A Chinese Cabbage cDNA with High Sequence Identity to Phospholipid Hydroperoxide Glutathione Peroxidases Encodes a Novel Isoform of Thioredoxin-dependent Peroxidase*

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A cDNA, PHCC-TPx, specifying a protein highly homologous to known phospholipid hydroperoxide glutathione peroxidases was isolated from a Chinese cabbage cDNA library. PHCC-TPx codes a preprotein of 232 amino acids containing a putative N-terminal chloroplast targeting sequence and three conserved Cys residues (Cys\textsuperscript{107}, Cys\textsuperscript{136} and Cys\textsuperscript{155}). The mature form of enzyme without the signal peptide was expressed in Escherichia coli, and the recombinant protein was found to utilize thioredoxin (Trx) but not GSH as an electron donor. In the presence of a Trx system, the protein efficiently reduces \( \text{H}_2\text{O}_2 \) and organic hydroperoxides. Complementation analysis shows that overexpression of the PHCC-TPx restores resistance to oxidative stress in yeast mutants lacking GSH but fails to complement mutant lacking Trx, suggesting that the reducing agent of PHCC-TPx is not GSH but is Trx. Mutational analysis of the three Cys residues individually replaced with Ser shows that Cys\textsuperscript{107} is the primary attacking site by peroxide, and oxidized Cys\textsuperscript{107} reacts with Cys\textsuperscript{155}-SH to make an intramolecular disulfide bond, which is reduced eventually by Trx. Tryptic peptide analysis by matrix-assisted laser desorption and ionization time of flight mass spectrometry shows that Cys\textsuperscript{155} can form a disulfide bond with either Cys\textsuperscript{107} or Cys\textsuperscript{136}.

The generation of reactive oxygen species, which cause serious damage to biological macromolecules, is an inevitable process in all aerobic organisms (1). The challenge may be even greater for plants than for other eukaryotes because plants not only occupy fixed positions in constantly changing environments but also consume \( \text{O}_2 \) during respiration and generate it during photosynthesis (2). To cope with the deleterious effects of reactive oxygen species, aerobic organisms are equipped with antioxidant enzymes, which include catalases, superoxide dismutases, and ascorbate- and thioredoxin (Trx)-dependent peroxidases (APx and TPx, respectively) (3). In addition, most eukaryotes contain antioxidant proteins termed glutathione peroxidases (GPx), which reduce hydroperoxides with glutathione (GSH) as an electron donor. There are at least five GPx isoforms in mammals, such as including the classical and cytoplasmatic GPx (called eGPx or GPx1), gastrointestinal GPx (called pGPx or GPx2), extracellular plasma GPx (called pGPx or GPx3), phospholipid hydroperoxide GPx (called eGPx or GPx4), and epididymis-specific secretory GPx (called eGPx or GPx5) (4). These isozymes differ with respect to structure, substrate specificity, and tissue distribution. All animal GPx family members, except for the recently described Cys-containing pGPx (5) and eGPx isoforms (6), possess selenocysteine (SeCys) at their active site. Whereas PHGPx and eGPx exist as a monomer, the other GPx isoforms form tetramers of identical subunits. Whereas the preferred PHGPx substrates are phospholipid hydroperoxides, eGPx and pGPx can reduce \( \text{H}_2\text{O}_2 \) and fatty acid hydroperoxides more effectively than phospholipid hydroperoxides (7). However, it is noteworthy that the in vitro reduction of hydroperoxides by pGPx and PHGPx proteins requires nonphysiologically high concentrations of GSH (4).

Besides the mammalian GPx families, several plant GPx cDNAs have been isolated from diverse plant sources. It has been demonstrated that most plant GPx proteins have a primary structure similar to those of animal PHGPx enzymes, except that the SeCys in the catalytic site is replaced by Cys in plant enzymes (8, 9). Although the existence of many plant GPx proteins has been inferred from primary nucleotide sequences, only one protein, from Citrus sinensis, has been experimentally shown to possess PHGPx activity (10). In this study, phosphatidylcholine hydroperoxide was used as a substrate, and the activity of this enzyme was found to be unusually low (11). The protein also is not able to reduce \( \text{H}_2\text{O}_2 \) with GSH as an electron donor under various assay conditions. Similar properties have also been reported for mammalian PHGPx proteins, for which the rate constant for phospholipid hydroperoxide as a substrate.

The abbreviations used are: Trx, thioredoxin; GPx, GSH-dependent peroxidase; eGPx, cytoplasmic GPx; eGPx, epididymis-specific secretory GPx; giGPx, gastrointestinal GPx; pGPx, plasma GPx; PHGPx, phospholipid hydroperoxide GPx; GS, glutamine synthetase; MALDI-TOF MS, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry; PHCC-TPx, PHGPx homologue of Chinese cabbage with TPx activity; SeCys, selenocysteine; TPx, Trx-dependent peroxidase; 1Cys-TPx, TPx containing one active cysteine; 2Cys-TPx, TPx containing two active cysteine residues; TR, Trx reductase; DTT, dithiothreitol; WT, wild type.

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is an order of magnitude smaller than that for cGPx (12). We were intrigued by these results and therefore decided to identify the physiologically relevant electron donor for plant non-SeCys PHGPx enzymes.

In this report we show that Trx is an efficient electron donor for the PHGPx homologue of Chinese cabbage in the reduction of \( \text{H}_2\text{O}_2 \) and organic hydroperoxides. Unlike previously characterized 1Cys- and 2Cys-TPx proteins (recently renamed Prx enzymes), the Trx-dependent peroxidase (TPx) activity of this enzyme is significantly affected by the mutation of each of the three Cys residues individually replaced with Ser, and it maintains monomeric state during the catalytic process of \( \text{H}_2\text{O}_2 \) reduction. Therefore, we designate the enzyme PHCC-TPx, for PHGPx homologue of Chinese cabbage with TPx activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—\( \text{H}_2\text{O}_2 \), cumene hydroperoxide, \( \text{t}-\text{butyl} \) hydroperoxide, GSH, yeast GSH reductase (GR), and bovine cGPx were purchased from Sigma. Yeast Trx, Trx reductase (TR), glutaredoxin (Grx), and glutathione, yeast GSH reductase (GR), and bovine cGPx were purchased from Sigma. Yeast Trx, Trx reductase (TR), glutaredoxin (Grx), and glutathione, yeast GSH reductase (GR), and bovine cGPx were purchased from Sigma. Yeast Trx, Trx reductase (TR), glutaredoxin (Grx), and glutathione, yeast GSH reductase (GR), and bovine cGPx were purchased from Sigma.

**Cloning and Expression of the PHCC-TPx in \( E. \text{coli} \)—**A full-length cDNA with extensive homology to the Arabidopsis thaliana PHGPx was identified in the course of a random sequencing of flower-specific cDNAs with extensive homology to the Arabidopsis thaliana PHGPx (14). To produce recombinant protein, the PHCC-TPx open reading frame was subcloned into the expression vector pGEX and expressed in the \( E. \text{coli} \) strain BL21 (DE3)pLysS. The protein was purified by the procedure described previously (15).

**Enzyme Assays**—The antioxidant activity of PHCC-TPx was demonstrated by monitoring the ability of the protein to inhibit the inactivation of GSH in the presence of DTT/Fe\(^{3+}/\text{O}_2 \) as described (16). The peroxidase activity was measured by the decrease in the absorption of A\(_{440} \) in a reaction mixture containing 50 mM Hepes, pH 7.0, 1 mM \( \text{H}_2\text{O}_2 \), various amounts of protein, and an electron donor, either the Trx system consisting of 0.3 mM NADPH, 10 \( \mu \text{M} \) Trx, 1 \( \mu \text{M} \) TR or the GSH system containing 0.3 mM GSH, 1 \( \mu \text{M} \) GR, and 0.3 mM NADPH (13). For the investigation of substrate specificity, GSH was replaced with various concentrations of \( \text{t}-\text{butyl} \) hydroperoxide, cumene hydroperoxide, or phosphatidylcholine hydroperoxide. Data represent the mean of at least three independent experiments.

**Site-directed Mutagenesis of PHCC-TPx—**Point mutation of specific amino acids in PHCC-TPx was performed by a PCR-mediated mutagenesis technique as described (17). After the mutations were verified by nucleotide sequencing, the mutant genes were expressed in \( E. \text{coli} \) using the pGEX expression vector. The yield of recombinant protein expressed from the full-length PHCC-TPx cDNA was low, and most protein was found in inclusion bodies (data not shown). Thus, we analyzed the biochemical properties of PHCC-TPx with the soluble, mature protein that lacks the N-terminal transit peptide and contains 170 amino acid residues, from amino acid positions 63 (Leu) to 232 (Ala).

**Substrate Specificity of PHCC-TPx—**Although the SeCys-containing GPx proteins specifically use GSH as an electron donor, there have been few reports on the GSH-dependent reduction of \( \text{H}_2\text{O}_2 \) by Cys-containing PHGPx proteins. Indeed, during the course of this work, a paper appeared which showed that the Plasmodium falciparum PHGPx utilizes Trx, but not GSH, as an electron donor for \( \text{H}_2\text{O}_2 \) reduction (28). Also, Björnstedt et al. (29) demonstrated that the mammalian isoform of nonselenium pGPx uses Trx and Trx reductase (TR), instead of GSH, to reduce hydroperoxides. Therefore, we compared the efficiency of the TRx (Trx, TR, and NADPH) and GSH (GSH, GR, and NADPH) systems in providing reducing power for PHCC-TPx in the reduction of \( \text{H}_2\text{O}_2 \).

The velocity of peroxidase-linked NADPH oxidation in the presence of the Trx system exhibits characteristic first-order kinetics (Fig. 3), and the reaction rate is proportional to the concentration of PHCC-TPx (data not shown). With the Trx system, PHCC-TPx catalyzes the reduction of \( \text{H}_2\text{O}_2 \) more efficiently than does yeast TPx1 used as a control (Fig. 3A). However, virtually no peroxidase activity is detected for PHCC-TPx in the presence of the GSH system, even when large amounts of GSH are added (Fig. 3B). On the contrary, bovine cGPx shows...
a significant catalytic activity toward H$_2$O$_2$ in the presence of a physiologically relevant concentration of GSH (10 $\mu$M), but the activity is completely lost when the GSH system is replaced with the Trx system. Moreover, the oxidation of NADPH by PHCC-TPx is negligible in the absence of any of the three components of the Trx system, as compared with the extent of...
oxidation observed in the presence of all three components (data not shown), which is a property typical to TPx proteins (16). Based on these characteristics, we measured the initial rates of NADPH oxidation catalyzed by PHCC-TPx with various concentrations of Trx and compared the kinetic parameters with those reported for yeast TPx1, which are similar to the values obtained in our assay conditions (22). Lineweaver-Burk plots (data not shown) revealed that the $V_{\text{max}}$ and $K_m$ values for Trx are 35 μmol/min/mg of protein and 4.2 μM for the reaction catalyzed by PHCC-TPx, respectively (Table I). These values are about 6- and 2-fold higher, respectively, than those for yeast TPx1. Moreover, the $K_m$ values of PHCC-TPx toward $H_2O_2$, cumene hydroperoxide, and t-butyl hydroperoxide, are 4.9, 5.5, and 11.7 μM, respectively, which are slightly higher than those described for yeast TPx1 (22). Also, the $V_{\text{max}}$ values for PHCC-TPx measured with each of the three peroxides are 17.2, 7.6, and 14.3 μmol/min/mg of protein for the reduction of hydroperoxides. The catalytic efficiencies ($V_{\text{max}}/K_m$) of PHCC-TPx are generally higher than those of yeast TPx1 for all of the substrates used in this experiment. However, only negligible PHCC-TPx activity was detected when phosphatidylcholine hydroperoxide was used as a substrate (data not shown), which is consistent with results reported for the $P$. $faciatus$ PHGPx (28).

Effect of Cys Mutation on the Catalytic Activity of PHCC-TPx—By having established that Trx is an electron donor for PHCC-TPx, we were intrigued by the absolute conservation of the three Cys residues in PHCC-TPx. To explore this observation further, we investigated the role of each Cys residue in catalytic activity by replacing each individually with Ser. And an additional amino acid, Gln (272), reported to be a part of catalytic triad of mammalian GPx proteins (25) was also included to analyze its role in $H_2O_2$ reduction systems. As shown in Fig. 4A, the extent of GS protection increases in a saturable, dose-dependent manner in the presence of WT PHCC-TPx. In contrast, the C107S mutant protein is unable to protect GS regardless of the nature of the reducing equivalents (DTT or Trx), whereas the C136S and C155S mutants are fully active in the presence of DTT (Fig. 4B). The complete absence of GS protection by the C107S mutant strongly suggests that Cys$^{107}$ is the primary site of attack by $H_2O_2$ yielding Cys$^{107}$-SOH and H$2O$. In the presence of DTT and in the absence of either Cys$^{136}$ or Cys$^{155}$, Cys$^{107}$-SOH may react with one of the DTT thiol groups to form a mixed disulfide bond, and the second DTT thiol group may attack the free thiol Cys$^{107}$-SH and oxidized DTT. This may explain why the C136S and C155S mutants protect GS from oxidation by the DTT-containing mixed function oxidation system. When DTT is re-placed with a non-thiol reducing equivalent such as ascorbate, PHCC-TPx no longer protects GS from oxidation, confirming that its antioxidant activity is tightly linked to a thiol-specific reducing group (data not shown).

Analysis of Disulfide Bond Formation in PHCC-TPx—According to the catalytic model developed for several TPx isoforms (17), the proteins belonging to 1Cys- and 2Cys-TPx families formed monomer and dimers during $H_2O_2$ catalysis, which can be detected by non-reducing SDS-PAGE. Because PHCC-TPx contains three conserved Cys residues, we investigated whether PHCC-TPx forms inter- or intramolecular disulfide bonds during the catalytic cycle and, if so, which Cys residues are involved in this bond formation. To test for dimer formation, we examined the electro-mobility of PHCC-TPx in the presence or absence of 10 mM DTT by SDS-PAGE with yeast TPx1 as a control. In the absence of DTT, Cys residues of PHCC-TPx are expected to be oxidized by treatment at 95 °C for 5 min for SDS-PAGE analysis. As reported previously (16), yeast TPx1 forms dimers in non-reducing SDS-PAGE conditions, but PHCC-TPx is present at a molecular size corresponding to the monomeric form, regardless of the presence or absence of DTT (Fig. 5A). These results suggest that, unlike what is observed for 2Cys-TPx isoforms (16), the PHCC-TPx might form intramolecular disulfide linkages upon oxidation. In the absence of DTT, two protein bands (the upper band is faint) can be observed for the PHCC-TPx protein (Fig. 5A), and most of the protein in the lower band shifts to the upper band after treatment with DTT. To analyze disulfide bonds in the protein, gel slices containing each band separated on SDS-PAGE without the presence of DTT were treated with trypsin, and the resulting peptides were subjected to MALDI-TOF MS. The data obtained are summarized in Fig. 5. Molecular masses equiva-
lent to those of tryptic peptides of amino acids 107–123 (calculated m/z 1992.92 and observed m/z 1992.93) and of amino acids 126–151 (calculated m/z 2950.45) were clearly detected for both WT bands, indicating that a fraction of both Cys107 and Cys136 residues are present in a reduced state (SH form). On the contrary, the molecular mass corresponding to the free SH form of Cys155 (amino acids 153–157, with expected m/z 696.35) is not detected in the spectrum of peptides prepared from the lower band (Fig. 5, B1) but is clearly present in peptides from the upper minor band (m/z

| TABLE I |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| Substrate          | PHCC-TPx          | Yeast TPx1*       |
|                    | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $V_{max}/K_m$ | $K_m$ (μM) | $V_{max}$ (μmol/min/mg) | $V_{max}/K_m$ |
| Trx                 | 4.2           | 34.9               | 8.3           | 2            | 5.5                    | 2.8          |
| H$_2$O$_2$          | 4.9           | 17.2               | 3.5           | 4            | 4.3                    | 1.6          |
| Cumene hydroperoxide| 5.5           | 7.6                | 1.4           | 4            | 2.2                    | 0.55         |
| tert-Butyl hydroperoxide| 11.7     | 14.3               | 1.2           | 10          | 2.4                    | 0.24         |

* Data are drawn from the results of Ref. 22.

Fig. 4. Effect of point mutations on the antioxidant function of PHCC-TPx. A, TPx activities of WT and point-mutated PHCC-TPx proteins were analyzed under the same conditions as described in the legend of Fig. 2A. B, GS protection activities of WT PHCC-TPx (●) and mutant proteins, C107S (□), C136S (△), C155S (○), and Q142G (◆), were measured as described under “Experimental Procedures.” The extent of changes in activity is expressed as a percentage relative to the maximal activity of the enzyme.

Fig. 5. SDS-PAGE analysis and disulfide bond analysis of PHCC-TPx by MALDI-TOF mass spectrometry. A, purified proteins (0.5 μg/10 μl) of PHCC-TPx (lanes 1 and 3) and yeast TPx1 (lanes 2 and 4) were mixed with an equal volume of reducing (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10 mM DTT) or non-reducing (the reducing buffer minus DTT) buffer and heated at 95 °C for 5 min. The proteins were separated by SDS-PAGE on a 12% gel and visualized by silver staining. The lane with an arrowhead was loaded with molecular standards whose molecular weights are indicated on the left. B, analysis of disulfide bonds by MALDI-TOF MS. Bands from nonreducing gels were excised and treated with trypsin. Molecular masses of the resulting peptides were determined by MALDI-TOF MS. Only the relevant portion of each mass spectrum is shown. Peptides derived by tryptic digestion are as follows: B1 and B3 and B5 were obtained from the WT lower band; B2 was from the minor WT upper band; B4 and B6 were from the C155S mutant; and B7 was from the C107S mutant. The peaks with m/z 2544.22 and m/z 2863.36 in B3 were from dipeptides linked by a disulfide bond between Cys107 and Cys155. Peaks with 3501.65 (B5) (or 3501.75 in B7) and 3657.87 (B5) (or 3657.88 in B7) are from dipeptides linked by a disulfide bond between Cys155 and Cys136. Please note that the peak of m/z 696.352 from a tryptic peptide containing Cys155 is very strong in B2 but absent in B1. C, assignment of mass peaks appearing in the mass spectra.
conclude that Cys 155 forms a disulfide bond with both Cys 107 and that they are hypersensitive to H2O2 (18). Also, the disulfide bond between Cys155 and Cys107 is significantly decreased when they are cultured in minimal medium without GSH. Yeast Mutants Lacking GSH but not Trx

TRX1 and TRX2 disruption of two yeast Trx genes, not GSH, is responsible for the reduction of PHCC-TPx activity. These results clearly demonstrate that Trx, but not GSH, acts in conjunction with GPx3 but not with PHCC-TPx. If Cys136-Cys155 disulfide bond. Because Cys 107 of PHCC-TPx is the primary oxidation site by H2O2 and C107S and C155S mutants is similar to that of WT enzyme in the presence of DTT but not in Trx (Fig. 4), it can be concluded that Cys107 is the primary site attacked by H2O2 to produce H2O and H2O2 reduction. Overexpression of PHCC-TPx Rescues the H2O2 Sensitivity of Yeast Mutants Lacking GSH but not Trx—It has been shown that the growth rate of GSH-deficient yeast mutants is significantly decreased when they are cultured in minimal medium and that they are hypersensitive to H2O2 (18). Also, the disruption of two yeast Trx genes, TRX1 and TRX2, results in pleiotropic effects that include H2O2 hypersensitivity (19). The hypersensitivity of the two yeast mutants to oxidizing compounds may be accounted for by the fact that antioxidant enzymes in yeast mutants are not able to function in the absence of the electron donor of Trx or GSH. Thus, if the activity of PHCC-TPx is dependent on Trx in vivo, as suggested by the in vitro data, a plasmid overexpressing PHCC-TPx is expected to complement the phenotype of yeast mutants lacking GSH, but not Trx, because the GSH null mutant has normal level of Trx. To identify the actual gene, we conclude that there is an intramolecular disulfide bond between Cys155 and Cys107 in most of the proteins. Next, we examined the possibility of disulfide formation between Cys155 and Cys136. The molecular weight of the disulfide formed by this linkage was calculated to be 3502.64. For the lower band of WT protein separated on non-reducing SDS-PAGE gel, there is such a peak with m/z 3501.65 (Fig. 5, B6), but the same peak with a stronger intensity is observed for the C107S mutant (Fig. 5, B7; note that the scales of intensity are different in two panels), suggesting that disulfide bond between Cys136 and Cys155 can be formed, and the formation is facilitated by the absence of Cys107. Taken together, we conclude that Cys155 forms a disulfide bond with both Cys107 and Cys136 in most of the individual enzymes and that the presence of Cys107 is not prerequisite for the formation of Cys136-Cys155 disulfide bond. Because Cys107 of PHCC-TPx is the primary oxidation site by H2O2 and C107S and C155S mutants completely lose the TPx activity (Fig. 4), it may be reasonable to conclude that Cys107-Cys155 is the primary disulfide bond formed during the catalytic cycle of H2O2 reduction.

We have shown both in vitro and in vivo that PHCC-TPx, which shares high sequence identity with plant PHGPx proteins, specifically uses Trx, but not GSH, in the catalysis of hydroperoxides. Considering the high amino acid sequence homologies among the PHGPx proteins, it can be proposed that most PHGPx proteins of plant sources would have similar substrate specificity on Trx. Like other TPx enzymes, PHCC-TPx uses redox-sensitive Cys residues in the process of H2O2 reduction, whereas most peroxidases use redox cofactors contained at their active sites (9). Although PHCC-TPx can be considered as a new TPx isoform, it is quite distinctly related to other TPx families with less than 10% amino acid sequence identity. This low level of sequence homology is also observed among proteins belonging to other subfamilies of TPx proteins, although proteins in the same subfamily share high sequence similarity to one another (16).

With respect to the number of conserved Cys residues and their catalytic processes, the six mammalian TPx isoforms fall largely into two subgroups. Each of the four members of the 2Cys-TPx subfamily (TPx isoforms I–IV) forms homodimers in which the two subunits are joined by an intermolecular disulfide bond, but the other two TPx isoforms, V–VI, are monomeric proteins. Thus, the PHCC-TPx can be classified into the latter group of TPx proteins, because it functions as a monomer during H2O2 reduction. Based on the data obtained in our experiments, a reaction process of PHCC-TPx-mediated reduction of peroxides can be proposed as follows, which is different from those reported for the mammalian 2Cys-TPx or SeCys-dependent GPx proteins (17). From the facts that C107S mutant is incapable of protecting GS activity with the help of any electron donor of DTT or Trx and that the activity of C136S and C155S mutants is similar to that of WT enzyme in the presence of DTT but not in Trx (Fig. 4), it can be concluded that Cys107 is the primary site attacked by H2O2 to produce H2O and H2O2 reduction. In contrast to the reaction intermediate of Cys47-SOH in yeast TPx1 which immediately reacts with Cys175-SH of the other subunit to form intermolecular disulfide bond (16), the oxidized intermediate Cys107-SOH of PHCC-TPx forms intramolecular disulfide bond with Cys155-SH, which is reduced eventually by electrons donated from Trx and used for the next round of H2O2 catalysis. However, it is not clear whether the third Cys residue, Cys136, is involved in the catalytic reaction or not. The C136S mutant exhibits only 30% of peroxidase activity of the WT protein by using Trx as an electron donor (Fig. 4), suggesting it is important for the enzymatic activity. In addition, we could detect intramolecular disulfide bond between Cys136 and the one of the active site Cys residue (Cys155).
Such observations tempted us to raise the possibility that in the catalytic process there may be disulfide bond exchange between three Cys residues, similar to that observed in the methionine sulfoxide reductase (30). It is interesting to note that the third Cys residue was not absolutely required for the methionine sulfoxide reductase activity (30). However, it is also possible that, rather than direct involvement of Cys136 residue in the catalytic reaction, the reduced peroxidase activity of C136S might be attributable to a structural effect caused by the mutation. Further studies are needed to clarify the role of Cys136 in PHCC-TPx enzyme.

It has been generally recognized that in mammalian cells GPx plays a major role in eliminating cytotoxic H2O2, because catalase, the strong antioxidant peroxisomal enzyme, has a low affinity for H2O2 ($K_m = 0.05–1.1 \times 10^7$ M) (31). However, in plant systems, this role is assumed by APx proteins, which exist as several isoforms and are distributed in the cytosol, chloroplasts, and glyoxysomes. Based on the kinetic parameters of PHCC-TPx analyzed with a yeast Trx system in H2O2 reduction (Table 1), we speculate that the high catalytic efficiency ($V_{max}/K_m$) of PHCC-TPx for H2O2 reduction means that it is another strong candidate for the removal of hydroperoxides, especially in plant chloroplasts. To test this speculation, additional studies are required by using a plant-derived Trx system instead of yeast proteins to re-establish the kinetic parameters of the plant enzyme, PHCC-TPx, because the interaction between Trx and its electron acceptor often represents species-specific (32) and isoform-specific properties (33).

The defensive role of PHCC-TPx in plant cells can also be surmised from observations that its homologues are induced to remarkable levels by various stress treatments, such as the exposure of tobacco protoplasts to high salt, soybean to bacterial infection, and *Avena fatua* embryos to imbibition (9). Moreover, diverse roles of plant PHGPx proteins can be proposed by the differential expression of PHGPx isoforms in barley (8); the expression of barley PHGPx1 and -2 is greatly increased in leaves exposed to stress, while the PHGPx3 mRNA is down-regulated in response to the same stress. Furthermore, a possible involvement of PHGPx in signal transduction pathways may be suggested by studies of the mitochondrial PHGPx, which functions as an anti-apoptotic agent in mitochondrial death signaling (34). In summary, although the exact function of the new isoform of plant TPx remains to be determined, our identification of Trx as the reducing agent of PHCC-TPx will greatly help us in the future to study the role of this novel enzyme in plant cells.

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