

Common Mechanism of Lignification of Compression Wood in Conifers and *Buxus*

Hideto Hiraide, Masato Yoshida, Saori Sato, Hiroyuki Yamamoto

Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan
Email: hiraide.hideto@e.mbox.nagoya-u.ac.jp

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Abstract

Woody plants develop a specialized secondary xylem known as reaction wood to enable formation of an ideal shape. Reaction wood in coniferous species is known as compression wood, and that of woody angiosperms as tension wood. However, the genus *Buxus* which is classified as an angiosperm, forms compression-wood-like reaction wood. We investigated the mechanism of lignification in coniferous compression wood and *Buxus* reaction wood: 1) Several lignin synthesis genes were upregulated in differentiating reaction wood of *Buxus microphylla*; 2) *B. microphylla* possesses a specific laccase gene that is expressed specifically in differentiating reaction wood (*BmLac4*); 3) laccase activity localization was closely related to lignification of reaction wood, and laccase activity was high in the secondary wall middle layer; 4) in reaction wood cell walls, galactan was present in the outer portion of the secondary wall middle layer, and the level of xylan was reduced. These findings suggest that lignification in *B. microphylla* reaction wood is identical to that of coniferous compression wood. These may represent general mechanisms of increasing lignin content in various reaction woods.

Keywords

Lignification, Laccase, *Buxus*, Reaction Wood, Compression Wood

1. Introduction

Woody plants develop a specialized secondary xylem known as reaction wood to enable formation of an ideal shape [1] [2]. In general, woody gymnosperms (conifers) develop reaction wood on the lower side of inclined stems and branches, which is known as compression wood [3] [4]. Compression wood generates compressive growth stress, which pushes the inclined stem and branches upward [5]. Compression wood has chemical and anatomical features different from normal wood; it shows eccentric growth on the lower side of inclined stems

and branches; the xylem is a darker brown than normal wood; its tracheids in the transverse plane are circular and have a larger microfibril angle and higher lignin content than those of normal wood; the tracheid cell wall lacks a secondary wall inner layer (S3), and has helical ribs on the luminal side; and so on [4] [6].

In this study, we focused on reaction wood of the genus *Buxus*. *Buxus* species are classified as angiosperms. Woody angiosperms generally develop reaction wood known as tension wood on the upper side of inclined stems and branches; tension wood is characterized by the presence of G fibers which develop a cellulose-rich cell wall layer (the G layer) with a very small microfibril angle. However, *Buxus* species form no tension wood, and instead develop compression-wood-like reaction wood on the lower side of inclined stems and branches [7], which generates compressive growth stress [8]. *Buxus* reaction wood is similar in chemical and anatomical features to compression wood [7] [8]; it shows eccentric growth on the lower side of inclined stems and branches; the xylem is darker brown than normal wood; the wood fiber and vessels in the transverse plane are circular; and the wood fiber has a large microfibril angle in the secondary wall, and an increased lignin content in the cell wall. Interestingly, the cell wall develops a highly lignified region in the outer portion of the secondary wall middle layer (S2) [9], which is a remarkable characteristic of coniferous compression wood and known as S2L.

We investigated whether the lignification mechanism of *Buxus* reaction wood is identical to that of compression wood by assessing the lignification process in both *Buxus* reaction wood and coniferous compression wood. Molecular biological approaches have been used in previous studies to assess the mechanism of formation of compression wood [10]-[15]. We demonstrated that: 1) expression of a laccase (*CoLac1*) in *Chamaecyparis obtusa* (a coniferous species) is very low in differentiating normal wood but very high in differentiating compression wood; 2) laccase activity is higher in the S2L region than in other parts of the S2 layer; 3) *CoLac1* is expressed at the onset of secondary wall thickening and is localized to the S2L region [16] [17]. Based on these results, we conclude that a specific laccase (*CoLac1*) is localized in the S2L region, which results in a high lignin concentration in the S2L region in *C. obtusa*. Other previous studies suggested that the difference in hemicellulose type and distribution in compression wood might be involved in lignification [18] [19]. Supposing the lignification mechanism of *Buxus* reaction wood to be identical to that of compression wood, this would be difficult to explain by the theory of convergent evolution. In this case, *Buxus* reaction wood also cannot be explained by this theory. In addition, our study provides fundamental information regarding compression wood formation and compressive growth stress generation, because the common mechanisms might be important for generating compressive growth stress.

In this study, we assessed the commonality of lignification by determining whether *Buxus* (*Buxus microphylla* var. *japonica*) possesses a specific laccase gene that is expressed specifically in differentiating reaction wood; whether *in situ* laccase activity in differentiating reaction wood is higher than that in normal wood, and if it is higher in the S2L region than the other S2 regions; and whether the distribution of hemicellulose (which is related to the lignin distribution) in the reaction wood cell wall is altered.

2. Materials and Methods

2.1. Plant Material

For cloning of genes involved in lignin synthesis, we used *Buxus microphylla* var. *japonica* grown at Nagoya University (~1.85 m tall). In mid-June, after bark had been removed from a branch, differentiating xylem, which is a darker brown, was harvested from the lower side of the branch using a chisel, and was used as the reaction wood sample. Differentiating xylem was harvested from the upper side of the branch, and was used as the normal wood sample. In the same way, differentiating reaction and normal woods were harvested from four other branches for quantitative reverse transcript polymerase chain reaction (qRT-PCR) analysis.

For evaluation of laccase activity and ultraviolet microscopy, we harvested xylem blocks including differentiating reaction wood from the lower side of branches. Xylem blocks including differentiating normal wood were harvested from the upper side of these branches. The blocks were divided into small blocks of several millimeters square using a razor blade. To analyze similar sections of differentiating xylem for evaluation of laccase activity and for ultra violet microscopy, the small blocks were divided into two pieces in the transverse plane.

Buxus microphylla (~120-cm height, 5-cm base diameter) grown with the stem tilted at ~30° to induce formation of reaction wood was used for immunolabeling. Another plant was grown with the stem vertical to induce formation of normal wood. Xylem blocks including mature reaction wood were harvested from the lower side of the stem. Xylem blocks including mature normal wood were harvested from the vertical stems. Blocks were di-

vided into small blocks of several millimeters square using a razor blade.

2.2. Gene Cloning

Total RNA was purified from the differentiating xylem, and cDNA was synthesized using Primescript (TaKaRa, Japan). The target gene was amplified using degenerate primers [16]. The *B. microphylla* translation initiation factor (*BmTIF*, AB762681) gene was also amplified as a reference gene. The amplification product was inserted into a plasmid for DNA sequencing. The similarity of the sequence compared to that of *Arabidopsis thaliana* was investigated using The Arabidopsis Information Resource (TAIR) BlastX program. New sequences were submitted to the DNA Data Bank of Japan.

2.3. qRT-PCR

To investigate transcript abundance in differentiating normal wood and reaction wood, we performed qRT-PCR analysis. We harvested four branches from a *B. microphylla* tree, and shaved off the differentiating xylem from both the lower and upper side of the stem using a chisel. The samples were immediately snap-frozen in liquid nitrogen, and the frozen differentiating xylem was stored at -80°C until RNA extraction. Total RNA was extracted from 50 mg of differentiating xylem using the RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's protocols, and treated with DNase I (TaKaRa, Japan) to remove contaminating genomic DNA. Absence of contaminating genomic DNA was confirmed by the failure of PCR amplification of the cyclophilin housekeeping gene (accession number, AB762680). The RNA concentration was estimated spectrophotometrically using Gene Quant (Amersham, Germany). cDNAs were synthesized at 37°C for 30 min with 500 ng of total RNA in a volume of 15 μL using PrimeScript RT Master Mix (Perfect Real Time; TaKaRa, Japan); the cDNAs were then diluted 3:10 with water.

Gene-specific primers were designed using the Primer 3 Plus program (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>); the sequences are shown in **Table 1**. Amplification was performed in triplicate using the StepOnePlus Real Time PCR System (Applied Biosystems, USA). The baseline and threshold cycles were determined automatically using the Step One software ver. 2.1 (Applied Biosystems, USA). The 20- μL reaction mixture consisted of 0.5 μL cDNA, 200 nM sense and anti-sense primers, and 10 μL POWER SYBR Green PCR Master Mix (Applied Biosystems, USA). The reaction conditions were as follows: 95°C for 10 min (95°C for 15 s, 58°C for 60 s) \times 40. To detect any additional products, after the melting curve was analyzed, we performed agarose gel electrophoresis and confirmed the absence of additional bands. The *BmTIF* gene was used as a reference gene; transcript abundance is expressed as the ratio of the target gene transcript to that of *BmTIF*.

2.4. Laccase Activity Staining

To analyze *in situ* laccase activity in differentiating xylem, we performed laccase activity staining using 3,3'-diaminobenzidine tetrahydrochloride (DAB) [17]. One of the twin blocks (see plant materials) was treated with catalase in sodium acetate-acetic acid buffer (pH 5.0) for 30 min to remove H_2O_2 , preventing peroxidase activity.

Table 1. Primer pairs used for qRT-PCR.

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')	Product size
<i>BmTIF</i>	AAGGAATCTGCTGCAATGG	ACAATTCAGCCTGCACAAG	116 bp
<i>BmPAL1</i>	ATCCCTGCAGTGGCACTTAC	ATGCAATTCTCGCACTCTCC	180 bp
<i>BmCAD1</i>	GTGACGGTTTACAGCCCACT	GACCCAGAATTCCTCTCTCA	70 bp
<i>BmLac1</i>	CGCTTTAGCAGCTCAACTCC	GCTTTGGCAGTTCTTCTTGG	181 bp
<i>BmLac2</i>	CGTCTTCCACTCTTCAAACCAAC	CTTGGCAGGTTTGGTTCACTG	190 bp
<i>BmLac3</i>	CAGCCATTACTCTCACCAACAC	AACAACACGGCTGCCATTAG	194 bp
<i>BmLac4</i>	ATCGTCCCATTCTCTTCCT	TGAAGAAGAAACGCCTGTCC	182 bp
<i>BmLac5</i>	CAAGAACGGCGTGTCCATA	GTGTTACTGGTGGGCAGTT	183 bp

The blocks were immersed overnight in laccase activity staining buffer (0.5 mM DAB, 100 µg/mL catalase, in sodium acetate-acetic acid buffer [pH 5.0]) at 30°C. After activity staining, the blocks were fixed with 3% glutaraldehyde in phosphate buffer (pH 7.0), and embedded in LR White resin. Two-micrometer-thick sections were cut from the top of the transverse plane of the blocks, and observed under a light microscope. In addition, the same section was observed under a polarizing microscope to visualize cells that showed secondary wall thickening [20] [21].

2.5. Ultraviolet Microscopic Analysis

The other of the twin blocks (see plant materials) was fixed with 3% glutaraldehyde in phosphate buffer (pH 7.0), and embedded in epoxy resin. One-micrometer-thick sections were cut from the block and subjected to ultraviolet microscopy. Ultraviolet photomicrographs were taken at a wavelength of 280 nm using an ultraviolet microscopic spectrophotometer (MPM800, Carl Zeiss, Germany) equipped with an ultraviolet-sensitive CCD camera (CM-140 GE-UV, JAI, Denmark).

In addition, the same section was observed under a polarizing microscope to visualize cells that showed secondary wall thickening [20] [21].

2.6. Immunolabeling

Stem blocks of several millimeters square were frozen rapidly by immersion in liquid chlorofluorocarbon cooled with liquid nitrogen. The blocks were transferred into acetone containing 0.7% glutaraldehyde cooled to -80°C, and incubated for at least 2 d for freeze substitution. The blocks were incubated at -20°C for 2 h and then at 4°C overnight. After the blocks had been warmed to room temperature, they were immediately washed in acetone three times for 10 min each, and then embedded in LR White resin.

One-micrometer-thick sections were cut from the embedded blocks, and used for immunolabeling. The sections were first immersed in 50 mM glycine/phosphate-buffered saline (PBS) for 15 min, and then washed three times for 5 min each in PBS. Sections were then immersed in 0.8% (w/v) bovine serum albumin/PBS for 1 h at room temperature to block nonspecific binding, and washed three times for 5 min each in PBS. Sections were incubated for 2 days at 4°C with LM5, LM10, or LM22 primary antibody (Plant Probes) diluted 50-fold in PBS, and washed three times for 10 min each in PBS, followed by incubation for 1 h at 35°C with the secondary antibody (Alexa Fluor 647 Goat Anti-Rat IgG (H + L) Antibody, Life Technologies) diluted 100-fold in PBS containing 0.1% Tween 20 (PBS-T). During and after this treatment, the sections were protected from light. The sections were washed three times for 10 min each in PBS-T, and developed with Fluoromount/Plus (Diagnostic BioSystems). Alexa647 fluorescence was visualized by confocal laser microscopy (FLUOVIEW FV10i, Olympus).

3. Results

3.1. Similarity of Cloned *B. microphylla* Genes to Genes in the *Arabidopsis thaliana* Genome

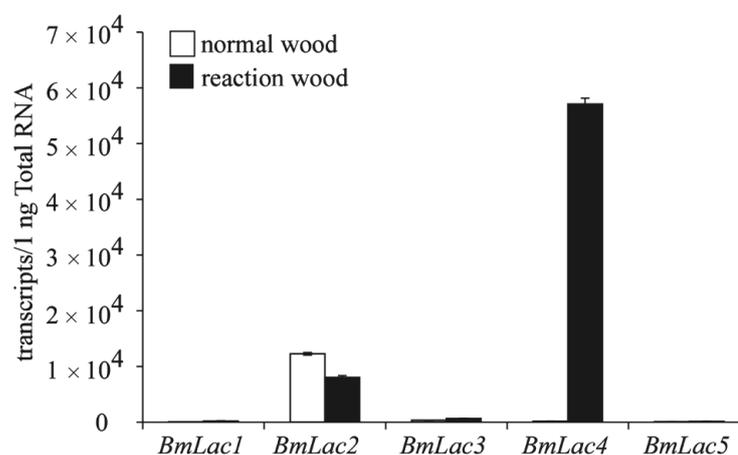
To estimate the functions of cloned *B. microphylla* genes, we investigated their sequence similarity to genes in the *A. thaliana* genome (Table 2). The cDNA sequences demonstrated similarity to genes encoding enzymes involved in lignin precursor (monolignol) synthesis, laccase, and TIF SUI1 family protein (63% - 93% identities; E-value, < E-5). The cloned sequences are available under the following accession numbers: *BmLac1* (AB762671), *BmLac2* (AB762672), *BmLac3* (AB762673), *BmLac4* (AB762674), *BmLac5* (AB762675), *BmPAL1* (AB762676), *BmCAD1* (AB762677), and *BmTIF* (AB762681).

3.2. Comparison of Transcript Abundance between Differentiating Reaction and Normal Woods

To compare the gene expression levels in differentiating reaction wood with those in differentiating normal wood, we performed qRT-PCR. Among laccase genes (*BmLac1-5*), *BmLac2* and *BmLac4* were expressed mainly in differentiating normal and reaction woods (Figure 1). Further analysis, which was more precise due to the use of four biological replicates, demonstrated a difference in transcript abundance between differentiating normal

Table 2. Similarity of cloned *B. microphylla* genes to genes in the *Arabidopsis thaliana* genome.

Gene name	Accession	Identity	E-value	Most similar gene	Locus
<i>BmTIF</i>	AB762681	93%	5E-45	Translation initiation factor SU11 family protein	AT1G54290
<i>BmPAL</i>	AB762676	78%	6E-70	PAL2	AT3G53260
<i>BmCAD</i>	AB762677	71%	E-44	CAD4	AT3G19450
<i>BmLac1</i>	AB762671	64%	E-132	Laccase 6	AT2G46570
<i>BmLac2</i>	AB762672	69%	E-143	Laccase 17	AT5G60020
<i>BmLac3</i>	AB762673	69%	E-134	IRX12, LAC4	AT2G38080
<i>BmLac4</i>	AB762674	71%	E-144	Laccase 17	AT5G60020
<i>BmLac5</i>	AB762675	67%	E-134	Laccase 5	AT2G40370

**Figure 1.** Expression of five laccase genes in differentiating xylem. Transcript abundance is the copy number of the tested gene per 1 ng of total RNA. Values are means \pm SD of three independent measurements for each gene.

and reaction woods (**Figure 2**). The transcript abundance of *BmPAL* and *BmCAD* was higher in differentiating reaction wood (**Figure 2**). In contrast, the transcript abundance of *BmLac2* was not different between the two types of wood. The transcript abundance of *BmLac4* was very low in differentiating normal wood, but very high in differentiating reaction wood.

3.3. Laccase Activity Staining and Lignin Deposition in Differentiating Reaction Wood

To investigate lignin deposition in differentiating reaction wood, we performed ultraviolet microscopy. In the micrographs, lignin appears dark; thus darker regions indicate higher lignin concentrations [22]–[24]. Asterisks indicate cells that have begun secondary wall thickening (confirmed by polarizing microscopy [20] [21]). In addition, to investigate *in situ* laccase activity in differentiating reaction wood, we performed laccase activity staining (**Figure 3(A)**, **Figure 3(B)**, **Figure 3(D)**, **Figure 3(F)**), in which a brown color shows laccase activity [17]. The negative control was autoclaved to inactivate laccase activity; no staining was observed in sections prepared in this manner (**Figure 3(A)** & **Figure 3(D)**).

Laccase activity was initiated in the compound middle lamella and cell wall layers of secondary walls in all regions prior to the onset of lignin deposition, as described below. In differentiating reaction wood, staining was observed in the compound middle lamella before the onset of secondary wall thickening (**Figure 3(B): c, e**), while lignin deposition was not observed (**Figure 3(C): c, e**). Staining was observed in the secondary wall outer layer (S1) immediately after the onset of the secondary wall thickening (**Figure 3(B), asterisk**), when lignin was deposited in the compound middle lamella (**Figure 3(B): asterisk**). Staining appeared in the secondary wall middle layer (S2) during secondary wall thickening (**Figure 3(B), s**), when lignin was being deposited in the secondary wall (**Figure 3(C): s**). The staining in the cell wall was reduced with maturation after disappearance

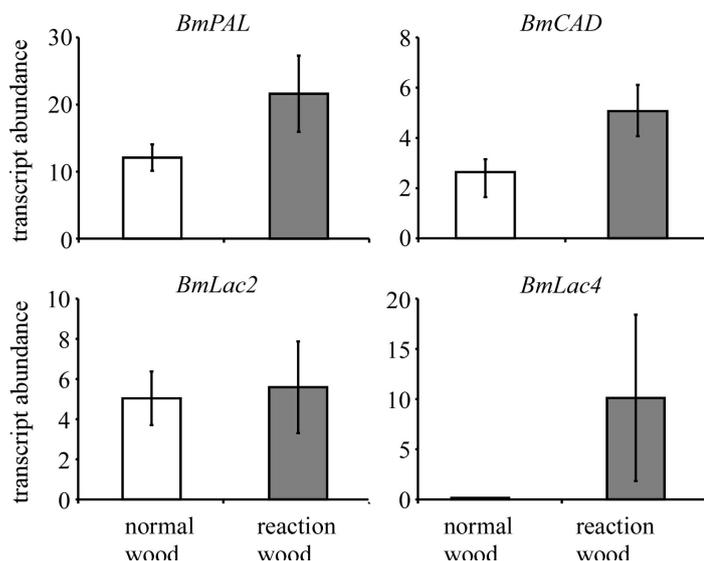


Figure 2. PAL, CAD, and laccase expression in differentiating xylem. Transcript abundance is the ratio of the copy number of the tested gene to that of a reference gene (*BmTIF*). Values are means \pm SD of four biological replicates for each gene.

of protoplasm (**Figure 3(B): mx**), when lignin deposition was completed (**Figure 3(C): mx**). In mature cells, the outer region of S2 showed a higher lignin concentration than the inner region in mature cells (**Figure 3(C): mx**).

In differentiating normal wood, staining was observed in the compound middle lamella before the onset of secondary wall thickening (**Figure 3(E): c, e**), when lignin deposition was not observed (**Figure 3(F): c, e**). Staining was observed in the secondary wall immediately after the onset of secondary wall thickening (**Figure 3(E): asterisk**), when lignin was being deposited in the secondary wall (**Figure 3(F): asterisk**). Staining was evident in the secondary wall middle layer (S2) during secondary wall thickening (**Figure 3(B), s**), when lignin was being deposited in newly formed cell walls (**Figure 3(C): s**). The staining in the cell wall was reduced with maturation after disappearance of protoplasm (**Figure 3(E): mx**), when lignin deposition was completed (**Figure 3(F): mx**). During secondary wall thickening of reaction wood, the cell wall showed higher laccase activity in the secondary wall than that of normal wood (**Figure 3(B), E: s**). During secondary wall thickening of normal wood, the cell wall showed higher laccase activity in the innermost portion of the cell wall compared to other parts of the cell wall (**Figure 3(B) & Figure 3(E): s**).

3.4. Distribution of Hemicellulose in Reaction Wood

To investigate hemicellulose distribution in the cell wall, we performed immunolabeling using LM5, LM10, and LM21 antibodies, which are specific for galactan, xylan, and mannan, respectively.

In mature reaction wood, LM5 labeling was observed not in S1, but in the outer part of S2 (**Figure 4(A) & Figure 4(C)**). In mature normal wood, little LM5 label was observed around the primary wall (**Figure 4(B) & Figure 4(D)**).

In mature reaction wood, LM10 was observed in S1 and S2, but the labeling density was low in the outer part of S2 (**Figure 4(E) & Figure 4(G)**). In mature normal wood, LM10 labeling was observed in S1 and S2, and the labeling density in S1 was high (**Figure 4(F) & Figure 4(H)**).

In both mature normal and reaction wood, LM21 was observed in S2 (**Figures 4(I)-(L)**); however, the labeling density was low in the central part of S2 in mature reaction wood (**Figure 4(J) & Figure 4(L)**).

4. Discussion

4.1. Differential Expression of Lignin Synthesis Genes between Reaction and Normal Wood

Among the laccases of *B. microphylla* (*BmLac1-5*), *BmLac2* and *BmLac4* were expressed mainly in differentiating

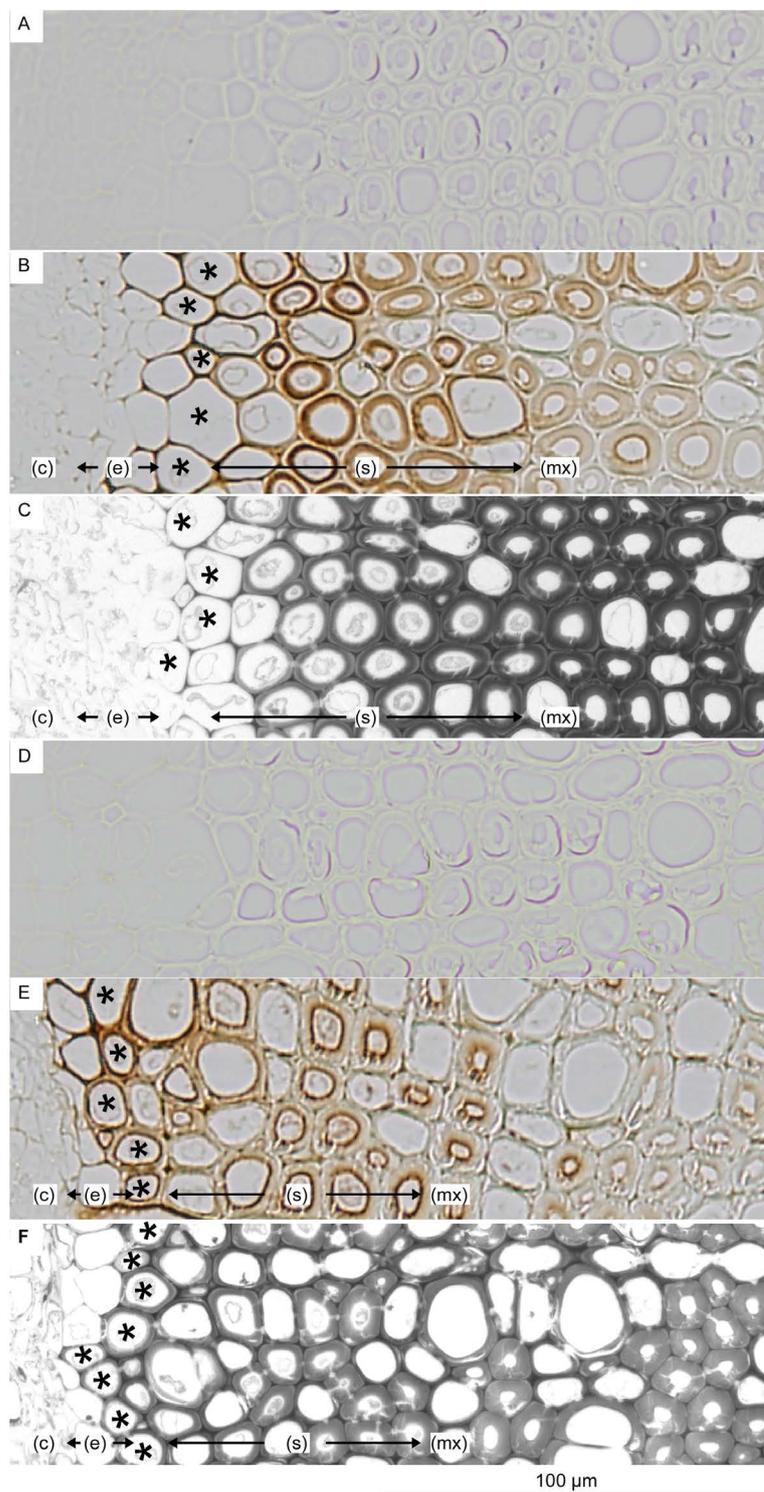


Figure 3. Localization of laccase activity and lignin deposition in differentiating xylem, and their relationship. (A) Reaction wood section autoclaved and stained for laccase activity. (B) Reaction wood section stained for laccase activity. (C) Lignin deposition in differentiating reaction wood section. (D) Normal wood section autoclaved and stained for laccase activity. (E) Normal wood section stained for laccase activity. (F) Lignin deposition in a differentiating normal wood section. In each image, the cambium is on the left, and the pith is on the right. Asterisks indicate cells that have begun secondary wall thickening (confirmed by polarizing microscopy [20] [21]). (c) cambial zone; (e) expansion zone, cells before onset of secondary wall thickening; (s) cells during secondary wall formation; (mx) mature cells.

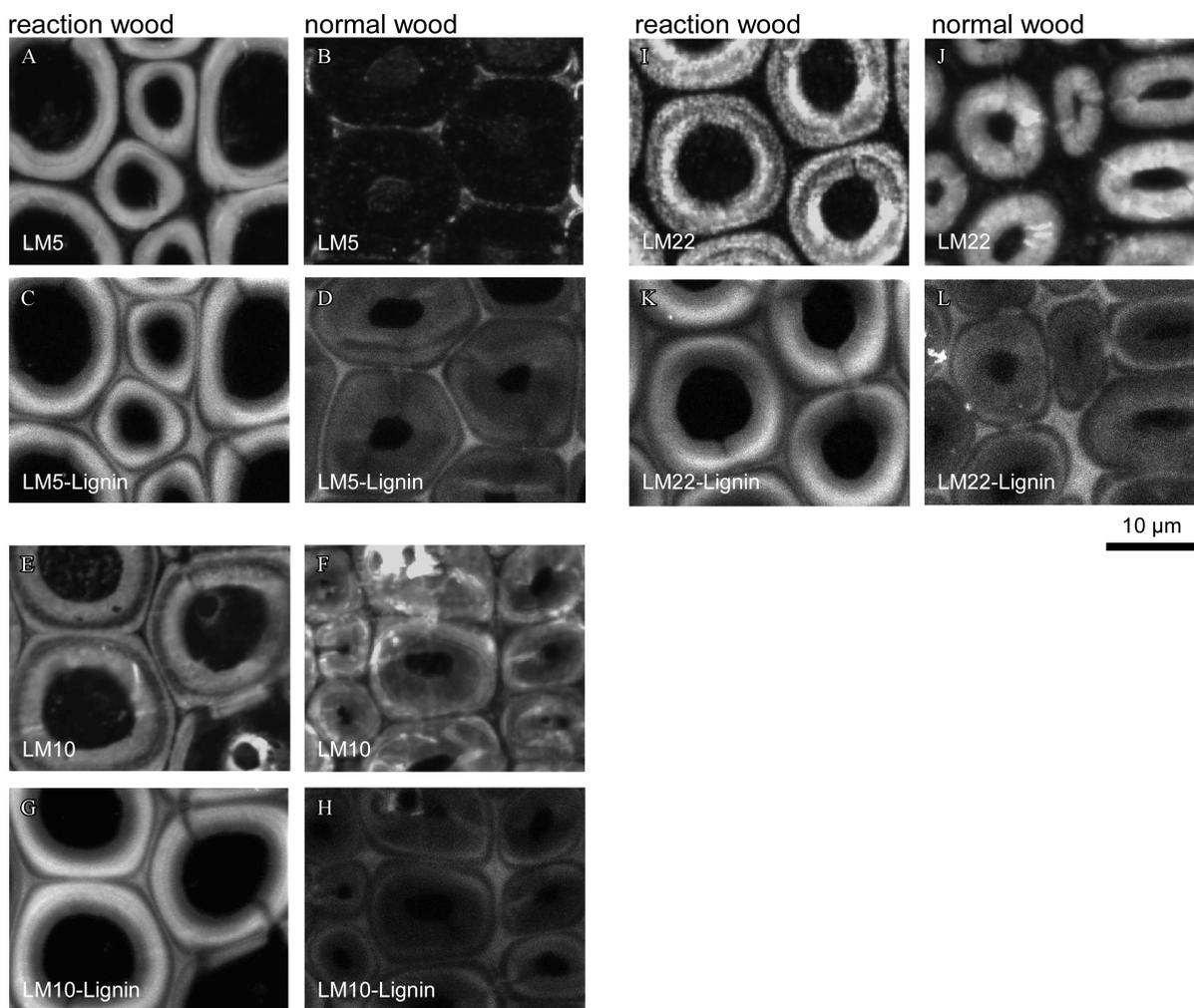


Figure 4. Immunolocalization of hemicelluloses in mature xylem. (A), (B) LM5 labeling in reaction and normal woods. (C), (D) Lignin distribution in A and B. (E), (F) LM10 labeling in reaction and normal woods. (G), (H) Lignin distribution in (E) and (F). (I), (J) LM22 labeling in reaction and normal woods. (K), (L) Lignin distribution in I and J.

normal and reaction wood (**Figure 1**). This result suggested that laccases encoded by *BmLac2* and *BmLac4* function in differentiation into normal and reaction woods. A more-detailed analysis demonstrated that *BmLac2* expression is similar between normal and compression woods, but *BmLac4* expression is considerably higher in differentiating reaction wood than in differentiating normal wood (**Figure 2**). This result demonstrated that *B. microphylla* possesses a reaction-wood-specific laccase gene that is expressed during differentiation. In several coniferous species, laccase RNAs or proteins are upregulated in differentiating compression wood [12]-[15]. Moreover, a compression-wood-specific laccase was found in *C. obtusa* and *Pinusradiata* [16] [25]. Thus, development of a specific laccase is common to *B. microphylla* and coniferous species.

Expression of *BmPAL* and *BmCAD* was higher in differentiating reaction wood than in differentiating normal wood (**Figure 2**). This suggests that differentiating reaction wood supplies a greater quantity of monolignol to increase lignin content. In coniferous species, the expression of genes involved in monolignol synthesis is upregulated in differentiating compression wood compared to that in normal wood [10] [11] [16]. Thus, upregulation of monolignol synthesis genes is common to *B. microphylla* and coniferous species.

4.2. Relationship of Laccase Activity to Lignin Deposition and Lignin Distribution in the Cell Wall in Reaction Wood

In differentiating reaction wood, lignin deposition was not observed in any region before secondary wall thick-

ening (**Figure 3(C): c, e**). At the onset of secondary wall thickening, lignin began to be deposited in compound middle lamella (**Figure 3(C): asterisk**). During secondary wall thickening, lignin was being deposited to form the secondary wall (**Figure 3(C): s**). This lignin deposition process seems identical to that of compression wood of *Cryptomeria japonica* [20]. However, lignification in compound middle lamella is the exception; lignin is not deposited in the cell corner (intercellular space) in coniferous compression wood [4], while lignin was deposited in this region in *Buxus* reaction wood (**Figure 3(C): mx**).

Lignin deposition was initiated after laccase activity appeared in the compound middle lamella, S1, and S2 in differentiating reaction wood (**Figure 3(B) & Figure 3(C)**), suggesting that laccase functions in lignification during differentiation into reaction wood in *B. microphylla*. We found that, in *C. obtusa*, laccase activity appeared prior to lignification in all cell wall layers in differentiating normal and compression wood [17]. This previous report coincides with the case of *B. microphylla*. Thus, participation of laccase oxidization in reaction wood lignification is a common feature of *Buxus* and coniferous species.

In differentiating reaction wood, laccase activity during lignification in the secondary wall was higher compared to that in normal wood (**Figure 3(B) & Figure 3(F): s**). Our qRT-PCR analysis of a laccase gene (**Figure 2: BmLac4**) indicated that laccase activity in the reaction wood cell wall was higher than that in normal wood due to *BmLac4* expression, as demonstrated by laccase activity staining (**Figure 3(B) & Figure 3(E): s**). The activity staining suggested that the higher laccase activity in the S2 layer is involved in the increase in lignin content in this region of reaction wood. In differentiating compression wood in *C. obtusa*, laccase activity is higher in the outer region of the S2 layer within the secondary wall, which results in a high lignin concentration in the S2L region [17]. Thus, the relationship between laccase activity intensity and lignin deposition in the secondary wall is common to *B. microphylla* and coniferous species. However, there is a slight difference; laccase activity was high in a broad range of S2 in differentiating reaction wood of *B. microphylla* (**Figure 3(B): s**). S2L in *B. microphylla* reaction wood has a mild gradient of lignin concentration in the S2 layer; the S2L region occupies a broad region of the S2 layer (**Figure 3(C): mx**). Thus, laccase activity coincides with the lignin distribution in the secondary wall in *Buxus* reaction wood.

4.3. Hemicellulose Distribution in the Cell Wall of Reaction Wood

LM5, LM10, and LM22 antibodies label galactan [(1-4)- β -D-galactosyl residues], xylan [(1-4)- β -D-xylosyl residues of unsubstituted and relatively low-substituted xylans], and mannan [β -linked mannan polysaccharides/ β -(1 \rightarrow 4)-manno-oligosaccharides], respectively.

LM5 labeling was weak around the compound middle lamella in normal wood fibers (**Figure 3(B) & Figure 3(D)**). This indicates that galactan is localized to the compound middle lamella in normal wood fibers. LM5 labeling was not detected in normal wood xylem of poplar (*Populustrichocarpa* Torr. & A. Gray \times *P. koreana*-Rehder) [26], and was sparse in normal wood cell walls of willow (*Salix* spp.) [27]. In *P. radiata*, LM5 labeling was localized to the primary wall (around the compound middle lamella) [18]. Thus, our result coincides with that in conifer species. LM5 labeling was evident in the outer region of the S2 layer in reaction wood fibers in *B. microphylla* (**Figure 4(A) & Figure 4(C)**). This result indicates that galactan is localized to the outer region of the S2 layer in reaction wood fibers. Coniferous compression wood is rich in galactan, and is localized to the outer region of the S2 layer in the cell wall [18] [19]. Tension wood is also rich in galactan, and is localized to the G layer [26] [27]. Although *Buxus* reaction wood also contains abundant galactan, like tension and compression woods, the distribution is similar to that in compression wood. Galactan is generally not localized to the secondary wall in woody angiosperms, but was detected in the secondary wall in *B. microphylla* reaction wood. This result suggests that galactan plays a key role in reaction wood. Moreover, this galactan distribution in the reaction wood cell wall is common to both *B. microphylla* and coniferous species. Thus, the function of galactan deposition is likely also common among them, and might be crucial for generating compressive growth stress.

LM10 labeling was localized to the entire secondary wall in normal wood fibers, and was abundant in the S1 layer (**Figure 4(F) & Figure 4(H)**). Xylan was localized to the entire secondary wall, and was abundant in the S1 layer. In poplar [28], *Zinnia elegans* [29], and *Fagus crenata* [30], xylan is localized to the secondary wall. Thus, xylan localization in *B. microphylla* normal wood is similar to that in other angiosperms. In contrast, in reaction wood fibers, LM10 labeling was localized to the S1 layer and inner region of the S2 layer, and was sparse in the outer region of S2 (**Figure 4(F) & Figure 4(H)**). Thus, xylan is localized to these regions in the reaction wood cell wall. In compression wood of *P. radiata*, xylan is localized to the compound middle lamella,

S1 layer, and inner region of the S2 layer, but not the outer part of the S2 layer [18]. Xylan localization is common between *B. microphylla* and *P. radiata* in that the S2L region contains little xylan. This commonality suggests that the reduction in the xylan content in the outer region of the S2 layer might be involved in S2L formation. LM11, which also labels xylan, was used to investigate xylan localization. The distribution of LM11 labeling was similar to that of LM10 (data not shown). Xylan in woody angiosperms is *O*-acetyl-4-*O*-methylglucuronoxylan; in the case of coniferous species, xylan is arabino-4-*O*-methylglucuronoxylans [31]. LM10 binds the former, and LM11 labels both the former and latter. The lack of a difference in the results of LM10 and LM11 labeling suggests that *B. microphylla* xylem likely does not contain arabino-4-*O*-methylglucuronoxylan, or that the two types of xylan have an identical localization.

LM22 labeling was localized to the S2 layer in normal and reaction woods. Xylem of woody angiosperms (hard wood) is mainly *O*-acetyl-4-*O*-methylglucuronoxylan with little glucomannan [31]; LM 22 labels glucomannan. Thus, glucomannan is likely localized to the S2 layer in normal and reaction woods. In reaction wood, however, labeling was slight in the central region of the S2 layer; LM22 labeling was slight on the inner side of S2L (Figure 4(I) & Figure 4(K)). The glucomannan content of compression wood is half that of normal wood [32]. In *P. radiata*, the glucomannan content is lower in the outer portion of S2 in compression wood than in normal wood [18]. The reduction in the amount of glucomannan in *B. microphylla* reaction wood is similar to that in compression wood, but the distribution in the cell wall is different. LM21, which also labels mannan, was used to investigate mannan localization. The distribution of LM21 labeling was similar to that of LM22 (data not shown). Donaldson and Knox (2012) [18] reported that LM21 and 22 labeled lignified cells within *P. radiata* xylem, but the labeling was localized to relatively low lignification regions; e.g., the secondary wall of normal wood and inner portion of S2 of compression wood. Based on this result, the authors assumed that glucomannan functions in limiting lignification. The localization of glucomannan did not coincide with the S2L region. Thus, the function of mannan in *B. microphylla* reaction wood may be slightly different from that in compression wood.

4.4. General Discussion

1) Common points among *B. microphylla* and coniferous species: In this study, we investigated the factors involved in the increased lignin content in *B. microphylla* reaction wood; e.g., expression of genes involved in lignin synthesis, laccase activity localization, and hemicellulose distribution in the cell wall. The majority of the results indicated commonalities between *B. microphylla* reaction wood and compression wood. These findings suggest that lignification in *B. microphylla* reaction wood is basically identical to that in coniferous compression wood.

2) Development of reaction woods during plant evolution: *Buxus* reaction wood resembles coniferous compression wood in anatomy and functionality, likely due to convergent evolution. However, the factors involved in the increased lignin content are almost identical, which cannot be explained by convergent evolution. Thus, development of *Buxus* reaction wood also cannot be explained by this theory. Rather than convergent evolution, our findings suggest that *Buxus* reaction wood has a common origin with compression wood. The genus *Buxus* is classified as a eudicot, and many woody species included in this clade form typical tension wood. Therefore, we assume that after angiosperms diverged from gymnosperms, the ancestor of *Buxus* might have had a latent ability to produce compression wood, which was subsequently re-expressed due to unknown factors.

3) Compressive growth stress and generation of *Buxus* reaction wood: *Buxus* reaction wood generates compressive growth stress [8]. The lignin swelling hypothesis [33] suggests that this stress is due to the combination of a large microfibril angle and high lignin content. Yamamoto *et al.* [34] reported that microfibril angle and lignin content are correlated with compressive growth stress in coniferous species. Moreover, Okuyama *et al.* [34] confirmed that the lignin concentration in S2L is correlated positively with compressive growth stress. The lignin swelling hypothesis is applicable to *Buxus* reaction wood because it has an elevated lignin content in the secondary wall and a large microfibril angle [9]. Our results suggest that the lignification process in *B. microphylla* reaction wood is basically identical to that in compression wood. Thus, the mechanism of generation of compressive growth stress might be identical between the two types of wood.

5. Conclusion

We found common points between coniferous compression wood and compression-wood-like reaction wood of

an angiosperm species: 1) Several lignin synthesis genes were up-regulated in differentiating reaction wood; 2) *B. microphylla* possesses a specific laccase gene which is expressed specifically in differentiating reaction wood (*BmLac4*); 3) laccase activity localization was closely related to lignification of reaction wood, and laccase activity was high in the S2 layer; 4) in the reaction wood cell wall, galactan appeared in the S2L region, and the amount of xylan was reduced. These findings suggest that lignification in *B. microphylla* reaction wood is basically identical to that in coniferous compression wood. These common points may represent mechanisms underlying the increased lignin content in reaction woods.

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