

Plant biology and pathology / Biologie et pathologie végétales

The regulation from guaiacyl to syringyl lignin in the differentiating xylem of *Robinia pseudoacacia* ☆

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Abstract

¹³C- and deuterium (D)-labeled ferulic acid and sinapic acid ([8-¹³C, 3-OCD₃]-ferulic acid and [8-¹³C, 3,5-OCD₃]-sinapic acid) were administered to robinia (*Robinia pseudoacacia* L.) shoots. To estimate the distribution of the label from administered ferulic or sinapic acid, continuous 50-μm-thick tangential sections cut from the cambium of robinia were subjected to lignin chemical analysis by the DFRC method. Labeled ferulic acid was incorporated into guaiacyl and syringyl lignin. The incorporation of labeled ferulic acid into syringyl units was observed only in the later stage of lignification. Labeled sinapic acid was incorporated into syringyl lignin in the early stage and the later stage of lignification. In general, syringyl lignin was deposited in the later stage of cell wall lignification. Thus, the incorporation of sinapic acid to syringyl lignin in the early stage of lignification was abnormal. Taken together, the aromatic ring-modifying reactions (the conversion from guaiacyl to syringyl moiety, including the hydroxylation and methylation) were more important for the regulation of the sinapyl alcohol biosynthesis than the reducing reactions (the reduction of acids to alcohols) in the differentiating xylem. **To cite this article:** K. Yamauchi, K. Fukushima, *C. R. Biologies* 327 (2004).

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Résumé

Régulation du passage de lignines de type guaiacyle vers des lignines de type syringyle dans le xylème en voie de différenciation de *Robinia pseudoacacia* Des doses d'acides férulique et sinapique marqués au ¹³C- et au deutérium (D) ([8-¹³C, 3-OCD₃]-acide férulique et [8-¹³C, 3,5-OCD₃]-acide sinapique) ont été administrés à de jeunes pousses de robinier (*Robinia pseudoacacia* L.). Pour estimer la distribution du marquage de l'acide férulique ou sinapique administré, des coupes tangentielles sériées de 50 μm, obtenues à partir du cambium du robinier, ont été soumises à une analyse chimique de leur lignine par la méthode DFRC. L'acide férulique marqué s'est incorporé aux lignines guaiacyle et syringyle. L'incorporation de l'acide férulique marqué dans les unités syringyles a été seulement observée lors de la dernière étape de la lignification. L'acide sinapique marqué s'est incorporé à la lignine syringyle lors des première et dernière étapes de la lignification. En général, la

☆ This article is dedicated to a pioneer who has fixed his eyes upon the lignin structures and distributions from the new angle, that is, morphological aspect, differentiation of the cell or evolution of plant.

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lignine syringyle se dépose lors de la dernière étape de la lignification des parois cellulaires. Ainsi, l'incorporation de l'acide sinapique à la lignine syringyle lors du premier stade de lignification est-elle anormale. Prises ensemble, les réactions de modification du noyau aromatique (la conversion de la fonction guaiacyl en syringyle, avec l'hydroxylation et la méthylation) sont plus importantes pour la régulation de la biosynthèse de l'alcool sinapylique que les réactions de réduction (des acides en alcools) dans le xylème en cours de différenciation. **Pour citer cet article : K. Yamauchi, K. Fukushima, C. R. Biologies 327 (2004).**

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Keywords: robinia; tracer experiment; DFRC method; syringyl lignin biosynthesis; lignification

Mots-clés : robinier ; expérience de marquage ; méthode DFRC ; biosynthèse de la lignine syringyle ; lignification

Abbreviations

DFRC, Derivatization followed by reductive cleavage; GC-MS, gas spectrometry–mass spectrometer; G, guaiacyl; S, syringyl; SIM, selected ion monitoring.

1. Introduction

Lignin is one of the major components of woody plant cell walls, and it gives rigidity to plants and allows water conduction. Lignin molecules in angiosperms are composed of guaiacyl (G) and syringyl (S) units, which are derived from coniferyl alcohol and sinapyl alcohol, respectively [6]. A difference in the ratio of G to S units in different morphological regions of some angiosperms has been shown by UV microscopy [2,3,10], bromination-TEM-EDXA [13], and the chemical characterization of various tissue fractions [1,7]. In addition, the selective radio labeling of specific units in lignin and visualization of the labeled units by microautoradiography is useful for studying the formation and structure of lignin with respect to its location in wood tissues [11,12,17]. These studies demonstrated that G lignin is deposited mainly in the early stage of xylem differentiation on vessel walls and the compound middle lamella of the fiber wall, while S lignin is deposited on fiber secondary cell walls in the later stage of differentiation.

Recent studies suggest that the precursor of syringyl lignin, sinapyl alcohol, is biosynthesized via feruloyl–CoA thioester, coniferaldehyde, and sinapaldehyde without forming sinapic acid [4,5,8,14]. However, we previously demonstrated that exogenous ferulic acid was incorporated into G and S lignin, and exogenous sinapic acid was also incorporated into S lignin in robinia [9,15]. This suggests that sinapic acid is an intermediate of syringyl lignin. Sinapyl alcohol

is formed from ferulic acid via the aromatic ring modifying reaction (the hydroxylation and the methylation) and the reducing reactions (the reduction of acids to alcohols), whereas it formed from sinapic acid only via the reducing reductions. It is important to clarify the differences in the regions where sinapic and ferulic acid are incorporated in differentiating xylem in order to elucidate the enzymatic systems responsible for modifying the aromatic ring and reducing the side chain.

In this study, we prepared different stage of continuous sections of newly formed xylem from robinia fed labeled ferulic and sinapic acids, to reveal where these compounds are incorporated into S lignin in the differentiating xylem. We estimated the distribution of the labeled lignin by using the derivatization followed by reductive cleavage method (DFRC), followed by gas chromatography–mass spectrometry (GC-MS). The difference in the *in situ* incorporation of the labeled precursors is discussed in the regulation of sinapyl alcohol biosynthesis in the differentiating xylem.

2. Material and methods

2.1. Synthesis of labeled precursors

[8-¹³C, 3-OCD₃]-ferulic acid and [8-¹³C, 3,5-OCD₃]-sinapic acid (Fig. 1) were synthesized as described previously [9,15].

2.2. Plant material and administration of precursors

The upper parts of 2-year-old shoots of robinia (*Robinia pseudoacacia* L.) trees that were growing on the campus of the Nagoya University were cut off in July 2000. A small depression was made at the

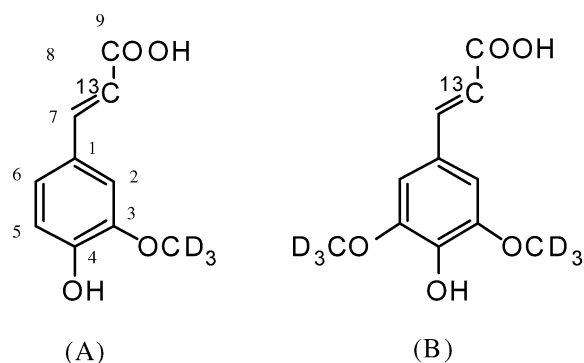


Fig. 1. Precursors labeled with stable isotopes. (A) Ferulic acid- $[8-^{13}\text{C}, 3\text{-OCD}_3]$. (B) Sinapic acid- $[8-^{13}\text{C}, 3, 5\text{-OCD}_3]$.

top of the remaining stem and was filled with 30 ml of a 2-mM aqueous solution of each precursor dissolved in a phosphate buffer (pH. 7.01, 25 °C). After 9 days, each shoot was harvested and was soaked in 80% ethanol. And then 50- μm -thick tangential sections were cut from the surface of the bark-free xylem with a sliding microtome. All section was treated by ethanol-benzene (1:2) for 8 h to remove the labeled precursor and low molecular phenolic compounds. The analyses of lignin by the DFRC method were per-

formed using these sections, as shown in Fig. 2 and described previously [15].

2.3. Analysis of lignin

To degrade and detect the labeled lignin, each section (approximately 1 mg) was applied to the DFRC method [16] followed by GC-MS, with some modification as followed; AcBr stock solution: acetyl bromide/acetic acid (1:4, v/v); Acidic reduction solvent: dioxane/acetic acid/water (5:4:1, v/v/v). To wood section was added the AcBr stock solution (0.5 ml). The mixture was gently stirred at 50 °C for 3 h. The solvent was completely removed by blowing down of N_2 gas. The residue was dissolved in the acidic reduction solvent (0.5 ml). Zinc powder (10 mg) was added to a well-stirred solution. Stirring was continued for 30 min. To the mixture were added dichloromethane (1 ml), a saturated ammonium chloride solution (1 ml), and an internal standard (docosane in dichloromethane). The pH of the aqueous phase was adjusted to less than 3 by adding diluted HCl. The aqueous phase was extracted twice more with dichloromethane. The combined dichloromethane fractions were dried over anhydrous Na_2SO_4 , and

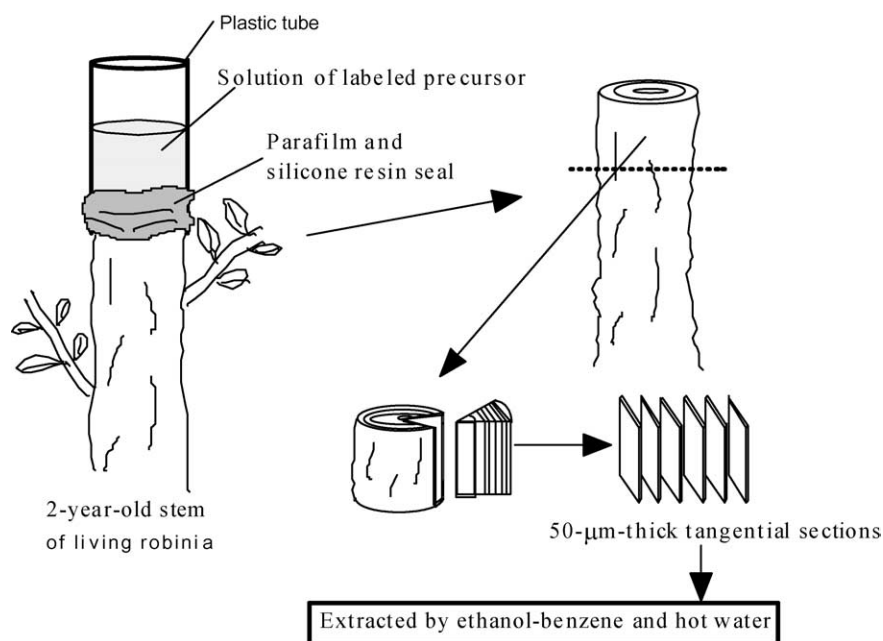


Fig. 2. Administration of labeled precursors and sample preparation.

evaporated in vacuo. The residue was acetylated for 40 min in 1.5 ml of dichloromethane containing 0.2 ml of acetic anhydride and 0.2 ml of pyridine. All volatile components were removed completely by co-evaporation with ethanol in vacuo.

Acetylated DFRC products were analyzed by GC-MS. Mass spectra were recorded at 70 eV with a GCMS-OP2010 (Shimadzu, Kyoto, Japan), with a fused silica capillary column (DB-1, 30 m \times 0.32 mm i.d.). The sample (1 μ l) was injected at 220 °C. The temperature was programmed to increase from 150 to 280 °C at 20 °C min⁻¹ and, after 5 min, maintained at 280 °C. The carrier gas was helium. Labeled DFRC products were determined by using selected ion monitoring (SIM), according to the previous method [15].

3. Results and discussion

Fig. 3 shows the distribution of the DFRC degradation products (G_{DFRC} , S_{DFRC}) in the robinia given labeled ferulic acid. DFRC method only produces these products from 8-O-4' structures in lignin [17]. Compared with non-labeled shoots, the lignin composition was not affected by the administration of ferulic acid (data not shown). The low yields of the sections near the cambium indicated that these tissues were in the initial stage of lignification. G lignin began to form in the early stage of cell-wall lignification, and the proportion of S lignin increased gradually as lignifica-

tion proceeded. This indicates that lignin composition is clearly regulated by the differentiation of cell wall in robinia. Previous studies using microautoradiography demonstrated that G lignin was deposited in the early stage of xylem differentiation on vessel walls, followed by the deposition of S lignin on the fiber cell wall, in magnolia, lilac, and beech [12,17]. Our results for robinia were similar to theirs.

Fig. 4 shows the partial mass spectra of each DFRC product (G_{DFRC} and S_{DFRC}) in controls (A) and robinia fed with labeled ferulic (B) or sinapic (C) acid. Each figure shows where the labeled precursor was incorporated into lignin. The molecular ion peaks for G_{DFRC} and S_{DFRC} monomers are at m/z 264 and 294, respectively. In general, aromatic acetates easily lose a ketene group (m 42). Therefore, the mass spectra of G_{DFRC} and S_{DFRC} have base peaks (m) at m/z 222 and 252, respectively. As described previously [15], when fed with [8-¹³C, 3-OCD₃]-ferulic acid, mass peaks were detected at m/z 226 (222 + 4; m + 4) in G_{DFRC} and at m/z 256 (252 + 4; m + 4) in S_{DFRC} (Fig. 4B). When fed with [8-¹³C, 3,5-OCD₃]-sinapic acid, a mass peak was detected at m/z 259 (252 + 7; m + 7) in S_{DFRC} (Fig. 4C). Examination of the areas of the m + 4, m + 7, and m peaks on selected ion-monitoring (SIM) chromatograms allowed us to estimate the amount of labeled lignin subunits in each section. Fig. 5 shows the distribution of the yields of labeled DFRC products in each section of robinia fed with labeled ferulic (A) or sinapic (B)

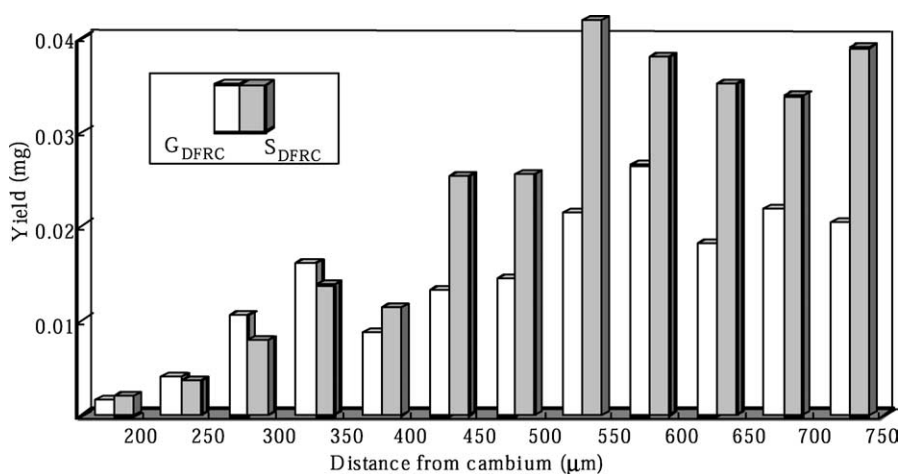


Fig. 3. Distribution of the DFRC products (G_{DFRC} , S_{DFRC}) in the robinia given labeled ferulic acid.

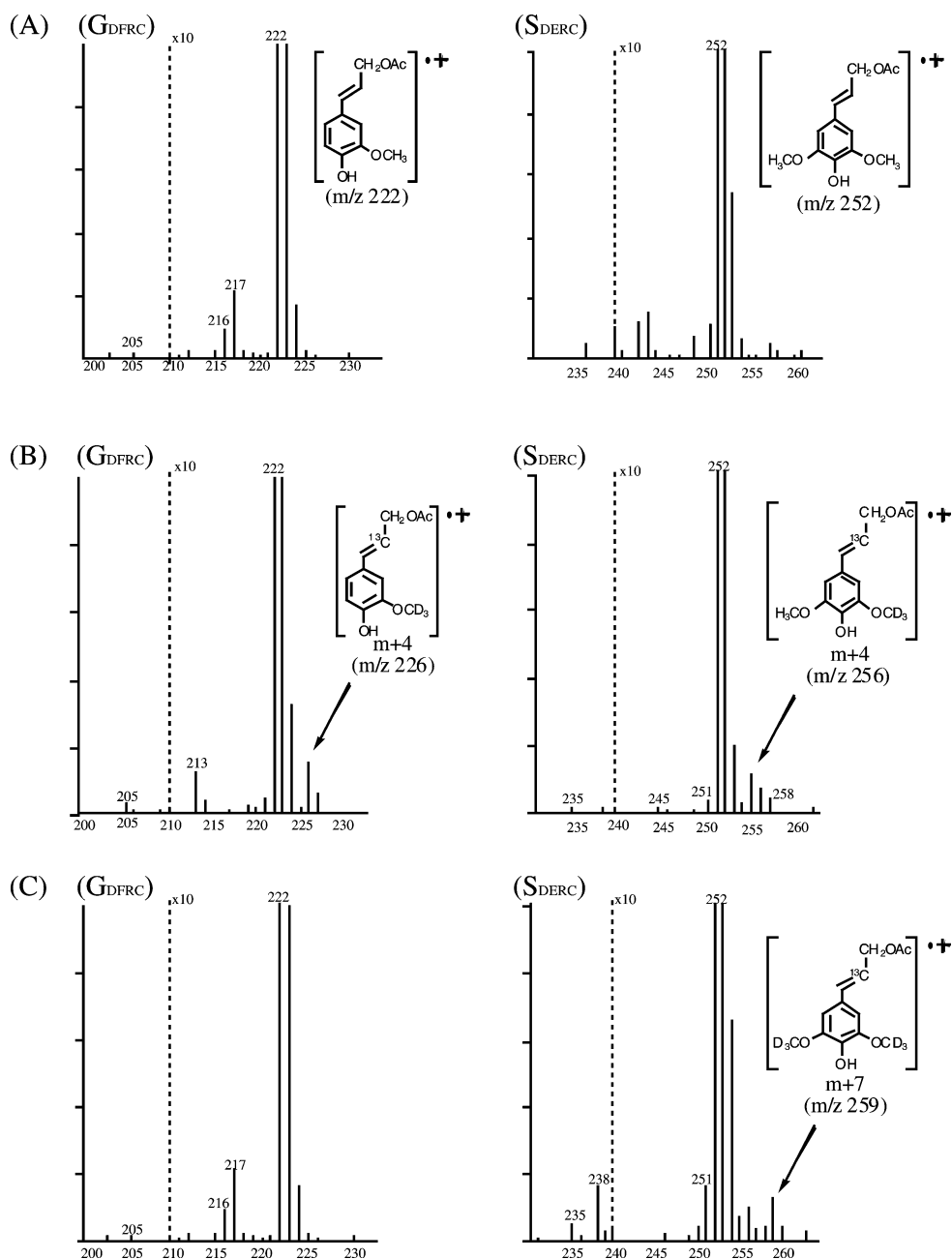


Fig. 4. Partial mass spectra of each DFRC product in robinia. (A) Control, no feeding; (B) fed with labeled ferulic acid; (C) fed with labeled sinapic acid.

acid. When fed with $[8-^{13}C, 3-OCD_3]$ -ferulic acid, labeled G_{DFRC} was located 250–350 and 550–700 μm from the cambium. The distribution of labeled G_{DFRC} increased gradually as cell-wall lignification

proceeded, and the labeled S_{DFRC} was located 450–600 μm from the cambium. When fed with $[8-^{13}C, 3,5-OCD_3]$ -sinapic acid, labeled S_{DFRC} was located 150–250 and 450–700 μm from the cambium. Little

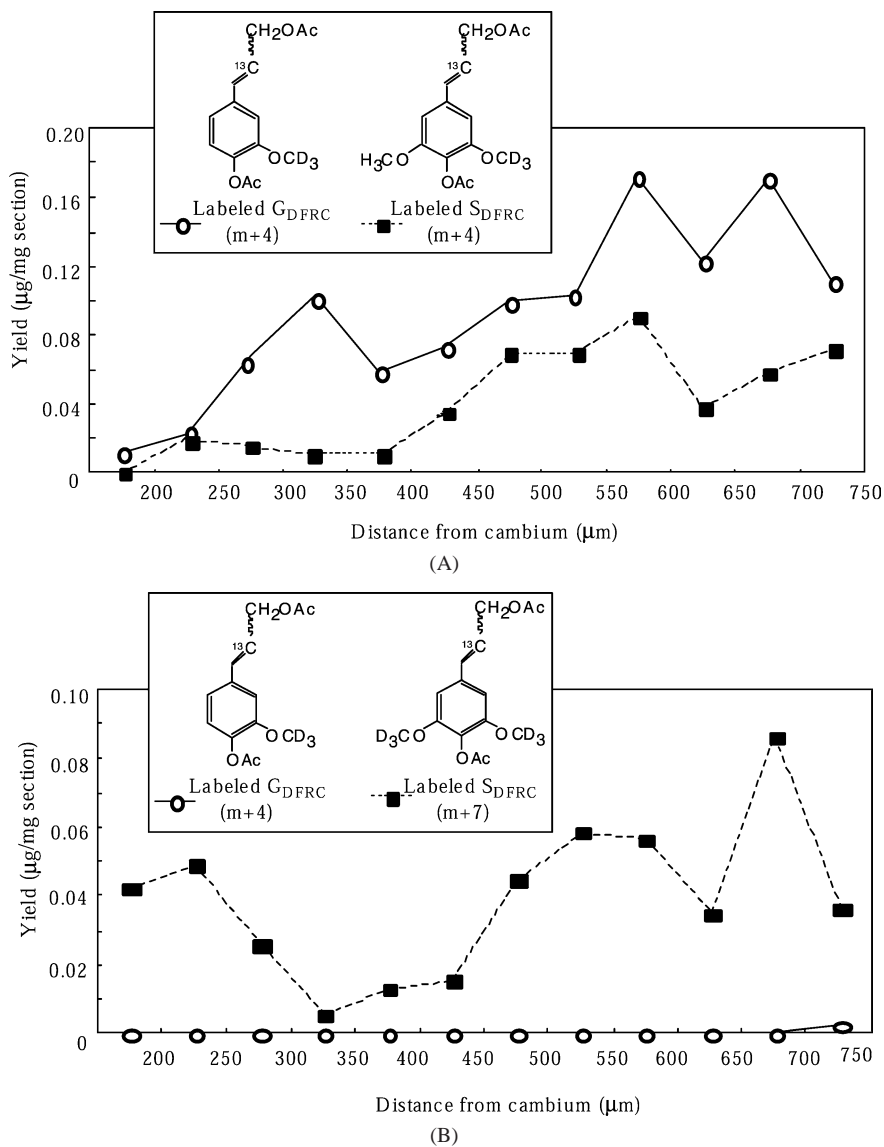


Fig. 5. Distribution of the yield of labeled DFRC products. (A) Labeled G_{DFRC} ($m+4$) and S_{DFRC} ($m+4$) in robinia fed with labeled ferulic acid. (B) Labeled G_{DFRC} ($m+4$) and S_{DFRC} ($m+7$) in robinia fed with labeled sinapic acid.

of the label from sinapic acid was incorporated into G_{DFRC} in any section.

Labeled ferulic acid has begun to incorporate into G lignin in the early stages of cell-wall lignification and the incorporations increased as cell-wall lignification proceeded. And the labeled ferulic acid is also incorporated into S lignin during the late stage of lignification. These localizations corresponded with the heterogeneous deposition of lignin moieties. La-

beled sinapic acid was incorporated into S lignin in the early and late stages of lignification. However, the deposition of S lignin occurred mainly in the later stage of lignification. Thus, the incorporation of labeled sinapic acid into syringyl lignin in the early stage would be abnormal conversion.

These results indicated that the exogenous ferulic acid was incorporated in the normal lignin biosynthetic process, including the regulation of coniferyl

and sinapyl alcohol biosynthesis, whereas the exogenous sinapic acid was not. The conversion of ferulic acid to sinapyl alcohol goes via the aromatic ring modifying reaction (the hydroxylation and the methylation) and the reducing reactions (the reduction of acids to alcohols). The conversion of sinapic acid to sinapyl alcohol goes only via the reducing reactions (the reduction of acids to alcohols). Therefore, the regulation of the syringyl lignin biosynthetic process would occur in the aromatic ring modifying reaction (the conversion from guaiacyl to syringyl moiety) in the differentiating xylem.

4. Conclusions

In robinia, sinapyl alcohols are generated mainly in the later stage in the differentiating xylem. For the regulation of the sinapyl alcohol biosynthesis, the aromatic ring-modifying reactions including the hydroxylation and methylation (the conversion from guaiacyl to syringyl moiety) are more important than the reducing reactions (the reduction from sinapic acid to sinapyl alcohol).

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References

- [1] T. Higuchi, Lignin biochemistry: biosynthesis and biodegradation, *Wood Sci. Technol.* 24 (1990) 23–63.
- [2] B.J. Fergus, D.A.I. Goring, The location of guaiacyl and syringyl lignins in birch xylem tissue, *Holzforschung* 24 (1970) 113–117.
- [3] B.J. Fergus, D.A.I. Goring, The distribution of lignin in birch wood as determined by ultraviolet microscopy, *Holzforschung* 24 (1970) 118–124.
- [4] Y. Musha, D.A.I. Goring, Distribution of syringyl and guaiacyl moieties in hardwoods as indicated by ultraviolet microscopy, *Wood Chem. Technol.* 9 (1975) 45–58.
- [5] S. Saka, D.A.I. Goring, Localization of lignins in wood cell wall, in: T. Higuchi (Ed.), *Biosynthesis and Biodegradation of Wood Component*, Academic Press Inc., Orlando, FL, USA, 1985, pp. 51–62.
- [6] N.S. Cho, L.Y. Lee, G. Meshitsuka, J. Nakano, On the characteristics of hardwood compound middle lamella lignin, *Mokuzai Gakkaishi* 26 (1980) 527–533.
- [7] H.L. Hardell, G.J. Leary, M. Stoll, U. Westermark, Variation in lignin structures in defined morphological parts of birch, *Svensk Papperstidn.* 83 (1980) 71–74.
- [8] N. Terashima, K. Fukushima, S. Tsuchiya, K. Takabe, Heterogeneity in formation of lignin. VII. An autoradiographic study on the formations of guaiacyl and syringyl lignin in poplar, *J. Wood Chem. Technol.* 6 (1986) 495–504.
- [9] N. Terashima, K. Fukushima, K. Takabe, Heterogeneity in formation of lignin. VIII. An autoradiographic study on the formations of guaiacyl and syringyl lignin in *Magnolia kobus* DC, *Holzforschung (Suppl.)* 40 (1986) 101–105.
- [10] K. Fukushima, N. Terashima, Heterogeneity in formation of lignin. XIII. Formations of *p*-hydroxyphenyl lignin in various hardwood visualized by microautoradiography, *J. Wood Chem. Technol.* 10 (1990) 413–533.
- [11] J.M. Humphreys, M.R. Hemm, C. Chapple, New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase, *Proc. Natl Acad. Sci. USA* 96 (1999) 10045–10050.
- [12] L. Li, J.L. Popko, T. Umezawa, V.L. Chiang, 5-Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms, *J. Biol. Chem.* 275 (2000) 6537–6545.
- [13] D. Guo, F. Chen, K. Inoue, J.W. Blount, R.A. Dixon, Down-regulation of caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase in transgenic alfalfa: impacts on lignin structure and implications for the biosynthesis of G and S lignin, *Plant Cell* 13 (2001) 73–88.
- [14] G. Schoch, S. Goepfert, M. Morant, A. Hehn, D. Meyer, P. Ullmann, D. Werck-Reichhart, CYP98A3 from *Arabidopsis thaliana* is a 3'-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway, *J. Biol. Chem.* 276 (2001) 36566–36574.
- [15] K. Yamauchi, S. Yasuda, K. Fukushima, Evidence for the biosynthetic pathway from sinapic acid to syringyl lignin using labeled sinapic acid with stable isotope at both methoxy groups in *Robinia pseudoacacia* and *Nerium indicum*, *J. Agric. Food Chem.* 50 (2002) 3222–3227.
- [16] K. Yamauchi, S. Yasuda, K. Hamada, Y. Tsutsumi, K. Fukushima, Multiform biosynthetic pathway of syringyl lignin in angiosperms, *Planta* 216 (2003) 496–501.
- [17] F. Lu, J. Ralph, Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers, *J. Agric. Food Chem.* 45 (1997) 2590–2592.