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Monolignol glucosides as intermediate compounds in lignin biosynthesis. Revisiting the cell wall lignification and new ^{13}C-tracer experiments with *Ginkgo biloba* and *Magnolia liliiflora*

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Abstract: A large amount of monolignol glucosides (MLGs: *p*-glucocoumaryl alcohol, coniferin, syringin) are found in lignifying soft xylem near cambium and they disappear with the progress of lignification. Recently, it became a matter of debate whether those MLGs are real intermediates in lignin biosynthesis or only a storage form of monolignols outside of the main biosynthetic pathway. The latter is partly based on a misinterpretation of ^{14}C-tracer experiments and partly on the simple generalization of the results of gene manipulation experiments concerning the flexible and complex lignification. In the present paper, it could be confirmed by the most reliable ^{13}C-tracer method that MLGs are real intermediates in the pathway from L-phenylalanine to macromolecular lignin-polysaccharides complexes in the cell walls. This pathway via MLGs is essential for transport and programmed delivery of specific monolignols in a stable form from intracellular space to specific lignifying sites within the cell wall. The pool size of MLGs is large in most gymnosperm trees and some angiosperm species that emerged in an early stage of phylogeny, while the pool size is small in most angiosperms. This difference in pool size is reasonably understandable from the viewpoint of plant evolution, in the course of which the role of MLGs changed to meet variation in type of major cells, reaction wood formation, and postmortem lignification.

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Introduction

It is well-known that monolignol glucosides (MLGs) are formed in lignifying soft xylem near cambium of gymnosperms such as spruce, pine, and ginkgo, and they disappear with the progress of lignification. Freudenberg et al. (1955), Freudenberg and Torres-Serres (1967), and Marcinowski and Grisebach (1977) administered ^{14}C-phenylalanine (Figure 1, **1**) to growing spruce or pine, and *p*-glucocoumaryl alcohol (Figure 1, **3a**) and coniferin (Figure 1, **3b**), and subsequently both MLGs and lignin were isolated from the stem and branch. Both MLGs and lignin were radioactive. When radioactive coniferin was administered to those growing trees, lignin became radioactive too (Freudenberg et al. 1955) without any structural conversion of coniferyl alcohol moiety (Kratzl et al. 1957). Various MLGs specifically labeled with ^{3}H, ^{14}C, or specifically enriched with ^{13}C have been fed to a variety of plants including ginkgo, pine, spruce, magnolia, lilac, poplar, oleander, wheat, etc., and the lignin formation process and structure of the labeled lignins were examined by nondestructive methods including double labeling techniques, microautoradiography, and difference nuclear magnetic resonance (NMR) spectroscopy focusing on differences between ^{13}C-enriched and unenriched specimens in solid and in solution state. The structure of the labeled lignin showed the same characteristic features as the untreated lignin (Fukushima and Terashima 1990, 1991a,b; Terashima et al. 1993, 2002, 2009a; Xie et al. 1994a,b; Eglinton et al. 2000). The results of the quoted papers indicate that MLGs are the real intermediates in biosynthesis of lignin in plant cell walls as presented in

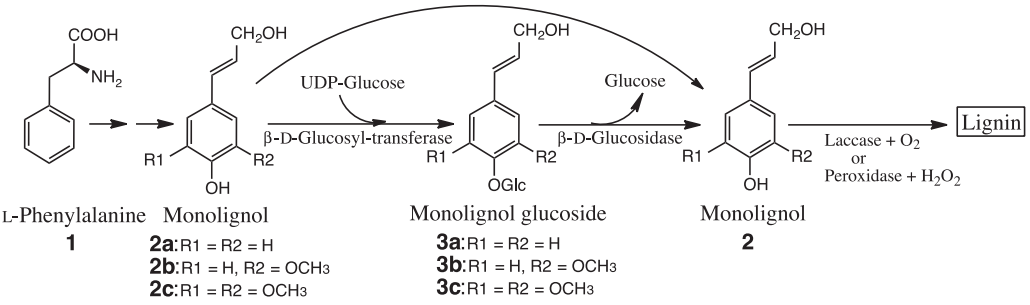

Figure 1: Biosynthetic pathway from phenylalanine to lignin.

Figure 1. The same glucosides are found also in cambium and surrounding tissue of angiosperm trees belonging to Magnoliaceae and Oleaceae (Terazawa et al. 1984).

The MLGs are considered to be storage and transport form of the monolignols, and the glucosidation and deglucosidation steps may regulate lignin biosynthesis, while the former is mediated by a uridine diphosphate glucose (UDP-glucose): monolignol glucosyltransferase and the latter is induced by 4-*O*-β-D-glucosidase (Ibrahim and Grisebach 1976; Ibrahim 1977; Terazawa et al. 1984; Dharmawardhana et al. 1995; Dharmawardhana and Ellis 1998; Steeves et al. 2001; Samuels et al. 2002; Lanot et al. 2006; Tsuyama and Takabe 2015).

Recently, however, it became a matter of debate whether the MLGs are the real essential intermediates (as presented in Figure 1) or only an unessential storage form of monolignols outside of the main biosynthetic pathway of lignin. This view is derived from the following observations and explanations:

1. Kaneda et al. (2008) fed ^{3}H-phenylalanine (Phe-[^{3}H]) (Figure 1, **1**) to dissected cambium and developing wood of lodgepole pine (*Pinus contorta* var *latifolia* Englem.) seedlings for 4 h, allowing uptake and metabolism, followed by rapid freezing of the cells. Then autoradiography was performed to detect the locations of the monolignols responsible for lignification. Parallel experiments showed that radioactivity was incorporated into the polymeric lignin. A methanol-soluble pool was characterized by high-performance liquid chromatography (HPLC) combined with liquid scintillation counting of radioactivity. Radioactivity from the Phe-[^{3}H] was incorporated into the expected lignin precursors, such as *p*-coumaryl alcohol (Figure 1, **2a**) and coniferyl alcohol (Figure 1, **2b**), as well as pinoresinol. Coniferin (Figure 1, **3b**), the glucoside of coniferyl alcohol, was detected by UV absorption on the chromatogram, but the radioactivity was not detected by scintillation counting of the HPLC fraction corresponding to coniferin. Therefore, it was concluded that the major part of coniferin is not the precursor of lignin biosynthesis, but instead it is a storage form associated with the metabolism of other phenylpropanoids such as pinoresinol. These experimental results and conclusions of Kaneda et al. (2008) are quite contrary to those described by Freudenberg and Torres-Serres (1967), Marcinowski and Grisebach (1977), Terashima et al. (1993, 1998), and Fukushima et al. (1997).
2. Down-regulation of monolignol glucosyltransferase, necessary for conversion of monolignols (Figure 1, **2**) to their glucosides (Figure 1, **3**), did not inhibit lignification in *Arabidopsis* (Lanot et al. 2006). Knockout mutants of *Arabidopsis* for genes supposed to have affinity to β-glucosidase displayed a significant increase in coniferin content but did not belong to a lignin-deficient phenotype. So MLGs were supposed to be a storage form of monolignols in *Arabidopsis* and not to be the direct precursors of lignin (Chapelle et al. 2012).
3. Glucosidation of monolignol is thought to be necessary to transfer hydrophobic monolignols to lignifying cell wall across the plasma membrane. However, there is no reasonable explanation for the fact that a large amount of MLGs is found in the lignifying secondary wall of gymnosperm tracheid (Morikawa et al. 2010) and that their turnover is slow (Marcinowski and Grisebach 1977). MLGs were not found, or only in small amounts, in lignifying xylem of most angiosperm trees except for Magnoliaceae and Oleaceae (Terazawa et al. 1984).

The reliability of lignin research by isotope-labeling method, in which labeled MLGs are employed as precursors of native lignin formation, would be highly questionable, if MLGs would be unessential intermediates beyond the main pathway of lignin biosynthesis in the cell walls.

In this paper, the experiments described in paragraph (1) above will be revisited by means of a more reliable

^{13}C-tracer method than the ^{14}C-tracer method employed by Kaneda et al. (2008). *Ginkgo biloba* Linn. and *Magnolia liliiflora* Desr. have been selected for the tracer experiments. The question will be in focus whether the MLGs are derived from the precursor L-phenylalanine in lignifying shoot of ginkgo and magnolia. Hypothetical explanations will be proposed for a reasonable interpretation of the observations described in paragraphs (2) and (3) above, and the most essential steps of lignification will be reviewed also from the point of view of reaction wood formation.

Materials and methods

Feeding of L-phenylalanine and L-phenylalanine-[2-^{13}C] to lignifying plant cell walls: Fresh shoots (diameter: 4–5 mm) of *G. biloba* and *M. liliiflora* were harvested in late May and early June, respectively, and they were cut short (4 cm) with one trimmed leaf (about 3×3 cm^2) as shown in Figure 2. The shoots were fed with aqueous solutions (5 mg/200 µl water) of L-phenylalanine-[2-^{13}C] (99%, Cambridge Isotope Laboratories, MA, USA) or L-phenylalanine (Merck & Co., NJ, USA), or with 200 µl of water as an unfed control. After the precursor solution was adsorbed within a few hours, water was added and allowed to metabolize for 24 h under light (12 h) and dark (12 h) at room temperature.

Extraction of coniferin and syringin from the plant tissue: The leaf was removed, and the shoot was cut short and extracted with hot ethanol-water (1:1, v/v) and hot water. The combined extract was concentrated under reduced pressure and subjected to separation by preparative silica gel TLC (Kieselgel 60PF$_{254}$, Merck, Germany) and a mixture of acetone:ethyl acetate:water (10:10:1, v/v) as a developer. Those developed bands were collected and extracted with hot water, which corresponded to retardation factor (Rf) values and UV fluorescence of authentic coniferin (Rf: 0.35) and syringin (Rf: 0.28) prepared by the method of Terashima et al. (1996). The crude coniferin and syringin fractions were obtained after evaporation of water under reduced pressure.

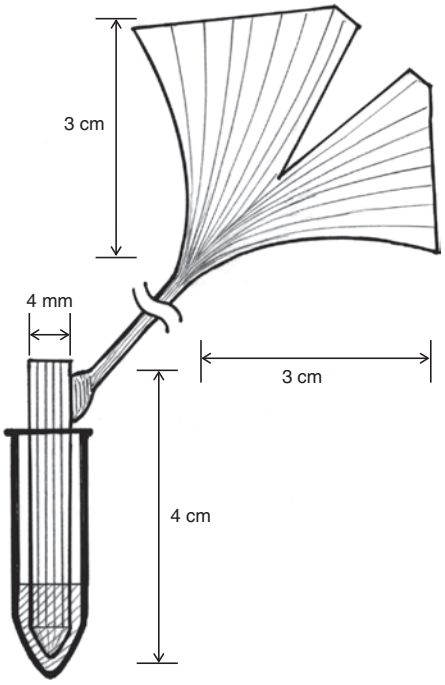

Figure 2: Feeding of aqueous solution of ^{13}C-L-phenylalanine or L-phenylalanine to a short cut shoot (4 mm×4 cm) of *Ginkgo biloba* with a trimmed leaf (about 3×3 cm).

Proof of incorporation of ^{13}C-phenylalanine into MLGs by LC-MS/MS: The incorporation of ^{13}C-phenylalanine into MLGs was confirmed by subjecting the isolated coniferin and syringin fractions to the analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-20AD (SHIMADZU, Japan) was used as an HPLC system. The mobile phase was a gradient flow of water and acetonitrile/methanol (1/6, v/v). A Luna 5 µm Phenyl-Hexyl 150×2.0 mm (Phenomenex, CA, USA) column was applied. Flow rate: 0.2 ml min^{-1}; column temperature: 40°C. MS was carried out on API2000 (AB SCIEX, CA, USA) instrument equipped with an electrospray ionization interface. The instrument was operated in positive ion mode with the parameters of curtain gas 20 psi; ion spray voltage 5500 V; temperature 400°C; ion source gas (1) 70 psi; ion source gas (2) 80 psi; interface heater ON; collision gas 6; and deflector 6 V. The other parameters were optimized individually.

LC-MS/MS can provide highly sensitive quantitation by multiple reaction monitoring (MRM), in which a precursor ion (on MS1) and its product ion (on MS2) are selected to detect a target compound selectively. Coniferin and syringin in the crude fractions from the plant tissue were identified by the same LC-retention time and mass spectra derived from MRM with those obtained on the authentic compounds (Figure 3) prepared by the method of Terashima et al. (1996). According to the theory of LC-MS/MS (Gross 2004) as described in Supplement 1, the ^{13}C-contents were estimated by comparing the relative amount of ^{13}C-labeled MLGs provided by the MRM ion intensities of ^{13}C-labeled MLGs against the unlabeled MLGs between the crude MLGs fractions isolated from the shoot fed with ^{13}C-enriched and unenriched phenylalanine.

Results and discussion

Conversion of L-phenylalanine to lignin via MLGs

The MRM transitions monitored were *m/z* 360 to *m/z* 131 for coniferin and *m/z* 390 to *m/z* 161 for syringin, based on the MS/MS analyses (Figure 3). The MRM data for ^{13}C-coniferin and ^{13}C-syringin were *m/z* 361 to *m/z* 132 and *m/z* 391 to *m/z* 162, respectively. Figure 4 shows LC-MS/MS profiles of coniferin isolated from the shoots of ginkgo fed with L-phenylalanine-[2-^{13}C] (Figure 4b) and unenriched L-phenylalanine (Figure 4a). The analytical data of coniferin isolated from the unfed shoots were identical with those fed with L-phenylalanine (not shown). The ^{13}C-enrichment estimated from the relative amount

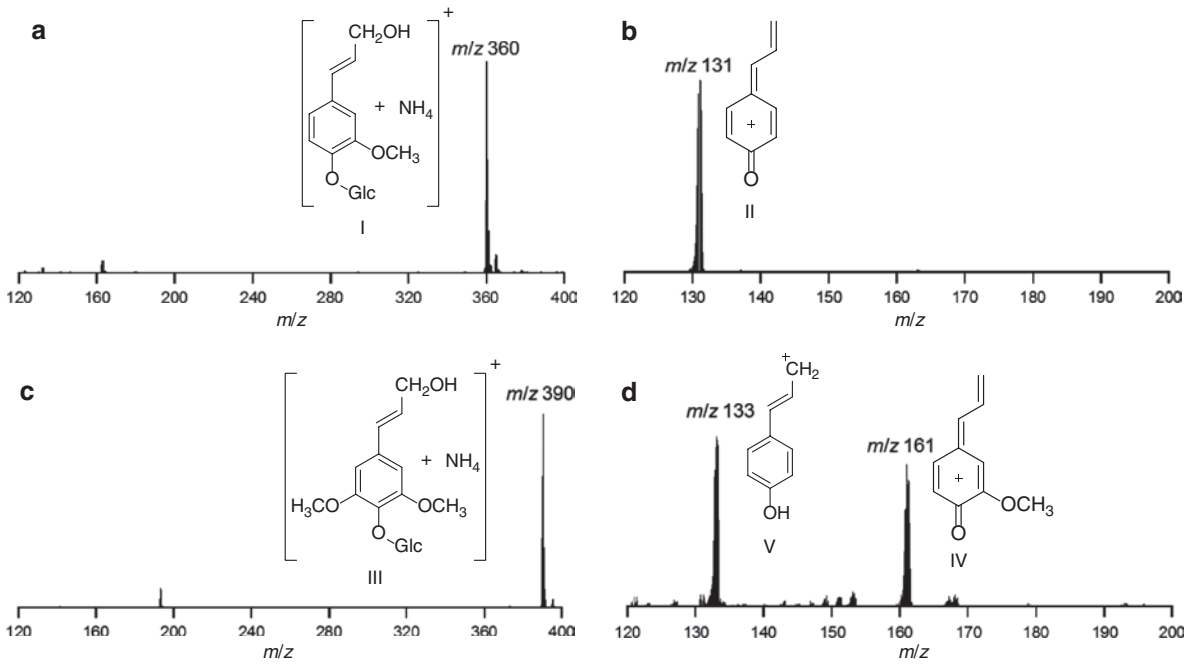

Figure 3: Mass spectra of authentic coniferin and syringin. MS spectrum of coniferin (a) and MS/MS spectrum (b) of fragment peak at *m/z* 360 of coniferin, and MS spectrum of syringin (c) and MS/MS spectrum (d) of fragment peak at *m/z* 390 of syringin.

of ^{13}C-coniferin (*m/z* 361) isolated from the ginkgo shoot fed with ^{13}C-L-phenylalanine (b) was 3.5 times higher than that isolated from the shoot fed with unenriched phenylalanine (a) and unfed shoot. The ^{13}C-enrichment of coniferin and syringin isolated from magnolia shoot fed with L-phenylalanine-[2-^{13}C] (Figure 5b and d) were 2.1 and 4.4 times higher than those fed with L-phenylalanine (Figure 5a and c), respectively.

As the shoots of ginkgo and magnolia originally contain a large amount of MLGs, newly formed ^{13}C-coniferin and ^{13}C-syringin from the precursor ^{13}C-L-phenylalanine were diluted to the ^{13}C-enrichment level of 2–5 times of the natural abundance (about 1.1%). This rate of ^{13}C-incorporation into coniferin from the 99% ^{13}C-enriched phenylalanine in ginkgo shoot is sufficiently high compared to the ^{14}C-recovery rate (0.3% in case of coniferin) from ^{14}C-L-phenylalanine due to more extensive dilution in the experiments of Freudenberg and Torres-Serres (1967), who employed a young spruce tree.

In the present experiment, it was confirmed that L-phenylalanine is effectively converted to MLGs in differentiating xylem of ginkgo and magnolia. It has been

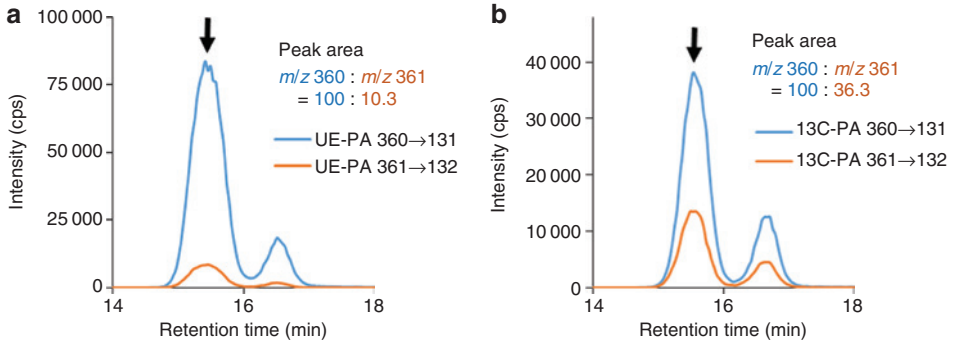

Figure 4: LC-MS/MS profiles of coniferin isolated from the shoots of ginkgo fed with unenriched L-phenylalanine (UE-PA) (a) and L-phenylalanine-[2-^{13}C] (b) by MRM. The peak at 15.5 min shows coniferin (indicated by the arrow). The peak area of MRM transition (precursor ion→product ion) for ^{13}C-coniferin (*m/z* 361→*m/z* 132) relative to unlabeled coniferin (*m/z* 360→*m/z* 131) isolated from the ginkgo shoot fed with ^{13}C-L-phenylalanine (b) is 3.5 times higher than that isolated from the shoot fed with unenriched phenylalanine (a) and unfed shoot (not shown).

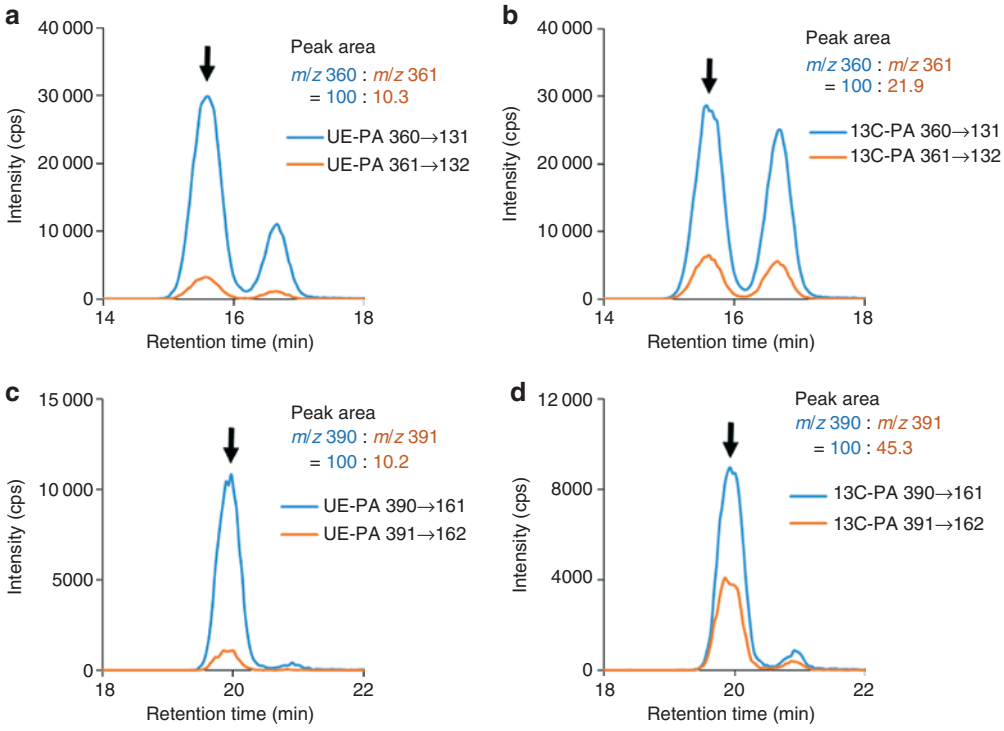

Figure 5: LC-MS/MS profiles of coniferin (a and b) and syringin (c and d) isolated from the shoots of magnolia fed with unenriched L-phenylalanine (a and c) and L-phenylalanine-[2-^{13}C] (b and d) by MRM. The peak at 15.5 min shows coniferin and that at 19.9 min shows syringin (indicated by the arrows). The peak area of MRM transition (precursor ion→product ion) for ^{13}C-coniferin (*m/z* 361→*m/z* 132) relative to unlabeled coniferin (*m/z* 360→*m/z* 131) (b) and that of ^{13}C-syringin (*m/z* 391→*m/z* 162) relative to unlabeled syringin (*m/z* 390→*m/z* 161) (d) isolated from the magnolia shoots fed with ^{13}C-L-phenylalanine are 2.1 times and 4.4 times higher than those isolated from the shoots fed with unenriched phenylalanine (a and c) and unfed shoot (not shown), respectively.

already shown that a variety of ^{14}C-labeled MLGs as well as ^{13}C-labeled MLGs were efficiently incorporated into cell wall lignin without modification of C_6-C_3 skeleton, when they were fed to differentiating xylem of various plants (Terashima et al. 1993, 2002, 2009a,b; Eglinton et al. 2000; Evtuguin et al. 2003). Therefore, it can be safely concluded that MLGs are real intermediate compounds in lignin biosynthesis. Because coniferin is insoluble in toluene in the solution cocktail of scintillation counting of radioactivity, Freudenberg and Torres-Serres (1967) employed a cocktail modified by addition of methanol to dissolve ^{14}C-coniferin for determination of its radioactivity. Supposedly, if Kaneda et al. (2008) tried to modify scintillation counting cocktail for dissolving coniferin, radioactivity of coniferin might have been detected as well as that of free monolignols and pinoresinol that are soluble in unmodified toluene.

Pool size of MLGs as intermediates in lignin biosynthesis

Pulse labeling experiments employing pine seedlings and $^{14}CO_2$ or L-phenylalanine-[U-^{14}C] showed a rapid synthesis of coniferin and a slow turnover of this compound (Marcinowski and Grisebach 1977). The concentration of coniferin in differentiating xylem of Japanese black pine (*Pinus thunbergii* Parl.) is highest at the beginning of May, before the most active lignifying season, June and July, and therefore, the apparent size of coniferin pool reflects the difference between production and consumption that depends on the stage of xylem differentiation (Fukushima et al. 1997). Morikawa et al. (2010) observed, by scanning electron microscope (SEM), a large amount of needle-like deposits of coniferin in the tracheid lumina of Japanese cypress (*Chamaecyparis obtusa* Sieb. et Zucc.) from the beginning of the formation stages proceeding from the outer secondary wall layer (S1) to the middle secondary wall layer (S2). Differential Raman spectra taken from the differentiating xylem of this tree before and after washing with water also showed that developing secondary walls contained coniferin during the S2 layer formation and also after the formation of the inner secondary wall layer (S3) (Morikawa et al. 2010). All of those observations indicated that the pool of coniferin intermediate in differentiating xylem of representative gymnosperm trees is very large.

Terazawa et al. (1984) isolated coniferin from the cambial sap of various gymnosperm trees, and both coniferin and syringin from a few species of angiosperm trees belonging to Magnoliaceae and Oleaceae. In the present work, it was reconfirmed that the pool of MLGs in *M. liliiflora* is quite large based on the following fact: the ^{13}C-enrichment of ^{13}C-MLGs derived from 99% ^{13}C-phenylalanine is 3–5 times as high than the natural abundance due to extensive dilution in the large pool of unenriched MLGs originally existed in the lignifying tissue.

Even in the angiosperm trees from which MLGs were not isolated, monolignol-glucosyltransferase is widely distributed (Ibrahim 1977). The universal presence of this enzyme in different taxa can be interpreted that it plays a pivotal role in the plant kingdom. Low levels of the enzyme activity were detectable in the bryophytes and pteridophytes, while highly specific activities of the enzyme were found in most of the woody angiosperms and almost all gymnosperm species (Ibrahim 1977). Wide distribution of β-glucosidase specific for MLGs (Marcinowski and Grisebach 1978; Marcinowski et al. 1979; Samuels et al. 2002; Chapelle et al. 2012; Tsuyama and Takabe 2015) suggests that glucosidation and deglucosidation steps are essential for the formation of macromolecular lignin in both gymnosperm and angiosperm xylem cell walls. However, the size of the intermediate pool differs among plant species. The pool size is large enough to allow its isolation in most gymnosperms and magnolia that emerged in an early stage of phylogeny (Soltis et al. 1999). The pool size is small in most angiosperm trees such as cherry, oak, and poplar (Terazawa et al. 1984; Tsuyama and Takabe 2014).

This difference in the pool size related to plant evolution can be interpreted that the role of MLGs in cell wall lignification differs between major tracheid walls in gymnosperms and major fiber walls, and minor vessel walls in angiosperms. The modification of cell type and lignification mode as well as lignin structure during the plant evolution provided advantages to angiosperms in terms of ready adaptability to environmental changes on the earth (Terashima 2013). The ability to reaction wood formation also belongs to the adaptation process, which helps growing plants to maintain their stem and branch in proper position (Terashima 2013). The difference in the pool size of MLGs can be reasonably explained from the role of MLGs in reaction wood formation as will be discussed below.

Role of MLGs in lignification of cell walls

The glucosidation and deglucosidation steps of monolignols were sometimes neglected in the flow chart of lignin biosynthesis. This is partly due to vague information and understanding of the observations concerning the unique behavior and role of MLGs in cell wall lignification. Glucosidation protects active phenolic hydroxyl group of monolignols and converts the water-insoluble monolignols into water-soluble ones. In addition, free monolignols are toxic to living plant cell (Väisänen et al. 2015). Roles of MLGs are related to temporal offset of these disadvantageous physico-chemical and toxic properties of monolignols for programmed assembly of macromolecular lignin and polysaccharides in the differentiating cell walls as described in the following four paragraphs.

(1) Transport of monolignols from intracellular space to actively lignifying site in the cell wall. It was shown by immunocytochemical localization that monolignols are synthesized in the cytosolic compartment (Takabe et al. 2001). The deposition of lignin occurs first in the previously deposited polysaccharides gel in the cell corner (CC) and compound middle lamella (CML) regions followed by S1, S2, and S3, successively (Takabe et al. 1981; Terashima et al. 1993, 1998). This gel is in oxidative environment as shown by Kratzl et al. (1956), who fed a spruce branch with ^{14}C-creosol or ^{14}C-vanillyl alcohol and found that these compounds were incorporated into the wood tissue. So it is essential to deliver the monolignols in their stabilized and water-soluble form through the plasma membrane and the thick polysaccharides gel to the actively lignifying site in the tracheid wall. Tsuyama et al. (2013) proposed a common mechanism of proton-dependent coniferin transport in the lignifying tissues of woody plants, both in angiosperms and gymnosperms. Morikawa et al. (2010) observed coniferin distribution in the CC middle lamella region and S2 layer of differentiating tracheid of Japanese cypress (*C. obtusa*).

(2) Programmed delivery of specific monolignols to specific morphological regions of lignifying xylem. It has been shown by microautoradiography that *p*-hydroxyphenyl-, guaiacyl-, and syringylpropane units are incorporated into different morphological regions in different lignification stages of various plant xylem (Terashima et al. 1986, 1993; Terashima and Fukushima 1988; Fukushima and Terashima 1990, 1991a,b) and by microscopic spectrophotometry of Japanese oak (Yoshinaga et al. 1997). This programmed monolignol delivery is reasonably understood by programmed supply of MLGs (Terashima et al. 1993, 1998) combined with localization of specific β-D-glucosidase for specific MLGs in the differentiating xylem. Coniferin specific β-glucosidase has been found in the secondary cell wall of pine (Dharmawardhana et al. 1995; Samuels et al. 2002) and poplar (Tsuyama and Takabe 2015).

(3) Quick supply of a large amount of monolignols from the large pool of MLGs in gymnosperms. For construction of 3D architecture of tracheid walls composed of about 70% polysaccharides and 30% lignin, a large pool of water-soluble MLGs is essential for quick supply of monolignols. Quick supply of MLGs is especially important for a rapid formation of lignin-rich layer in CC and CML regions between newly formed thin-walled cells near cambium. The supramolecular structures of lignin in those regions are grape-like clusters consisting of highly condensed polylignol micells (Terashima et al. 2012). This water-impermeable and mechanically strong layer is resistant to high turgor pressure in the night and allows the thin-walled cells to expand quickly in radial direction to normal size, and then the secondary wall layers can be formed by deposition of polysaccharides in the lumen side of the expanded cells followed by deposition of lignin as observed by microautoradiography (Terashima et al. 1988; Fukushima and Terashima 1991a) and electron microscopy (EM) (Terashima et al. 2004, 2009a,b).

Quick supply of MLGs is also important for maintaining the stem and branch in proper position by spontaneous formation of lignin-rich tracheid wall particularly at its outer S2 layer in compression wood (CW) (Parham and Côté 1971; Fujita et al. 1978; Okuyama et al. 1998). The CW lignin of pine contains higher ratio of *p*-hydroxyphenylpropane unit than normal wood lignin (Bland 1961; Westermark 1985; Fukushima and Terashima 1991b; Fukushima et al. 1997). This indicates that the large pool of specific MLG plays an important role in quick and programmed delivery of a specific monolignol for the various lignification regions.

On the other hand, less amounts of MLGs are necessary for lignification of the major fiber cells in angiosperm xylem than in case of lignification of tracheids in gymnosperms. Further decreased supply of MLGs keeps stem and branch in proper position by formation of gelatinous layer (G-layer) in fiber wall of tension wood (Okuyama et al. 1994). Yoshinaga et al. (2012) showed that lignification of S1 and S2 layers and of CML occurs during G-layer formation in poplar fiber cell walls. So a small pool of MLGs is sufficient for tension wood formation. Members of the genus *Magnolia* are considered to be "primitive" angiosperms lacking of G-fibers in the reaction wood (Okuyama et al. 1990, 1994). This fact provides one of the reasonable explanations for the large MLGs' pool in *M. liliiflora* as shown in the experiments of the present work.

(4) Postmortem lignification. The biological activity in the cytosol of differentiating conifer tracheid comes to an end before completion of secondary wall lignification in order to recover the essential elements such as nitrogen (mostly captured in enzymes) and other mineral elements from the mature cell for recycling them for the metabolism of new growing cells. The volume proportion of tracheid and ray parenchyma cells in conifer xylem is 90–95% and 5–10%, respectively (Spicer 2014). In the absence of a large MLGs' stock in the secondary wall of the tracheids, the tracheid must actively produce and supply the MLGs until completion of S layer lignification. The recovery of elements by the apoplastic pathway will be impossible through the thick lignified S layers of dead tracheids, and a recovery via the minor ray parenchyma cells will also be insufficient, because those cells do not facilitate radial water transport (Barnard et al. 2013). Thus, in most gymnosperms and some angiosperm trees including *Magnolia*, a large pool of MLGs is necessary for efficient recovery of essential elements via postmortem lignification.

On the other hand, lignification process in a typical hardwood xylem is quite different from that in a softwood xylem (Terashima and Fukushima 1989; Terashima et al. 1993). In a hardwood, lignification starts first at CC and CML regions of vessels followed by lignification of thin-walled S layers of vessels and CML region of the major fiber cells, and then lignification of the thick fiber S layers. Hardwood xylem consists of more than half (50–70%) of fiber cells with low lignin contents, which are especially low in tension wood. In addition, the proportion of ray and axial parenchyma cells is high (Spicer 2014), which may participate in supply and recovery of essential elements. Thus, storage of a large amount of MLGs is unnecessary in most angiosperms. Tsuyama and Takabe (2014, 2015) found free sinapyl alcohol in differentiating xylem of poplar wood along with a small amount of coniferin and coniferin-β-glucosidase. Considering the fact that the glucosidation and deglucosidation steps are universal in lignifying plant tissues, the existence of free monolignols in angiosperms can be rationalized by the small pool size of corresponding MLGs and the presence of β-glucosidase.

In postmortem lignification of xylem tracheary elements (TEs) of *Zinnia elegans*, parenchymatic xylem cells that surround TEs synthesize and transport lignin monomers and reactive oxygen species to the cell walls of dead TEs (Hosokawa et al. 2001; Pesquet et al. 2013). Yoshinaga et al. (2012) suggested possible supply of monolignols from ray cells during the lignification of fiber cell walls with G-layer in poplar tension wood. Considering the common occurrence and important role of MLGs in transport and programmed delivery of monolignols as well as the fact that free monolignols are toxic to living cell (Väisänen et al. 2015), it may be reasonable to suppose that monolignols are supplied in the form of MLGs in very

small pool size in the above mentioned non-cell autonomous lignification of angiosperms.

Flexibility and homeostasis in cell wall lignification

Because lignin is a kind of secondary metabolite, the lignification process and the lignin structure are variable depending on the plant species, type of the cell, stage of cell wall differentiation and cell wall layers, and also environment of the growing plant. On the other hand, cell wall lignification is the most basic metabolic process for survival of vascular plants. A variety of homeostatic system for defending the essential part of cell wall lignification against mechanical, biochemical, or genetic defects has been developed during the several hundred million years of evolution. In the study of basic lignification mechanism by transgenic techniques, precaution is needed if various results are compared, which were obtained from different plant species under different experimental conditions. Down regulation of cinnamyl alcohol dehydrogenase in transgenic poplar caused a red coloration of xylem tissue due to conjugated aldehyde group, but neither the lignin amount nor the monomer composition was significantly modified (Baucher et al. 1996). The red color was intense during the first growing season but disappeared or became much paler during the winter time. In the second year of growth, the red color was restricted to the young xylem and disappeared in the first growth ring (Baucher et al. 1996). This kind of flexibility and homeostasis of cell wall lignification may provide sometimes unclear or confusing explanations of the results of transgenic experiments related to glucosyltransferase (Lanot et al. 2006; Vanholme et al. 2010) and β-glucosidase (Chapelle et al. 2012). According to Zhao et al. (2013), lignin laccase activity is necessary and nonredundant with peroxidase activity for monolignol polymerization during plant vascular development. The laccase triple loss-of-function mutant lac4×lac17×lac11 of *Arabidopsis* has practically no xylem lignin but accumulates a large amount of coniferin and syringin. Accumulation of MLGs suggests that glucosidation of monolignols is an essential step in lignification of vascular cell walls.

Conclusions and future prospects

The experimental results with the ^{13}C-tracer method on *G. biloba* and *M. liliiflora* and the critical re-evaluation of the literature provided further evidence that MLGs play an important active role in lignification as intermediate compounds in the course of the programmed delivery of monolignols to the assembly site of macromolecular lignin and polysaccharides in the xylem cell wall. MLGs are more than simple storage chemicals for lignification. During the evolution of plants, significant diversification occurred in microanatomical features of stem and branch as well as the lignification process of cell walls. The pool size of MLGs varies along with diversification of the lignification process. Present hypothetical scenario on the role of MLGs is focused mainly on the lignification of tree xylem cell walls. The question, whether the pathway via MLGs is mandatory or not for lignification in the phloem, leaves, and roots of all vascular plants, is still waiting for clarification. Many details can be investigated by ^{13}C-labeling and LC-MS/MS analyses combined with genetic modulation of a variety of plants. The detailed mechanism for 3D assembly of lignin and polysaccharides in differentiating cell wall is not yet fully clarified. The elucidation of role and fate of the glucose liberated from MLGs by β-glucosidase would be needed for better understanding of the assembly mechanism.

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