Non-Destructive Approaches to Identify the Ultrastructure of Lignified Ginkgo Cell Walls

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ABSTRACT

Information on the lignification mechanism and 3D ultrastructural assembly of lignin and polysaccharides in plant cell walls is essential for better understanding of physical, chemical and biological properties of lignified plant tissue. *Ginkgo biloba* (ginkgo) is one of the most suitable plant species for lignification studies because it is the oldest still living trees on earth appeared in the early stage of evolution of trees, and it retains primitive characteristic features of lignified plant cell walls. Because conventional destructive analyses can provide only limited information on the 3D assembly of cellulose, pectin, hemicelluloses and lignin in cell walls, we examined the lignification process in ginkgo xylem by various non-destructive approaches: radioisotope tracer methods using $^3$H and $^{14}$C combined with a scintillation counting technique and microautoradiography; a stable isotope tracer method using $^2$H, $^{13}$C, combined with the techniques of mass spectrometry and differential NMR spectrometry; observation of lignifying cell wall under field-emission scanning electron microscopy combined with mild selective removal of lignin or polysaccharides from the cell walls retaining their morphological features. The combined results provided useful information on the ultrastructure of lignified cell walls not only in ginkgo xylem but also in xylems of most coniferous trees.

Keywords: $^{13}$C-NMR, isotope labeling, FE-SEM, lignin, microautoradiography, polysaccharide

Abbreviations: CC, cell corner; CMFs, cellulose microfibrils; $^{13}$C-NMR, difference NMR spectrum between spectra of $^{13}$C-enriched and unenriched compounds; FE-SEM, field emission scanning electron microscopy; GC-MS, gas chromatograph-mass spectrometry; LSC, liquid scintillation counting; MAR, microautoradiography; ML, middle lamella; MWL, milled wood lignin; NMR, nuclear magnetic resonance spectroscopy; PW, primary wall; SW, secondary wall; S1, S2 and S3, outer, middle and inner layer of secondary wall; TEM, transmission electron microscopy; Tof-SIMS, time-of-flight secondary ion mass spectrometry; UV, ultraviolet

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INTRODUCTION

In the course of plant evolution, some species acquired a metabolic pathway to produce lignin, and their cell walls were endowed with characteristic physical, chemical and biological properties as a result of lignification by: making cell walls hydrophobic so that aqueous nutrients can be conducted through the cells; making cell walls and tissues mechanically strong so that plants can grow higher and can extend branches to receive more sunlight; making cell walls resistant to biological attacks by microorganisms and animals; protecting living cells from physico-chemical damage by sun light. Those special properties of lignified cell walls enabled trees to grow and survive for many years. The largest portion of organic substances on earth – including humic substances in the soil – originated from the lignified cell walls of woody plants.

Those special properties are mainly derived from the unique property of lignin and its assembly with cellulose, hemicelluloses and pectin in the cell wall. Therefore, information on the macromolecular structure of lignin and polysaccharides, and their three dimensional (3D) assembly in the cell wall, the ultrastructure, is essential for a better understanding of the special properties of lignified plant tissue. However, we meet many difficulties in obtaining the necessary information since the structure of lignin and the ultrastructure of cell walls are heterogeneous in many respects. This review deals with the study on the formation and ultrastructure of cell walls in ginkgo xylem by the use of non-destructive approaches in order to overcome these difficulties.

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HETEROGENEITY IN FORMATION AND ULTRASTRUCTURE OF LIGNIFIED PLANT CELL WALLS

In some plant tissues or in some microorganisms, cellulose, hemicelluloses or pectin is formed without subsequent lignification. On the other hand, lignin is never formed in the absence of those polysaccharides in any plant tissue. Formation of lignin occurs only on the preformed network of polysaccharides, which regulates the formation and structure of the lignin macromolecule. This network differs greatly in relative amounts and mode of association of cellulose microfibrils (CMFs) with pectin and hemicelluloses depending on the stage of cell wall differentiation. In addition, the structure of lignin varies depending on the age of the cell because lignin is a kind of secondary metabolite. Thus, the 3D assembly of the cell wall polymers is a complex process regulated by the age-dependent formation of polysaccharide networks and lignin macromolecules (Terashima 1990; Terashima et al. 1993, 1998, Terashima 2000, 2001; Terashima et al. 2004).

In the first stage of cell wall formation in differentiating xylem of gymnosperms, the major network of polysaccharides in the middle lamella (ML) and cell corner (CC) of adjacent cells is composed of a large amount of pectic substances associated with CMFs that provides much space for succeeding deposition of a large amount of lignin. Both sides of this ML, primary wall (PW), outer, middle and inner layers of the secondary wall (S1, S2 and S3 of SW) are successively formed by laying specific networks of CMFs and hemicelluloses followed by deposition of lignin (Terashima and Fukushima 1988). The lignin deposition proceeds by irreversible polymerization of monolignols (Fig. 1A-C) to form globular macromolecules (modules) of slightly different sizes depending on the morphological region of the cell wall. These lignin modules fix the network of polysaccharides by formation of chemical bonds with polysaccharides, and they finally fill up the spaces in the network (Terashima et al. 2004; Terashima and Yoshida 2005). Thus, the ultrastructure of the lignified plant cell wall is not homogeneous in various respects, in structures of the preformed polysaccharide network, in the structure of lignin modules, in morphological regions of the cell wall, and in the kind of cell. The ultrastructure varies also depending on the kind of tissue and plant species. In addition, an abnormal wood xylem is formed on a leaning stem or a branch of a tree as a result of the reaction to gravity, and the ultrastructure of the reaction of a wood cell wall is significantly different from that of a normal wood cell wall. Those heterogeneities are the most significant features that endowed special properties to the lignified plant cell wall.

NECESSARY INFORMATION FOR ELUCIDATION OF ULTRASTRUCTURE

The heterogeneous structure of the lignin macromolecule and heterogeneous assembly of lignin and polysaccharides in the cell wall can be elucidated based on the following information:

(a) Total frequencies of different types of monomer units (p-hydroxyphenylpropane unit: I, guaiacylpropane unit: J and syringylpropane unit: K in Fig. 2) in lignin;
(b) Localization of I, J, K within a lignin macromolecule and in different cell wall layers (CC, ML, PW and SW);
(c) Total frequencies of inter-unit bonds and functional groups I-Y in lignin;
(d) Localization of I-Y in CC, ML, PW and SW;
(e) Localization and frequency of lignin-polysaccharide bonds, Ca-OR in Q within a lignin macromolecules and in CC, ML, PW and SW;
(f) Stereochemistry of side chain carbons of C6-C3 units, Q threo/erythro, and S, V, etc.;
(g) Higher order structure, shape and size of lignin macromolecule (module);
(h) Macromolecular structure of CMFs, pectin and hemicelluloses;

### Fig. 1 Precursors in biosynthesis of lignin and polysaccharides, and enzyme inhibitors useful for labeling with isotopes.

<table>
<thead>
<tr>
<th>Monolignols</th>
<th>Monolignol glucosides</th>
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<tr>
<td>A: R''=R''=H; p-Coumaryl alcohol</td>
<td>A-Glc: R''=R''=H; p-Glucoconaryl alcohol</td>
</tr>
<tr>
<td>B: R'=OCH3, R''=H; Coniferyl alcohol</td>
<td>B-Glc: R''=OCH3, R''=H; Coniferin</td>
</tr>
<tr>
<td>C: R'=R''=OCH3; Sinapyl alcohol</td>
<td>C-Glc: R'=R''=OCH3; Syringin</td>
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### Monolignol glucosides

- **A**
  - R''=R''=H
  - p-Glucoconaryl alcohol
- **B**
  - R'=OCH3, R''=H
  - Coniferin
- **C**
  - R'=R''=OCH3
  - Syringin

### Diagrams

- **D**
  - D-Glucuronic acid-[6-14C]
- **E**
  - myo-Inositol-[2-3H]
- **F**
  - L-α-Aminoxyβ-phenyl-propionic acid
- **G**
  - 2-Deoxy-d-glucose
Ultrastructure of ginkgo cell walls. Noritsugu Terashima

(i) 3D assembly of lignin, hemicellulose and cellulose in the cell walls;
(j) Variation of information (a)–(i) among different kinds of cells;
(k) Variation of information (a)–(j) between reaction wood cell and normal wood cell;
(l) Variation of information (a)–(k) among different plant species.

Because conventional destructive analyses of purified lignin or component polysaccharides obtained by separation from the cell wall can provide only information of (a), (c), (f) and (h) above, non-destructive methods must be used to obtain the information (b), (d), (e), (g), and (i). The difficulty in getting information (j), (k) and (l) can be circumvented by the use of differentiating xylem of ginkgo (Ginkgo biloba).

ADVANTAGES OF USING GINKGO FOR LIGNIFICATION STUDY

The use of ginkgo possesses many advantages over the use of other plant species in getting the above necessary formation:

(1) Ginkgo biloba is one of the oldest still living trees on earth, and it retains primitive characteristic features of gymnospermous trees that appeared in the early stage of their evolution.
(2) Except for the irregularity in size and shape of tracheids, the anatomical features of vascular tissue and xylem tissue of ginkgo are similar to those of representative gymnospermous tree, and conifers such as pine and spruce (Timell 1986). In the xylem part of those conifers, more than 95% of cells are tracheids. On the other hand, the xylem of angiospermous trees such as magnolia, beech and poplar contains a variety of cell types, vessels, fibers, ray cells and others. And their cell wall ultrastructure and lignin structure are significantly different. Therefore, the theoretical understanding of the properties of those tree xylem based on the different types of cells is more complex than that of ginkgo xylem composed mostly of tracheids. This means that information (j) can be simplified to a large extent by the use of ginkgo.
(3) A leaning stem or branch of conifers such as pine or spruce forms abnormally thick-walled compression wood cells, and the ultrastructure of the cell wall is significantly different from those of the normal wood cell. Ginkgo trees are less affected by gravity, and do not form representative compression wood. This also means that the information (k) can be almost neglected in the case of ginkgo.
When a tracer method is applied, an aqueous solution of Ginkgo lignin is a representative gymnosperm lignin. Ginkgo lignin is less difficult than that on magnolia, A-Glc, B-Glc, and C-Glc (Kawai et al. 2006). The activity of glucosidase specific to coniferin (B-Glc) was highest in the season when lignification proceeds most actively (Dharmawardhana et al. 1995, 1998). These findings strongly support the validity of the isotope-labeling method that the labeled precursors were prepared by replacing specific hydrogen or carbon with radioisotope, $^3$H or $^{14}$C (Terashima 1989; Terashima et al. 1986) or stable isotope, $^3$H or $^{13}$C can provide information, which cannot be obtained by any other methods. And it is a remarkable advantage that we can trace the labeled isotope even if the structure of the labeled component is converted to any other structure by chemical or biological treatments (Imai et al. 1997; Parkás et al. 2001, 2004a, 2004b).

The suitable precursors for labeling, detection and available information useful for ultrastructural study of ginkgo cell walls are summarized in Table 1 and Figs. 1 and 2.

Selective labeling of lignin

The monolignol glucosides accumulate in the cambial sap of all gymnosperms and in some angiosperms such as Magnolia (Freudenberg and Harkin 1963; Terazawa et al. 1986). Among various precursors of lignin biosynthesis, $p$-glucoumaryl alcohol (A-Glc), coniferin (B-Glc) and syringyl (C-Glc) were shown to be suitable precursors for selective labeling of protolignin in the cell walls of Magnolia (Terashima et al. 1986) and pine (Terashima et al. 1988; Fukushima and Terashima 1991b) and ginkgo (Fukushima and Terashima 1991a). It was suggested that the monolignols (A, B, C) biosynthesized in cytosol are transported through the plasma membrane to differentiating cell wall as their glucosides (A-Glc, B-Glc, C-Glc) (Kawai et al. 2006). The activity of glucosidase specific to coniferin (B-Glc) is best obtained by the ozonation method (Akiyama et al. 1986) such as electron microscopy, ultraviolet (UV) microscopy, polarized light microscopy, interference microscopy, infrared and Raman spectroscopy, solid state NMR and solution NMR of MWL. The tracer method includes radio- and stable isotope tracer methods, and an immunocytochemical technique method. Combinations of the techniques in the two categories can provide the most reliable and detailed information on (a)–(l) above. Information (f) on the stereochemistry of $Q$ (threo/erythro), S, and $V$ is best obtained by the ozonation method (Akiyama et al. 2002). Combinations of tracer technique with suitable destructive analyses such as mass spectrometry can also provide additional important information.

Tracer methods employing radio- and stable isotopes

Tracer methods using radioisotopes, $^3$H or $^{14}$C, and stable isotopes $^1$H or $^{13}$C can provide information, which cannot be obtained by any other methods. And it is a remarkable advantage that we can trace the labeled isotope even if the structure of the labeled component is converted to any other structure by chemical or biological treatments (Imai et al. 1997; Parkás et al. 2001, 2004a, 2004b).

The suitable precursors for labeling, detection and available information useful for ultrastructural study of ginkgo cell walls are summarized in Table 1 and Figs. 1 and 2.
Monolignol glucoside (A-Glc-B-Glc-C-Glc) ring-2'-H LSC Total number of labeled aromatic ring (I, J, K)
Monolignol glucoside (A-Glc-B-Glc-C-Glc) ring-2'-H MAR Localization in high resolution, and number of a labeled unit (I, J, K) in the cell wall
Monolignol glucoside (A-Glc-B-Glc) ring-5'-H LSC Total number of aromatic ring without bond at position 5 → total number of condensed substructure (S, T, U, 1/2W)
Monolignol glucoside (A-Glc-B-Glc) ring-5'-H MAR Localization and number of uncondensed substructure without bond at position 5 → number of condensed substructure (S, T, U, 1/2W)
Monolignol glucoside (A-Glc-B-Glc-C-Glc) Cy2'-H, 3H, 3H LSC, TA Biosynthetic pathway of monolignols
Monolignol glucoside (A-Glc-B-Glc-C-Glc) 13Cβ LSC Total number of labeled Cβ on C6-Cβ unit → lignin content
Monolignol glucoside (A-Glc-B-Glc-C-Glc) 13Cβ MAR Localization in low resolution, and number of labeled Cβ on C6-Cβ unit in the cell wall
Monolignol glucoside (B-Glc-C-Glc) O°CH3 LSC Total number of labeled O°CH3 → number of J, or K unit in lignin
D-Gluconic acid (D) 6°-14C MAR, LSC Localization in high resolution, and quantity of pectylacturonan, pectic substances
myo-Inositol (E) 2'-3H MAR, LSC Localization in high resolution, and amount of xylan
D-Mannose (F) + AOPP (G) + 2-DG (H) 2'-3H MAR, LSC Localization in high resolution, and amount of mannan
Coniferin (B-Glc) ring-1',3'C 13C-NMR qualitative and quantitative analyses of bonds involving ring-C3, in L, M, N, O, P, Q, R, S, T, U, V, Y
Coniferin (B-Glc) ring-3',5'C 13C-NMR qualitative and quantitative analyses of bonds involving ring-C3, in Q, R, S, T, U, V
Coniferin (B-Glc) ring-4',3'C 13C-NMR qualitative and quantitative analyses of bonds involving ring-C4, in L, M, N, O, Q, R, S, T, U, V
Coniferin (B-Glc) ring-5',3'C 13C-NMR qualitative and quantitative analyses of bonds involving ring-C5, in S, T, U, W, Q, R, S, T, U, V, Y
Coniferin (B-Glc) 13Cα 13C-NMR qualitative and quantitative analyses of bonds involving side-chain Cα, in L, M, N, O, P, Q, R, S, T, U, V, Y
Coniferin (B-Glc) 13Cβ 13C-NMR qualitative and quantitative analyses of bonds involving side-chain Cβ, in L, M, N, O, P, Q, R, S, T, U, V, Y
Coniferin (B-Glc) 13Cγ 13C-NMR qualitative and quantitative analyses of bonds involving side-chain Cγ, in L, M, N, O, P, Q, R, S, T, U, V, Y

Monolignol glucoside (A-Glc-B-Glc-C-Glc) Cy2'-H, 3H, 3H TA, GC-MS Biosynthetic pathway of monolignols

Selective labeling of polysaccharides

Pectin can be labeled by administration of its precursor UDP-glucuronic acid-[glucuronyl]-U-[14C] (Terashima et al. 1986; Fukushima and Terashima 1989) or glucuronic acid-[6,14C] (D) (Imai and Terashima 1991, 1992a). The glucuronic acid may be converted to galacturonic acid by isomerase and incorporated into pectylacturonan. The latter precursor gives higher selectivity of labeling for pectin. Xylan can be labeled also by administration of myo-inositol-[2-13C] (E) (Imai and Terashima 1992b). Selective radio-labeling of mannog in ginkgo was achieved by administration of D-mannose-[2-13C] (F) together with L-α-aminoxy-β-phenylpropionic acid (G) as an inhibitor of lignin biosynthesis, and 2-deoxy-D-glucose (H) as an inhibitor of cellulose synthesis (Imai et al. 1997).

Detection of isotopes

The radiolabeling technique combined with photographic detection of β-rays from the label under a microscope, microautoradiography, enables us to visualize the deposition process of the labeled polysaccharides or lignin in the differentiating cell walls (Fujita and Harada 1979; Takabe et al. 1981; Terashima et al. 1988; Terashima and Fukushima 1989; Imai and Terashima 1992a, 1992b; Imai et al. 1997). Selective labeling of cellulose is difficult, but the formation of CMFs can be visualized by observation of a cross section of differentiating xylem under a polarized light microscope (Takabe et al. 1981; Terashima et al. 1986).

Thus the order of successive deposition and assembly process of major cell wall polymers, pectin, xylan, mannan, CMFs and lignin during the cell wall formation can be visualized by a combination of microautoradiography and polarized light microscopy (Terashima et al. 1986, 1988; Terashima and Fukushima 1989; Terashima et al. 1993).

Formation of condensed substructures with C-C bond at position 5 of aromatic ring (S, T, U and W) causes the loss of labeled 13C at the position 5 of monolignol precursor (Table 1). Quantitative estimation of this loss is achieved by administration of the monolignol double-labeled with 13C and 14C to position 5 of ring hydrogen and side chain carbon respectively to lignifying tissue, and by subsequent determination of the change of 13C/14C ratio before and after incorporation of the monolignol into cell wall lignin. This double labeling technique based on the change of 13C/14C ratio is an effective nondestructive approach for determination of important lignin substructures irrespective of the method for estimating radioactivity. Quantitative determination of 13C/14C ratio in the double-labeled tissue or in the precursor can be achieved by scintillation counting of the β-rays from 13CH2O and 14CO2 produced by burning the tissue or the precursor (Terashima 1989).

Solid-state 13C-NMR analysis combined with the technique of selective 13C-enrichment of a specific carbon in protolignin is also a useful nondestructive approach for elucidation of the chemical structure of protolignin in the cell wall. By recording the difference 13C-NMR spectrum between the spectra of 13C-enriched lignin and unenriched lignin (δ13C-NMR), the assignment of signals and quantitative determination of signal intensity can be improved significantly though the resolution of the spectrum in solid-state is low compared with that in solution-state (Lewis et al. 1989; Eberhardt et al. 1993; Terashima et al. 1997; Hafren et al. 2002; Terashima et al. 2002). Solution-state NMR of specifically 13C-enriched MWL provides information on (a) and (c) in detail (Terashima and Seguchi 1991; Xie and Terashima 1991; Xie et al. 1994; Robert et al. 1998; Terashima et al. 1990).
CONCLUSION AND FUTURE PROSPECTS

Ginkgo is a suitable tree species for the study of ultrastructural assembly of lignin and polysaccharides in cell walls by isotope tracer methods. Necessary information for the study of the ultrastructure of tracheid walls in ginkgo can be obtained by visualization of successive incorporation of CMFs, pectin, hemicelluloses and lignin into lignifying cell walls by microautoradiography, nondestructive determination of macromolecular structure of lignin by differential $^{13}$C-NMR spectroscopy combined with $^{13}$C-enrichment technique of specific carbon in lignin, and observation of lignin modules on the network of polysaccharides by FE-SEM. The basic feature of the ultrastructure of tracheid walls in ginkgo xylem is commonly observed in xylems of other gymnosperms such as pine or spruce.

Non-destructive approaches have been applied also for study of lignin structure and ultrastructural assembly of lignin and polysaccharides in xylem cell walls of common angiospermous trees such as poplar (Joseleau et al. 2004; Ruel et al. 2006), beech (Awano et al. 1998, 200, 2001, 2002) and eucalyptus (Etvuguin et al. 2003). However, the xylem of these trees is composed of vessel, fiber, and ray cells and parenchyma, and the macromolecular structure of lignin and its assembly with polysaccharides differ among different cell types. Therefore, the physical and chemical properties of xylem tissue of angiosperms must be understood based on combinations of different cell wall ultrastructures. In the future, development of the technique for collecting cells of same type is necessary to apply some nondestructive analytical methods such as differential $^{13}$C-NMR.

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