analyzed on
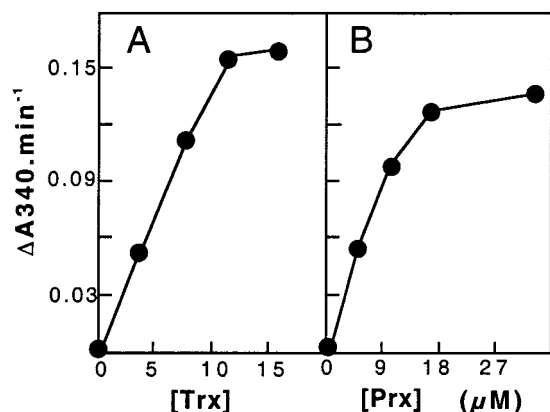
Figure 4. Trx and Prx dependency of H_2O_2 reduction. The concentrations of the other components are as follows: 150 μM NADPH, 1.6 μM NADPH Trx reductase (NTR), and 100 μM H_2O_2. A, Prx concentration is 5 μM; B, Trx concentration is 4 μM.

ACA44 gel filtration, the poplar Prx also behaved as a monomer (data not shown), suggesting that type C Prx is monomeric.

Catalytic Properties of Type C Poplar Prx

One of the most widely used tests for the detection of Prx activity is the protection of plasmids in the presence of a thiol metal-catalyzed oxidation (MCO) generating system (generally DTT and reduced Fe; Fig. 3). Although the strong oxidizing agents generated by the MCO system are able to destroy the plasmid, the addition of recombinant Prx clearly has a protective effect that cannot be reproduced by the addition of similar amounts of an unrelated protein, BSA. When DTT was replaced by reduced ascorbate, the plasmid was not protected and degraded, confirming the need for a thiol as a reductant of Prx.

The poplar Prx is able to reduce H_2O_2 in the presence of external hydrogen donors, Trx (Fig. 4) or Grx (Fig. 5). As shown in Table I, the Prx can use reduced poplar Trx *h* as a proton donor generated by the addition of NADPH and NTR. Omitting either the NTR or the Trx *h* components as well as the substrate H_2O_2 results in a complete loss of the NADPH oxidizing activity. Table I also shows that the C type poplar Prx can alternatively use reduced poplar Grx as a proton donor to carry out its catalytic reaction. In this case, the generating system is composed of NADPH, GR, glutathione, and Grx. Because reduced glutathione directly reduces H_2O_2, a background activity is observed when either Grx or Prx are omitted. Nevertheless, a full activity is only observed when all the components of the GSH/Grx chain are supplied, strongly suggesting that Grx can serve as an alternate proton donor for type C Prx.

Figure 4A shows the Prx linked reduction of H_2O_2 in the presence of increasing Trx *h* concentrations. The reaction rate increases as a function of Trx concentrations and saturates at about 10 μM (Fig. 4A).

The reaction rate was also examined as a function of the Prx concentration (Fig. 4B). As expected, the catalytic activity goes up with increasing Prx concentrations in the range 0 to 15 μM. The rate of the reaction was also estimated as a function of the Grx concentrations when reduced GSH was the donor (Fig. 5). Similar to what was observed with Trx, the reaction rate increases as a function of the Grx concentration and saturates at about 30 μM (Fig. 5A). With glutathione and Grx as electron donor, the reaction rate increased with increasing Prx concentrations in the range 0 to 80 μM (Fig. 5B).

The capacity of recombinant poplar Prx to reduce various other peroxides has also been evaluated. Table II shows that besides H_2O_2, cumene hydroperoxide and *tert*-butyl hydroperoxide can also be used by this enzyme, with similar efficiency. However, cumene hydroperoxide is clearly detrimental to proteins of the Trx system because the activity recorded drops to about 35% of those obtained with either H_2O_2 or *tert*-butyl hydroperoxide, whereas this activity remains nearly constant with the Grx system.

Expression and Localization in Planta

We have also generated evidence that the type C Prx gene is efficiently transcribed and translated in planta. Figure 6A shows the results of a northern experiment made with RNAs isolated from three different poplar organs: leaves, stems, and roots. A single transcript of about 1 kb was detected in all organs, and the level of transcription was high in leaves (lane L), lower in roots (lane R), and weak in stems (lane S). The use of specific polyclonal antibodies allowed us to detect the presence of the Prx polypeptide in all organs as well (Fig. 6B). The western-blot experiment matches the results of the northern-blot experiment, with higher expression in leaves and
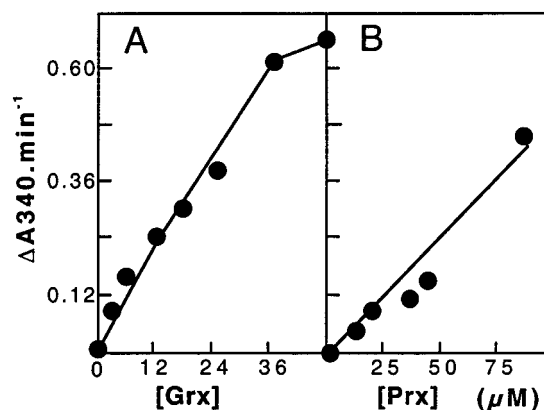

Figure 5. Grx and Prx dependency of H_2O_2 reduction. The concentrations/amounts of the other components are as follows: 150 μM NADPH, 0.5 units glutathione reductase (GR), 1 mM GSH, and 100 μM H_2O_2. A, Prx concentration is 40 μM; B, Grx concentration is 12 μM. The background activities due to reduced glutathione alone (about 0.1 Δ OD/min) have been substracted.

Table I. *Proton donor requirements for the peroxiredoxin-linked reduction of H_2O_2*

All reactions were carried out at 30°C in a total volume of 500 μL and followed spectrophotometrically using a Cary 50 spectrophometer (Varian Mulgrave, Victoria, Australia). The results are expressed in ΔA_{340} per minute. The concentrations/amounts of the various components were as follows: A, 1.6 μM NTR, 15 μM Trx h, 17 μM Prx, 100 μM H_2O_2 and B, 0.5 units GR, 1 mM GSH, 50 μM Grx, 43 μM Prx; 100 μM H_2O_2.

A		B	
Complete	0.522	Complete	0.662
−NTR	0.006	−GR	0.000
−Trx h	0.005	−GSH	0.000
−Prx	0.004	−Grx	0.095
−H_2O_2	0.016	−Prx	0.095
		−H_2O_2	0.011

roots. It was estimated that there is approximately 500 ng Prx mg protein^{-1} in leaves based on a standard curve established with increasing amounts of the recombinant protein (data not shown). A single polypeptide was detected in plant extracts with a size identical to the recombinant protein. Again, this indicates that there is no cleavage of a signal sequence in the protein. Nevertheless, immunolocalization experiments in leaves indicate the presence of large amounts of type C Prx in the sieve tubes of the phloem. The gold particle labeling was extremely specific and essentially restricted to plastid-like structures present in these cells (Fig. 7).

DISCUSSION

The Prx sequence isolated here is neither a so-called 2-Cys Prx (referred to in this work as type A), nor a 1-Cys Prx (referred here as type B). The poplar Prx is the shortest among the many isotypes characterized so far (only 162 amino acids). It lacks especially a C-terminal extension consistently present in isotypes A and B and there is no N-terminal extension. The presence in all ESTs of a stop codon in frame 9 triplets upstream of the initiation codon precludes the existence of a transit peptide in this protein. Despite the apparent absence of a signal sequence, the protein was shown by immunochemistry to be located in plastids of the sieve elements of the phloem. Thus, either there is a signal internal to the sequence or the protein is transported through the vesicular pathway from the companion cell via the branched plasmodesmata. It is interesting that both Trx and Grx are major soluble protein components of the phloem sap (Szederkenyi et al., 1997; Ishiwatari et al., 1998). This has prompted speculation that both proteins are involved in redox based regulation in phloem cells. It is clear that the Prx isolated here could participate to such a regulation because it uses both Trx and Grx as a proton donor. It is interesting that it has been proposed that upon injury, the sieve element plastids disintegrate and

release their protein content at the level of the sieve plate (Knoblauch and van Bel, 1998). Because type C Prx is specifically located in those structures, it is tempting to speculate that is should be involved in the response to oxidative stress generated in such conditions.

A high expression of Prx was obtained by cotransformation with the plasmid pSBET and the protein could then be purified with a very high yield. The native enzyme is monomeric in solution and its two Cys residues are in the reduced form, irrespective of its mode of purification (inclusion of a reductant as DTT or not). It is interesting that type A Prx are reported to be dimeric enzymes with a disulfide bridge connecting the catalytic Cys of one subunit and another Cys of the other subunit. Because this additional Cys is in the C terminus extension, a similar organization cannot apply to the type C Prx described in this work.

We propose that the short length of the sequence is a first criterion of a type C Prx. Other criteria are the presence of an additional invariant Cys at position 76 and the capacity to use either Trx or Grx as a proton donor for the reaction. The poplar enzyme reduces alkyl hydroperoxides, and especially H_2O_2, in the presence of an exogenous proton donor. This was demonstrated both kinetically, following the NADPH oxidation linked to the catalytic process, and by the observation that externally added H_2O_2 is able to oxidize critical Cys residues, even at low concentrations. The capacity to reduce H_2O_2 was also confirmed with a widely used plasmid protection assay. The biochemical data suggest that the poplar Prx is able to use either Trx or Grx as proton donors, a result that has not been reported before (Fig. 8). This raises the question of the identity of the physiological proton donor of this new type C Prx.

The biochemical data presented here do not permit us to determine the identity of the physiological proton donor in plants because both systems (Trx and Grx) display similar efficiencies. A further complication is the high number of Trx h variants in plants (Buchanan et al., 1994; Rivera-Madrid et al., 1995), because the different isoforms could display varying affinities toward type C Prx. We could not test all isoforms as proton donors at this point, but could

Table II. *Comparison of various alkylhydroperoxides as substrates of poplar Prx*

Reactions are performed at 30°C in 50 mM phosphate buffer, pH 7, with 150 μM NADPH, 100 μM substrates, and in the presence of 1.6 μM NTR, 15 μM Trx, and 25 μM Prx for the Trx system and 0.5 units of GR, 1 mM GSH, 12.5 μM Grx, and 25 μM Prx for the Grx system. Reactions were started after 1 min by adding substrates.

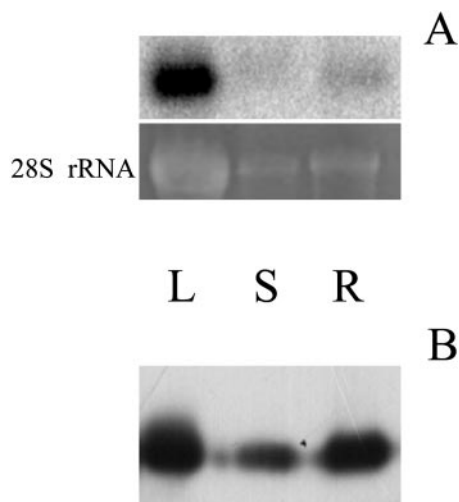| Substrate	Proton Donor	
	Trx system	Grx system
H_2O_2	0.453	0.311
Cumene hydroperoxide	0.174	0.278
tert-butyl hydroperoxide	0.409	0.256

Figure 6. : Detection of Prx mRNA and polypeptide in planta. L, Leaves, S, stems; R, roots. A, Northern-blot analysis: as a control, 28S rRNA stained by Radiant Red RNA gel stain from Bio-Rad before blotting is shown. B, Western-blot analysis with purified anti-Prx antibody.

observe that the distant *Chlamydomonas reinhardtii* Trx *h* shows similar reactivity to the poplar Trx *h* used in this work (data not shown). This could be an indication that there should not be a high specificity between the Trx *h* isoforms versus type C Prx. In an earlier work, using a Trx modified by site-directed mutagenesis, a yeast Prx (YLR109) has been identified as an in vivo target of Trx (Verdoucq et al., 1999). It should be stressed, however, that YLR109 presents lower homologies to type C Prx because it possesses several sequence insertions and lacks the additional conserved Cys typical of all plant sequences. As a consequence, it is not clear whether this result can be extended to the type C plant Prx exemplified by the sequence studied in this work.

A very interesting piece of information about the nature of the proton donor can be obtained from sequences available in the nucleotide data bank. Three of those sequences (*Hemophilus influenzae, Neisseria meningitidis*, and *Vibrio cholerae* with accession nos. AAC22230, CAB94403, and AE004330, respectively) exhibit a natural fusion between a Prx motif present in the N terminus of the sequence and a Grx motif that constitutes the C terminus of the sequence. The N terminus displays strong homology (over 50%) with the poplar Prx sequence, with no sign of a N terminus extension. In addition, two of these se-
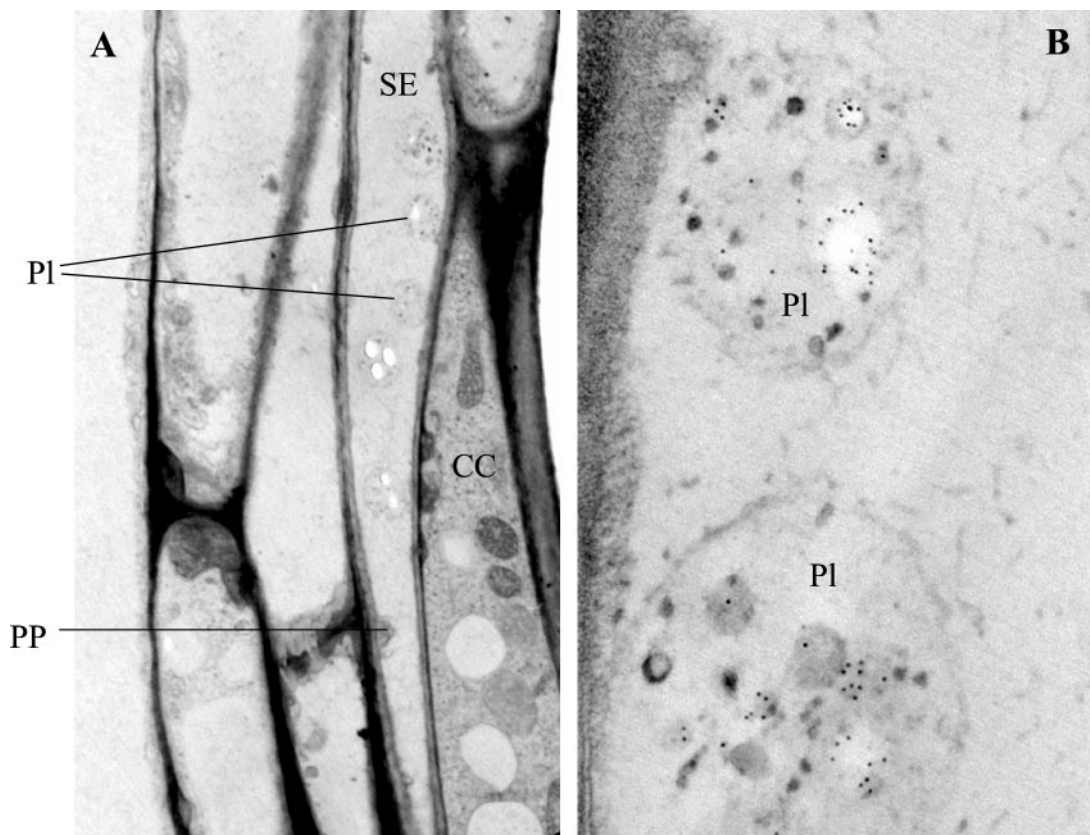

Figure 7. Electron micrographs of poplar sieve elements. A, One sieve element (SE) containing sieve element plastids (Pl) and parietal P proteins (PP) shown with companion cell (CC). B, Prx labeling in sieve element plastids.
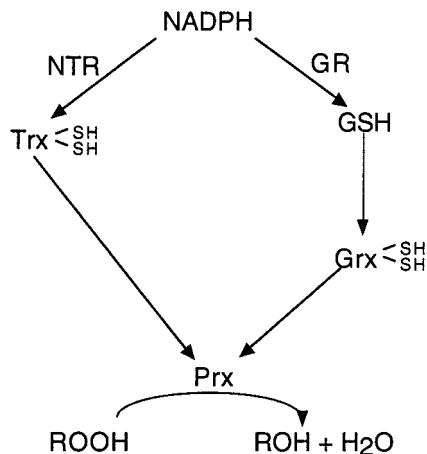
Figure 8. The two pathways involved in type C Prx catalysis.

quences (*V. cholerae* and *N. meningitidis*) possess the conserved additional Cys (equivalent to Cys 76). This definitely classifies those sequences as a putative ancestor of type C Prx. The existence of these prokaryotic sequences strongly suggests that a Grx could serve as a physiological proton donor to type C Prx, in a manner analogous to the natural fusion protein that exists between Trx reductase and Trx in *Mycobacterium leprae* (Wieles et al., 1995). Moreover, recent analyses indicate that in prokaryotes, gene fusion events could reasonably indicate a functional association of these proteins (Enright et al., 1999; Marcotte et al., 1999).

MATERIALS AND METHODS

Cloning of the cDNA Sequences, Expression in *Escherichia coli* of the Recombinant Proteins, and Purification Procedures

The expression and purification of *Chlamydomonas reinhardtii* and poplar (*Populus trichocarpa* × *deltoides*) Trx *h* as well as of Arabidopsis NTR have been described already (Jacquot et al., 1994; Stein et al., 1995; Behm and Jacquot., 2000). The procedure for the overexpression and purification of Grx will be described in a separate paper.

Several ESTs corresponding to poplar Prx were identified in GenBank (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Nucleotide) using the program Advanced Blast 2.0 (http://www.ncbi.nlm.nih.gov:80/BLAST/). Two primers were designed to amplify the full-length cDNA by PCR using as a template a phage cDNA xylem/phloem library from poplar provided by Drs. Wout Boerjan and Hugo Meyermans (University of Gent, Belgium). The forward oligonucleotide (GGGGCCATGGC-CCCGATTGCTGTTGGT) contains an NcoI restriction site (underlined) with the initial ATG followed by the 5′ sequence of the open reading frame, and the reverse primer (GGGGGGATCCTCAAAGATCCTTGAGATATCC) contains a BamHI restriction site followed by sequences complementary to the stop codon and matching the 3′ end of the open reading frame. The fragment coding for Prx was

amplified by PCR, using 2.5 μL of cDNA library as a template. The cDNA library was first denatured by an initial step of 10 min at 95°C. *Taq* polymerase was then added and 35 cycles of amplification were performed (1 min at 95°C, 2 min at 47°C, and 3 min at 72°C), followed by a final extension step of 10 min. Overall, the PCR reaction (100 μL) contained 1 unit of Goldstar DNA polymerase, 200 nM of each primer, 2 mM MgCl$_2$, and 160 μM deoxyribonucleotide triphosphate).

The PCR product of about 500 bp was then purified, digested, and cloned into the pET-3d vector to generate the construction pET-Prx that was used to transform BL21(DE3). An initial small-scale expression gave very poor yields, presumably because of the existence of codons poorly adapted to *E. coli* translation machinery. It is well known that several triplets are not frequently used in *E. coli*, presumably because of low amounts of the corresponding tRNAs (Makrides, 1996). A thorough examination of the poplar Prx sequence indicates that a tandem of AGG triplets coding for the doublet R128-R129 should be extremely unfavorable for a high yield expression. In accordance, the *E. coli* BL21 (DE3) strain was cotransformed by pET-Prx and by the plasmid pSBET that encodes the tRNA needed to recognize the AGG and AGA triplets (Schenk et al., 1995). The *E. coli* BL21(DE3) strain, cotransformed with pSBET (Schenk et al., 1995), was transformed by electroporation by pET-Prx. One ampicillin- and kanamycin-resistant clone was amplified up to 5 L in Luria-Bertani medium at 25°C and induced during 4 h with 100 μM isopropyl-β-D-thiogalactopyranoside. The bacterial cells were collected by centrifugation (5,000g, 15 min) and resuspended in TE buffer (30 mM Tris-HCl, pH8, and 1 mM EDTA) to a final volume of about 80 mL. The cell suspension was sonicated on ice by 30-mL batches for 3 min using an Ultrasonic XL Sonicator with an output of 4 and a duty cycle of 50. Solid ammonium sulfate was then added and the proteins precipitating between 40% and 80% (w/v) of the saturation were collected by centrifugation (30,000g, 20 min). The protein pellet was then resuspended in about 20 mL of TE. The purification of recombinant poplar Prx requires two chromatographic steps. First, the sample was loaded onto an ACA44 column (70 × 5 cm) and then the fractions containing the recombinant protein were pooled and laid onto a DEAE Sepharose fast-flow column (10 × 2.5 cm). The protein was eluted with a 0 to 0.4 M NaCl gradient in buffer TE (250–250 mL), and then concentrated and dialyzed against TE buffer on a cell (Amicon, Beverly, MA) equipped with a YM10 membrane. The protein was kept frozen at −20°C and stored as 50-μL aliquots at a concentration of about 30 mg mL^{-1}.

In Vitro Peroxidase Assays

The reduction of H$_2$O$_2$ or alkyl hydroperoxides was first estimated indirectly by measuring NADPH oxidation at 340 nm. It is based on the property that Prx uses a hydrogen donor to carry out catalysis (most often reduced Trx generated via NADPH and NTR), but also in this study, reduced Grx in the presence of glutathione, which is itself maintained reduced by NADPH and GR.

The reaction mixture (500 μL) for the Trx-dependent assay was as follows: 50 mM Na-K phosphate buffer, pH 7.0; 150 μM NADPH; 0 to 50 μM poplar or *C. reinhardtii* Trx *h*; 0 to 50 μM Prx; and 1.5 μM Arabidopsis Trx reductase. The reaction was initiated by adding 1 mM H_2O_2 and the NADPH oxidation was followed spectrophotometrically at 340 nm using a Cary 50 apparatus at 30°C.

When Grx was tested as the proton donor, the reaction medium had the following composition: 50 mM Na-K phosphate buffer, pH 7.0; 150 μM NADPH; 1 mM reduced glutathione; 0.5 units GR; 0 to 50 μM Grx; and 0 to 80 μM Prx. As described above, the reaction was carried out in 500 μL, initiated with H_2O_2, and its rate followed spectrophotometrically at 340 nm.

A second possible test is the protection of plasmids against radicals generated by MCO. H_2O_2 can be formed by a reaction occurring between DTT, Fe^{3+}, and O_2, and then transformed into hydroxyl radical by the Fenton reaction (Halliwell and Gutteridge, 1990). These radicals are able to damage nucleic acids or inactivate enzymes as Gln synthetase.

Plasmid pLBR19 was used to measure the capacity of Prx to eliminate H_2O_2 before its tranformation in radicals. A reaction mixture of 20 μL includes 3.3 μM $FeCl_3$, 10 mM DTT for the thiol MCO system, or 10 mM ascorbate for the non-thiol MCO system, different Prx concentrations (10–40 μM), or 20 μM BSA, in 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-[NaOH], pH 7.2. The reaction was initiated by incubating the mixture for 40 min before adding about 1 μg of plasmid for 7 h at 37°C. The plasmid degradation was evaluated on 1% (w/v) agarose gel after staining with ethidium bromide (Sambrook et al., 1989).

Thiol Content Titration

The free thiol content of recombinant poplar Prx was estimated using DTNB as described by Jacquot et al. (1984). One milligram of Prx dissolved in 1 mL of Tris-HCl (100 mM, pH 8.0) was reacted with 10 μL of 20 mM DTNB in the presence of various H_2O_2 concentrations (ranging from 100 μM to 1 mM). An assay was performed in the presence of 1% (w/v) SDS to denature the protein and estimate whether it contains buried thiol groups. The cleavage of DTNB to TNB^- was followed by measuring the A_{412} after 30 min reaction in the dark, and the thiol content was determined using a molar extinction coefficient of 13,600 M^{-1} cm^{-1} at 412 nm.

Protein Extraction from Poplar Tissues

Four-hundred milligrams of 2-month-old poplar leaves, stems, and roots was used for protein extraction. Tissues were ground into powder in the presence of liquid nitrogen and suspended in the extraction buffer (50 mM Tris-HCl, pH 7.5; 14 mM phenylmethylsulfonyl fluoride; and 0.05% [v/v] β-mercaptoethanol). Soluble proteins were obtained in the supernatant by two successive centrifugation steps (14,000g, 10 min) and then precipitated in the presence of

acetone (4 volumes). Proteins were pelleted by centrifugation, the pellet was then washed by a novel centrifugation step in the presence of acetone, and dried after eliminating the supernatant. Proteins were resuspended in a solubilization buffer composed of 125 mM Tris HCl, pH 6.8; 20% (v/v) glycerol; 2% (w/v) SDS; 5% (v/v) β-mercaptoethanol; and 0.05% (w/v) bromophenol blue. Protein concentrations in the extracts were determined by the Bradford reaction as described in the Bio-Rad kit.

Antibody Purification

The antibodies have been purified from the serum by immuno-adsorption on a Prx-Sepharose column generated by coupling 35 mg of Prx to 2.5 g of CNBr Sepharose gel (Amersham Pharmacia Biotech, Uppsala). Fractions of 4 mL of the rabbit serum were applied onto the column (1 × 5 cm) and the antibodies were allowed to react with the matrix for 30 min. After washing the column with TE buffer, antibodies were eluted with 50 mM acetic acid in fractions of 1 mL in tubes containing 200 μL of 1 M Tris-HCl, pH 8. Fractions containing the antibodies were pooled and dialyzed against TE buffer.

SDS-PAGE and Western Blotting

Proteins from poplar tissues (10 μg) were separated by 14% (w/v) SDS-PAGE following the procedure of Laemmli (1970) and transferred onto a polyvinylidene difluoride membrane during the night in the presence of transfer buffer (25 mM Tris, 192 mM Gly, and 20% [v/v] ethanol). Blocking of the membrane was achieved at room temperature during 4 h with the blocking buffer (20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.05% [w/v] milk powder; and 0.05% [v/v] Tween 20). The membrane was then incubated with purified anti-Prx antibodies (equivalent to 1.10^{-3} OD at 280 nm) in 15 mL of blocking buffer during at least 2 h, and extensively washed before reaction with anti-rabbit secondary antibodies provided in the Immune Star Goat Anti Rabbit Detection Kit from Bio-Rad. The bioluminescence reaction was done according to the instructions of the manufacturer.

RNA Extraction and Northern Blotting

Total RNA was extracted from 100 mg of leaves, stems, and roots from 2-month-old poplar grown in hydroponic medium, with the RNeasy Plant Mini Kit (Qiagen USA, Valencia, CA) with addition of polyethylene glycol 8000 to the extraction buffer at a concentration of 20 mg mL^{-1}. Ten micrograms of total RNA was separated and blotted by capillarity onto a nylon membrane from Amersham Pharmacia Biotech. The probe, consisting of about 100 ng of a PCR fragment (identical to the Prx fragment cloned), was labeled with ^{32}P dATP following the instructions provided in the kit Prime a Gene Labeling System (Promega, Madison, WI). The probe was then purified with the QIAquick Nucleotides Removal Kit from Qiagen, and was estimated to contain 30 million cpm. Hybridization was performed at

42°C in the presence of 5× SSPE and 0.5% (w/v) SDS, and washing at 65°C with a solution of 2× SSPE and 0.1% (w/v) SDS.

Immunolocalization Procedures

Samples were dissected from young poplar leaves in a drop of fixation solution and then fixed by immersion 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. Leaf samples were subsequently rinsed in 0.1 M of the above buffer, treated with 2% (w/v) OsO_4, dehydrated in a series of alcohol and propylene oxide, embedded in Durcupan, and finally polymerized at 56° for 48 h.

Ultrathin sections were successively floated for 15 min in a 0.05 M Gly, 0.05 M Tris-phosphate-buffered saline (TPBS) solution, 30 min on a blocking solution of 1% (w/v) BSA in TPBS (TPBS/BSA), and then treated for 2 h with the purified rabbit Prx-antibodies (diluted 1:100 [v/v] in TPBS/BSA). This step was omitted in the controls. After washing, sections were incubated for 2 h in a solution of 0.05 M Tris-HCl buffer containing 0.05% (w/v) polyethylene glycol with gold-labeled goat anti-rabbit IgG (10 nm, dilution 1:20 [v/v], Sigma, St. Louis). Sections were then rinsed, dried, and stained with uranyl acetate and lead citrate.

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