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Putative Cationic Cell-Wall-Bound Peroxidase Homologues in *Arabidopsis*, AtPrx2, AtPrx25, and AtPrx71, Are Involved in Lignification

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Supporting Information

ABSTRACT: The final step of lignin biosynthesis, which is catalyzed by a plant peroxidase, is the oxidative coupling of the monolignols to growing lignin polymers. Cationic cell-wall-bound peroxidase (CWPO-C) from poplar callus is a unique enzyme that has oxidative activity for both monolignols and synthetic lignin polymers. This study shows that putative CWPO-C homologues in *Arabidopsis*, AtPrx2, AtPrx25, and AtPrx71, are involved in lignin biosynthesis. Analysis of stem tissue using the acetyl bromide method and derivatization followed by the reductive cleavage method revealed a significant decrease in the total lignin content of *ATPRX2* and *ATPRX25* deficient mutants and altered lignin structures in *ATPRX2*, *ATPRX25*, and *ATPRX71* deficient mutants. Among *Arabidopsis* peroxidases, AtPrx2 and AtPrx25 conserve a tyrosine residue on the protein surface, and this tyrosine may act as a substrate oxidation site as in the case of CWPO-C. AtPrx71 has the highest amino acid identity with CWPO-C. The results suggest a role for CWPO-C and CWPO-C-like peroxidases in the lignification of vascular plant cell walls.

KEYWORDS: *Arabidopsis thaliana, CWPO-C, lignin biosynthesis, plant peroxidase*

INTRODUCTION

Lignin is a polymer of hydroxylated and methoxylated phenylpropane units (monolignols) that is accumulated most abundantly in the secondary cell walls of vascular plants. In angiosperms, lignins are composed of two major monolignols, coniferyl and sinapyl alcohols, that polymerize through at least five different linkage types and result in guaiacyl (G) and syringyl (S) units, respectively.[1] Many studies on the lignin biosynthesis mechanism resulted in the clarification of basic monolignol biosynthesis. However, the polymerization mechanism of monolignols in a matrix of cellulose and hemicellulose outside the cell is still unclear. Lignin polymerization is generally accepted to be derived from the oxidative coupling by class III peroxidases (plant peroxidases; EC 1.11.1.7) of a monolignol and the growing polymer.[2] Higher plants contain two heme-containing peroxidase classes, termed class I and class III, which differ from each other in primary structure and reaction specificity.[3] Plant peroxidases that are located in the cell wall and vacuoles[4] have large numbers of isoforms and broad specificities for oxidizing substrates in the presence of hydrogen peroxide.[5] However, most plant peroxidases, which have been well characterized in flowering plants, such as horseradish peroxidase (HRP) and *Arabidopsis thaliana* peroxidase (ATP) A2, can oxidize only coniferyl alcohol efficiently, but are inefficient at oxidizing sinapyl alcohol.[6,7] The reason is considered to be the steric hindrance caused by the overlap of sinapyl alcohol's 5-methoxy group with the Pro-139 residue in the substrate binding site.[6] Because the Pro-139 residue is highly conserved in plant peroxidases, most of them appear unable to utilize sinapyl alcohol as a good substrate. The lack of oxidation activity for sinapyl alcohol and the lignin polymer in known plant peroxidases is a barrier to elucidating the lignin polymerization reaction.

Cationic cell-wall-bound peroxidase (CWPO-C) from *Populus alba* L. is the only enzyme that has been verified to oxidize not only monolignols efficiently but also lignin oligomers directly.[8] It was revealed that CWPO-C oxidizes substrates though the Tyr74 and Tyr177 residues, which are located near the heme (<14 Å) and exposed on the protein surface.[9,10] The substrate oxidation site being on the protein surface enables CWPO-C to oxidize a variety of compounds that cannot come into the heme pocket, such as sinapyl alcohol. The unsolved riddle in the oxidative coupling of monolignols, including sinapyl alcohol, and growing lignin polymers by plant peroxidase can be explained by the oxidation mechanism of CWPO-C. The *CWPO-C* gene is constitutively expressed in shoot tissues, including the developing xylem, and is not affected by many stress treatments. The protein localizes to the middle lamellae, cell corners, and secondary cell walls of the fiber cells during lignification.[11] The oxidation activity and tissue distribution of CWPO-C allow us to speculate that CWPO-C or a CWPO-C homologue (isoenzyme) is a specific peroxidase responsible for the lignification in the cell walls of vascular plants including poplar. According to plant genomic information (www.phytozome.net), almost all plants contain peroxidases that have high amino acid identities of >60% with CWPO-C. These peroxidases usually have a tyrosine residue that corresponds to the Tyr74 or Tyr177 of CWPO-C. It is expected that these peroxidases have a function similar to that of CWPO-C. *A. thaliana* (*Arabidopsis*) also contains some candidate CWPO-C isoenzymes. In this study, we focused on

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seven *Arabidopsis* plant peroxidases with relatively high amino acid sequence identities to CWPO-C and/or with Tyr74 or Tyr177 of CWPO-C. To investigate their physiological functions, we isolated homozygous mutant lines in which T-DNA insertions were used to knock out the expression of each peroxidase. Our subsequent analysis of the mutant phenotypes provides in vivo evidence that three putative CWPO-C homologues, AtPrx2, AtPrx25, and AtPrx71, are involved in stem lignification. This study may help to explain the elusive mechanism of lignin polymerization by plant peroxidases and identify the peroxidase isoforms responsible for lignification in vascular plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions. Wild-type *Arabidopsis* plants (*A. thaliana* ecotype Colombia) and eight mutant lines, purchased as described below, were used in this study. Surface-sterilized seeds were sown on 0.8% (w/v) agarose plates in plastic Petri dishes with Murashige and Skoog (MS) salt plus 3% (w/v) sucrose, pH 5.6. Petri dishes were placed in a growth chamber (MLR-351; Sanyo Electric Co., Tokyo, Japan) and cultured at 22 ± 1 °C under 16 h light (120 μmol photons $m^{-2}s^{-1}$) and 8 h dark conditions. Seedlings cultured for 3 weeks were transferred to pots containing vermiculite and perlite (1:1, v/v) and irrigated every 4 days with 0.1% Hyponex (Hyponex Japan, Osaka, Japan).

Reverse Transcription PCR (RT-PCR). Total RNA was extracted from roots and leaves of 6-week-old plants with Plant RNA Isolation Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After DNase (RQ1 RNase-Free DNase, Promega, Tokyo, Japan) treatment, a 1 μg aliquot from each RNA preparation was denatured at 65 °C for 10 min and then reverse-transcribed at 45 °C for 90 min with AMV Reverse Transcriptase XL (Takara Shuzo, Otsu, Japan) and oligo (dT) primers in a reaction volume of 20 μL. Following heat denaturation at 95 °C for 5 min, the resulting first-strand cDNAs were diluted 100-fold, and 1−4 μL of these samples was subjected to PCR in a final volume of 25 μL using *TaKaRa Ex Taq* (Takara Shuzo, Otsu, Japan) and the gene-specific primers described in Table S1 in the Supporting Information. The following PCR protocol was used: 98 °C for 10 s; a variable number of cycles (20, 25 or 30; to ensure that PCR assays were in a linear range) at 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 60 s. To check that an equal amount of cDNA was used for RT-PCR, the cDNA of β-tubulin was also amplified. PCR products were run on 2% (w/v) agarose gels and stained with ethidium bromide.

Isolation of T-DNA Insertion Lines. Homologous amino acid sequences to CWPO-C in *Arabidopsis* were obtained by sequence searching using TAIR BLAST 2.2.8 (http://www.arabidopsis.org/index.jsp). *Arabidopsis* T-DNA insertion mutants, SALK_085028 (*atprx2*), SALK-100989 (*atprx13*), SALK-047689 (*atprx43*), SALK-151762 (*atprx62S*), SALK-137991 (*atprx69*), and SALK-091561 (*atprx71*), were purchased from the *Arabidopsis* Biological Resource Center. AL751641 (*atprx25*) and AL766099 (*atprx62A*) were purchased from GABI-Kat collections. Selection of homozygous T-DNA knockout plants was performed by PCR using gene-specific primers. According to the T-DNA insertion point information provided by TAIR, the primer pairs shown in Table S2 in the Supporting Information were designed. The following PCR protocol was used: 98 °C for 10 s; 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 60 s. PCR products were run on 1% (w/v) agarose gels and stained with ethidium bromide. Each homozygous line was then self-fertilized, and seeds were collected in bulk from the dried fruits.

Determination of Acetyl Bromide Soluble Lignin (ABSL). Whole stem samples were harvested from 6-week-old the wild type and each mutant. They were ground to a meal in liquid nitrogen and then extracted with methanol at room temperature for 6 h. The same extraction procedure was repeated three times. The same extraction procedure was repeated three times. The extractive-free sample obtained was oven-dried and used for further lignin analyses. The acetyl bromide lignin assay was performed as described by Fukushima and Hatfield.[12] The extractive-free, oven-dried sample (ca. 10 mg) was digested with 10 mL of acetic acid solution containing 25% acetyl bromide at 70 °C for 30 min in a glass. After cooling, the reaction mixture was transferred to a tube containing 20 mL of acetic acid and 9 mL of 2 M sodium hydroxide. After mixing, 1 mL of 7.5 M hydroxylamine hydrochloride solution was added, and the volume was adjusted to 50 mL with acetic acid. The absorbance at 280 nm of the mixture was recorded using a spectrophotometer (Jasco V-530, Japan). A blank sample was run as a control. The content percentage of lignin = $100V(A_{sample} - A_{blank})/a \cdot W \cdot d$ [V = volume of the final solution (L); A = absorbance; a = molar extinction coefficient ($g^{-1} \cdot L \cdot cm^{-1}$); W = dry weight of the sample (g); d = cell thickness (cm)]. A molar extinction coefficient of 23.35 $g^{-1} \cdot L \cdot cm^{-1}$ was used for the calculation of percent ABSL content.[13]

Derivatization followed by Reductive Cleavage (DFRC) Method. The DFRC method was performed as described by Lu and Ralph.[14] Briefly, 10 mg of extractive-free sample in 3 mL of acetyl bromide reagent (acetyl bromide/acetic acid, 20:80, v/v) was stirred gently at 50 °C for 3 h. The solvent was removed by rotary evaporation below 50 °C. The evaporation residue was resuspended in 7.5 mL of acidic reduction solvent (dioxane/acetic acid/water, 5:4:1, v/v/v). Following the addition of 50 mg of zinc dust, the mixture was stirred for 30 min at room temperature. This mixture was transferred into a separatory funnel with 10 mL of dichloromethane and saturated ammonium chloride. The internal standard, 0.1 mg of *n*-octacosane, was added, and the aqueous phase was adjusted to between pH 2 and 3 using 3% HCl. After vigorous mixing, the organic layer was collected, and the extraction was repeated twice with 5 mL of dichloromethane. The organic layer was dehydrated in sodium sulfuric anhydride, redissolved in 1.5 mL of dichloromethane, and acetylated overnight with 0.2 mL of acetic anhydride and 0.2 mL of pyridine. After coevaporation with ethanol under reduced pressure, which was repeated until all of the acetic anhydride and pyridine was removed, the samples were dissolved in dichloromethane and subjected to GC-MS analysis. GC-MS analysis was performed using a Shimadzu GC-17A gas chromatograph coupled with a Shimadzu QP5050 mass spectrometer (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a DB-WAX column (0.25 mm × 60 cm; J&W Scientific, Folsom, CA, USA). The chromatograph was programmed at a rate of 3 °C min^{-1} from 150 to 234 °C and at 8 °C min^{-1} from 234 to 300 °C. The final temperature (300 °C) was held for 3 min. The amounts of coniferyl alcohol and sinapyl alcohol were determined using calibration curves derived from acetylated pure coniferyl alcohol and sinapyl alcohol, respectively.

Histochemical Assay. Stem sections (1 cm from the base) were cut from 6-week-old wild-type, *atprx2*, and *atprx25* plants. For paraffin sectioning, samples were fixed overnight at 4 °C in FAA (formalin/glacial acetic acid/70% ethanol, 1:1:18) and dehydrated using a graded ethanol series. Following substitution with chloroform, the samples were embedded in paraffin, sectioned at 10 μm thickness using a microtome, and put on slide glass. To remove paraffin, the slides were immersed in xylene for 30 min twice, air-dried at room temperature, and dewaxed using a graded ethanol series (5 min in 100%, twice for 5 min in 95%, 5 min in 80%, 5 min in 70%, and 5 min in 50% ethanol). After washing with water twice, the cross sections were stained in 0.02% toluidine blue for 1 min. After washing with water, the cross sections were dried and observed with a light microscope. Three individual plants of wild type and each mutant were used, and five cross sections were prepared from each individual plant. Three or four areas (50 μm × 50 μm) of interfascicular fiber region per a cross section were subjected to determination. Each area contained approximately 20 interfascicular fiber cells.

RESULTS

Selection of *Arabidopsis* Peroxidases That May Contribute to Lignification. A database search using TAIR with the poplar CWPO-C protein as the probe allowed us to find seven peroxidases, AtPrx13, AtPrx25, AtPrx43, AtPrx62,

Table 1. Putative Cationic Cell-Wall-Bound Peroxidase (CWPO-C) Homologues in *Arabidopsis*, AtPrxs, and Their Mutant Lines Used in This Study

protein	AA identity to CWPO-C (%)	TAIR gene no.	mutant line	remarks
AtPrx2	44	At1g05250	SALK_085028 (*atprx2*)	has Tyr74; cell wall protein (Bayer et al.[22])
AtPrx3	48	At1g77100	SALK_100989 (*atprx13*)	
AtPrx25	64	At2g41480	AL751641 (*atprx25*)	has Tyr177
AtPrx43	50	At4g25980	SALK_047689 (*atprx43*)	
AtPrx62	66	At5g39580	SALK_151762 (*atprx62S*)	different T-DNA insertion points
			AL766099 (*atprx62A*)	
AtPrx69	59	At5g64100	SALK_137991 (*atprx69*)	cell wall protein (Borderies et al.[15])
AtPrx71	68	At5g64120	SALK_091561 (*atprx71*)	cell wall protein (Borderies et al.[15] and Rouet et al.[16])

AtPrx69, AtPrx70, and AtPrx71, which share >48% amino acid sequence identity with CWPO-C. AtPrx71, which shares the maximum amino acid sequence identity (68%) with CWPO-C of all the *Arabidopsis* proteins, has been reported to be localized in the cell wall.[15,16] AtPrx25, which shares 64% amino acid sequence identity, conserves Tyr177, which functions as an oxidation site on the protein surface of CWPO-C. In addition, cell wall peroxidases AtPrx1 and AtPrx2, which have exactly the same sequence and share 44% amino acid identity with CWPO-C, were added to the list for conserving Tyr74 (Tyr78 in the case of AtPrx1 and AtPrx2), which also functions as an oxidation site in CWPO-C. The structural analysis using homology modeling showed that Tyr78 in AtPrx2 and Tyr177 in AtPrx25 were exposed on the protein surface similar to Tyr74 and Tyr177 in CWPO-C (see Figure S1 in the Supporting Information).

Of the nine peroxidases we focused on seven that are listed in Table 1, AtPrx2, AtPrx13, AtPrx25, AtPrx43, AtPrx62, AtPrx69, and AtPrx71, because their T-DNA insertion mutants are available. RT-PCR was performed using mRNA from wild-type *Arabidopsis* root, leaf, and stem as templates. Figure 1 shows the transcript levels of these peroxidases in wild-type *Arabidopsis* and that of the β-tubulin transcripts run as constitutive controls. The seven peroxidases appeared to be expressed in root, leaf, and stem, sometimes strongly, like *ATPRX71* in stem and *ATPRX1/ATPRX2* in root. It was not possible to discriminate between *ATPRX1* and *ATPRX2*.

To obtain plant lines with a disruption in these genes, we screened the collection of T-DNA insertion mutants of the TAIR knockout facility (http://www.arabidopsis.org) and purchased the mutants from the SALK or GABI-Kat collections. After self-crossing, the position of the T-DNA insertion and the homozygosity were verified by PCR using gene-specific primer pairs and T-DNA specific reverse primers. The gene structure and T-DNA insertion points for each gene are shown in Figure 2A. The *atprx62S* and *atprx62A* mutants have different T-DNA insertion points in the *ATPRX62* gene. T-DNA disruption of the gene expression in each mutant was verified by RT-PCR (Figure 2B). The combined *ATPRX1* and *ATPRX2* expression (*ATPRX*/*ATPRX2*) in *atprx2* stem tissue was apparently suppressed (Figure 2b), but not in root and leaf tissues (see Figure S2 in the Supporting Information).

Effects of AtPrx2 and AtPrx25 Deficiencies in Stem Formation. To study the effects of gene disruption on the development of *Arabidopsis* seedlings, the mutant seedlings were compared with wild-type plants under standard nutrient medium and soil conditions. The appearances of 3- and 6-week-old T-DNA inserted mutant seedlings were visually indistinguishable from that of wild-type seedlings, including root elongation. Prominent lignification of plant cell wall occurs in stem tissues. Thus, we performed more detailed observations focused on the progression of lignification in stems using 6-week-old wild-type and mutant seedlings. After length, dry weight, and stem diameter measurements, a significant difference was found between two mutants and wild-type in the dry weight of the main stem. The dry weights of *atprx2* and *atprx25* were 69 and 83% of wild-type, respectively ($P < 0.03$ for both, Table 2). These results suggested that AtPrx2 and AtPrx25 have some role in stem formation.

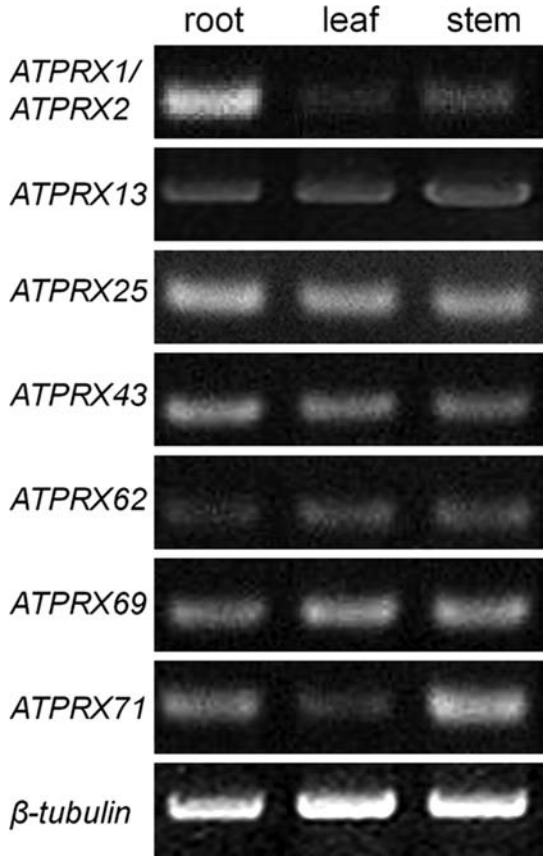

Figure 1. Expression analysis of *Arabidopsis* peroxidase by semi-quantitative RT-PCR. cDNA was generated from the root, leaf, and stem of a 6-week-old wild-type plant. The *β-tubulin* gene was used as a cDNA quantity control.

AtPrx2, AtPrx25, and AtPrx71 Are Involved in Lignification. If some peroxidases had a function on lignin polymerization, gene deficiencies would result in the alteration of lignin content and/or composition. To measure the lignin

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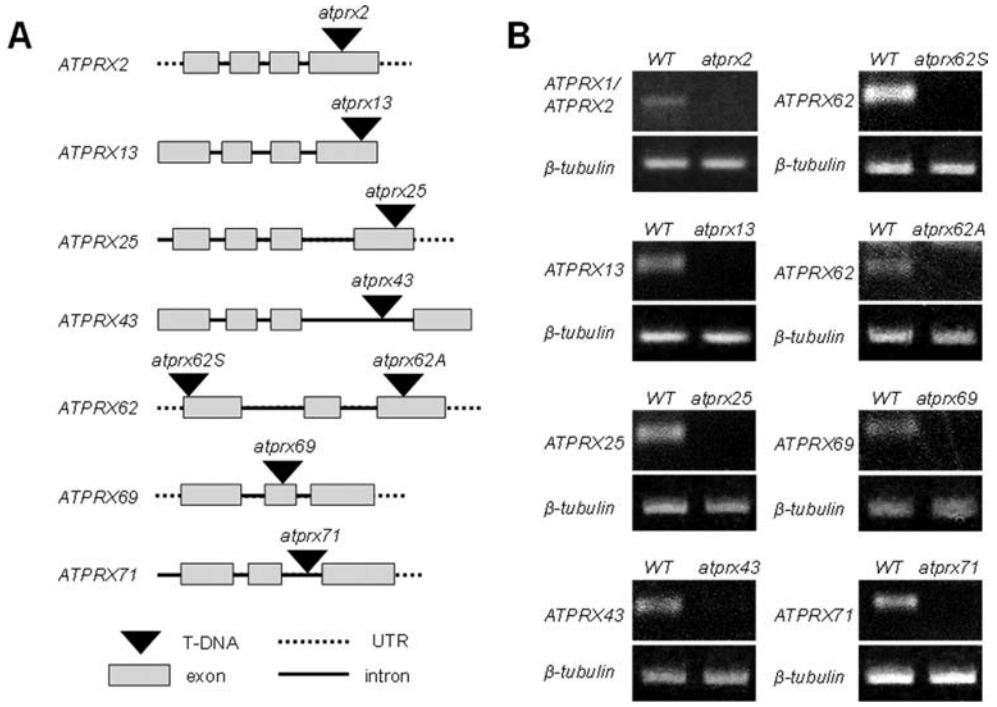

Figure 2. Conformation of gene knockouts by T-DNA insertion in the *Arabidopsis* peroxidases: (A) schematic representation of the AtPrxs and their T-DNA insertion sites; (B) semiquantitative RT-PCR for the AtPrx genes in wild-type and mutants. cDNA was generated from the stems of 6-week-old wild-type and mutant plants. The PCR was performed with 25 cycles for each AtPrx gene and with 20 cycles for the *β-tubulin* gene that served as the cDNA quantity control.

Table 2. Stem Growth and Development of 6-Week-Old Putative Cationic Cell-Wall-Bound Peroxidase (CWPO-C) Mutant and Wild-Type Arabidopsis Plants[a]

line	stem length (cm)	dry wt (mg plant^{-1})	diameter of cross section (mm)	no. of lateral branch per plant	max length of lateral branch (cm)
atprx2	20.7 ± 4.9	5.84 ± 2.42*	0.65 ± 0.09	2.40 ± 1.20	12.9 ± 5.4
atprx13	23.5 ± 3.6	8.79 ± 2.16	0.66 ± 0.11	3.18 ± 1.01	12.7 ± 5.1
atprx25	22.2 ± 4.6	7.05 ± 2.15*	0.70 ± 0.08	3.00 ± 0.89	12.2 ± 5.1
atprx43	25.0 ± 3.2	9.70 ± 3.19	0.65 ± 0.07	2.83 ± 0.94	12.3 ± 4.8
atprx62S	23.7 ± 4.9	8.16 ± 2.64	0.68 ± 0.07	2.54 ± 0.66	14.3 ± 3.6
atprx62A	25.5 ± 3.3	9.64 ± 2.77	0.62 ± 0.09	2.31 ± 0.79	13.0 ± 3.2
atprx69	22.2 ± 4.8	8.27 ± 2.69	0.62 ± 0.12	2.44 ± 0.96	10.1 ± 6.7
atprx71	23.2 ± 2.6	8.23 ± 1.93	0.73 ± 0.09	3.00 ± 0.71	13.3 ± 5.8
wild type	22.5 ± 4.0	8.47 ± 3.66	0.64 ± 0.09	2.78 ± 1.00	14.6 ± 3.0

[a]Data represent the mean ± SD (n = 10). Asterisks indicate statistical significance vs the wild-type control in each case (*, $P < 0.03$, Student's t test).

Table 3. Lignin Content and Monomer Composition Based on Derivatization Followed by Reductive Cleavage (DFRC) Method in *Arabidopsis* Putative Cationic Cell-Wall-Bound Peroxidase (CWPO-C) Mutants[a]

line	lignin (%)	μmol G/g lignin	μmol S/g lignin	S/G	μmol (G + S)/g lignin
atprx2	17.3 ± 1.4*	639 ± 108	232 ± 33*	0.36 ± 0.02*	870 ± 139*
atprx13	21.2 ± 0.4	486 ± 103	166 ± 49	0.34 ± 0.09	653 ± 15
atprx25	18.0 ± 1.7*	745 ± 88*	258 ± 56*	0.34 ± 0.04*	1003 ± 143*
atprx43	21.1 ± 1.5	510 ± 122	164 ± 61	0.32 ± 0.04	674 ± 83
atprx62S	18.4 ± 2.6	497 ± 154	174 ± 7	0.37 ± 0.13	672 ± 148
atprx62A	19.1 ± 1.6	508 ± 100	152 ± 9	0.31 ± 0.05	660 ± 109
atprx69	21.5 ± 1.7	503 ± 107	159 ± 2	0.32 ± 0.05	661 ± 104
atprx71	19.3 ± 2.1	748 ± 101*	276 ± 56*	0.37 ± 0.03*	1025 ± 157*
wild type	20.1 ± 1.4	590 ± 45	171 ± 20	0.28 ± 0.02	761 ± 56

[a]Data represent the mean ± SD (n = 4). Asterisks indicate statistical significance vs a wild-type control in each case (*, $P < 0.01$, Student's t test). The polymerization of the monolignols, coniferyl and sinapyl alcohol, result in guaiacyl (G) and syringyl (S) units, respectively.

content and composition, main stems were harvested from 6-week-old wild-type plants and mutants. Table 3 shows the lignin content determined by the acetyl bromide method and the arylglycerol-β-aryl (β-O-4) ether lignin composition determined by derivatization followed by the reductive cleavage method.[14] Significant reductions of acetyl bromide lignin levels

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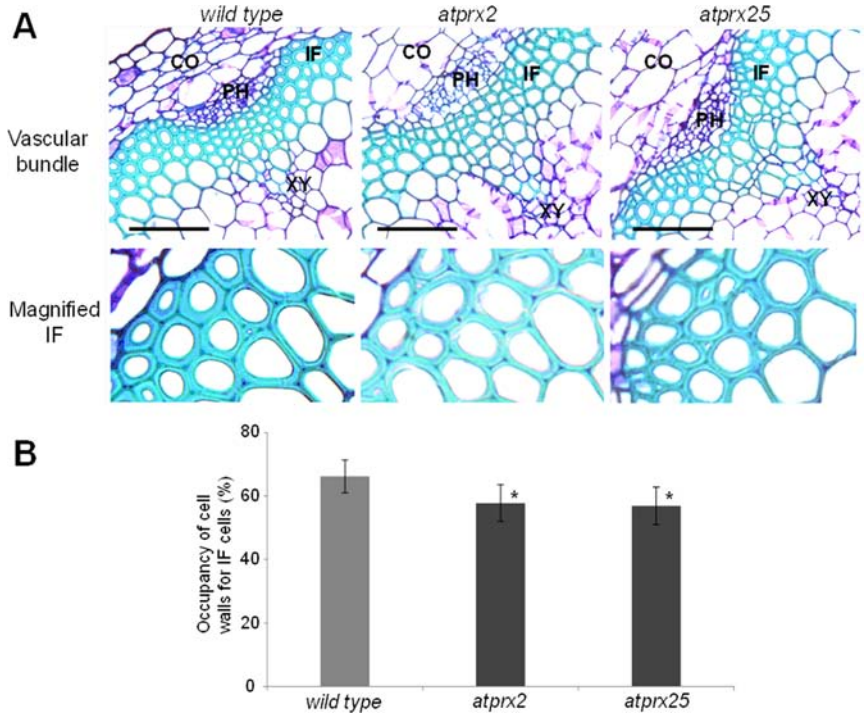

Figure 3. Histological analysis of wild-type and two putative cationic cell-wall-bound peroxidase mutants, *atprx2* and *atprx25*, in *Arabidopsis* stem cross sections: (A) microscopic observations of the cross section stained by toluidine blue (one representative result of eight independently performed experiments is shown; CO, cortex; IF, interfascicular fiber; PH, phloem; XY, xylem; bar = 50 μm); (B) quantitative analysis of cell wall area for interfascicular fiber cells measured by the analyzing software Winroof . ∗, $P < 0.01$, compared with wild-type. Data represent the results of three independent experiments. Error bars represent standard deviations.

($P < 0.03$) were found in *atprx2* and *atprx25* mutant lines, but not in other mutant lines. In contrast to the decrease of the acetyl bromide lignin levels, the level of the *β-O*-4 linkage-type lignin was increased. In the *β-O*-4 linkage-type lignin of *atprx2* and *atprx25*, the increase in S monomer volume was higher than that of G monomer, and the ratio of S and G units (S/G ratio) rose. A very similar pattern was seen for the *atprx71* mutant line, although no significant difference was found in the acetyl bromide lignin level. These results indicate that a knockout of *ATPRX2* or *ATPRX25* results in a reduction of lignin content, and a knockout of *ATPRX2*, *ATPRX25*, or *ATPRX71* causes changes in the *β-O*-4 bonded lignin structure.

Thinning of the Cell Walls in *atprx2* and *atprx25*. Following the observation that deficiencies of *ATPRX2* or *ATPRX25* could decrease the acetyl bromide lignin content, we used light microscopy to perform detailed observations and further characterize the cell wall structure. Stem cross sections were prepared from equivalent regions of the 6-week-old wild-type, *atprx2*, and *atprx25* plants. When cross sections were stained with toluidine blue (Figure 3A), a prominent anatomical feature in the fiber cells of the interfascicular region of the stems of *atprx2* and *atprx25* was observed, which showed marked lignification. As shown in Figure3A, interfascicular fiber cells with thin cell walls were observed in the cross sections of *atprx2* and *atprx25*. We quantified the cell wall areas in the microscope light field by calculating the percentage of cells that were defined as occupied in the toluidine blue stained area. Figure 3B shows the cell wall occupancy of interfascicular fiber cells measured by the analyzing software Winroof (Mitani, Fukui, Japan). When compared with wild-type, the occupancy of cells walls was decreased by 13% in *atprx2* ($P < 0.01$) and 14% in *atprx25* ($P < 0.01$).

DISCUSSION

Lignin, end-wise polymers containing *β-O*-4 linkages at a high frequency, can be formed by coupling individual monolignols to a growing polymer;[5] therefore, oxidation of the monolignols and growing polymer is an absolute necessity. Unlike known peroxidases, represented by HRP and ATP A2, CWPO-C from poplar can oxidize both, because CWPO-C can make use of its Tyr74 and Tyr177 residues on the proteins' surface as oxidation sites instead of the heme pocket.[10] The protein surface oxidation of CWPO-C is a good mechanism for the coupling reaction being catalyzed by plant peroxidases. Thus, we thought that the CWPO-C-like peroxidases of vascular plants would be involved in lignin biosynthesis. In this study, we focused on *Arabidopsis* plant peroxidases that shared an amino acid identity of >48% with CWPO-C and had a conserved tyrosine residue on the protein surface corresponding to position 74 and/or 177 of CWPO-C. In *Arabidopsis*, 73 plant peroxidases have been identified.[17] AtPrx71 has the highest amino acid sequence identity (68%) with CWPO-C in *Arabidopsis* and has 66% amino acid sequence identity with tobacco peroxidase isoenzyme TP-60, which has been shown to be a lignification-specific peroxidase.[18] AtPrx2 is a paralogous gene of AtPrx1 with an identical sequence; they share the highest amino acid identity (44%) of the seven *Arabidopsis* peroxidases that have a Tyr residue (Tyr78) corresponding to Tyr74 with CWPO-C. AtPrx25, which shares a 64% amino acid identity with CWPO-C, is the sole peroxidase that conserves Tyr177 in *Arabidopsis*. To our knowledge, except for CWPO-C, there are only three plant peroxidase isozymes, which have been reported to be involved in lignin biosynthesis, that oxidize sinapyl alcohol as their preferred substrate rather than coniferyl alcohol. Interestingly, Tyr74 occurs in two of these

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Table 4. Representative Candidates of Cationic Cell-Wall-Bound Peroxidase (CWPO-C) Isoenzyme in Various Plant Species

species	protein name	identity (%)	Tyr74	Tyr177
woody plants				
C. papaya	supercontig_468.4	74.5	–	–
C. papaya	supercontig_75.24	61.5	–	○
C. clementina	clementine0.9_015745m	77.2	○	○
C. sinensis	orange1.1g020511m	76.8	○	○
E. grandis	Eucgr.L02460.1	77.6	○	○
M. esculenta	cassava4.1_011945m	82.5	–	○
M. domestica	MDP0000684133	75.8	–	○
S. moellendorffii	97402 (e_gw1.19.288.1)	59.7	–	○
P. persica	ppa008723m	63.6	–	○
V. vinifera	GSVIVT01029771001	70.1	○	○
herbaceous plants				
A. coerulea	Aquca_007_00386	78.2	○	○
B. distachyon	Bradi2g04490.1	61.7	–	○
B. rapa	Bra000228	64.7	–	○
C. rubella	Carubv10023605m	63.4	–	○
C. sativus	Cucsa.217820.1	72.2	–	○
G. max	Glyma13g23620.1	64.0	–	○
L. usitatissimum	Lus10007051	72.2	○	○
M. truncatula	Medtr2g008770.1	75.8	–	–
M. truncatula	Medtr8g120300.1	63.3	–	○
M. guttatus	gv1a009839m	76.0	○	○
O. sativa	LOC_Os01g07770.1	61.4	–	○
P. vulgaris	Phvulv091014893m	69.9	○	○
R. communis	29989.m000432	85.4	○	○
S. italica	Si002216m	62.4	–	○
S. bicolor	Sb03g004380.1	61.1	–	○
T. halophila	Thhalv10016924m	64.8	–	○
Z. mays	GRMZM2G081928_T01	49.3	–	–
Z. mays	GRMZM2G136158_T02	44.1	○	–

(ZePrx33.70 and ZePrx33.44 purified from *Zinnia elegans*[19]). The amino acid sequence of the third isozyme (BPX1 purified from *Betula pendula*[20]) is unknown. If AtPrx2 and AtPrx25 have an oxidation site on the protein surface like CWPO-C, these tyrosine residues are noteworthy candidates that may become a substrate oxidation site. In addition, *P. chrysosporium* Lip and *T. cervina* Lip use different amino acids in different localities as substrate oxidation sites.[21] It cannot be ruled out that protein surface tyrosine residues, or tyrosine residues other than Tyr78 of AtPrx2 and Tyr177 of AtPrx25, may act as oxidation sites in AtPrx2, AtPrx25, and AtPrx71. In either case, the peroxidases that are responsible for lignin polymerization are expected to have unusual peroxidase activities, like CWPO-C. Characterizing these activities will be interesting and is being pursued.

Potential peroxidase isoenzymes that are involved in lignification can be extrapolated to localize in stem tissue, especially in the cell wall. We found an accumulation of all the peroxidase isoenzymes' mRNAs in stems from 6-week-old wild-type seedlings (Figure 1). AtPrx1/AtPrx2, AtPrx69, and AtPrx71 were recognized as cell wall peroxidases by previous characterization and proteomic analysis.[15,16,22] Knockouts of each target gene in each mutant line, except for *atprx2*, were confirmed by RT-PCR (Figure 2). The mutant *atprx2* with T-DNA inserted into the exon of *ATPRX2* could be termed an *ATPRX2* knockout mutant. *ATPRX1*/*ATPRX2* expression in *atprx2* was suppressed only in the stem, likely because *ATPRX2* expresses principally in the stem, whereas *ATPRX1* expresses principally in the root and leaf (Figure 2 and Figure S2 in the Supporting Information).

Despite the lack of these genes, we could not find any visual differences between the appearance of the mutant lines and the wild type grown for 3 and 6 weeks under the same conditions. Plant peroxidases are thought to be involved in a broad range of physiological processes in plants because of their catalytic versatility and the great number of isoforms.[23] A deficiency in only one peroxidase gene may be complemented by other peroxidases. Multiple studies have shown that the down-regulation of a target gene is an effective strategy to evaluate the genes' contribution to lignification. Down-regulation of the cationic peroxidase TP-60 crucially reduced lignin content in tobacco,[18] and up to a 20% reduction of lignin content was observed in a PrxA3a down-regulated hybrid aspen (*Populus sieboldii* × *P. gradidentata*).[24] In the *atprx2* and *atprx25* mutants, the lignin content of the stem was significantly decreased by about 14 and 11%, respectively (Table 3). In addition, the stems of both mutants had altered lignin structures and compositions that were analyzed by the DFRC method and GC-MS. Because DFRC monomers are restricted to those involved in *β-O*-4 ether, the most frequent interunit linkages in lignin,[14] the increased levels of DFRC monomers in the stems of the two mutants revealed altered lignin structures. Lignin composition, as reflected by the S/G ratio, was also altered in the mutants. The higher S/G ratio of the three mutants resulted principally from the higher recovery of S monomers. A similar phenotype was also observed in the stem of *atprx71*; however, the lignin content was not affected. These results indicate that AtPrx2 and AtPrx25 may have similar

contributions, which are higher than that of AtPrx71, to lignin biosynthesis.

Lignification occurs only in certain types of plant cells, such as xylem and interfascicular fibers. Because of the high negative correlation between the lignin content of tracheid cell walls and corresponding wall thickness,[25] lignification is associated with secondary cell wall thickening. As expected in a comparison with wild type, a smaller volume of the interfascicular fiber cell wall was observed in the mutants *atprx2* and *atprx25* (Figure 3). The reduction of dry weight in both lines may, in part, be attributed to the reduced cell wall volume.

This study demonstrates that AtPrx2, AtPrx25, and AtPrx71, which have high amino acid sequence identities with CWPO-C and/or the oxidation site of CWPO-C in the protein surface, are involved in the lignin biosynthesis of *Arabidopsis* stems. Thus, the amino acid sequence of CWPO-C is useful for the identification of lignification-specific peroxidases. Candidate CWPO-C isoenzymes in 8 woody plants and 17 herbaceous plants, which were identified using databases, are indicated in Table 4. All plant species, regardless of woody or grassy, contain at least one peroxidase having a very high amino acid sequence identity (~ >60%) with CWPO-C and contain Tyr74 and/or Tyr177, except *Zea mays* (maize). Although the identity is relatively low, maize also contains some plant peroxidases containing Tyr74 or Tyr177, such as GRMZM2G136158 listed in Table4. These peroxidases may be involved in lignification through uncommon oxidation abilities, like CWPO-C. It is now of interest to clarify the activities and physical functions of these peroxidases.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CWPO-C, cationic cell-wall-bound peroxidase; RT-PCR, reverse transcription PCR; β-*O*-4, arylglycerol-β-aryl; DFRC, derivatization followed by reductive cleavage

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