International Journal of Plant Developmental Biology ©2007 Global Science Books

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

Noritsugu Terashima

2-610 Uedayama, Tenpaku, Nagoya, 468-0001 Japan

Correspondence: **norteras@quartz.ocn.ne.jp**

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; **Δ^{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

CONTENTS

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.

Received: 28 February, 2007. Accepted: 2 April, 2007.

Invited Review

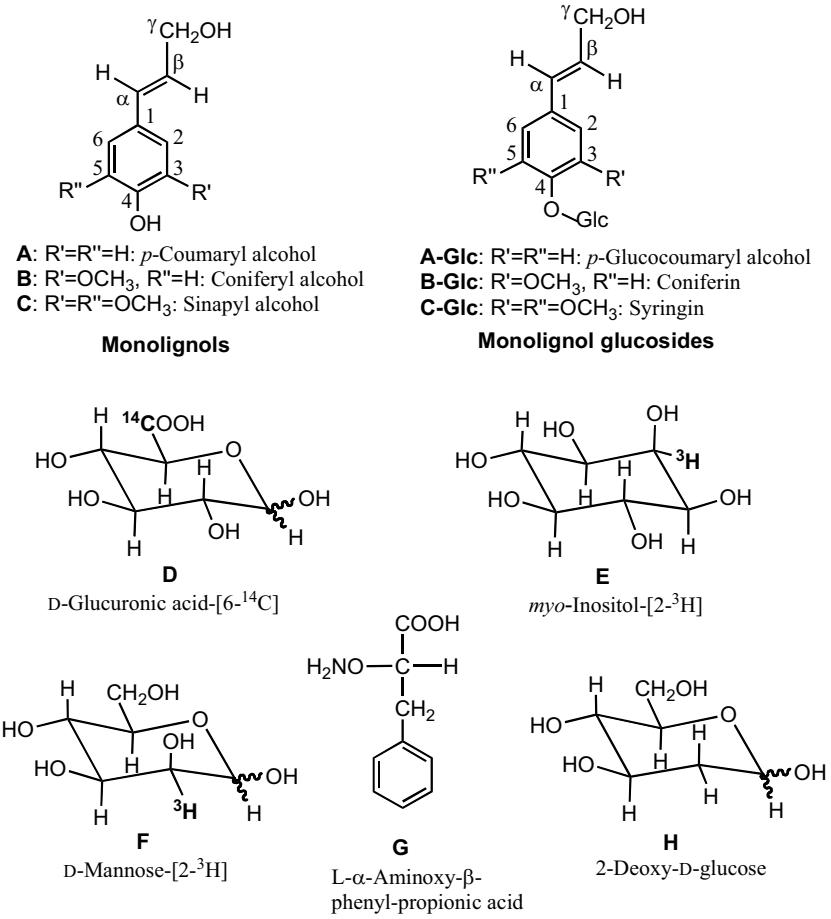

Fig. 1 Precursors in biosynthesis of lignin and polysaccharides, and enzyme inhibitors useful for labeling with isotopes.

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, Cα-OR in **Q** within a lignin macromolecules and in CC, ML, PW and SW;
(f) Stereochemistry of side chain carbons of C_6-C_3 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;

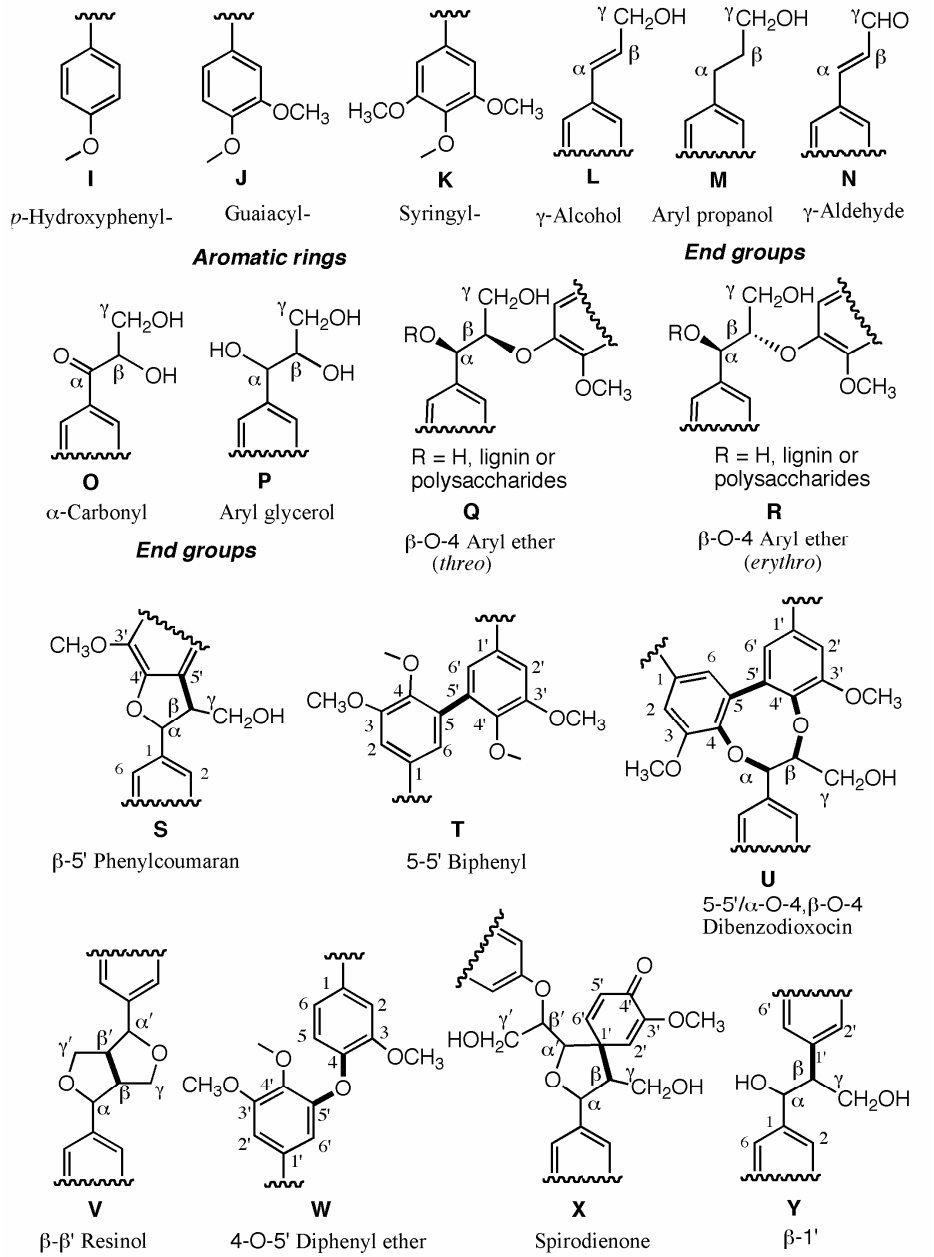

Fig. 2 Aromatic rings, end groups and inter-unit bonds in ginkgo lignin.

(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.

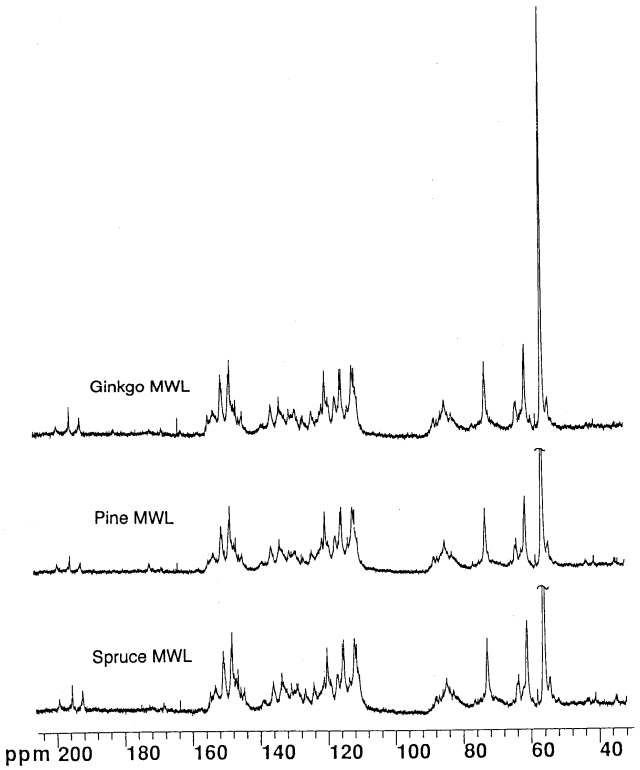

Fig. 3 Carbon 13 NMR spectra of MWLs of ginkgo, pine and spruce.

(4) Ginkgo lignin is a representative gymnosperm lignin close to conifer lignin in structure. **Fig. 3** shows a ^{13}C-NMR spectra of milled wood lignin (MWL) prepared from xylems of ginkgo, spruce and pine. The fact that those spectra closely resemble each other indicates that the structure of ginkgo lignin is almost identical with that of representative coniferous trees. The deposition of lignin in differentiating ginkgo xylem proceeds also in the same way as it does in conifers (Fukushima and Terashima 1991a, 1991b). In addition, more than 95% of monomeric units in ginkgo lignin is guaiacyl unit (**J**), while the content of the *p*-hydroxyphenyl (**I**) and syringyl (**K**) units is low (Obst and Landucci 1986; Fukushima and Terashima 1991a). This means that the structure of ginkgo lignin is less complicated than that of lignins composed of comparable ratio of (**I**), (**J**) and (**K**) in angiospermous trees. Thus, getting information (a)~(d) on ginkgo is less difficult than that on magnolia, beech or poplar.

(5) When a tracer method is applied, an aqueous solution of isotope-labeled precursor of lignin, pectin or hemicellulose is administered to a cut shoot of tree. The cut end of the shoot is put in the solution to grow for more than several weeks to form new xylem cell walls so that the precursor is incorporated into the corresponding cell wall polymers. It is difficult for the cut shoots of pine and spruce to survive in solution for more than 2-3 weeks, and effective adsorption of aqueous precursor solution is sometimes prevented by secretion of resinous material at the cut end of the stem. On the other hand, a cut shoot of ginkgo stem does not secrete resin, and the shoot can survive more than one month in aqueous precursor solution or in water. The cut ginkgo shoot forms new xylem tissue normally, and sprout additional new leaves within 3-4 weeks. It is easy to grow cloned trees from 15~20 cm cuttings of 2 year shoots by potting them in moist sand.

NON-DESTRUCTIVE APPROACHES TO THE HETEROGENEOUS ULTRASTRUCTURE OF LIGNIFIED PLANT CELL WALLS

One of the most effective approaches is to observe the lignification process of differentiating cell walls from their early stage to the final stage by non-destructive methods. The available non-destructive methods are divided into two categories: microscopy-spectroscopic methods and tracer methods. The methods in the former category are various characterization techniques in a solid state (Lin and Dence 1992) such as electron microscopy, ultraviolet (UV) microspectroscopy, 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 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 isotope ^{2}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; Parkås *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*-glucocoumaryl alcohol (**A-Glc**), coniferin (**B-Glc**) and syringin (**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**) 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 monolignols (**A**, **B**, **C**) administered as their glucosides (**A-Glc**, **B-Glc**, **C-Glc**) are incorporated into protolignin in the cell wall via the natural biosynthetic pathway.

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, ^{2}H or ^{13}C (Terashima *et al*. 1991; Matsui *et al*. 1994a, 1994b; Terashima *et al*. 1995, 1996, 2003) and the labeled precursor is administered to a growing stem of a tree. For radioisotope tracer experiments combined with microautoradiography, a V-shaped groove is made in circumferential direction on the growing stem, and fine glass wool is packed in the groove. The labeled precursor solution is added to the wool so that the precursor is supplied to lignifying xylem part. For ^{13}C-enrichment of specific lignin carbon, a cut shoot is put in aqueous precursor solution so that the precursor is absorbed from the cut end of the shoot. In the lignifying cell wall, the

Table 1 Precursors for specific labeling of lignin and polysaccharides by isotopes, and available information.

Precursor (Fig. 1)	Label	Detection	Available information (substructure in Fig. 2)
Monolignol glucoside (**A-Glc**,**B-Glc**,**C-Glc**)	ring-2-^{3}H	LSC	Total number of labeled aromatic ring (**I**, **J**, **K**)
Monolignol glucoside (**A-Glc**,**B-Glc**,**C-Glc**)	ring-2-^{3}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-^{3}H	LSC	Total number of aromatic ring without bond at position 5 → total number of condensed substructure (**S**, **T**, **U**, 1/2**W**)
Monolignol glucoside (**A-Glc**,**B-Glc**)	ring-5-^{3}H	MAR	Localization and number of uncondensed substructure without bond at position 5 → number of condensed substructure (**S**, **T**, **U**, 1/2**W**)
Monolignol glucoside (**A-Glc**,**B-Glc**,**C-Glc**)	Cγ-^{3}H,^{3}H	LSC, TA	Biosynthetic pathway of monolignols
Monolignol glucoside (**A-Glc**,**B-Glc**,**C-Glc**)	^{14}Cb	LSC	Total number of labeled Cβ on C_6-C_3 unit → lignin content
Monolignol glucoside (**A-Glc**,**B-Glc**,**C-Glc**)	^{14}Cb	MAR	Localization in low resolution, and number of labeled Cβ on C_6-C_3 unit in the cell wall
Monolignol glucoside (**B-Glc**,**C-Glc**)	O^{14}CH$_3$	LSC	Total number of labeled O^{14}CH$_3$ → number of **J**, or **K** unit in lignin
D-Glucuronic acid (**D**)	6-^{14}C	MAR, LSC	Localization in high resolution, and quantity of polygalacturonan, pectic substances
myo-Inositol (**E**)	2-^{3}H	MAR, LSC	Localization in high resolution, and amount of xylan
D-Mannose (**F**) + AOPP (**G**) + 2-DG (**H**)	2-^{3}H	MAR, LSC	Localization in high resolution, and amount of mannan
Coniferin (**B-Glc**)	ring-1-^{13}C	Δ^{13}C-NMR	qualitative and quantitative analyses of bonds involving ring-C3, in **L**, **M**, **N**, **O**, **P**, **Q**/**R**, **S**, **T**, **U**, **X**, **Y**
Coniferin (**B-Glc**)	ring-3-^{13}C	Δ^{13}C-NMR	qualitative and quantitative analyses of bonds involving ring-C3, in **Q**/**R**, **S**, **T**, **U**, **V**
Coniferin (**B-Glc**)	ring-4-^{13}C	Δ^{13}C-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-^{13}C	Δ^{13}C-NMR	qualitative and quantitative analyses of bonds involving ring-C5, in **S**, **T**, **U**, **W**, **Q**/**R**
Coniferin (**B-Glc**)	^{13}Cα	Δ^{13}C-NMR	qualitative and quantitative analyses of bonds involving side-chain Cα, in **L**, **M**, **N**, **O**, **P**, **Q**/**R**, **T**, **U**, **V**, **X**, **Y**
Coniferin (**B-Glc**)	^{13}Cβ	Δ^{13}C-NMR	qualitative and quantitative analyses of bonds involving side-chain Cβ, in **L**, **M**, **N**, **O**, **P**, **Q**/**R**, **S**, **U**, **V**, **X**, **Y**
Coniferin (**B-Glc**)	^{13}Cγ	Δ^{13}C-NMR	qualitative and quantitative analyses of bonds involving side-chain Cγ, in **L**, **M**, **N**, **O**, **P**, **Q**/**R**, **S**, **V**, **Y**
Monolignol glucoside (**A-Glc**,**B-Glc**,**C-Glc**)	Cγ^{2}H,^{2}H	TA, GC-MS	Biosynthetic pathway of monolignols

LSC: Liquid scintillation counting of sample, or scintilation counting of $^{3}H_2O$ and/or$^{14}CO_2$ produced by burning the labeld sample; MAR: Microautoradiography; TA: Thioacidolysis; AOPP: L-α-aminooxy-β-phenylpropionic acid; 2-DG: 2-deoxy-D-glucose; Δ^{13}C-NMR: Difference NMR spectrum between the spectrum of specifically ^{13}C-enriched sample and unenriched sample in solution state or in solid state; GC-MS: Gas chromatograph-mass spectrometry.

glucoside is hydrolyzed by a specific glucosidase, and the liberated labeled monolignol is incorporated into the newly formed lignin macromolecule, which is specifically labeled at a specific hydrogen or carbon corresponding to the administered monolignol glucosides.

Selective labeling of polysaccharides

Pectin can be labeled by administration of its precursor UDP-glucuronic acid-[glucuronyl-U-^{14}C] (Terashima *et al*. 1988; Fukushima and Terashima 1990) or glucuronic acid-[6-^{14}C] (**D**) (Imai and Terashima 1991, 1992a). The glucuronic acid may be converted to galacturonic acid by isomerase and incorporated into polygalaturonan. The latter precursor gives higher selectivity of labeling for pectin. Xylan can be labeled also by administration of *myo*-inositol-[2-^{3}H] (**E**) (Imai and Terashima 1992b). Selective radio-labeling of mannan in ginkgo was achieved by administration of D-mannose-[2-^{3}H] (**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 ^{3}H at the position 5 of monolignol precursor (**Table 1**). Quantitative estimation of this loss is achieved by administration of the monolignol double-labeled with ^{3}H and ^{14}C at position 5 of ring hydrogen and side chain carbon respectively to lignifying tissue, and by subsequent determination of the change of ^{3}H/^{14}C ratio before and after incorporation of the monolignol into cell wall lignin. This double labeling technique based on the change of ^{3}H/^{14}C ratio is an effective nondestructive approach for determination of important lignin substructures irrespective of the method for estimating radioactivity. Quantitative determination of ^{3}H/^{14}C ratio in the double-labeled tissue or in the precursor can be achieved by scintillation counting of the β-rays from $^{3}H_2O$ and $^{14}CO_2$ produced by burning the tissue or the precursor (Terashima 1989).

Solid-state ^{13}C-NMR analysis combined with the technique of selective ^{13}C-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 ^{13}C-NMR spectrum between the spectra of ^{13}C-enriched lignin and unenriched lignin (Δ^{13}C-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; Hafrén *et al*. 2002; Terashima *et al*. 2002). Solution-state NMR of specifically ^{13}C-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. 1999, 2001; Evtuguin *et al*. 2003; Terashima *et al*. 2003; Evtuguin *et al*. 2005) though the MWL does not represent all protolignin in the cell wall.

Δ^{13}C-NMR possesses additional great advantages over the conventional NMR in tracing structural changes involving the specific carbon before and after reaction (Parkås *et al*. 2001, 2004a, 2004b).

Non-destructive approaches by microscopy and spectroscopy

Transmission electron microscopy (TEM) and field emission scanning electron microscopy (FE-SEM) provide necessary information (g), (h), and (i) on the shape and size of lignin modules, polysaccharides, and their assembly in the cell wall (Nakashima *et al*. 1997; Hafrén *et al*. 1999, 2000; Awano *et al*. 2001, 2002; Terashima *et al*. 2004). When combined with the specific immunochemical labeling of lignin substructures (Ruel *et al*. 1994; Joseleau and Ruel 1997; Burlat *et al*. 2000; Joseleau *et al*. 2004; Kukkola *et al*. 2004; Ruel *et al*. 2006) and specific hemicellulose (Baba *et al*. 1994; Awano *et al*. 1998, 2000; Maeda *et al*. 2000; Hosoo *et al*. 2002; Ruel *et al*. 2006), it also provides useful information on (b), (e), (i) and (j).

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) can also provide information on (a), (b), (c) and (e) on the localization of specific lignin substructures **J**, **K**, **Q**, **S**, **V**, **Y** except for **T** and **U** with respect to the morphological region of the cell wall (Saito *et al*. 2005a, 2005b).

Raman spectroscopy can provide information on (g) and (i) on regulated orientation of lignin aromatic ring in the cell walls (Atalla and Agarwal 1985; Agarwal and Atalla 1986). This information is not obtained by other methods.

Non-destructive analyses of lignified tissue in solid state by UV microscopy, Fourier transformed infrared spectroscopy, interference microscopy and cross polarization/ magic angle spinning nuclear magnetic resonance spectroscopy provide information on (a), (b), (c) and (e) (Lin and Dence 1992). However, it is not easy to obtain sufficient information on (g), (h) and (i) necessary to elucidate the structure of the lignin macromolecule and its assembly with polysaccharides due to the heterogeneous nature of the ultrastructure. The resolution of solid state NMR can improve significantly by non-destructive dissolution of whole lignified tissue (Fachuang and Ralph 2003).

LIGNIFICATION IN GINKGO XYLEM AND ULTRASTRUCTURE OF GINKGO CELL WALL

Tracer methods using ^{3}H and ^{14}C combined with liquid scintillation counting technique (LSC) and microautoradiography (MAR), and stable isotope tracer method using ^{2}H, ^{13}C, combined with the techniques of gaschromatograph-mass spectrometry (GC-MS) and Δ^{13}C-NMR are summarized in **Table 1**. Combined results on ginkgo lignin provided information on (a)~(e) (Terashima 2000; Terashima and Fukushima 2000; Terashima 2001; Terashima *et al*. 2003). Observation of differentiating xylem of ginkgo under FE-SEM indicated that globular lignin modules deposit randomly on the pectin-CMFs network in CC and ML, while bead-like modules of lignin hemicellulose complex surrounding CMFs deposit in the S2 layer. The size of the bead-like modules is estimated to be about 16 ± 2 nm in length, about 25 ± 1 nm in outer diameter, with a thickness of 4 ± 2 nm, and the modules in the S1 layer were larger and longer than those in S2 (Terashima *et al*. 2004). FE-SEM observation of ginkgo cell walls after mild selective removal of lignin or polysaccharides retaining their morphological features provided additional information on (g), (h) and (i) on the 3D architecture of lignin modules and polysaccharides (Terashima and Yoshida 2005). The chemical structure of lignin macromolecule and its ultrastructural assembly with polysaccharides in ginkgo cell walls are considered to be representative for those in most conifer cell walls.

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.

The 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 (Evtuguin *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.

REFERENCES

Akiyama T, Sugimoto T, Matsumoto Y, Meshitsuka G (2002) Erythro/threo ratio of β-*O*-4 structures as an important structural characteristic of lignin. I: Improvement of ozonation method for the quantitative analysis of lignin side-chain structure. *Journal of Wood Science* **48**, 210-215

Atalla RH, Agarwal UP (1985) Raman microprobe evidence for lignin orientation in cell walls of native woody tissue. *Science* **227**, 636-638

Agarwal UP, Atalla RH (1986) *In situ* Raman microprobe studies of plant cell walls: macromolecular organization and compositional variability in the secondary wall of *Picea mariana* (Mill) B.S.P. *Planta* **169**, 325-332

Awano T, Takabe K, Fujita M (1998) Localization of glucuronoxylans in Japanese beech visualized by immunogold labelling. *Plotoplasma* **202**, 213-222

Awano T, Takabe K, Fujita M, Daniel G (2000) Deposition and localization of glucuronoxylans in the secondary cell wall of Japanese beech as observed using immuno FE-SEM. *Plotoplasma* **212**, 72-79

Awano T, Takabe K, Fujita M (2001) Xylan and lignin deposition on the secondary wall of *Fagus crenata* fibers. In: Morohoshi N, Komamine A (Eds) *Progress in Biotechnology* (Vol 18), "*Molecular Breeding of Woody Plants*", Elsevier Science BV, Amsterdam, pp 137-142

Awano T, Takabe K, Fujita M (2002) Xylan deposition on secondary wall of *Fagus crenata* fiber, *Protoplasma* **219**, 106-115

Baba K, Sone Y, Kaku H, Misaki A, Sibuya N, Itoh T (1994) Localization o hemicelluloses in the cell walls of some woody plants using immuno-gold electron microscopy. *Holzforschung* **48**, 297-300

Burlat V, Joseleau J-P, Ruel K (2000) Topochemistry and microdiversity of lignin in plant cell walls. In: Yoon SK (Ed) *New Horizons in Wood Anatomy*, Chonnam National University Press, pp 181-188

Dharmawardhana DP, Ellis BE, Carlson JE (1995) A β-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin. *Plant Physiology* **107**, 331-339

Dharmawardhana DP, Ellis BE (1998) β-Glucosidase and glucosyltransferases in lignifying tissues. In: Lewis NG, Sarkanen S (Ed) *Lignin and Lignan Biosynthesis*, *ACS Symposium Series* **697**, pp 76-83

Eberhardt T, Bernards MA, He L-F, Davin LB, Wooten JB, Lewis NG (1993) Lignification in cell suspension culture of *Pinus taeda*. *In situ* characterization of a gymnosperm lignin. *The Journal of Biological Chemistry* **268**, 21088-21096

Evtuguin DV, Balaksin MY, Terashima N, Neto CP, Silva AMS (2003) New complementary information on the *E. globulus* lignin structure obtained by ^{13}C-selective labelling and advanced NMR techniques. *Proceedings of 12th International Symposium on Wood and Pulping Chemistry* (Vol 3), Madison, USA, pp 177-180

Evtuguin DV, Goodfellow BJ, Neto CP, Terashima N (2005) Characterization of lignin-carbohydrate linkages in *Eucalyptus globulus* by 2D/3D NMR spectroscopy using specific carbon-13 labelling technique. *Proceedings of 13th*

International Symposium on Wood, Fiber and Pulping Chemistry (Vol 2), Auckland, New Zealand, pp 439-444

Fachuang Lu, Ralph J (2003) Non-degradative dissolution and acetylation of balled-milled plant cell walls: high resolution-state NMR. *The Plant Journal* **36**, 535-544

Freudenberg K, Harkin JM (1963) The glucosides of cambial sap of spruce. *Phytochemistry* **2**, 189-193

Fujita M, Harada H (1979) Autoradiographic investigation of cell wall development II. Tritiated phenylalanine and ferulic acid assimilation in relation to lignification. *Mokuzai Gakkaishi* **25**, 89-94

Fukushima K, Terashima N (1990) Heterogeneity in formation of lignin. XIII. Formation of *p*-hydroxyphenyl lignin in various hardwoods visualized by microautoradiography. *Journal of Wood Chemistry and Technol*ogy **10**, 413-433

Fukushima K, Terashima N (1991a) Heterogeneity in formation of lignin. XIV. Formation and structure of lignin in differentiating xylem of *Ginkgo biloba*. *Holzforschung* **45**, 87-94

Fukushima K, Terashima N (1991b) Heterogeneity in formation of lignin. XV. Formation and structure of lignin in compression wood of *Pinus thunbergii* studied by microautoradiography. *Wood Science and Technology* **25**, 371-381

Hafrén J, Fujino T, Itoh T (1999) Changes in the cell wall architecture of differentiating tracheids of *Pinus thunbergii* during lignification. *Plant and Cell Physiol*ogy **40**, 532-541

Hafrén J, Fujino T, Itoh T, Westermark U, Terashima N (2000) Ultrastructural change in the compound middle lamella of *Pinus thunbergii* during lignification and lignin removal *Holzforschung* **54**, 234-240

Hafrén J, Westermark U, Lennholm H, Terashima N (2002) Formation of ^{13}C-enriched cell-wall DHP using isolated soft xylem from *Picea abies*. *Holzforschung* **56**, 585-591

Hosoo Y, Yoshida M, Imai T, Okuyama T (2002) Diurnal difference in the amount of immunogold-labelled glucomannans detected with field emission scanning electron microscopy at the innermost surface of developing secondary walls of differentiating conifer tracheids, *Planta* **215**, 1006-1012

Imai T, Terashima N (1991) Determination of the distribution and reaction of polysaccharides in wood cell walls by the isotope tracer technique. II. Selective radio-labeling of pectic substances in mitsumata (*Edgeworthia papprifera*). *Mokuzai Gakkaishi* **37**, 733-740

Imai T, Terashima N (1992a) Determination of the distribution and reaction of polysaccharides in wood cell walls by the isotope tracer technique. III. Visualization of the deposition and distribution of galacturonan in the cell wall of magnolia (*Magnolia kobus* DC) xylem by microautoradiography. *Mokuzai Gakkaishi* **38**, 475-481

Imai T, Terashima N (1992b) Determination of the distribution and reaction of polysaccharides in wood cell walls by the isotope tracer technique. IV. Selective radio-labeling of xylan in magnolia (*Magnolia kobus*) and visualization of its distribution in differentiating xylem by microautoradiography. *Mokuzai Gakkaishi* **38**, 693-699

Imai T, Yasuda S, Terashima N (1997) Determination of the distribution and reaction of polysaccharides in wood cell walls by the isotope tracer technique. V. Behavior of xylan during kraft pulping studied by the radiotracer technique. *Mokuzai Gakkaishi* **43**, 241-246

Imai T, Terashima N, Yasuda S (1997) Determination of the distribution and reaction of polysaccharides in wood cell walls by the isotope tracer technique VI. Selective radio-labeling of mannan in ginkgo (*Ginkgo biloba*). *Mokuzai Gakkaishi* **43**, 342-348

Joseleau J-P, Ruel K (1997) Study of lignification by non-invasive techniques in growing maze internodes. An investigation by FTIR, CP/MAS ^{13}C NMR and immunocytochemical transmission electron microscopy. *Plant Physiology* **114**, 1123-1133

Joseleau J-P, Faix O, Kuroda K, Ruel K (2004) A polyclonal antibody directed against syringyl propane epitopes of native lignins. *Comptes Rendus Biologie* **327**, 809-815

Kawai R, Takabe K, Fujita M, Matoh T, Shitan N, Yazaki K (2007) Mechanism of monolignol transportation through plasma membrane in poplar. *Proceedings of the 51st Lignin Symposium*, Sapporo, Japan, pp 14-17

Kukkola EM, Koutaniemi S, Pöllänen, Gustafsson M, Karhunen P, Lundell TK, Saranpää P, Kilpeläinen I, Teeri TH, Fagerstedt KV (2004) The dibendodioxocin lignin substructure is abundant in the inner part of the secondary wall in Norway spruce and silver birch xylem. *Planta* **218**, 497-500

Lewis NG, Razal RA, Yamamoto E, Bokelman GH, Wooten JB (1989) Carbon-13 specific labeling of lignin in intact plants. In: Lewis NG, Paice MG (Eds) *Plant Cell Wall Polymers, Biogenesis and Biodegradation*; ACS Symposium Series 399, pp 169-181

Lin SY, Dence CW (1992) *Methods in Lignin Chemistry*, Springer, Berlin, pp 81-213

Maeda Y, Awano T, Takabe K, Fujita M (2000) Immunolocalization of glucomannan in the cell wall of differentiating tracheids in *Chamaecyparis obtusa*. *Protoplasma* **213**, 148-156

Matsui N, Fukushima K, Kamada K, Nishikawa Y, Yasuda S, Terashima N (1994a) On the behavior of monolignol glucosides in lignin biosynthesis. I. Synthesis of monolignol glucosides labeled with ^{2}H at the hydroxymethyl group of side chain, and polymerization of the labeled monolignols *in vitro*. *Holzforschung* **48**, 215-221

Matsui N, Fukushima K, Kamada K, Nishikawa Y, Yasuda S, Terashima N (1994b) On the behavior of monolignol glucosides in lignin biosynthesis. II. Synthesis of monolignol glucosides labeled with ^{3}H at the hydroxymethyl group of side chain, and incorporation of the label into magnolia and ginkgo lignin. *Holzforschung* **48**, 375-380

Nakashima J, Mizuno T, Takabe K, Fujita M, Saiki H (1997) Direct visualization of lignifying secondary wall thickenings in *Zinnia elegans* cells in culture. *Plant and Cell Physiology* **38**, 818-827

Obst JR, Landucci LL (1986) The syringyl content of soft wood lignin. *Journal of Wood Chemistry and Technology* **6**, 311-327

Parkås J, Paulsson M, Westermark U, Terashima N (2001) Solid state NMR analysis of β-^{13}C-enriched lignocellulosic material during light-induced yellowing. *Holzforschung* **55**, 276-282

Parkås J, Paulsson M, Terashima N, Westermark U, Ralph S (2004a) Light-induced yellowing of selectively ^{13}C-enriched dehydrogenation polymers (DHPs). Part 1. Side-chain ^{13}C-enriched DHP [-α-, β-, and γ-^{13}C]. *Nordic Pulp and Paper Research Journal* **19**, 29-36

Parkås J, Paulsson M, Terashima N, Westermark U, Ralph S (2004b) Light-induced yellowing of selectively ^{13}C-enriched dehydrogenation polymers (DHPs). Part 2. NMR assignments and photoyellowing of aromatic ring 1-, 3-, 4-, and 5-^{13}C DHPs. *Nordic Pulp and Paper Research Journal* **19**, 44-52

Robert D, Ämmälahti E, Bardet M, Brunow G, Kilpeläinen I, Lundquist K, Neirinck V, Terashima N (1998) Improvement in NMR structural studies of lignin through two-and three dimensional NMR detection and isotope enrichment. In: Lewis N, Sarkanen S (Eds) *Lignin and Lignan Biosyntheses*. ACS Symposium Series **697**, Am. Chem. Soc. Washington DC, pp 237-254

Ruel K, Faix O, Joseleau J-P (1994) New immonogold probes for studying the distribution of the different lignin types during plant cell wall biogenesis. *Journal of Trace and Microprobe Techniques* **12**, 267-276

Ruel K,Chevalier-billosta V, Guillemin F, Sierra JB, Josleau J-P (2006) The wood cell wall at the ultrastructural scale - Formation and topochemical organization. *Maderas. Cientia y Tecnologia* **8**, 107-116

Saito K, Kato T, Tsuji Y, Fukushima K (2005a) Identifying the characteristic ions of lignin polymer using ToF-SIMS. *Biomacromolecules* **6**, 678-683

Saito K, Kato T, Takamori H, Kishimoto T, Fukushima K (2005b) A new analysis of the depolymerised fragments of lignin polymer using ToF-SIMS. *Biomacromolecules* **6**, 2688-2696

Takabe K, Fujita M, Harada H, Saiki H (1981) Lignification process of Japanese black pine (*Pinus thunbergii* Parl) tracheids. *Mokuzai Gakkaishi* **27**, 813-820

Terashima N (1989) An improved radiotracer method to study formation and structure of lignin. In: Lewis NG, Paice MG (Eds) *Plant Cell Wall Polymers, Biogenesis and Biodegradation*: *ACS Symposium Series* **399**, Am. Chem. Soc., Washington DC, pp 148-159

Terashima N (1990) A new mechanism for formation of a structurally ordered protolignin macromolecule in the cell wall of tree xylem. *Journal of Pulp and Paper Science* **16**, J150-J155

Terashima N (2000) Formation and ultrastructure of lignified plant cell walls. In: Yoon SK (Ed) *New Horizons in Wood Anatomy*, Chonnam National University Press, pp 169-180

Terashima N (2001) Possible approaches for studying three dimensional structure of lignin. In: Morohoshi N, Komamine A (Ed) *Progress in Biotechnology* (Vol 18), *Molecular Breeding of Woody Plants*, Elsevier Science BV., Amsterdam, pp 257-262

Terashima N, Fukushima K, Takabe K (1986) Heterogeneity in formation of lignin. VIII. An autoradiographic study on the formation of guaiacyl and syringyl lignin in *Magnolia kobus*. *Holzforschung* **40 (Suppl)**, 101-105

Terashima N, Fukushima K, Sano Y, Takabe K (1988) Heterogeneity in formation of lignin. X. Visualization of lignification process in differentiating xylem of pine by microautoradiography. *Holzforschung* **42**, 347-350

Terashima N, Fukushima K (1988) Heterogeneity in formation of lignin. XI. An autoradiographic study of the heterogeneous formation and structure of pine lignin. *Wood Science and Technology* **22**, 259-270

Terashima N, Fukushima K (1989) Biogenesis and structure of macromolecular lignin in the cell wall of tree xylem as studied by microautoradiography. In: Lewis NG, Paice MG (Eds) *Plant Cell Wall Polymers, Biogenesis and Biodegradation*: *ACS Symposium Series* **399**, American Chemical Society, Washington DC, pp 160-168

Terashima N, Seguchi Y, Robert D (1991) Selective ^{13}C enrichment of side chain carbons of uaiacyl lignin in pine. *Holzforschung* **45 (Suppl)**, 35-39

Terashima N, Fukushima K, He L-F, Takabe K (1993) Comprehensive model of the lignified plant cell wall. In: Jung HG, Buxton DR, Hatfield RD, Ralph J (Eds) *Forage Cell Wall Structure and Digestibility*, American Society of Agronomy, Madison, USA, pp 247-270

Terashima N, Yoshida M (2005) Ultrastructural assembly of lignin and polysaccharides in lignifying plant cell walls. *Proceedings of 13th International Symposium on Wood, Fiber and Pulping Chemistry* (Vol 2), Auckland, New Zealand, pp 423-426

Terashima N, Atalla RH, Ralph SA, Landucci LL, Lapierre C, Monties B (1995) New preparations of lignin polymer models under conditions that approximate cell wall lignification. I. Synthesis of the lignin polymer models and their structural characterization by ^{13}C NMR. *Holzforschung* **49**, 521-527

Terashima N, Ralph SA, Landucci LL (1996) New facile syntheses of monolignol glucosides; *p*-glucocoumaryl alcohol, coniferin and syringin. *Holzfor-*

schung **50**, 151-155

Terashima N, Atalla RH, Van der Hart DL (1997) Solid state NMR spectroscopy of specifically ^{13}C-enriched lignin in wheat straw from coniferin. *Phytochemistry* **46**, 863-970

Terashima N, Nakashima J, Takabe K (1998) Proposed structure for protolignin in plant cell walls In: Lewis N, Sarkanen S (Eds) *Lignin and Lignan Biosyntheses*. *ACS Symposium Series* **697**, American Chemical Society, Washington DC, pp 180-193

Terashima N, Hafrén J, Westermark U, Xie Y, Fukushima K, Lapierre C, van der Hart D (1999) Proposed 3D structure for softwood lignin. *Proceedings of 10th International Symposium on Wood and Pulping Chemistry* **1**, Yokohama, Japan, pp 106-109

Terashima N, Fukushima K (2000) Lignification of xylem cell walls in *Ginkgo biloba*. In: Teris A, van Beek (Eds) *Medicinal and Aromatic Plants – Industrial Profiles* (Vol 12), *Ginkgo biloba*, Harwood Academic Publishers, Chur, Switzerland, pp 49-62

Terashima N, Hafrén J, Westermark U, van der Hart DL (2002) Nondestructive analysis of lignin structure by NMR spectroscopy of specifically ^{13}C-enriched lignins 1: Solid state study of ginkgo wood. *Holzforschung* **56**, 43-50

Terashima N, Evtuguin DV, Neto CP, Parkås J (2003) Synthesis of coniferins ^{13}C-enriched at position 4 or 5 of the guaiacyl ring. *Holzforschung* **57**, 485-488

Terashima N, Evtuguin D, Pascoal Neto C, Parkås J, Paulsson M, Westermark U, Ralph S, Ralph J (2003) An improved ^{13}C-tracer method for the study of lignin structure and reactions. -Differential ^{13}C-NMR-. *Proceedings of 12th International Symposium on Wood and Pulping Chemistry* (Vol 1), Madison, USA, pp 175-178

Terashima N, Awano T, Takabe T, Yoshida M (2004) Formation of macromolecular lignin in ginkgo xylem cell walls as observed by field emission scanning electron microscopy. *Comptes Rendus Biologie* **327**, 903-910

Terazawa M, Okuyama H, Miyake M (1986) Phenolic compounds in living tissue of woods. I. Phenolic glucosides of 4-hydroxycinnamyl alcohol derivatives in the cambial sap of woods. *Mokuzai Gakkaishi* **30**, 322-328

Timell TE (1986) Origin and evolution of compression wood. In: Timell TE (Ed) *Compression Wood in Gymnosperms* (Vol 1), Springer-Verlag, Berlin, pp 597-621

Xie Y, Terashima N (1991) Selective carbon 13-enrichment of side chain carbons of ginkgo lignin traced by carbon 13 nuclear magnetic resonance. *Mokuzai Gakkaishi* **37**, 935-941

Xie Y, Robert D, Terashima N (1994) Selective carbon 13-enrichment of side chain carbons of ginkgo lignin traced by carbon 13 nuclear magnetic resonance. *Plant Physiology and Biochemistry* **32**, 243-249