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ORIGINAL ARTICLE

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Immunolocalization of an anionic peroxidase in differentiating poplar xylem

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Abstract Peroxidases are the major candidate enzymes involved in dehydrogenative polymerization of monolignols. Peroxidases have the signal sequence at their N-terminus and this suggests that they are transported to extracellular spaces or developing cell walls. In this study, we focused on an anionic peroxidase isozyme encoded by *prxA3a*, which seems to be related to lignification. To investigate the localization of peroxidase in differentiating xylem cells of poplar (*Populus sieboldii* × *Populus grandidentata*), anti-PRX3 antibody was raised against the anionic peroxidase. Western blotting and peroxidase activity inhibition assay showed specificity of the antibody. Labeling by anti-PRX3 antibody was localized in vessels and fibers during the secondary wall formation and was observed along the plasma membrane beside the microtubules. The labeling was not seen in the cell wall, where localization of peroxidases was expected during lignification. The peroxidase isozyme, which is suggested to be involved in monolignol polymerization, is localized on the plasma membrane and its localization might be regulated by microtubules.

Key words Poplar · Lignin · Peroxidase · Immunolabeling

Introduction

Peroxidases are widely distributed in the plant kingdom and are involved in many biochemical plant processes. They are also thought to catalyze the polymerization of monolignols.

Lignin, a major cell wall component in vascular plants, is composed of aromatic polymers. Lignin is composed of three different precursors (monolignols) – *p*-coumaryl, coniferyl, and sinapyl alcohols. These monolignols are polymerized by dehydrogenative polymerization in the presence of peroxidases and hydrogen peroxide.

Because peroxidases are suggested to be involved in polymerization of monolignols,[1] a number of peroxidase isozymes have been isolated and characterized from many plants.[2–7] In spite of extensive studies, it is still not known which peroxidase specifically regulates lignification.

Peroxidase isozymes in higher plants are classified into anionic, neutral, and cationic enzymes. Anionic peroxidases have been the focus of studies attempting to identify a peroxidase which is involved in lignification.[3,7–9] Anionic peroxidases catalyze the oxidation of hydroxycinnamyl alcohols more effectively than the cationic isozymes.[10] Specific expression of anionic peroxidase genes was also reported in poplar xylem,[8,11] suggesting involvement of the peroxidases in lignification. The cell wall-bound peroxidase isozymes are also good candidates for lignin-forming enzymes.[3]

In the present study, we investigated the localization of peroxidase as an enzyme involved in lignin biosynthesis in differentiating poplar xylem cells. For this study, an anionic peroxidase isozyme encoded by *prxA3a* (D38050)[11,12] was selected from the database. Immunolocalization of the enzyme in differentiating poplar xylem was observed using specific antibodies raised against the anionic peroxidase.

Material and methods

Plant materials

Samples were taken from the stem of poplar (*Populus sieboldii* × *Populus grandidentata*) grown in the Experimental Forest of Nippon Paper Industries at Akita, Japan. Small pieces containing differentiating xylem were cut with

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a razor blade and immediately immersed into liquid propane cooled with liquid nitrogen. The specimens were then transferred to 0.5% glutaraldehyde in acetone cooled to −80°C for 2 days for freeze substitution. After washing with acetone followed by substitution with ethanol, the specimens were embedded in LR white resin (London Resin, Basingstoke, UK).

Enzyme extraction

Differentiating poplar xylem was scraped away with a knife and frozen in liquid nitrogen. The tissue was ground in liquid nitrogen and homogenized in the extraction buffer [0.1 M Tris-HCl buffer (pH 7.0) containing 5 mM mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride] with 2.5% (w/v) polyvinylpolypyrrolidone. The extract was filtered through nylon mesh and centrifuged at 3000 *g* for 10 min at 4°C. The supernatant was then centrifuged again at 10 000 *g* at 4°C for 20 min and concentrated using a Microcon (Millipore, Bedford, MA).

Western blotting

Antibodies prepared by Takeuchi et al.[13] were used in this study. Three types of peptides, PRX1 (NTDTIESEKE), PRX2 (SGSGNPDPTL), and PRX3 (NCRRVNDNST), were synthesized according to the amino acid sequence of the anionic peroxidase encoded by the *prxA3a* gene and used as antigens. The anionic peroxidase *prxA3a* is composed of 347 amino acid residues and its predicted molecular mass is 37.2 kDa.[11] The antibodies were purified on CNBr-activated Sepharose 4B gel (Pharmacia Biotech, Uppsala, Sweden) coupled with each peptide according to the instructions from Pharmacia. Concentration of the purified antibodies was determined according to the method of Bradford.[14] After affinity purification, the specificity of the purified antibodies was examined by Western blot analysis against the crude extract from differentiating poplar xylem. All the procedures described below were performed at room temperature. The crude extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and blotted onto a polyvinylidine difluoride membrane, which was pretreated with methanol followed by incubation with blotting buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. The membrane was incubated with 3% skim milk in tris-buffered saline (TBS) for 30 min to block nonspecific antibody binding. After the membrane was washed with TBS-T [TBS containing 0.1% (v/v) Tween-20], it was incubated with the antibodies diluted in blocking solution for 1 h, followed by washing three times in TBS-T for 5 min each. Then the membrane was incubated with alkaline phosphatase-conjugated goat antirabbit IgG (Cappel, Aurora, OH) diluted 1:10 000 in blocking solution for 1 h. After three washes in TBS-T for 5 min each, the antibody reaction was detected with *p*-nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Assay of peroxidase activity

Total peroxidase activity was determined spectrophotometrically at 470 nm (ε = 5570 $M^{-1}cm^{-1}$), by measuring the absorbance of the reaction mixture with guaiacol as a substrate. To confirm the reaction between the antibodies and peroxidase, peroxidase activity inhibition assay was examined with the antibody. For this experiment, 0, 25, 50, and 100 μg of antibody was mixed with 120 μg of the crude extract from the differentiating poplar xylem and incubated for 1 h at 4°C.[15] This mixture was then incubated with MagnaBind goat anti-rabbit IgG (Pierce Chemical, Rockford), which is the secondary antibody coupled with silanized iron oxide beads, for 15 min at 4°C. After centrifugation, the supernatant was used for peroxidase assay. The supernatant was added to the reaction mixture containing 50 mM guaiacol in 50 mM phosphate buffer (pH 7.4). The reaction was initiated by addition of 5 mM H_2O_2 and the increase in absorbance at 470 nm was measured.

Immunolabeling

Ultrathin sections were cut from LR White-embedded specimens using the Ultracut E (Reichert-Jung) with a diamond knife, and mounted on nickel grids for immunoelectron microscopy. All preparations were subjected to the following treatments: (1) incubation for 15 min in 50 mM glycine in phosphate buffered saline (PBS) followed by three washes for 5 min each in PBS; (2) incubation for 30 min in blocking buffer, which contained 0.8% bovine serum albumin (BSA), 0.1% gelatin, 5% normal goat serum, and 2 mM NaN_3 in PBS, followed by three washes for 5 min each in washing buffer that contained the same components as the blocking buffer with the exception of goat serum; (3) overnight incubation with purified antibody against the peroxidase peptides at 4°C, followed by three washes for 5 min each in washing buffer. For the control experiment, serial sections were incubated in pre-immune serum or blocking buffer instead of the primary antibody; (4) incubation with 15-nm colloidal gold-conjugated goat antiserum against rabbit IgG for 2 h followed by three washes for 5 min each in washing buffer and then a wash in PBS; (5) postfixation in 2% glutaraldehyde for 5 min; and (6) three washes for 5 min each in PBS and then one wash in distilled water. All treatments were performed in a wet box. All sections were stained with 2% uranyl acetate for 10 min, then with Reynolds' lead citrate for 1 min, and finally examined under a transmission electron microscope (JEM 1220, Jeol, Japan). Sections (1 μm thick) were prepared and subjected to treatments (1) through (6). After silver enhancement, the sections were observed under a light microscope.

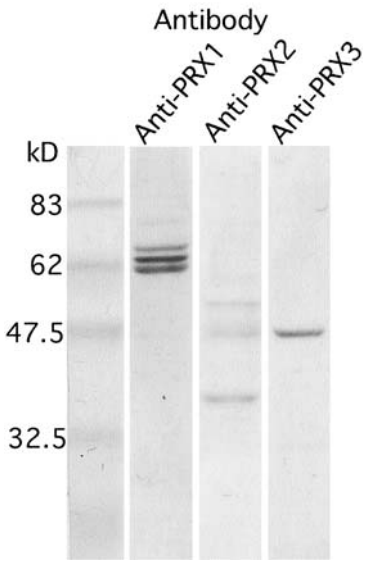

Fig. 1. Western blot analysis of antibodies against extract from *Populus sieboldii* × *Populus grandidentata*. Lanes *1*, *2*, and *3* show the reaction with antibodies against PRX1, PRX2, and PRX3, respectively. All antibodies reacted to 47 kDa protein, which was suspected as a peroxidase isozyme encoded by *prxA3a*

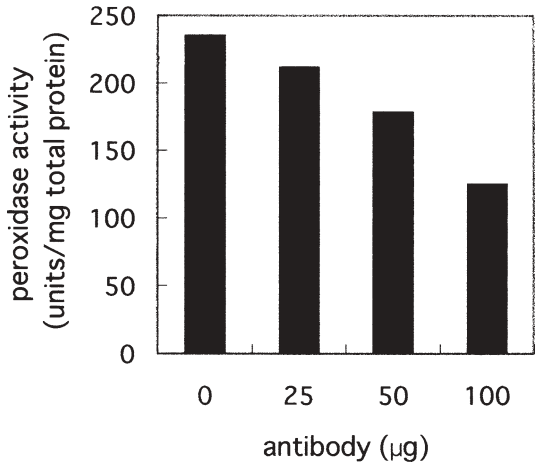

Fig. 2. Effect of the anti-PRX3 antibody on peroxidase activities of extract from stem tissues of poplar. The antibody inhibited peroxidase activities

Results

Each antibody raised against different peptides showed different cross reaction to the crude extract from differentiating xylem of poplar in the Western blot analysis (Fig. 1). Two of the antibodies showed cross reaction. Anti-PRX1 antibody showed strong bands at 60 to 70 kDa and a weak band at 47 kDa. Anti-PRX2 antibody recognized 35, 47, and 52 kDa proteins. In contrast, anti-PRX3 antibody reacted only to the 47 kDa protein, which was also detected by the other two antibodies. To confirm that the antibodies reacted to peroxidase, an inhibition assay of peroxidase activity was performed. Total peroxidase activity was determined by oxidation of guaiacol with H_2O_2. When anti-PRX3 antibody was added to the extract, it reacted with a target protein and produced an antibody–protein complex. Then, secondary antibody coupled with a heavy bead reacted to the first antibody and the complex was removed easily from the extract by centrifugation. The effect of anti-PRX3 antibody on peroxidase activity is shown in Fig. 2. Peroxidase activity was determined by absorbance of the reaction mixture at 470 nm 10 min after addition of H_2O_2 with anti-PRX3 antibody. The activity decreased when the antibody was added to the extract. This result suggests that the anti-PRX3 antibody inhibited peroxidase activity by removing peroxidase from the extract of poplar differentiating xylem. Anti-PRX1 and anti-PRX2 antibodies also inhibited peroxidase activity (data not shown).

Anti-PRX3 antibody was used for immunolabeling because it recognized a single protein in the poplar xylem extract in Western blot analysis. The secondary wall-forming stage was determined by polarizing microscopy.

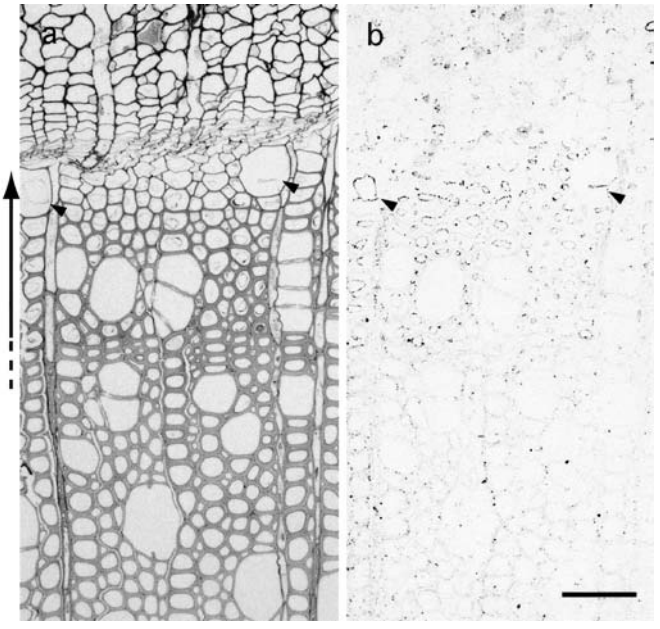

Fig. 3. Serial cross sections of differentiating poplar stained by safranine (**a**) and labeled with anti-PRX3 antibody (**b**). The *arrow* in **a** indicates the stage of secondary wall formation. Silver grains of peroxidase labeling were seen in fibers and vessels during secondary wall formation. The vessels marked with *arrowheads* were situated only in the earlier stage of cell differentiation. *Bar* 100 μm

Immunolight microscopic observation revealed marked labeling in vessels and fibers during the secondary wall formation (Fig. 3). The arrow in Fig. 3 indicates secondary wall-forming stage of fibers. Because vessels complete their cell wall formation much more quickly than fibers, only the newly formed vessels (arrowheads) were labeled. The labeling disappeared in vessels and fibers at the mature stage, when cytoplasm was not recognized under the light microscope. Electron microscopic observation revealed that the labeling was localized close to the plasma membrane of

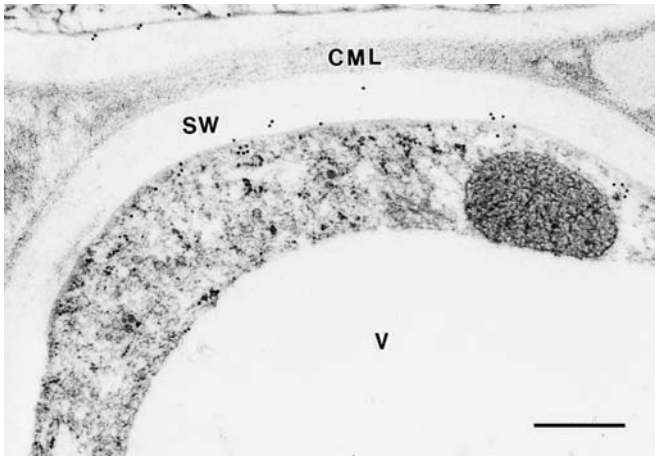

Fig. 4. Immunolabeling with anti-PRX3 antibody in a fiber cell in secondary wall formation. Labeling was observed in the vicinity of the plasma membrane. *SW*, secondary wall; *CML*, compound middle lamella; *V*, vacuole. *Bar* 0.5 μm

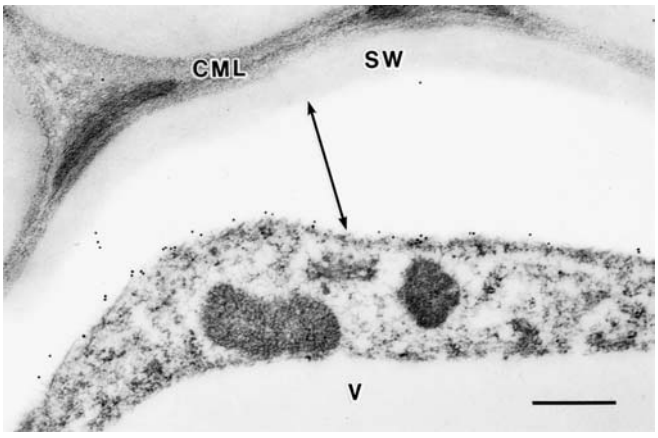

Fig. 6. Immunolabeling with anti-PRX3 antibody in a secondary wall-forming fiber cell, which was probably plasmolyzed during sample preparation. The differentiating stage of the fiber is similar to the fiber shown in Fig. 4. A large space (*double-headed arrow*) caused by plasmolysis was observed. The labeling was mainly seen on the outer surface of the plasma membrane. *Bar* 0.5 μm

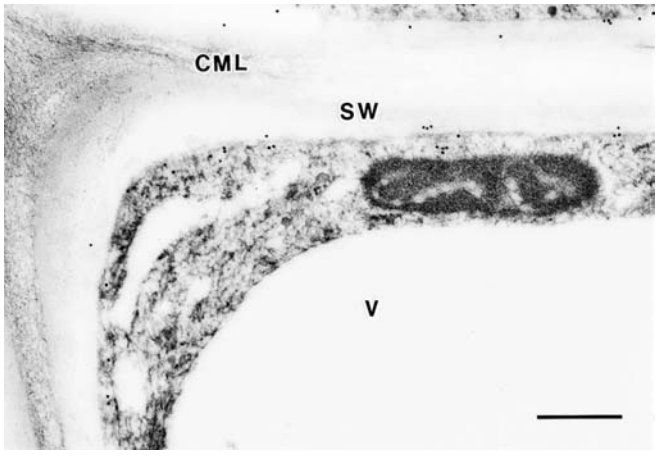

Fig. 5. Immunolabeling with anti-PRX3 antibody in a vessel cell undergoing secondary wall formation. *Bar* 0.5 μm

differentiating fibers at the beginning of S_2 formation (Fig. 4). The labeling was also observed close to the plasma membrane of the differentiating vessels at the early stage of secondary wall formation (Fig. 5). The gold particles seemed to be present on both sides of the plasma membrane, and it was difficult to discriminate whether the gold particles were localized in the plasma membrane or in the cell walls. Because some of the cells in the sections were plasmolyzed, probably during sample preparation, the plasma membrane and the cell wall were separated from each other in the cells. The gold particles were mainly localized on the plasma membrane surface that faced toward the cell wall (Fig. 6). Labeling was not observed, however, on the developing secondary cell wall or on the compound middle lamella. In longitudinal sections of fibers at the early stage of secondary wall formation, the labeling was localized close to the plasma membrane. In particular, labeling was localized in the boundary between the protoplast and the cell wall in close vicinity to the cortical microtubules (Fig. 7). Gold particles were seldom detected in the control sections treated with normal rabbit IgG.

Discussion

Peroxidase has been studied with regard to substrate specificity,[2,3,5,16,17] expression in tissues,[11] and seasonal changes of expression[18] to identify peroxidase involved in lignification. Anionic peroxidases are thought to be involved in lignification.[3,7,9] Osakabe et al.[11,12] characterized two anionic peroxidase genes, *prxA3a* and *prxA4a*, from *Populus kitakamiensis* (*Populus sieboldii* × *Populus grandidentata*) and revealed mRNA expression of *prxA3a* in differentiating and lignifying cells and that *prxA4a* was confined to shoot apices. They concluded that the anionic peroxidase encoded by the *prxA3a* gene is involved in lignification of cell walls. Furthermore, Li et al.[19] showed that the *prxA3a* promoter was mainly active in lignifying cells of stem tissue in transgenic tobacco plants. Tsuji et al.,[20] Morohoshi and Kajita,[21] and Li et al.[19] reported that transgenic poplars introduced with the antisense *prxA3a* gene had low peroxidase activity and low lignin content. The composition of lignin was also modified. We therefore selected *prxA3a* as a candidate gene involved in lignification. We synthesized three peptides (PRX1, PRX2, and PRX3), composed of 10 amino acid residues according to the amino acid sequence encoded by the *prxA3a* gene to raise the antibody. The three antibodies raised against the three peptides were expected to recognize the targeting peroxidase. These antibodies inhibited the oxidation of guaiacol (Fig. 2), confirming that the antibodies reacted with peroxidase isozymes in the crude extracts from the differentiating poplar xylem.

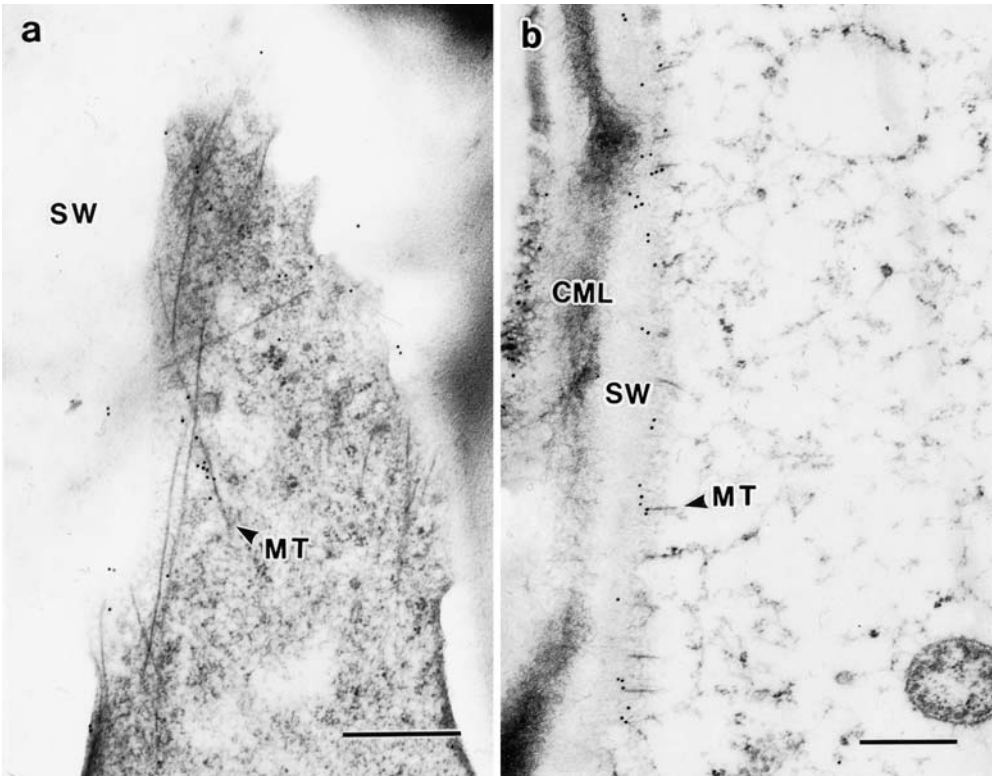

Fig. 7. Immunolabeling with anti-PRX3 antibody in an oblique section (**a**) and a longitudinal section (**b**) in secondary wall formation. *MT*, microtubule. *Bar* 0.5 μm

The antibodies showed distinct reaction from each other to the extract in the Western blot analysis. The antibody against PRX1 reacted with proteins of higher molecular mass than the predicted size of anionic peroxidases. There was also a weak reaction with the 47 kDa protein. The antibody against PRX2 reacted with 35, 47, and 52 kDa proteins. The deduced protein encoded by the *prxA3a* gene is composed of 318 amino acid residues and its molecular mass is 37.2 kDa.[19] Due to its amino acid sequence, the product of *prxA3* may be glycosylated. Christensen et al.[7] isolated six anionic peroxidase isozymes from *Populus trichocarpa* and their size ranges from 46 to 54 kDa. The amino acid sequence of one of these isozymes (PXP1) has high homology to the deduced product of *prxA3a*. PXP1, with molecular mass of 50.3 and 54.3 kDa, became 37 kDa after deglycosylation.[7] Therefore, the antibody against PRX2, which showed three bands in Fig. 1 might recognize both glycosylated and nonglycosylated products of *prxA3a* in the extracts from *P. sieboldii* × *P. grandidentata*. The antibody against PRX3 recognized only the 47 kDa protein.

According to the amino acid sequences of several poplar anionic peroxidase isozymes, PRX1 and PRX2 peptides are common sequences among some poplar anionic peroxidase isozymes; PRX1 (Asn86-Glu95) is found in PXP1 and PXP3–4[7] as well as PnC26.[22] PRX2 (Ser188-Leu197) is also found in PXP1. On the other hand, the PRX3 sequence (Asn299-Thr308), which is located near the C-terminus, is not found in published sequences of other anionic peroxidase isozymes. This indicates that the antibody against PRX3 recognizes the anionic peroxidase encoded by *prxA3a*. Therefore, this antibody enables visualization of the anionic peroxidase encoded by *prxA3a*.

The most interesting feature of the anionic peroxidase encoded by *prxA3a* is that the immunolabeling was localized close to the plasma membrane during secondary wall formation. Sparse labeling is observed on the cytoplasm, or the developing secondary walls. It has been thought that lignin-forming peroxidases are distributed in the lignifying cell walls. Peroxidases have been isolated from various plants, and extracellular or cell wall-bound peroxidases have been studied to identify peroxidases related to lignification.[3,5,10,16,23] Because they have a signal sequence at the N-terminus, they are expected to be transported to extracellular spaces or developing cell walls.[9] Our results, however, indicate that the anionic peroxidase which might be involved in lignification is localized near the plasma membrane. Although it was unclear if the labeling was localized "in" the plasma membrane in the cross sections, most of the gold particles were observed along the outer surface of the plasma membrane in the plasmolyzed cells (Fig. 6), suggesting the anionic peroxidase is located on the plasma membrane. Hydrogen peroxide that is indispensable for function of peroxidases might be generated in the plasma membrane.[24] The labelings were seen in the cells of the early stage of secondary wall formation, and it is reported that lignin deposition started from compound middle lamella at the start of the secondary wall formation.[25,26] Therefore, the peroxidase encoded by *prxA3a* might be involved in the early stage of lignification. Moreover, the immunolabeling of the anionic peroxidase was localized in the vicinity of the microtubules (Fig. 7a,b). The relation between microtu-

bules and cellulose microfibril deposition has been discussed for a long time. Although microtubules have not been discussed with respect to regulation of lignin deposition, they exist beneath cell walls, as shown in Fig. 7b, during secondary wall formation.[27] Microtubules might be involved in the regulation of the anionic peroxidase localization on the plasma membrane.

The anionic peroxidase encoded by *prxA3a* is a candidate for lignin-forming peroxidase. Peroxidases catalyze the formation of monolignol radicals in the presence of H_2O_2. Our results suggest that some of the monolignol radicals are formed near the plasma membrane in the presence of H_2O_2, dispersed to the cell wall, and finally coupled with each other to form macromolecular lignin. Cell wall-bound peroxidases were also isolated with regard to lignification.[3,22,28] Multiple enzymes may be involved in monolignol polymerization, and their distinct localization and substrate specificities must be revealed to understand more precisely the progress of lignin deposition and heterogeneity of lignin in the cell wall.

In this study, we focused only on an anionic peroxidase, however peroxidase consists of many isozymes. Cationic peroxidases[4,5] and laccase[29] are also possible agents of monolignol polymerization. Further investigation on the localization of other peroxidases and laccases might contribute to a better understanding of the final stage of lignification.

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