Original Article

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Lignin monomer transport in seed plants

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Abstract
Lignin biosynthesis consists of three steps: biosynthesis, transport, and polymerization of lignin precursors. Although biosynthesis of lignin precursors in the cell and polymerization of lignin precursors in the cell wall have been studied intensively, reports on the transport of lignin precursors are limited and the overall picture remains unresolved. This review summarizes studies on the transport and storage of monolignols, as well as the monolignol glucoside pathway in seed plants. Several mechanisms of lignin monomer transport may facilitate the plasticity of lignin structures and the spatiotemporal regulation of lignification.
Keywords: *ABC transporter; coniferin; lignification; passive diffusion; H^+-antiport*

1. Transport and Storage of Monolignols

Lignin is one of the major cell wall components of vascular plants and is essential for their survival on land, contributing to efficient water transport, morphological support, and defense against pathogens and UV irradiation. Lignin biosynthesis comprises three steps: biosynthesis, transport, and polymerization of lignin precursors. The biosynthesis of lignin precursors in the cells and the polymerization of lignin precursors in the cell walls have been extensively studied [1–6]. Intracellularly biosynthesized lignin precursors must be transported to the cell wall which is the site of polymerization. Although the transport of lignin precursors is an important step in the spatiotemporal regulation of lignin deposition, few studies have reported on this step and the overall picture remains unelucidated. Monolignols (*p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) are considered as typical and direct precursors of lignin polymerization. However, they are known to be cytotoxic [7,8]. Therefore, monolignol glucosides (*p*-glucocoumaryl alcohol, coniferin, and syringin) detected in the differentiating xylem are considered storage and transport forms of monolignols [1,2]. However, the storage of monolignols and their glucosides varies widely among plant species, and the direct supply of monolignols to the cell wall is also known to contribute to lignification [5,9].

Many studies have long reported the presence of substantial amounts of coniferin, a glucoside of coniferyl alcohol, in the differentiating xylem of conifers [1,10,11]. In the differentiating xylem of pine, coniferin content peaks in May and then gradually declines as lignification progresses [12,13]. When pine is administered with radiolabeled coniferin, the label localizes to the cell walls of xylem [14], suggesting that coniferin plays a role in xylem lignification. Furthermore, when radiolabeled phenylalanine is administered to *Ginkgo biloba* for few hours and incubated for 24 h, the radiolabel is incorporated into both lignin and coniferin [15]. When radiolabeled phenylalanine is administered to pine trees and incubated for 4 h, however, the radiolabel is incorporated into lignin but not coniferin [16]. These studies suggest that both long-term coniferin accumulation and short-term direct transport of monolignols contribute to lignification. *p*-Glucocoumaryl alcohol, a glucoside of *p*-coumaryl alcohol, has also been detected in the differentiating xylem of *Picea excelsa* [10]. When radiolabeled *p*-glucocoumaryl alcohol is administered to pine xylem, the radioactivity is incorporated into the cell walls in the early stages of lignification, indicating that *p*-glucocoumaryl alcohol may participate in the early stages of lignification [14,17].

Unlike gymnosperms (softwood species), only a few hardwood species, such as magnolias, store coniferin in their xylem, while many species contain little to no coniferin [11]. Previous studies have

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reported that poplar xylem contains sinapyl alcohol but little coniferin [18,19]. In Arabidopsis inflorescence stems, coniferin is present in greater quantities than coniferyl alcohol as a guaiacyl monomer, whereas sinapyl alcohol is more abundant than syringin, a glucoside of sinapyl alcohol, as a syringyl monomer [20]. These reports suggest that both the quantity and form of monolignol storage vary amoung plant species and lignin precursor type (e.g., guaiacyl vs. syringyl monomers). The presence of syringin in the phloem has been reported in *Magnolia kobus* [21] and poplar [19]. However, seasonal patterns of syringin storage suggest that stored syringin is not directly involved in xylem lignification [21,22]. Nevertheless, when radiolabeled monolignol glucosides are administered to various hardwoods, the radiolabels localize in the cell wall of the differentiating xylem [23], suggesting that monolignol glucosides may play a role in lignification not only in softwood but also in hardwood species.

In monocotyledonous bamboo, coniferin and syringin levels in the culms peak during periods of active elongation and growth, albeit in small amounts. *p*-Glucocoumaryl alcohol peaks during the active lignification phase, while monolignols, *p*-hydroxycinnamic aldehydes, and *p*-coumaric acid peak in August, when the lignin content is approximately 80% of that of mature bamboo, and their amounts are higher than those of their glucosides [24]. A previous study demonstrated that when rice plants were treated with radiolabeled *p*-glucocoumaryl alcohol, coniferin, or syringin, the radiolabels were found in the cell walls of the metaxylem and incorporated into lignin [25]. These data suggest that monolignol glucosides may serve as lignin precursors in monocotyledons.

2. Transport Mechanisms of Lignin Monomers

The mechanisms involved in the transport of lignin precursors remain largely elusive. Given that monolignol storage patterns and seasonal variations differ significantly among species and tissues, the modes of lignin precursor transport may also vary depending on the species, tissue type, and developmental stage of the plant. Possible mechanisms for lignin precursor transport include: 1. Passive diffusion; 2. Facilitated diffusion; 3. Primary transport (e.g., by ATP binding cassette [ABC] transporters); 4. Secondary transport (e.g., by H^+ antiporters belonging to multidrug and toxic compound extrusion [MATE] family or major facilitator superfamily [MFS]); and 5. Vesicle-mediated transport (Fig. 1) [9,26,27].

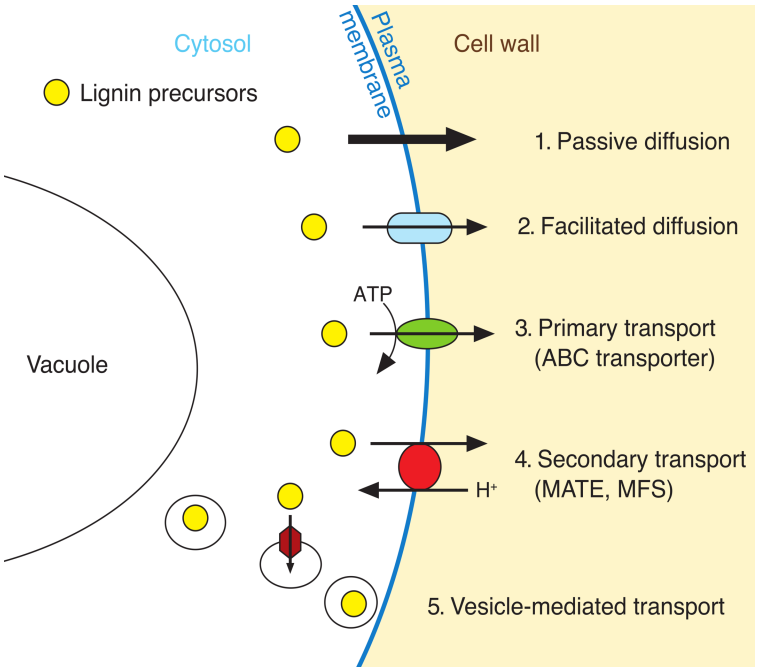

Fig. 1. Possible transport mechanisms for lignin precursors.

1. Passive diffusion; 2. Facilitated diffusion; 3. Primary transport (e.g., by ABC transporters); 4. secondary transport (e.g., by H^+ antiporters belonging to multidrug and toxic compound extrusion [MATE] family or major facilitator superfamily [MFS]),; and 5. Vesicle-mediated transport.

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Because lignin precursors are relatively hydrophobic and small molecules, the possibility of their passive diffusion across membranes has been considered. This possibility is supported by experiments using model compounds and artificial liposome membranes [28,29] and more recently by molecular dynamics simulations [30]. However, passive diffusivity of lignin precursor across biological membranes has not yet been demonstrated [27,31–34]. In an experimental system using artificial liposomal membrane vesicles encapsulating laccases, polymerization of coniferyl alcohol was observed within the vesicles. This report has led to the proposal of passive diffusion, driven by a concentration gradient between the cytoplasm and the cell wall, which continues to develop as polymerization progresses [35].

On the other hand, transport activities mediated by transporters have been observed in experiments using biological membranes. In microsomal membranes obtained from Arabidopsis rosette leaves, monolignols and monolignol glucosides are transported across the plasma membrane and the vacuolar membrane, respectively. These transport activities are associated with ABC-like transporters [31]. However, the observed transport activity may originate from parenchyma cells because rosette leaves contain little lignified tissues. An ABC transporter of *p*-coumaryl alcohol has been previously identified in Arabidopsis [7]. However, *p*-coumaryl alcohol is a precursor of the *p*-hydroxyphenyl unit, which constitutes only a minor component of the lignin typically synthesized in Arabidopsis. While extensive research has been conducted on ABC transporters responsible for transporting the precursors of guaiacyl and syringyl units (the predominant polymer units in lignin), the definitive identification of theses specific transporters remains elusive [33,36–40].

Membrane vesicles derived from actively lignifying tissues have demonstrated an alternative transport mechanism for lignin precursors. Experiments using microsomal membranes derived from actively lignifying poplar and cypress differentiating xylem have revealed ATP-dependent transport activities for both coniferin [27] and *p*-glucocoumaryl alcohol [32]. Inhibition experiments have indicated that these transport activities are not mediated by ABC transporters but are dependent on V-ATPases and the H^+ gradient, suggesting the involvement of coniferin/H^+ antiporters. In addition, ATP-dependent transport of coniferin and *p*-glucocoumaryl alcohol has also been detected in the differentiating xylem of spruce and tobacco BY-2 cells [33], as well as in growing bamboo culms [34]. Transport of *p*-glucocoumaryl alcohol has been suggested to be facilitated by the same transporter as coniferin [32,33]. These transport activities of monolignol glucosides are localized in the endomembrane system rather than the plasma membrane [27,32,33], potentially serving as a primary mechanism for coniferin storage in vacuoles. It is noted that coniferin transport activity has been observed even in angiosperms with limited coniferin accumulation. The conservation of these transport mechanisms across conifers, dicotyledons, and monocotyledons suggests that coniferin/H^+ antiporters may be ubiquitous in the lignifying tissues of various seed plants. No clear active or passive monolignol transport activity has been observed in microsomal vesicles derived from lignifying tissues [27,32–34].

Recent studies support vesicle-mediated transport of lignin monomers into the cell walls (Fig. 1). The above transport activities of monolignol glucosides in the endomembrane system may not be solely responsible for vacuole storage. The previous study has suggested that coniferin is transported to vacuoles or vesicles, where V-ATPase is localized, and that coniferin is then delivered to the cell wall via vesicle-mediated transport [27]. When pathogen infection induces autophagy-mediated lignification in Arabidopsis, monolignols is transported into the cell wall through autophagic membrane trafficking [41]. Furthermore, a very recent study using a lignin-forming cell suspension culture of Norway spruce reported that isolated extracellular vesicles contain phenolic compounds and enzymes related to lignin biosynthesis, suggesting that vesicles are involved in transport of lignin monomers [42].

3. Role of Monolignol Glucoside in Lignification

While coniferin is present in large amounts in the differentiating xylem of gymnosperms, its storage is limited in angiosperms, indicating that hardwoods may have evolved to lignify without excess monolignol storage [22]. Therefore, the role of coniferin in lignification may differ between gymnosperms

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and angiosperms. However, as mentioned above, coniferin transport is widely conserved among seed plants, and both the biosynthetic and hydrolytic enzymes related to coniferin have been identified in various seed plant species. Thus, the monolignol glucoside pathway, which include the glycosylation of monolignols, transport of monolignol glucosides, and their hydrolysis and polymerization, may be conserved across seed plants. In addition, several recent studies on mutants of genes involved in coniferin metabolism have suggested that the monolignol glucoside pathway plays an important role in regulating lignification in angiosperms.

The presence of UDP glycosyl transferases (UGTs) involved in coniferin biosynthesis has been reported in various plants [13,43–55]. Studies examining the downregulation or overexpression of coniferin *UGT* genes in angiosperms have shown varying results regarding their effects on coniferin levels [47–52,54,55]. In contrast, the impact on lignin levels appears to be more consistent. Specifically, suppressing coniferin UGT expression in Arabidopsis, poplar, flax, and pear has been shown to increase lignin content [49,50,52,54,55]. Additionally, the suppression of *AtUGT72B1* resulted in ectopic lignification, with significant effects on plant growth in Arabidopsis [49]. Overexpression of coniferin *UGT* in poplar and pear did not alter lignin levels [51,54].

The presence of coniferin β-glucosidase (BGLU), which is involved in the hydrolysis of coniferin, has been reported in various plant species [56–64]. In Arabidopsis, three coniferin *BGLU* genes have been reported [61]. The loss of a single gene does not affect lignin content but leads to increased coniferin levels, significantly influencing the levels of various secondary metabolites [20]. These results indicate that coniferin hydrolysis also occurs in Arabidopsis. In a double mutant of Arabidopsis laccases, a significant accumulation of coniferin in the vacuole was observed, accompanied by a decrease in lignin content [35], suggesting that coniferin storage is a regulatory process of lignification in Arabidopsis. When the expression of the coniferin *BGLU* genes in pear, *PbBGLU1* or *PbBGLU16*, was suppressed, lignin content remained unchanged, but coniferin levels increased, which is consistent with the results observed in Arabidopsis. In contrast, overexpression of *PbBGLU1* or *PbBGLU16* in both Arabidopsis and pear has been reported to increase lignin levels [64].

Taken together, when coniferin biosynthesis is inhibited or hydrolysis is enhanced (both of which result in decreased coniferin storage), lignin content increases. It is possible that coniferin is synthesized and transported, then gradually hydrolyzed to coniferyl alcohol, thereby regulating the appropriate degree of lignification. This monolignol glucoside pathway may be conserved among seed plants, as enzymes involved in coniferin biosynthesis and hydrolysis, as well as coniferin transport activity by H^+ antiporters in the endomembrane system, are conserved across a wide variety of seed plant species.

The monolignol glucoside pathway could serve as a regulatory mechanism for lignification, which is distinct from the direct transport of monolignols to the cell wall, due to the different localization of each enzyme and transporter. While coniferin UGT localizes intracellularly, its precise localization has been reported in several organelles, including the endoplasmic reticulum (ER) [51], chloroplasts [51], and cytoplasm [55]. Although coniferin UGT is not predicted to possess a transmembrane domain, it may be localized near the membrane through interactions with other membrane-bound proteins. Further studies are required to identify which proteins colocalize with coniferin UGT.

Synthesized cytoplasmic coniferin would be metabolized to coniferaldehyde glucosides, and subsequently coniferaldehyde [65]. Some studies have indicated that coniferin is metabolized by coniferin BGLU even when coniferin storage levels are low [20,64]. Coniferin BGLU is localized in the cell walls of conifers, hardwoods, and monocotyledons [62–64,66], suggesting that coniferin may undergo hydrolysis in the cell walls. Additionally, as an alternative pathway for coniferin metabolism, coniferin would be incorporated into lignin in the cell walls [67].

Although the involvement of H^+ antiporters [27,33,34] and ABC transporters [31] has been implicated in vacuolar accumulation, the mechanism by which coniferin is supplied to the cell walls remains unknown. It has also been considered that coniferin in vacuoles may diffuse after cell death [9], but in gymnosperms, the accumulated coniferin decreases while the cells are alive [12,19,27,68–70]. These findings

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imply that coniferin is transported to the cell walls while the cells are still alive. How is coniferin transported to the cell walls? One possibility is that multiple transporters mediate the movement of coniferin across both the tonoplast and the plasma membrane, facilitating its transport from vacuoles to the cell walls. However, coniferin transport activity has not been detected in the plasma membrane fraction [27,31]. An alternative hypothesis suggests that coniferin may be transported to the vacuoles or vesicles, where the V-ATPase is localized, followed by vesicle-mediated trafficking to the cell walls (Fig. 1) [27]. Vesicle transport is considered to play a role in the formation of the lignifying Casparian strip and in the transport of flavonoids [71,72]. Additionally, in response to infection, autophagic membrane trafficking is involved in monolignol secretion [41]. Furthermore, extracellular vesiculotubular structures are involved in the secretion of suberin monomers into the cell walls during suberin formation in Casparian strip [73]. Extracellular vesicles containing phenolic compounds have been isolated from a lignin-forming cell culture of Norway spruce [42]. These findings collectively support the hypothesis that endomembrane systems and extracellular vesicles could serve as mechanisms for transporting coniferin into the cell walls.

The mechanism by which the monolignol glucoside pathway influences lignification remains unclear. Monolignol glucoside levels may alter the expression of genes involved in the phenylpropanoid pathway [49]. Coniferin hydrolysis at the cell walls could delay the supply of coniferyl alcohol, thereby promoting the propagation of lignin polymer chains via β–O–4 bonds. The importance of monomer supply rate for the formation of β–O–4 bonds during the *in vitro* synthesis of artificial lignin (dehydrogenation polymer, DHP) has been well studied [1]. And indeed, in the poplar *ugt72b37* mutant, a decrease in the proportion of β–O–4 bonds within lignin was observed although the total lignin level increased [52]. The spatial distribution of lignin structures in the cell walls could influence the physical properties of the cell walls, which may, in turn, affect the expression of genes involved in lignin biosynthesis. Furthermore, although it is unclear whether directly or indirectly, the monolignol glucoside pathway is involved in various plant growth processes, including secondary cell wall formation [49,55], flowering and pollen maturation [49,50], responses to salt stress [74] and defense mechanisms against pathogen infection [75].

4. Plasticity of Monolignol Transport

In recent years, lignins derived from diverse lignin monomers, rather than from the conventional monolignols, have been identified in various plant species. Furthermore, lignins derived from unconventional monomers have been found in various mutants and transgenic plants in which genes related to the biosynthesis of lignin precursors are down- or up-regulated [4–6,76]. These observations suggest that the transport of lignin precursors is highly plastic, which may be explained by non-protein-mediated passive diffusion [30,35].

It is also possible that various lignin precursors are transported by ABC transporters. The plant ABC transporter family is divided into eight subfamilies based on its structure [77]. The ABCG family, one of the largest subfamilies, has been shown to play roles in the transport of various hydrophobic molecules [78,79]. Therefore, ABCG proteins are assumed to transport monolignols [39,40]. Indeed, AtABCG29 has been reported to be involved in the transport of *p*-coumaryl alcohol [7]. Furthermore, ABC transporter-like activity has been reported in Arabidopsis leaves [31]. Notably, in addition to full-size transporters, the ABCG family includes many half-size transporters that function as homodimers or heterodimers to export different substrates [77–79]. Many members of ABC transporters as well as their diverse combinations could enable the transport of diverse lignin precursors.

Seed plants may possess active transport mechanisms for the precise regulation of lignin biosynthesis, in addition to transport mechanisms by which diverse substrates can be transported to the cell walls. Further research is required to elucidate the storage and transport mechanisms of diverse lignin precursors, which enable seed plants to lignify their cell walls for development in diverse environments.

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