Deciphering the Enigma of Lignification: Precursor Transport, Oxidation, and the Topochemistry of Lignin Assembly

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ABSTRACT Plant lignification is a tightly regulated complex cellular process that occurs via three sequential steps: the synthesis of monolignols within the cytosol; the transport of monomeric precursors across plasma membrane; and the oxidative polymerization of monolignols to form lignin macromolecules within the cell wall. Although we have a reasonable understanding of monolignol biosynthesis, many aspects of lignin assembly remain elusive. These include the precursors’ transport and oxidation, and the initiation of lignin polymerization. This review describes our current knowledge of the molecular mechanisms underlying monolignol transport and oxidation, discusses the intriguing yet least-understood aspects of lignin assembly, and highlights the technologies potentially aiding in clarifying the enigma of plant lignification.

Key words: lignification; monolignol transport; ABC transporter; laccase; peroxidase.

INTRODUCTION

Lignin is complex phenypropanoid polymer derived primarily from three cinnamyl alcohols: p-coumaryl, coniferyl, and sinapyl alcohols (termed monolignols). It is a crucial structural component preserving the integrity of plant cell wall, imparting stiffness and strength of vascular plants, enabling the transport of water and solutes through the tracheary elements in the vasculature system, and affording physical barriers against invasions of phytopathogens, and other environmental stresses (Boerjan et al., 2003; Ralph et al., 2004a). However, its presence contributes to the recalcitrance of cell wall to degradation, and thus is detrimental to using cellulosic fibers in cattle feedstock, for pulping and paper making, and for producing liquid biofuels (Chen and Dixon, 2007; Li et al., 2008; Weng et al., 2008).

Plant lignification is a cellular process generating lignin polymer in the cell wall. In general, it occurs in three stages: the biosynthesis of monolignols in the cytosol; the transport of these monolignols to the cell wall; and their subsequent oxidative dehydrogenation and polymerization to form heterogeneous macromolecules. Over several decades, extensive studies have centered on monolignol biosynthesis, particularly on elucidating its pathways and the molecular regulation of the biosynthetic processes. The progresses have been intensively reviewed correspondingly (Anterola and Lewis, 2002; Boerjan et al., 2003; Ralph et al., 2004a; Ralph, 2007; Li et al., 2008; Vanholme et al., 2008; Zhong and Ye, 2009; Umezawa, 2010; Vanholme et al., 2010; Zhao and Dixon, 2011). Compared to the knowledge of monolignol biosynthesis, our understanding of lignin assembly, namely the transport of lignin precursors, their deposition, and subsequent activation and polymerization, is fragmentary. In this review, we outline the different speculations on the sequestration, transport, and subsequent spatial deposition of the monolignols, and describe recent progresses in molecular genetics and biochemical studies aimed at gaining an understanding of the underlying molecular mechanisms for translocating monolignols across cell membranes, advances in identifying the oxidative dehydrogenation enzymes, and progresses and issues in detailing the topochemistry of lignification. Meanwhile, we highlight the technological developments that might be applied productively to explore lignin assembly.

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Received 29 September 2011; accepted 13 December 2011
MONOLIGNOL BIOSYNTHESIS AND THE POTENTIAL PHYSICAL ORGANIZATION OF THE ENZYMES

Originating from the shikimate pathway, wherein plant produces aromatic amino acids, the biosynthesis of monolignols starts from the deamination of phenylalanine by the entry point enzyme phenylalanine ammonia lyase (PAL), and then undergoes subsequent aromatic-ring modifications via hydroxylation and methylation, and the transformation of the carboxylic moiety of the propane tail through esterification and reduction. This process yields primarily three hydroxycinnamoyl alcohols, \textit{p}-coumaryl, coniferyl, and sinapyl alcohols (i.e. monolignols) (Umezawa, 2010; Vanholme et al., 2010). More than 10 enzymes sequentially catalyze this synthetic pathway (Figure 1). Three of them, namely cinnamic acid 4-hydroxylase (C4H), \textit{p}-coumaroylshikimate 3’-hydroxylase (C3’H), and coniferaldehyde/ferulic acid 5-hydroxylase (FSH), are membrane-anchored cytochrome P450 proteins that predictably associate with the outer surface of the endoplasmic reticulum (ER) by virtue of their N-terminal membrane anchor (Li et al., 2008). Nevertheless, many other enzymes, such as phenylalanine ammonia lyase (PAL), 4-hydroxycinnamoyl CoA ligase (4CL), caffeoyl CoA O-methyltransferase (CCoAOMT), hydroxycinnamoyl CoA reductase (CCR), caffeic acid/5-hydroxyferulic acid 3/5-O-methyltransferase (COMT), and (hydroxy)cinnamyl alcohol dehydrogenase (CAD) found in diverse species operationally are soluble proteins and likely to be located within the cytosol (Takabe et al., 1985; Nakashima et al., 1997; Chen et al., 2000). Apparently, the different compartmental propensity of the monolignol biosynthetic enzymes, together with the exclusive plastidic localization of the shikimate pathway that leads to the synthesis of aromatic amino acids for phenylpropa-noids (Herrmann and Weaver, 1999; Rippert et al., 2009), implies either the occurrence of multiple subcellular sequestration of metabolic intermediates or the ideal physical organization of metabolic pathways.

\textbf{Figure 1.} The Scheme of the Simplified Shikimate–Phenylpropanoid–Lignin Biosynthetic Pathway, Illustrating Different Compartmentalization of the Biosynthesis.

CM, chorismate mutase; TYRA, arogenate dehydrogenase; ADT, arogenate dehydratase; PAT, prephenate aminotransferase; PAL, phenylalanine ammonia lyase; TAL, tyrosine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-hydroxycinnamoyl CoA ligase; HCT, hydroxycinnamoyl; CoA, shikimate/quinate hydroxycinnamoyltransferase; C3’H, p-coumaroylshikimate 3’-hydroxylase; CCoAOMT, caffeoyl CoA O-methyltransferase; CCR, cinnamoyl CoA reductase; CaldSH/FSH, coniferaldehyde/ferulate 5-hydroxylase; COMT, caffeic acid/5-hydroxyferulic acid O-methyltransferase; CAD, (hydroxy)cinnamyl alcohol dehydrogenase; POX, peroxidase; LAC, laccase.
the pathways to eliminate the phytotoxicity of aromatic intermediates and to enhance the transformation efficiency of the intermediate compounds. Such physical organization of metabolic pathways is variously termed ‘metabolic compartment’, ‘metabolic channeling’, or ‘metabolon’. The metabolic channels may contain arrays of consecutive, physically associated enzymes assembled on membranes or other physical structures, or interacted directly with each other. This association can channel biosynthetic intermediate from one enzyme to the next without releasing them into the total cellular metabolic pool (Srere, 1981; Hrazdina and Jensen, 1992). Earlier immunolocalization studies on monolignol enzymes in the lignifying mesophyll cells of *Zinnia elegans* and the differentiating xylem cells of *Eucalyptus* and *Populus* revealed that the isoforms of PAL, CAD, OMT, and 4CL could associate with ER-Golgi-derived vesicles, and then dispersed into the cytosol (Takabe et al., 2001; Takeuchi et al., 2001). In contrast, the peroxidase involved in the dehydrogenative polymerization of monolignols was synthesized in the rough ER and then transported to the lignifying cell wall (Takabe et al., 1985; Nakashima et al., 1997; Takabe et al., 2001; Takeuchi et al., 2001). Metabolic labeling experiments also revealed that a PAL isoform, PAL1, in tobacco was in close physical association with the membrane-bound P450 enzyme C4H on the microsomes for the synthesis of the active intermediate, p-coumaric acid (Czichi and Kindl, 1977; Hrazdina and Jensen, 1992; Deshpande et al., 1993; Rasmussen and Dixon, 1999; Dixon, 2000). Membrane fractionation and protein gel-blot analyses corroborated this observation. Furthermore, the transient coexpression of the PAL1 or PAL2 fused with green fluorescence protein in the cells expressing C4H demonstrated that two PAL isoforms exhibited different affinities in associating with the ER microsome and C4H likely organized the enzyme complex containing PAL1 and/or PAL2 (Achnine et al., 2004). These data suggest that monolignol biosynthetic enzymes are partially organized within the lignifying cells. Consequently, Dixon et al. (2001) speculated that there was a complicated metabolic channel for producing syringyl lignin, wherein three cytochrome P450 enzymes, C4H, C3'H, and FSH, anchor other operationally soluble enzymes, including COMT and perhaps specific forms of PAL, 4CL, and/or CCR, to the surface of ER. However, experimentally substantializing this appealing hypothesis proved challenging and technically demanding. Recently, many biochemical, biophysical, molecular, and cellular approaches were developed and applied for dissecting protein–protein interactions or protein complex organization (Lalonde et al., 2008; Miernyk and Thelen, 2008). Among them, several may offer the possibility to precisely evaluate the potential physical/spatial organization of lignin biosynthesis at cellular and subcellular levels. Particularly promising are the split ubiquitin membrane yeast two-hybrid screening (Obrdlik et al., 2004), surface resonance spectroscopy, fluorescence-based technology, such as bimolecular fluorescence complementation (BiFC) and the fluorescence energy resonance transfer (FRET), and three-dimensional super-solution microscopy. How lignin biosynthetic enzymes physically organized and/or compartmentalized and how such macromolecular organizations affect lignin deposition are intriguing aspects for future explorations.

**TRANSPORT OF MONOLIGNOLS ACROSS CELL MEMBRANE**

After monolignols are synthesized in the cytosol, they must be moved into the cell wall, where they are oxidized, and integrated into the matrix of the secondary cell wall. The exact mechanism whereby this occurs remains unclear; at least three models or pathways are envisaged, as illustrated in Figure 2: (1) exocytosis via ER-Golgi-derived vesicles; (2) passive diffusion via hydrophobic reactions through the plasma membrane; and (3) active transport via plasma membrane-located transporters or the other facilitators. We have discussed these potential mechanisms in detail in a recent review (Liu et al., 2011). To keep the integrity of this paper, we reiterate some related contents here but focus more on the up-to-date progresses on this subject.

**Monolignol Glucosylation and Deglucosylation**

Monolignols in cells occur both as free aglycones (non-sugar compounds) and as water-soluble 4-O-β-D-glucosides. The latter, namely p-coumaryl alcohol glucoside, coniferin, and syringin, are primarily found in gymnosperms (Terazawa and Miyake, 1984; Whetten and Sederoff, 1995). A set of angiosperm species also can generate those glucoconjugates in particular tissues; for instance, coniferin and syringin accumulate in *Arabidopsis* roots and their levels can be raised by light treatment (Hemm et al., 2004). In conifers, the build-up of coniferin correlates spatially and temporally with the beginning of secondary growth (Savidge, 1988, 1989). Presumably, these glucoconjugates are stored within the vacuoles of cambial cells (Leinhos and Savidge, 1993; Dharmawardhana et al., 1995). The possible presence of the glucosides of monolignols (or of cinnamaldehydes) in vacuoles of differentiating xylem cells led to the suggestion that those glucoconjugates may serve the storage and transport functions of monolignols at least in gymnosperms and some evolutionarily less-advanced angiosperms (Steeves et al., 2001; Tsuji and Fukushima, 2004). Presumably, those glucosides are seques-
trated first from the cytosol to vacuoles and then exported to the cell wall (Dharmawardhana et al., 1995, 1999; Samuels et al., 2002; Escamilla-Treviño et al., 2006).

Monolignol glucosylation is catalyzed by coniferyl/sinapyl alcohol glucosyltransferase (Steeves et al., 2001). The activity of this enzyme was detected in many gymnosperms and angiosperms; the woody angiosperms exhibited higher enzyme activities than did the herbaceous species (Ibrahim, 1977). On the other hand, monolignol deglucosylation, catalyzed by specific β-glucosidase, is thought to occur in the cell wall at the site of lignin polymerization. The apoplastic location of β-glucosidase was demonstrated by immunohistochemical studies (Marcinowski et al., 1979; Burmeister and Hosel, 1981). The enzymes were identified in Norway spruce (*Picea*...
abies) (Marcinowski and Grisebach, 1978), pinus species (Leinhos et al., 1994; Dharmawardhana et al., 1995), and A. thaliana (Escamilla-Treviño et al., 2006). The cDNAs encoding coniferin β-glucosidase were isolated from both lodgepole pine and Arabidopsis; their expression was proved highly specific at the site of lignification (Dharmawardhana et al., 1999; Escamilla-Treviño et al., 2006). Based on the discovery of UDP-glucose:coniferyl alcohol glucosyltransferase/coniferin β-glucosidase, a monolignol glucosyltransferase and β-glucosidase-mediated transport pathway was proposed, where the two enzymes coordinately regulate the storage and mobilization of monolignols for lignin biosynthesis (Dharmawardhana et al., 1995, 1999; Samuels et al., 2002; Escamilla-Treviño et al., 2006). However, recent experiments failed to support this speculation. A small cluster of glucosyltransferase genes were functionally characterized from Arabidopsis. The encoded enzymes exhibited ability to glucconjugate monolignols in vitro (Lim et al., 2001). However, disrupting those genes’ expression did not perturb lignin deposition (Vanholme et al., 2008), although a corresponding reduction or accumulation of soluble monolignol glucosides in the roots or leaves of transgenic plants was recorded (Lanot et al., 2006). Moreover, feeding [3H]-Phe to the dissected xylem of lodgepole pine (Pinus contorta) revealed that the radiolabels were incorporated directly into the monolignols and the lignins accumulated in the cell wall. The substantial amount of the radiolabels was associated neither with monolignol glucoside (coniferin) nor with the interior of the central vacuole, where, expectedly, coniferin is stored (Kaneda et al., 2008). These data argue against the proposed roles of monolignol glucosylation–deglycosylation in exporting lignin precursors across plasma membrane, and imply that monolignol aglycone is the chemical form used for such transport.

**Mechanisms for Transporting Monolignols across Cell Membranes**

**Exocytosis via Vesicles Derived from Endoplasmic Reticulum-Golgi Bodies**

The non-cellulosic polysaccharides of plant cell wall, such as pectin and hemicellulose, are known to be synthesized within the Golgi bodies and exported to the cell wall through an exocytosis mechanism (Lerouxel et al., 2006; Sandhu et al., 2009). Early autoradiographic and immunochemical studies implied a similar
mechanism for transporting lignin precursors. A vesicle traffic-
ing between cytosol and plasmalemma in the differentiating
tracheids of wheat xylem was observed (Pickett-Heaps, 1968).
Feeding the developing xylem with isotopically labeled Phe,
Tyr, and cinnamic acid resulted in their incorporation into the
rough ER, the Golgi apparatus, and some vesicles that were asso-
ciated with plasma membrane, or aggregated near the bands
of cell wall microtubules (Pickett-Heaps, 1968; Fujita and
Harada, 1979; Takabe et al., 1985). These findings suggested
that the ER-Golgi apparatus are likely to be involved in synthe-
sizing and transporting monolignols to the cell wall. However,
since the applied radiotracers are assimilated efficiently into li-
ginin and into proteins, it might complicate the interpretation
of these autoradiographic data. Indeed, in a recent experiment,
Kaneda et al. (2008) fed the [3H]-Phe to the cambium/develop-
ing xylem tissues of lodgepole pine that were isolated by cryo-
fixation and freezing substitution before sectioning (those
treatments substantially minimize the damage to the cells
and thus prevent pseudo-autographic imaging). By selectively
inhibiting phenylpropanoid or protein biosynthesis, they dis-
covered that the radioactivity presented in the ER-Golgi was
defined from in vitro partitioning experiments using immobi-
lized liposomes or lipid-bilayer discs as the model membranes.
Incubating monolignol-like compounds with artificial model
membranes caused them to freely partition into the mem-
brane phase due to hydrophobic–hydrophilic interactions
(Bojla et al., 2007, 2008). However, so far, we lack definitive
biochemical evidence for such transport in plant tissues.

### Passive Diffusion

Genetic engineering of lignin biosynthesis and chemical anal-
ysis of the resulting transgenic cell walls reveal that many non-
traditional phenolics can be incorporated into the lignin
polymer. In transgenic plants and/or mutants deficient in
monolignol biosynthesis, some metabolite intermediates
or precursors, such as 5-hydroxycinnamyl alcohol (Van
Doorselaere et al., 1995; Ralph et al., 2001), hydroxycinnamal-
dehydes (Baucher et al., 1996), and hydroxycinnamic acids
(Leple et al., 2007; Ralph et al., 2008), were integrated into li-
ginin. In addition, in monocot and some dicot species, monoli-
gnols are often further acylated with p-hydroxybenzoate,
p-coumarate (Lu et al., 2004), and acetate (Lu and Ralph,
2002). These derivatives occur in the cell wall lignin (del Rio
et al., 2007, 2008). Such accommodation of alternative mono-
mers in lignification implies a potential non-selective passive
diffusion of lignin monomers through plasma membrane
(Vanholme et al., 2008, 2010). This possibility was further evi-
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duced from in vitro partitioning experiments using immobi-

### Transporter-Mediated Active Sequestration and Export of
Monolignols

Global transcriptomic, proteomic studies, or functional genomics
in gymnosperms and angiosperms have frequently uncovered
highly expressed membrane transporters in lignifying or ligni-
ﬁed tissues. For example, several ESTs encoding for ATP-binding
cassette (ABC) transporters were disclosed from gene expression
profiling during wood transformation (Allona et al., 1998;
Hertzig et al., 2001; Kirst et al., 2003; Egertsdotter et al.,
2004). Transcriptional profiling based on the first-generation
loblolly pine microarray revealed the abundance of distinct
ABC transporter-like genes in the mature and compression
woods, wherein different types of lignins accumulated (Whetten
et al., 2001; Egertsdotter et al., 2004). In global transcript profiling
of A. thaliana stems, seven ABC transporter genes exhibited
similar expression patterns with the functionally known mono-
lignol biosynthetic genes (Ehling et al., 2005). ABC transporter
genes were also recognized in the Eucalyptus xylem-subtractive
library (Paux et al., 2004) and in the cultures of lignifying Zinnia
tracheary elements (Pesquet et al., 2005). Also, the cell wall
macro-array analysis of maize (Zea mays) brown mid-rib (bm)
mutants indicated that the lower levels of guaiacyl lignin in
bm2 mutants might reflect the decreased transcription of one
ABC transporter gene (Guillaumie et al., 2007). Furthermore,
in a recent proteomics study on plasma membranes from poplar
leaf, xylem, and cambium/phloem, a set of ABC transporters
showed a rather ‘specific’ tissue distribution; some members
of the ABC transporter subfamily B and G were identified only
in the cambium/phloem (Nilsson et al., 2010). These data imply
that ABC transporters might play a significant role in cell wall
lignification, presumably in transporting monolignols to the wall
(Samuels et al., 2002; Douglas and Ehling, 2005).

ABC transporters constitute a large, diverse family of proteins
that translocate a broad range of substances across cell mem-
branes (Higgins, 1992; Sánchez-Fernández et al., 2001; Verrier
et al., 2008). The genomes of Arabidopsis and rice (Oryza sativa)
each contain more than 120 putative ABC transporters (Sánchez-
Fernández et al., 2001; Jasinski et al., 2003). Many of these pro-
tein exhibited diverse transport activities to a variety of small
molecule metabolites, such as glutathione conjugates (Lu
et al., 1998; Tommasini et al., 1998), auxins (Geisler et al.,
2005; Strader and Bartel, 2009; Ruzicka et al., 2010), abscisic acid
(Kang et al., 2010; Kuromori et al., 2010), inorganic ions (Suh
et al., 2007), malate (Lee et al., 2008), wax and cutin precursors
(Pighin et al., 2004; McFarlane et al., 2010), defensive secondary
metabolites such as antifungal terpenoids (Jasinski et al.,
2001), tropane alkaloids (hyoscyamine and scopolamine)
(Goossens et al., 2003), benzylisouquinoline alkaloid (berberine)
(Shitan et al., 2003; Shitan and Yazaki, 2007), flavone glucuro-
nides (Klein et al., 2000), Frangne et al., 2002), isoflavone genis-
tein (Sugiyama et al., 2007), and many xenobiotics (Baerson
et al., 2005).

To ascertain whether the transport of monolignol is an ac-
tive process that involves ABC transporters, we recently
conducted the in vitro uptake assay to monolignols and/or their glucosides by using plasma and vacuolar membrane vesicles prepared from both Arabidopsis rosette leaves and poplar roots (Miao and Liu, 2010). Our study reveals several lines of evidence demonstrating the involvement of ABC-like transporters in monolignol sequestration and transport: (1) the transport of monolignols across either plasma or vacuolar membranes largely depends on the presence of nucleotide phosphates, particularly ATP; lacking it severely impairs the transport activity of either type of vesicles, suggesting that transport is an active process requiring energy; (2) a set of specific ABC transporter inhibitors largely reduce the uptake activity of both plasma and vacuolar membrane vesicles for lignin precursors in the presence of ATP; (3) the uptake of monolignols or their glucosides by membrane vesicles displays typical protein–ligand binding kinetics, indicating that it is a membrane protein-mediated biochemical process rather than passive diffusion; and (4) the uptake to lignin precursors by membrane vesicles shows obvious selectivity. In the presence of ATP, the vacuolar membrane vesicles actively sequester monolignol glucoujagulates, whereas the preference of the inverted plasma membrane vesicles is for the monolignol aglycones; this difference suggests that the glucosylation of monolignols is a prerequisite for vacuolar sequestration, while the aglycones are the form for direct export into cell walls. Also, the findings implicate the distinct classes of ABC transporters as being involved in either partitioning the polarized ‘storage form’ glucosides of monolignols into vacuoles or exporting the hydrophobic aglycones across plasmalemma. Compared to the vacuolar membrane vesicles, plasma membrane ones exhibit a more relaxed substrate specificity, being able to convey p-hydroxyphenyl ethanol and other related cinnamaldehydes, thereby suggesting the basis for the observed plasticity of lignin biosynthesis (Miao and Liu, 2010). Promiscuous substrate-transport activity and/or the low intrinsic level of plasmalemma diffusion may engender the deposition of non-classic lignin precursors in the cell wall. In addition, we found that the chemical character of the para-hydroxyl of monolignol substantially influences the nature of transport. While the 4-O-glucosylation of monolignols likely dominates the vacuolar sequestration, the etherification of the para-hydroxyl with a methyl moiety largely releases the dependence of monolignol transport on the ATP-energized mechanism. When incubated with 4-O-methyl coniferyl alcohol in the absence of ATP, the amount of this etherified monolignol derivative accumulates in the inverted plasma-membrane vesicles is comparable to that of conventional monolignols accumulated in the presence of ATP (Y. Miao and C.-J. Liu, unpublished data).

**Issues in Identifying Particular Transporters Responsible for Depositing Lignin**

To identify the specific ABC transporters functioning in lignification, Kaneda et al. (2011) analyzed a subset of Arabidopsis ABC transporters whose expressions have a good correlation with phenylpropanoid–lignin biosynthesis and are higher in the vascular or interfascicular tissues of the primary stem (Ehling et al., 2005). However, mutant lines singly deficient in those ABC transporter genes did not attenuate xylem lignification. Instead, two mutant lines respectively disrupting ABCB14 and ABCB15 impaired auxin polar transport in the stem, even though the former was recently characterized as a malate importer in leaf guard cells (Lee et al., 2008). These findings highlight the difficulty in characterizing the specific monolignol transporters. Since lignification is a very complicated cellular process, orchestrating a large set of gene expression during cell differentiation and secondary cell wall thickening, the ABC transporters found in vascular tissues and correlated with their lignification may be involved in distinct biological processes. Furthermore, lignin deposition and distribution are known to be tissue/cell-type specific, which may require a set of distinct membrane transporters. Many ABC transporter genes have overlapping tissue expression pattern and therefore possible functional redundancy. Analyzing plants with a single gene deletion might fail to distinguish its consequence on lignification, particularly when lignin content is measured in large-sized, pooled cell wall samples. In addition, ABC transporters typically exhibit low substrate specificity (Verrier et al., 2008; Ruzicka et al., 2010); some members may transport a set of unrelated molecules and fulfill disparate functions, as implied by the researches on ABCB14 (Lee et al., 2008; Kaneda et al., 2011). Besides creating multiple gene-deficient lines for the related ABC transporters, we must precisely measure the deficit of lignification in a tissue or cell-type-specific manner. Laser capture microdissection of vascular bundles and interfascicular fibers, followed by microthioacidolysis or pyrolytic GC/MS analysis (Patten et al., 2010) may offer a practical, higher-resolution strategy for identifying the particular set of ABC transporters or other membrane transporters involved in transporting monolignols and depositing lignin.

**ENZYMES RESPONSIBLE FOR OXIDATIVE DEHYDROGENATION**

After their deposition in the cell wall, monolignols undergo dehydrogenation, producing resonance-stabilized radicals. Then, the resulting phenoxy radicals couple each other or to the growing lignin polymer, if the polymer was also oxidized, to form different types of sub-linkages. Monolignol oxidation/dehydrogenation is proposed to be catalyzed by peroxidases, laccases, or other phenol oxidases. Those enzymes are widely found in lignin-forming tissues and can oxidize a variety of phenolics, including monolignols in vitro (Freudenberg, 1959; Ralph et al., 2004a; Davin et al., 2008).

Peroxidase uses hydrogen peroxide as its electron receptor to oxidize a variety of phenolics (Harkin and Obst, 1973). Different peroxidases have distinct predilection in oxidizing the conventional monolignols. Most peroxidases readily oxidize coniferyl alcohol, and the resulting radicals can transfer to,
and thus oxidize, sinapyl alcohol. Sinapyl alcohol itself is a poor substrate for oxidation, and is catalyzed only by a very specific peroxidase (Sasaki et al., 2006, 2008). These properties led to a radical mediation model to explain lignin polymerization, in which the coniferyl alcohol radicals generated through the interaction of the cell wall-bound peroxidases with monolignols diffuse to the growing lignin polymer; thereafter, they either transfer their higher oxidation state to a lignin molecule, if the lignin polymer is at a base oxidative state, or undergo a coupling reaction to form a covalent bond with the polymer, if the latter is at a higher oxidation state (Hatfield and Vermerris, 2001). Further, a complementary model was derived from this radical-transfer hypothesis by suggesting the operation of a diffusible redox shuttle-peroxidase system in which a membrane-bound peroxidase oxidizes manganese, the oxidized ions diffuse through small pores in the cell wall to the lignification site, and then they oxidize both the monolignols and growing lignin polymers. As the redox ions directly oxidize the monolignols and polymers, the radicals as the primary oxidation products from both constitute a high concentration, thus facilitating the coupling frequency of the monolignols with growing polymers (Onnerud et al., 2002). However, the need for having radical or redox-shuttle mediation was challenged by the identification of a Populus peroxidase specific for sinapyl alcohol that can directly and efficiently oxidize both sinapyl alcohol and the higher molecular polymers (Sasaki et al., 2004).

Peroxidases in plant are encoded by a multigene family. In Arabidopsis, 73 class III peroxidase genes were identified (Tognolli et al., 2002; Fagerstedt et al., 2010). Despite their high degree of sequence and function redundancy that complicate the identification of lignin-specific enzymes, the biological functions of class III plant peroxidases in lignification were demonstrated in a few genetic studies with altered expression of the peroxidase genes. Down-regulation of the gene coding for a cationic peroxidase NtPrx60 (TP60) in tobacco resulted in up to a 50% reduction in total acetyl bromide lignin, and the corresponding reduction in both the G and S units (Blee et al., 2003); down-regulation of an anionic peroxidase PkPrx03 (PrxA3a) in aspen resulted in a decline of up to 20% of total lignin content in transgenic lines (Li et al., 2003). These data suggest that species-specific oxidative enzymes are involved in plant lignification; on the other hand, different types of peroxidases may have overlapping biological functions in lignin polymerization.

Laccases (p-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) are copper-containing extracellular glycoproteins that require O2 as secondary substrate to oxidize various phenolic, inorganic, and/or aromatic amine substrates (Reinhammar and Malmstroem, 1981). Similar to peroxidase, laccases oxidize monolignols in vitro (Freudenberg, 1959; Sterjiades et al., 1992; Bao et al., 1993; Takahama, 1995; Richardson et al., 1997), and laccase-like activities were detected in the lignifying cell walls of differentiating xylem (Driouich et al., 1992; Bao et al., 1993; Liu et al., 1994; Richardson et al., 2000). Arabidopsis contains 17 putative laccase homologous genes, and their expressions closely concur across a wide variety of tissues/development stages (Turlapati et al., 2011). A few family members display unique tissue/cell-type expression patterns, suggesting their specific roles in either lignification or other oxidative processes (Pourcel et al., 2005; Turlapati et al., 2011). Determining the role of laccases in lignification yielded ambiguous findings until a recent genetic study wherein the functions of the Arabidopsis laccase genes LAC4 and LAC17 in xylem lignification were demonstrated conclusively (Berthet et al., 2011). About a 30–40% reduction of lignin content in the stem of mutant plants simultaneously disrupting LAC4 and LAC17 was registered. A deficiency of LAC17 primarily affected the deposition of guaiacyl lignin and resulted in a change of the S/G ratio in mutant lines, implying the preference of LAC17 for oxidizing coniferyl alcohol. However, definitive specifications of the biochemical properties of those LACs are lacking presently. It will be interesting to see whether the identified LACs are able to oxidize sinapyl alcohol and other higher molecule oligomers/polymers.

Interestingly, down-regulation of LAC broadly changed the phenylpropanoid biosynthetic pathway. Almost the entire batch of monolignol biosynthetic genes was suppressed coordinate with the disruption of LAC4 and 17 (Berthet et al., 2011). These data imply the existence of some form of feedback regulatory mechanisms at the plasma membrane/cell wall interior that prevents the accumulation/transport of monolignols when oxidative capacity is diminished. It is interesting to decipher whether the accumulated monolignols or their derivatives serve as signaling molecules, and how the set of biosynthetic genes in the pathway are organized as a particular ‘regulon’.

SPATIAL DISTRIBUTION OF LIGNIN AND TOPOCHEMISTRY OF LIGNIFICATION

Lignin is known to be selectively deposited in the walls of different cell types. For example, the vessels of birch wood are enriched in G units, while the fiber wall incorporates both S and G units (Fergus and Goring, 1970). This feature is also demonstrated in Arabidopsis stems, where the lignin of the vascular bundle rich in vessel cells primarily contains G units, while the interfascicular fibers are enriched in S units (Chapple et al., 1994). In a more subtle way, lignin is differentially deposited in discrete regions of the cell walls. In spruce wood, the lignin of the middle lamella embeds more p-coumaryl alcohol-derived units than does the secondary wall lignin that mainly is derived from coniferyl alcohol (Whiting and Goring, 1982). Feeding labeled monolignols into developing xylem reveals the preferential deposition of radiolabeled p-coumaryl alcohol in the middle lamella or cell corners, whereas coniferyl alcohol is located mostly in the secondary wall (Terashima et al., 1993). Moreover, during the development of secondary cell wall to form S1-, S2-, and S3-layers, the three kinds of monolignols exhibit sequential deposition, in the order of p-coumaryl alcohol (for the H unit), coniferyl alcohol (G unit), 
and sinapyl alcohol (S unit) (Terashima et al., 1986a, 1986b, 1993). Disturbing lignin biosynthesis by down-regulating particular monolignol biosynthetic genes, such as CCR, selectively affects the spatial deposition of lignin in the fiber and xylem cells, and even within the different sub-layers of secondary cell wall (Chabannes et al., 2001; Ruel et al., 2009; Thévenin et al., 2011). These data strongly suggest that lignification is tightly regulated during developmental programs and that lignin deposition within the wall is a highly organized process. Although the exact molecular mechanism in controlling lignin deposition remains elusive, the lignin composition of each individual cell appears to be controlled by a complex array of gene expression in the differentiating cells or in adjacent cells. This complexity would determine the number and type of lignin precursors deposited at lignification sites, which were predicted as the key factors controlling lignin structure (van Parijs et al., 2010). Accordingly, the spatial and temporal expression of the monolignol transporters and their substrate specificity may greatly contribute to the tissue and cell-type-specific lignification.

Once monolignols are laid down in the cell wall, they are proposed to diffuse to the initiation site for polymerization. Lignification, in general, begins at the cell corner of the middle lamella and the S1 region of the secondary cell wall before spreading across the secondary wall towards the lumen (Donaldson, 2001; Möller et al., 2006). Lignification of primary cell wall typically begins after the start of the formation of the secondary cell wall, while lignification of secondary cell wall usually starts when secondary cell wall formation is completed. Currently, it is unclear yet how the precise topochemistry of lignification occurs and is controlled. A type of non-enzymatic dirigent protein was characterized functioning as a template guiding the formation of the bond linkage of two monolignol molecules, and defining the stereochemistry of the resulted optically active compound lignan (Davin et al., 1997). This proteinaceous control mechanism was envisaged for lignin polymerization, where proteins bearing dirigent sites guide the phenoxy radical coupling within the cell wall (Davin and Lewis, 2000, 2005; Davin et al., 2008). The dirigent sites were then predicted to be the lignin initiation sites (Donaldson, 2001). However, lignin is structurally known as an optical inactive, racemic polymer containing many different types of bonds connecting monolignol residues in a somewhat random pattern (Vanholme et al., 2010). These facts apparently challenge the involvement of dirigent proteins in polymerization. In addition, although a set of dirigent homolog genes were found in plants, so far, none was definitively defined in lignin polymerization, either biochemically or genetically.

Because lignification starts at the region furthest from the protoplast, it was suggested that the initiation sites may have been bound to specific regions of the existing cell wall (Donaldson, 1994). The cell walls of monocot grasses and some dicot species, including Arabidopsis thaliana, contain significant quantities of ester-bound hydroxycinnamates, primarily $p$-coumarate and ferulate (Ishii, 1997; Ralph et al., 2004b). Particularly for ferulate, these ‘wall-bound’ phenolics can dimerize or polymerize with each other, or with other cell wall phenolics, forming ester-to-ether linkages to cross-link the adjacent polysaccharides, lignins, and/or structural proteins (Ralph et al., 2004a). These ‘wall-bound’ phenolics purportedly also act as nucleation sites for lignin polymerization (Ralph et al., 2004a; Ralph, 2010). Peroxidase activity was noted, catalyzing the formation of ferulate-mediated ester to ether linkages between polysaccharide and lignin composition, or the cross-links of ferulate residues in polysaccharides (Burr and Fry, 2009a, 2009b). However, conclusive evidence to establish the role of ‘wall-bound’ ferulate in initiating lignin polymerization is needed.

Since lignin assembly depends on the quantity and type of monolignol radicals within the particular region of the cell wall (Hatfield and Vermerris, 2001; van Parijs et al., 2010), the potential sub-compartmentalization of the radical-producing enzymes, namely peroxidase and laccase, within the cell wall may affect the initiation of lignin polymerization and control the spatial deposition of a particular type of lignin. Both enzymes are extracellular proteins, and are known from immunohistochemical analyses to locate in middle lamellae, cell corner, and secondary cell wall of the lignifying cells (Bao et al., 1993; Liu et al., 1994; Richardson et al., 2000; Sasaki et al., 2006). However, there is little detail about their potential sub-compartmentalization and spatial movement within the cell wall during developmental programming and the lignification process. An early study found that the sycamore maple laccase is far less active on phenolic substrates containing multiple aromatic rings than is peroxidase (Sterjiades et al., 1993). This leads to the assumption that laccases may polymerize monolignols into oligo-lignols during the early stages of lignification, whereas the cell wall peroxidases may function when $\text{H}_2\text{O}_2$ is generated during the later stages of xylem cell development or in response to environmental stresses (Sterjiades et al., 1993). Recently, intriguing findings from several pieces of cell wall immunohistochemical studies revealed the existence of diverse microenvironments within cell walls that are likely to influence the access of proteins or enzymes to specific targets (Lee et al., 2011). Some small-molecular-weight carbohydrate binding modules or immuno-epitopes can be effectively blocked or restricted to access to their ligands with the presence of pectic homogalacturonan (Herve et al., 2010; Marcus et al., 2010). It is possible that the distribution and localization of oxidative enzymes within the cell wall are affected with the similar ‘polymer masking’ effects. Along with characterizing lignin-specific laccases and peroxidases, it now might be able to evaluate this appealing hypothesis. Detailed biochemical analyses of the identified laccases and peroxidases should clarify whether different types of oxidative enzymes exhibit substrate specificity, particularly toward oxidizing high-molecule oligomers or polymers. Moreover, advanced microscopic techniques now enable us to dissect the details of single protein behaviors in a particular cell compartment, as recently demonstrated in a series of researches.
applying spin disc confocal microscopy to delineate the functional association of the cellulose synthase complex with cortical microtubules (Paredez et al., 2006; Gutierrez et al., 2009). Aided with this type of microscopic technique, fluorescently labeled cellulose synthase was clearly observed as motile punctates at the plasma membrane. They moved bi-directionally at constant rates in linear tracks aligned and coincident with cortical microtubules, whereby they orient nascent microfibrils in the secondary thickening cell walls (Paredez et al., 2006). In an earlier ultrastructural study employing transmission electron microscopy, Donaldson (1994) observed that, during the initiation of lignification in the cell wall of *Pinus radiata*, lignin particles deposited in the S1 sub-layer appeared as elongated structures, quite distinct from the round-shaped particles in the middle lamellae. This finding suggests that the orientation of lignin deposition in the secondary cell wall is affected by either the deposited cellulose microfibrils or the wall’s microtubules. The same approach for monitoring the mobility of cellulose synthase might be applicable to probing the subcellular action of oxidative enzymes during lignification. Moreover, the emergence of super-resolution fluorescence microscopy offers the capability of visualizing the three-dimensional fine structure of the cell and to efficiently detect a single nanosized fluorophor with the remarkable spatiotemporal resolution of <20 nm (Bates et al., 2008; Huang et al., 2009; Gutierrez et al., 2010). This technique permits the imaging of features that previously were only resolved via electron microscopy. Applying one type of super-resolution microscopy, Structured Illumination Microscopy (SIM), Fitzgibbon et al. (2010) were able to see the apertures of individual plasmodesmal pores and trace the fine structure of endoplasmic reticulum-derived tubules through the pores and between adjacent sieve element cells. With such spectroscopic techniques, we might be able to pinpoint the functional sites and action modes of the specific laccases, peroxidases, and any other types of cell wall proteins possibly related to lignification.

**CONCLUDING REMARKS**

Lignification is an extremely complicated but tightly controlled biological process, even though the bond formation in lignin polymerization is a random chemical process. Lignin deposition and assembly are spatially and temporally programmed in differentiating cell walls. Two important events therein are the transport of precursors across the protoplast membrane and the subsequent oxidative dehydrogenation in the cell walls. In concert with a complex array of transcriptional regulation (reviewed by Zhong and Ye, 2009; Umezawa, 2010; Zhao and Dixon, 2011) and potential metabolic organization of monolignol synthesis, spatially and temporally controlled monolignol transport and oxidation in distinct cell types and/or the particular sub-compartments of the cell wall are envisioned, which may play a critical role in controlling the elaborate processes of lignin deposition. Moving the monolignols across cell membranes takes an energy-requiring, selective transport process that involves ABC transporters. Despite the complexity of this process, research is underway towards clarifying and identifying particular monolignol transporters. Aided by modern microscopic technologies, together with the identification of the monolignol transporters and the specific oxidative enzymes for generating phenox radicals, the enigma of lignification in plants will be better elucidated.

**FUNDING**

This work was supported by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the US Department of Energy through Grant DEAC0298CH10886; the National Science Foundation through grant MCB-1051675; the Laboratory Directed Research and Development Program of Brookhaven National Laboratory (No 11-007); the CAS/SAFEA International Partnership Program for Creative Research Teams in Plant Metabolisms, and the National Science Foundation of China for Oversea Distinguished Young Scholars (31028003). No conflict of interest declared.

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