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Lignification and peroxidase in tension wood of *Eucalyptus viminalis* seedlings

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Abstract Seedlings of *Eucalyptus viminalis* were grown for 50 days with their stems bent so tension wood would form. Every 10 days the lignin content, monomeric composition, and peroxidase activity in the tension wood were compared with those in the lower side (opposite wood) and in vertically grown controls. The lignin content in the developing tension wood started to decrease after 10 days of bending and kept decreasing for 50 days, whereas those in control plants and opposite wood remained almost unchanged. The yields of syringaldehyde from tension wood by nitrobenzene oxidation increased, and consequently the syringyl/guaiacyl ratio of the lignin was higher in tension wood than in opposite wood and control plants. The peroxidase ionically bound to the cell walls (IPO) catalyzed oxidation of guaiacol and syringaldazine. The syringaldazine-oxidizing activity of IPO from tension wood increased, whereas the activities of IPO from opposite wood and control plants did not show any marked change. In tension wood the increase in syringaldazine-oxidizing activity of IPO was consistent with an increase in the syringaldehyde yield. This suggests that IPO contributes to syringyl lignin deposition as other enzymes involved in the monolignol biosynthesis do in tension wood formation.

Key words *Eucalyptus* · Tension wood · Lignin biosynthesis · Peroxidase · Substrate preference

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Introduction

Compression wood in gymnosperms and tension wood in angiosperms are types of reaction wood formed to return stems toward an upright position when trees are bent or tilted. They differ from normal wood anatomically, physically, and chemically. Tension wood has been generally characterized by anatomical features (e.g., gelatinous layer formation and less vessel frequency) and chemical features (e.g., less lignin and elevated cellulose content).[1–5] Most investigations of tension wood have been concerned with its occurrence, development, ultrastructure, and physical properties.

There are some discrepancies among the descriptions of the chemical composition of tension woods.[6–9] In relation to lignin, chemical analyses of tension woods in several plant species by Bland,[7] Bland and Scurfield,[8] and Timell[9] showed that tension wood of some angiosperms do not exhibit decreased lignin content. Some reports concerning monomeric compositions (syringyl/guaiacyl ratio) between tension wood lignin and normal wood lignin showed different results.[7,8,10,11] In contrast, the appearance of compression wood seems to be universal in gymnosperms, whereas tension wood formation is highly variable depending on the plant species. Disagreement about the characteristics of tension wood lignins is probably due to the fact that tension wood specimens for chemical analyses have been collected from trees grown naturally or from bent seedlings cultured for long periods. Neither sequential change in lignin content nor monomeric composition during tension wood formation has been reported in these studies.

During reaction wood formation the biosynthesis of wood constituents such as cellulose, lignin, and hemicelluloses may be dramatically affected. Reaction wood formation therefore is interesting in terms of biochemical and molecular biological studies on the biosyntheses of plant cell walls. Physiological changes during tension wood formation have received little attention. So far only Kutsuki and Higuchi investigated enzyme activities involved in lignin biosyn-

thesis in reaction wood of an angiosperm and gymnosperms, but changes in these enzymatic activities during development of reaction wood were not examined.[12] Wardrop and Scaife[13] and Scurfield and Wardrop[14] investigated peroxidase in tension wood histochemically and showed that the intensity of peroxidase activity was coincident with development of the gelatinous layer, an observation confirmed by Scurfield.[15] The relation of detected peroxidase(s) to lignification in tension wood is still unknown.

Oxidative polymerization of monolignols, the last step of lignin biosynthesis, may be catalyzed by peroxidase or laccase.[16,17] Many peroxidase isoenzymes have been recognized in plant cells, but the function of each isoenzyme remains unclear. In a series of studies,[18–21] we have examined the substrate utilization of peroxidase isoenzymes toward sinapyl alcohol and coniferyl alcohol. If each peroxidase isoenzyme has a substrate preference toward a certain monolignol, and such isoenzymes exist in angiosperms, not only the sequence of enzymes participating in the differentiation of syringyl and guaiacyl monolignols in the monolignol biosynthesis,[16] but also these peroxidases may contribute to regulating the deposition of heterogeneous lignin. Syringyl lignin locates mainly in secondary walls, whereas guaiacyl lignin is in primary walls and middle lamellae.[22,23] Here we investigated the lignin content, monomeric composition, and peroxidase activity toward syringyl and guaiacyl substrates during formation of tension wood of *Eucalyptus viminalis* seedlings by bending its stem for 50 days. Analyses of tension wood lignin and peroxidase activity were carried out every 10 days, and their changes are discussed in the context of lignification in tension wood.

Materials and methods

Plant materials

Eucalyptus viminalis seedlings were purchased from a plant breeding company (Tokai Forest Co.). *E. viminalis* seedlings (ca. 80 cm tall) grown in pots were transferred to the nursery of Shizuoka University at the beginning of April and then were grown under natural conditions. At the beginning of June, reaction wood was caused to form along the upper sides of the stems by bending until their apical buds were fully reversed. They were fastened to prevent recovery and then allowed to grow for 50 days. The control seedlings were fastened upright and grown under the same conditions.

Sixteen seedlings each of bent and control stems were subjected to chemical and enzyme analysis every 10 days as described below. The reaction wood samples (ca. 6 cm in length) were harvested from the bent part of stems. Samples were longitudinally divided into those obtained from the upper side and those from the lower side. Control samples were collected from the same internodes and used without dividing.

Preparation of peroxidase

Ten of sixteen samples from each upper side or lower side were debarked, and the developing xylem tissue was immediately scraped from each side using razor blades, frozen in liquid nitrogen, and bulked separately. Developing xylem tissues were also obtained from 10 control samples in the same manner. Xylem tissue was homogenized in 50 mM Tris-HCl buffer (pH 7.5) in a Waring blender at 4°C. After centrifugation at 18000 *g* at 0°C for 20 min, the supernatant was designated soluble peroxidase (SPO). The cell wall residue was subjected to three sequential cycles of resuspension and centrifugation in the same buffer and then was homogenized with 0.6 M NaCl in 50 mM Tris-HCl buffer (pH 7.5) using the same methods. The supernatant obtained after centrifugation was used as ionically bound cell wall peroxidase (IPO). SPO and IPO were concentrated by an ultrafiltration unit (MW > 10000; Advantec, Japan) at 4°C and then were assayed for peroxidase activity.

Assay for peroxidases

Peroxidase activity was assayed using guaiacol and syringaldazine as substrates according to described methods.[19] Oxidation of guaiacol and syringaldazine was monitored spectrophotometrically at 470 and 530 nm, respectively, with activities computed per gram of fresh cells using extinction coefficients (ε) of 5570 l/mol/cm for guaiacol and 65001/mol/cm for syringaldazine.[24]

Lignin determination and alkaline nitrobenzene oxidation

Six of sixteen samples from each upper side, lower side, and control were debarked and oven-dried at 80°C. Wood meals prepared from each sample were extracted with ethanol/benzene (1:2, v/v) for 6 h in a Soxhlet extractor. The lignin content in the wood meals was determined by the acetyl bromide method.[25] Alkaline nitrobenzene oxidation followed by gas chromatography analysis was performed according to the method described previously,[26] except that a TC-1 column (0.25 mm $\phi \times$ 30 m; GL Science, Japan) and program temperatures (initial temperature 120°C, final temperature 240°C; program rate 4°C/min) were used. Oxidation products, vanillin, vanillic acid, syringaldehyde, and syringic acid were determined. We did not detect *p*-hydroxybenzaldehyde or *p*-hydroxybenzoic acid in our experiment.

Results and discussion

Changes in lignin content and monomeric composition in tension wood of *Eucalyptus* seedlings

We used *E. viminalis* seedlings to study changes in lignin and peroxidase activity during tension wood formation because formation of tension wood in *Eucalyptus* species has

been confirmed by the presence of a gelatinous layer, elevated cellulose, and reduced lignin content.[1,5,9,15] Because young seedlings do not have xylem cells that were formed during preceding years, analytical data on tension wood lignin can be restricted to lignin in cells developed during the current year. It was difficult to analyze individual seedlings because of the limited amount of sample obtained from each plant. We therefore ran the lignin analysis and peroxidase assays on the bulk sample of tension wood for comparison with those from opposite wood and control specimens. In this study, we used seedlings generated by the seedling producer. In the nursery, cross-pollination with different varieties were minimized. Therefore, we believed that the genetic variation among individual seedlings was not considerable, although they were not clones.

The lignin content in the upper side of *E. viminalis* seedlings gradually decreased from 19.9% to 12.9% over the 50-day bending period, whereas lignin content in its lower side and in vertical stems (control) remained almost unchanged during this period (Fig. 1). The reduced lignin content in the upper side clearly indicates that tension wood developed along the upper side of the bent stem within 50 days, though we did not conduct anatomical studies to confirm the appearance of the gelatinous layer in the fibers. The lignin content overall decreased from 19.9% to 16.6% in tension wood after 20 days of bending (Fig. 1). This confirmed results from preliminary experiments where the lignin content of tension wood of *E. viminalis* seedlings decreased from 21.7% to 16.3% during a 20-day bending period and to 15.1% by 40 days.

The yield of syringaldehyde by alkaline nitrobenzene oxidation was higher from the tension wood lignin after 30 days of bending and kept increasing thereafter until 50 days with continuous bending of the stems (Fig. 2). The increase in the yield of syringaldehyde followed the decrease in lignin content, with a lag of 10 days (Figs. 1, 2). In contrast, the yield of vanillin from tension wood remained almost the same or decreased slightly (Fig. 3). The yields of vanillic acid and syringic acid from tension wood, opposite wood, and controls were less than 10% of yields for each corresponding aldehyde; and the patterns of their sequential changes were similar to those of the aldehydes (data not shown). The yields of syringaldehyde or vanillin from controls fluctuated slightly but did not follow a trend through the experimental period (Figs. 2, 3). Similarly, the yields of syringaldehyde (S) or vanillin (V) from opposite wood did not change significantly (Figs. 2, 3). We computed the S/V molar ratio from the yields of syringaldehyde plus syringic acid and vanillin plus vanillic acid. The S/V ratio in the tension wood lignin after 50 days of bending was markedly higher (S/V = 4.5) than that of the control plant (S/V = 2.8), the opposite wood (S/V = 3.5), or the stems before bending (S/V = 3.0).

Previous papers reported that tension wood contains less lignin than the vertical stem (control) and contains syringyl and guaiacyl residues in normal or slightly higher ratios.[7,8,11,27] Bland[7] and Bland and Scurfield[8] emphasized that lignin in opposite wood had a lower syringyl/guaiacyl ratio than either normal wood or tension wood. However, the reported S/V ratios of tension wood, opposite wood, and normal wood of *E. camaldulensis* seedlings in the literature[8] were 3.0, 2.4, and 2.6, respectively, after tension wood was induced by bending their stems for 6 months. Furthermore, higher S/V ratios in tension woods compared to vertical

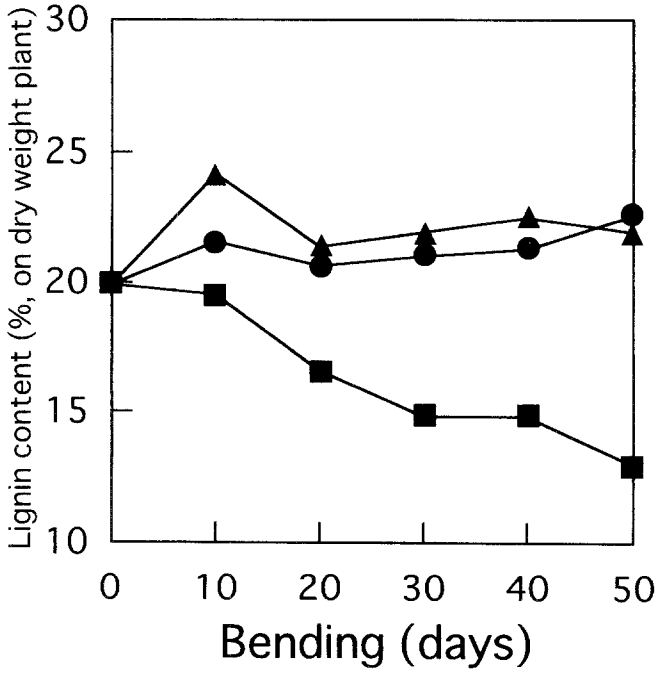

Fig. 1. Changes in lignin content in *Eucalyptus viminalis* seedlings during bending treatment. *Squares*, tension wood; *circles*, opposite wood; *triangles*, control

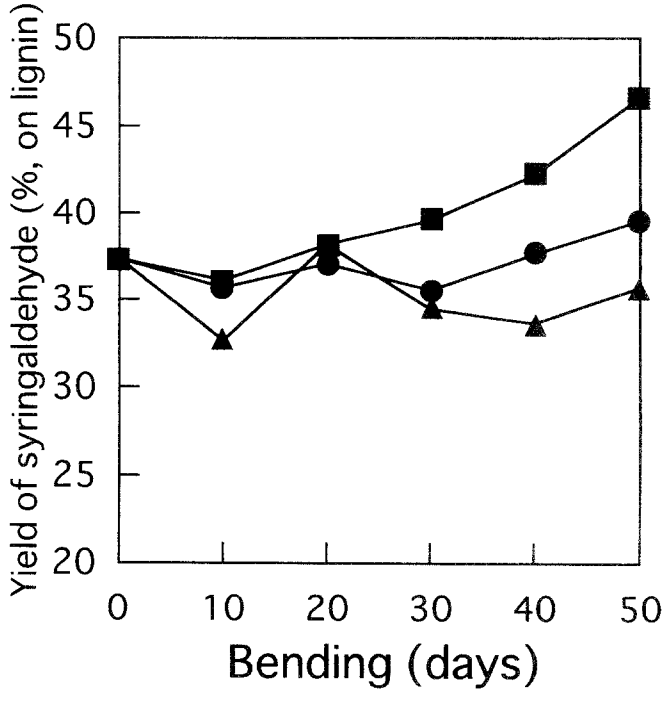

Fig. 2. Changes in the yield of syringaldehyde from *E. viminalis* seedlings on nitrobenzene oxidation. *Squares*, tension wood; *circles*, opposite wood; *triangles*, control

stems were also observed in other plant species: *Tristania conferta* (2.5 vs. 1.9) and *Lagunaria patersoni* (3.7 vs. 2.8).[8] More recently, Bailleres et al. investigated tension wood in a *Eucalyptus* hybrid clone and found that the syringyl/guaiacyl ratio as determined by thioacidolysis clearly increased in tension wood.[10] On the other hand, Baba et al. reported no difference in syringyl/guaiacyl ratios as determined by thioacidolysis between 3-year-old *E. camaldulensis* normal wood and tension wood induced by a 2-week inclination.[11] Our results suggest that the syringyl/guaiacyl ratio in tension wood lignin generally tends to increase. In our experiment, an apparently high S/V ratio in the tension wood was observed after 40 days of bending, and the lignin contents decreased within a 20-day bending period. Baba et al. observed gelatinous layer formation in a fiber in the upper side of the stem inclined for 2 weeks.[11] Therefore, the decrease in the lignin content of the tension wood appearing at the early stage of the bending may be ascribed to formation of the gelatinous layer and a consequent increase in the cellulose contents in fibers. It appears that the syringyl lignin content of tension wood decreased 19% when computed against dry plant weight after 50 days of bending (Table 1). By contrast, there is a greater reduction in both lignin (35.2%) and vanillin (44.5%). This suggests that carbon flow in lignin biosynthesis may be directed toward syringyl lignin rather than guaiacyl lignin during tension wood formation.

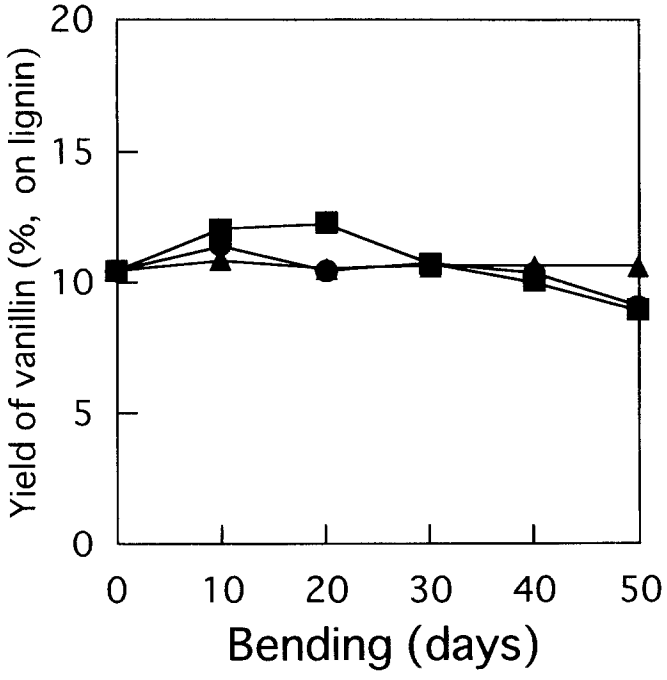

Fig. 3. Changes in the yield of vanillin from *E. viminalis* seedlings on nitrobenzene oxidation. *Squares*, tension wood; *circles*, opposite wood; *triangles*, control

Changes in peroxidase activity in tension wood of *Eucalyptus* seedlings

We determined peroxidase activity using two substrates, syringaldazine and guaiacol, which have the same substitution pattern on their aromatic moieties as do sinapyl and coniferyl alcohols, respectively. We previously investigated oxidation and dehydrogenative polymerization of sinapyl and coniferyl alcohols by peroxidase preparations from poplar, Japanese cedar, and horseradish peroxidase and found that the peroxidases with a large preference for sinapyl alcohol but small preference for coniferyl alcohol exhibited a large preference for syringaldazine but not guaiacol. Thus, we concluded that the substrate preference of peroxidase with respect to sinapyl and coniferyl alcohols can be predicted by assaying the activity toward syringaldazine and guaiacol, respectively.[18–21]

Peroxidases from *E. viminalis* seedlings were fractionated into two fractions – soluble peroxidase (SPO) and ionically bound cell wall peroxidase (IPO) – by means of successive extraction from developing xylem with buffer, followed with the same buffer containing 0.6M NaCl. SPO prepared from tension wood, opposite wood, or control plants showed guaiacol-oxidizing (Fig. 4) but not syringaldazine-oxidizing activity (data not shown). This suggests that SPOs in *E. viminalis* have no activity toward syringyl substrate. Guaiacol-oxidizing activity of SPO from tension wood increased over 50 days, whereas the activity of SPO from the opposite wood decreased, and that from the control plants remained essentially constant. Changes in guaiacol-oxidizing activities of IPO from each sample of tension wood, opposite wood, or control plants did not follow a constant trend during the bending of their stems (Fig. 5). The activity of IPO from opposite wood and from control plants toward syringaldazine was unaffected by the bending treatment throughout the experimental period (Fig. 6). It should be noted that syringaldazine-oxidizing activity of IPO from tension wood increased after 20 days of bending. Accordingly, only IPO from tension wood had a

Table 1. Lignin content and yield of nitrobenzene oxidation products in tension wood of *Eucalyptus viminalis* seedlings

Duration of bending (days)	Lignin content		Yield of vanillin		Yield of syringaldehyde	
	% In plant[a]	Relative %[b]	% In plant	Relative %	% In plant	Relative %
0	19.9	100	2.1	100	7.4	100
40	14.8	74.4	1.5	71.4	6.2	83.8
50	12.9	64.8	1.1	52.4	6.0	81.1

[a] Percentage was computed based on the dry weight of the plant material
[b] Relative percentage was computed on the basis that the data on day 0 is 100%

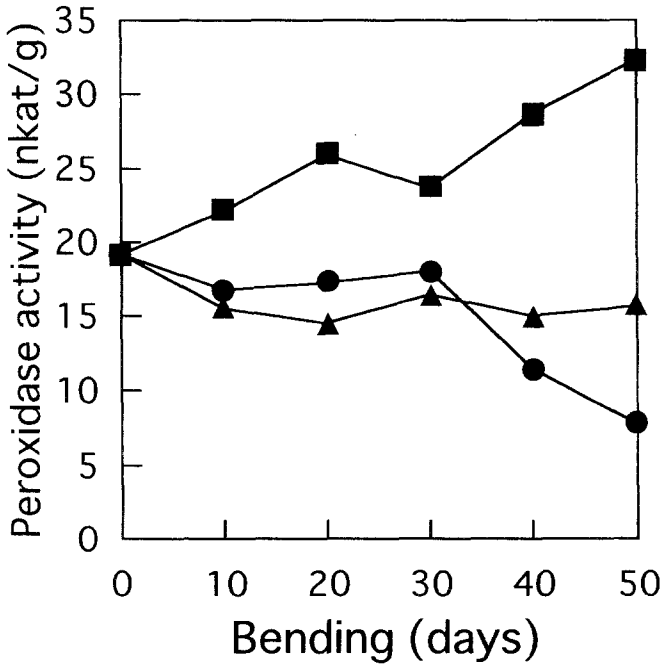

Fig. 4. Changes in peroxidase activity of soluble peroxidase (SPO) from *E. viminalis* seedlings toward guaiacol. *Squares*, tension wood; *circles*, opposite wood; *triangles*, control

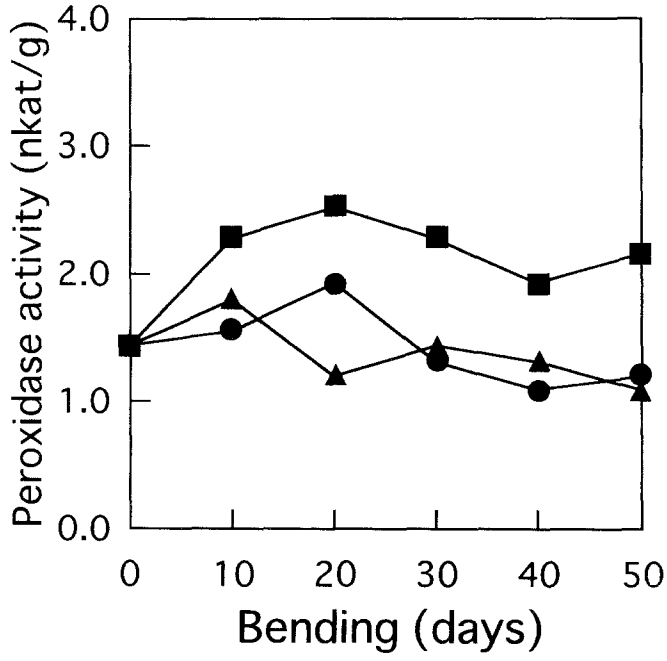

Fig. 5. Changes in peroxidase activity of ionically bound peroxidase (IPO) from *E. viminalis* seedlings toward guaiacol. *Squares*, tension wood; *circles*, opposite wood; *triangles*, control

strong substrate preference for syringaldazine compared to all SPOs or IPOs from opposite wood and control plants. The increase in syringaldazine-oxidizing activity seems consistent with the increase in the yield of syringaldehyde from tension wood lignin (Figs. 2, 6).

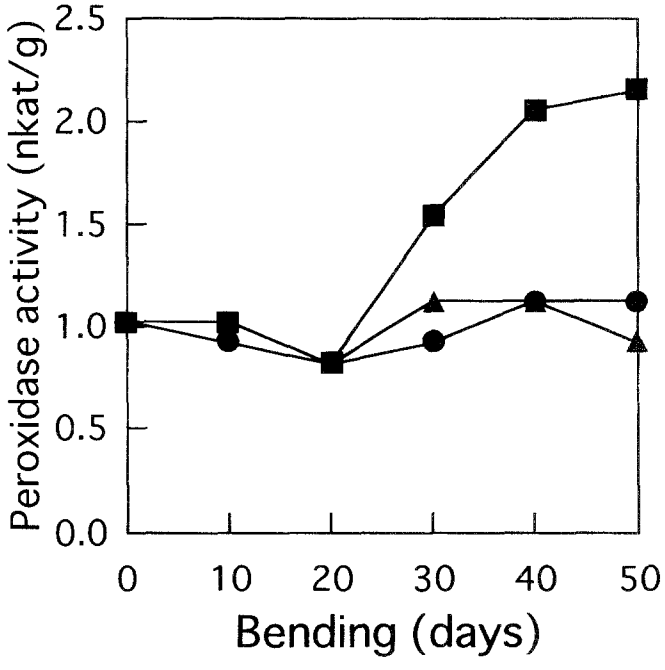

Fig. 6. Changes in peroxidase activity of IPO from *E. viminalis* seedlings toward syringaldazine. *Squares*, tension wood; *circles*, opposite wood; *triangles*, control

A functional role of plant peroxidase for the oxidative polymerization of monolignols in lignin biosynthesis has been suggested,[28] and laccase has been considered as a functional catalyst during lignification.[17] Peroxidase or laccase participation in the deposition of lignin has been under discussion,[16,17] but in our enzyme assay system we detected no oxidation of syringaldazine and guaiacol by either SPO or IPO from tension wood, opposite wood, or control plants in the absence of exogenous hydrogen peroxide. We therefore concluded that peroxidase is responsible for polymerization of monolignols in *E. viminalis* seedlings.

The IPO from *E. viminalis* showed oxidizing activity for both guaiacol and syringaldazine, but SPO from this species did not show syringaldazine-oxidizing activity. A number of peroxidase isoenzymes have been discussed in relation to lignification. Following the report that peroxidase-dependent syringaldazine-oxidizing activity was restricted to lignifying cells in woody plants,[28] supporting results have been reported.[29-31] Thus, this substance is often used to investigate the lignification-specific peroxidase isoenzyme. Regarding localization of lignification-specific peroxidases, cell wall-bound peroxidase isoenzymes have been reported to be responsible for lignin deposition in several plant species, such as *Zinnia elegans*,[32] tobacco,[33] and poplar.[29] Accordingly, we assume that the lignification-specific peroxidase isoenzyme may be included in IPO because this peroxidase has a large preference for syringaldazine and is presumably localized in the cell walls.

Peroxidase isoenzyme isolated from poplar callus cell walls has a substrate preference for sinapyl alcohol and syringaldazine over coniferyl alcohol and guaiacol, respectively.[18,20] This syringyl-specific isoenzyme ionically binds to

the cell walls and catalyzes dehydrogenative polymerization of sinapyl alcohol in vitro.[18] More recently, we reported that the IPO from poplar xylem also showed a larger substrate preference for sinapyl alcohol and syringaldazine than SPO from poplar xylem or SPO and IPO from Japanese cedar xylem.[21] The poplar xylem IPO also catalyzed dehydrogenative polymerization of sinapyl alcohol in vitro.[21] In this study, IPO from *E. viminalis* seedlings catalyzed the oxidation of syringaldazine, whereas the SPO hardly touched it, suggesting that IPOs from *E. viminalis* and poplar might have similar substrate preferences.

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