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Heterogeneity in formation of lignin – XI: An autoradiographic study of the heterogeneous formation and structure of pine lignin

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Summary. Selective labeling of p-hydroxyphenyl-, guaiacyl- and syringylpropane moieties in protolignin was achieved by administration of corresponding ^{3}H-labeled monolignol glucosides to differentiating xylem of pine. The growing process of the protolignin macromolecule in the specific morphological region was visualized by application of high resolution microautoradiography to the selectively labeled wood tissue.

p-Hydroxyphenyl lignin is formed mainly in the compound middle lamella and cell corner in an early stage of cell wall differentiation. There are two peaks of deposition of guaiacyl lignin in the compound middle lamella at an early stage and in the secondary wall at a late stage. The content of condensed guaiacyl units is higher in the middle lamella than in the secondary wall lignin. Syringyl lignin is formed mainly in the inner layer of the secondary wall in a late stage as a minor structural moiety. During the formation of the cell wall, protolignin grows under definite biological regulations to a heterogeneous macromolecule which consists of various structural moieties arranged in a regular manner. The origin of the heterogeneous structure was explained as a result of the biogenesis of protolignin in the cell wall.

Introduction

It is difficult to investigate the structure of protolignin in the cell wall, because it is impossible to isolate protolignin in an intact state or to depolymerize it quantitatively into its building units. Non-degradative analytical methods such as UV, IR, Raman and CP/MAS-NMR spectroscopy and SEM-EDXA provide useful information on the macromolecular structure and distribution of protolignin in the cell wall.

A combination of high resolution microautoradiography with a technique of selective labeling of the specific structural moieties in lignin also provides useful information, which is not obtained by other methods (Terashima et al. 1986). In this method, a growing tree is administered a lignin precursor which is labeled at a specific position with ^{3}H. Monolignol glucosides are suitable precursors for selective labeling of the specific moieties. The lignification process can be visualized by taking a microautoradiograph of a cross section of the newly formed xylem which contains cells at various stages of differentiation, and the location and amount of the specific structural units in different morphological regions can be determined by image analysis of the microautoradiogram. The structural hetero-

geneity of the protolignin macromolecule is visually demonstrated by integrating the deposition patterns of p-hydroxyphenyl, condensed and non-condensed guaiacyl, and syringyl moieties in the cell walls at various lignification stages. In this paper, the heterogeneous nature of pine lignin was visualized by microautoradiography, and the origin of the heterogeneity was described.

Experimental

Materials

Three-year-old shoots were obtained from *Pinus thumbergii* Parl. grown on the campus of Nagoya University.

Coniferin-[arom.ring-2-^{3}H], coniferin-[arom.ring-5-^{3}H] and syringin-[arom.ring-2-^{3}H] were synthesized from vanillin-[ring-2-^{3}H] (Terashima et al. 1979a), vanillin-[ring-5-^{3}H] (Terashima et al. 1979b) and syringaldehyde-[ring-2-^{3}H] (Tomimura, Terashima 1979) respectively.

p-Coumaric acid-[ring-2-^{3}H] and p-coumaryl-β-D-glucoside-[arom.ring-2-^{3}H] were synthesized from 2-bromo-4-hydroxybenzaldehyde by the same method as that described for labeled ferulic acid (Terashima et al. 1979a) and coniferin (Kratzl, Billek 1953; Fuchs 1955), respectively.

Administration of precursors

A V-shaped groove, 2 mm wide and 5 mm long, was made in circumferential direction on the cut shoot of a pine with a razor blade. The bottom of the groove reached to the differentiating xylem. Fine glass wool was packed in the groove. A solution of glucoside (about 1 mg, 8–10 μCi) in water (100 μl) was added dropwise to the fine glass wool, and metabolized for 3 hours. A drop of 3% glutaraldehyde in phosphate buffer (pH, 7.0) was added to the groove, and a small block of xylem tissue near the groove was cut and fixed again in 3% glutaraldehyde overnight in the refrigerater. A part of the xylem tissue was dehydrated through a graded ethanol series by a conventional method and embedded in epoxy resin prepared by mixing Quetol 812 (100 g, Nissin EM Co. Ltd, Tokyo), MNA (89 g) and DMP-30 (1.7 g). Another part of the tissue was used for radioassay.

Radioassay

Xylem tissue was milled (80 mesh) and extracted thoroughly with ethanol-benzene and hot water. The dried wood meal was subjected to alkaline nitrobenzene oxidation, and aromatic aldehydes were separated by silica gel thin layer chromatography, using a mixture of n-hexane : isoamyl alcohol : acetic acid (400 : 64 : 1) as a developer. p-Hydroxybenzaldehyde, vanillin and syringaldehyde were extracted from the gel with methanol. The radioactivity of the aldehydes were determined by a scintillation counting method.

Autoradiography

Two-μm-thick transverse sections were cut on a Sorvall JB-4 microtome equipped with a glass knife from the embedded segment of xylem tissue. They were mounted on glass-slides and covered with Kodak AR-10 stripping film. The glass slides were stored in a refrigerator for 3 weeks to 1 year. They were developed with Kodak D-19 and fixed with Fuji Fix. The sections were stained with toluidine blue O, and microphotographs were taken with ZEISS photomicroscope III. The analysis of silver grain distribution in the microphotographs were made with the aid of ZEISS IBAS 1 image analyzer. A polarization microscope Olympus POS was used for observation of secondary wall formation.

Results and discussion

Selectivity of labeling

The results of the radioassay by nitrobenzene oxidation are shown in Table 1. The total activity of aromatic aldehydes was only 8–19% of the labeled wood meal. This means that the information obtained by this degradative method is restricted to only a part of the lignin macromolecule. However, it is possible to estimate the selectivity of labeling by measuring the distribution of radioactivity in three kinds of aldehydes. The radioactivity of the aldehydes from the wood tissue administered with p-coumaric acid indicates that this acid is one of the common precursors of p-hydroxyphenyl, guaiacyl and syringyl lignins, as expected from their biosynthetic pathway, while the radioactivity of p-coumaryl-β-D-glucoside was incorporated mainly into p-hydroxyphenyl lignin, and only a small part into guaiacyl and syringyl lignins. There was some biochemical interconversion from guaiacyl to p-hydroxyphenyl units occurred partly, but conversions from guaiacyl to syringyl units and from syringyl to guaiacyl units occurred to a very small extent.

These results indicate that biochemical interconversions between lignin precursors do not occur extensively under the present experimental conditions, and the

Table 1. Incorporation of radioactivity from labeled precursors into newly formed lignin of black pine as shown by nitrobenzene oxidation

		Precursors administered			
		p-Coumaric acid-[r.-2-^{3}H]	p-Coumaryl-β-D-glucoside-[a.r.-2-^{3}H]	Coniferin-[a.r.-2-^{3}H]	Syringin-[a.r.-2-^{3}H]
^{3}H dpm[a] in oxidation products	W[b]	25,790	31,070	20,570	2,380
	H	950	3,450	1,430	0
	V	2,450	330	2,490	0
	S	80	40	70	200

[a] From 10 mg of extractive free wood meal
[b] W: Extractive free wood meal ; H: p-hydroxybenzaldehyde; V: vanillin; S: syringaldehyde

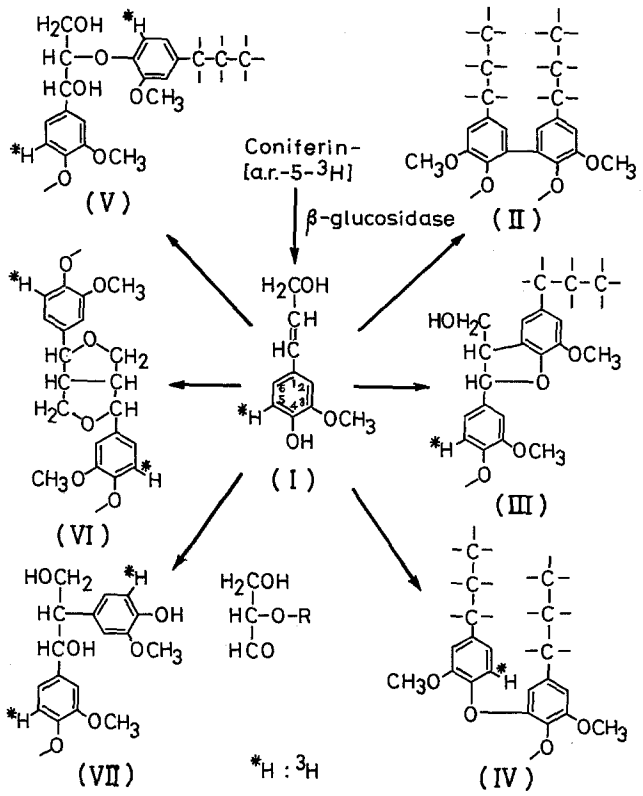

Fig. 1. Removal of ^3H at position 5 of guaiacyl ring of coniferyl alcohol (I) by formation of condensed structures (II, III, IV) during dehydrogenative polymerization. Non-condensed structures (V, VI, VII) retain the ^3H. If ^3H is at position 2, no removal of ^3H occurs

specific structural moiety is labeled selectively. Accordingly, a large part of the siver grains in the autoradiogram are assigned to the specific structural moiety corresponding to the monolignol glucoside administered.

Formation and morphological distribution of condensed guaiacyl structures in pine lignin

It has been shown by radiotracer experiments on lignification of pine that the amount of condensed guaiacyl units formed in an early stage of xylem differentiation was larger than those formed in a later stage (Terashima et al. 1979b; Fukushima 1987), and the factors affecting the formation of condensed structures has been studied in vitro (Terashima, Seguchi 1987).

This inhomogeneous formation of condensed structures can be visualized by microautoradiography employing coniferin-[arom.ring-5-^3H]. As shown in Fig. 1, the ^3H at position 5 of the guaiacyl ring of coniferyl alcohol (I) derived from coniferin is removed by formation of condensed structures (II, III, IV) with linkages

at this position, while the ^3H is retained in the non-condensed structures (V, VI, VII). On the other hand, when coniferin-[arom.ring-2-^3H] is administered, the ^3H at position 2 is expected to remain almost intact during the dehydrogenative polymerization, because condensed structures with a bond at this position is rarely formed. Almost no elimination of ^3H has been observed in the pine tissue administered ferulic acid-[ring-2-^3H] (Tomimura, Terashima 1979).

Because the tissue section labeled by low-energy beta emitter ^3H can provide a high resolution autoradiogram, it is possible to estimate semiquantitatively the activity in different morphological regions by silver grain counting (Terashima et al. 1986). Since the number of silver grains produced by the ^3H at position 2 (Fig. 2a) and position 5 (Fig. 2b) is proportional to the number of total and non-condensed guaiacyl units, respectively, the difference between these two values corresponds to the number of condensed structures. The distribution of silver grains in these autoradiograms were shown in Figs. 3c and 3d. The difference between these two graphs indicates that the lignin formed in the middle lamella regions at an early stage of xylem differentiation contains more condensed units than that formed at a later stage in secondary wall.

One of the factors favorable to the formation of condensed guaiacyl structures may be the fact that dehydrogenative polymerization proceeds in a carbohydrate gel and the kind of gel changes from pectic substances in the early stage to hemicelluloses in the later stages (Terashima et al. 1988; Terashima, Seguchi 1987). One of the other factors may be the participation of p-coumaryl alcohol in dehydrogenative polymerization. Because the p-hydroxyphenyl unit is capable of forming condensed units at both the 3 and 5 positions with another p-hydroxy-phenyl or guaiacyl units, the supply of p-coumaryl alcohol in the early stage (Figs. 3b, 4b, and 5) is favorable to the formation of condesed guaiacyl stuctures.

It is noteworthy that the deposition of guaiacyl lignin proceeds in three distinct stages (Fig. 3c). The first stage occurs at the cell corners and in the middle lamella region after the start of deposition of carbohydrates in S_1. The second is the slow lignification stage. During this phase, the carbohydrates are deposited in the S_2 layer. The main lignification occurs in the third stage after the start of S_3 formation. The same pattern with two lignification peaks has been observed in a microauto-radiogram of pine xylem administered with coniferin-[β-^{14}C] (Terashima et al. 1988).

Formation and morphological distribution of p-hydroxyphenyl and syringylpropane units

The process of formation of p-hydroxyphenyl and syringyl lignins was examined by administration of p-coumaryl-β-D-glucoside-[arom.ring-2-^3H] and syringin-[arom. ring-2-^3H] respectively. p-Coumaric acid-[arom.ring-2-^3H] was also administered to compare its incorporation into protolignin with the other precursors. Incorporation of p-hydroxy-phenylpropane units (Fig. 4b) was observed in an earlier stage of cell wall differentiation than that of guaiacylpropane units (Fig. 2a and 3c), and a large portion of p-hydroxyphenyl lignin was located in the compound middle lamella (Fig. 3b).

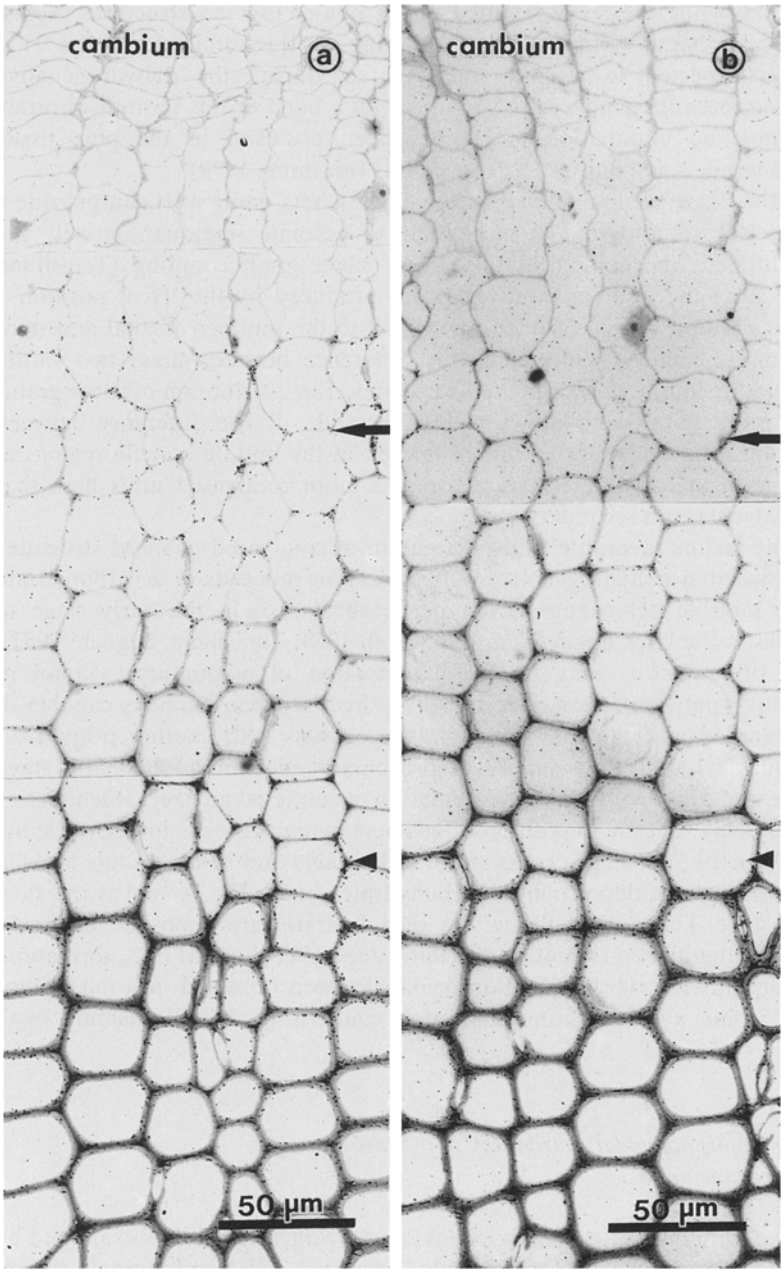

Fig. 2a and b. Microautoradiograms of differentiating xylem of pine administered with coniferin-[arom.ring-2-^{3}H]: **a** coniferin-[arom.ring-5-^{3}H]: **b** Arrow and arrowhead indicate start of S_1 and S_3 formation respectively

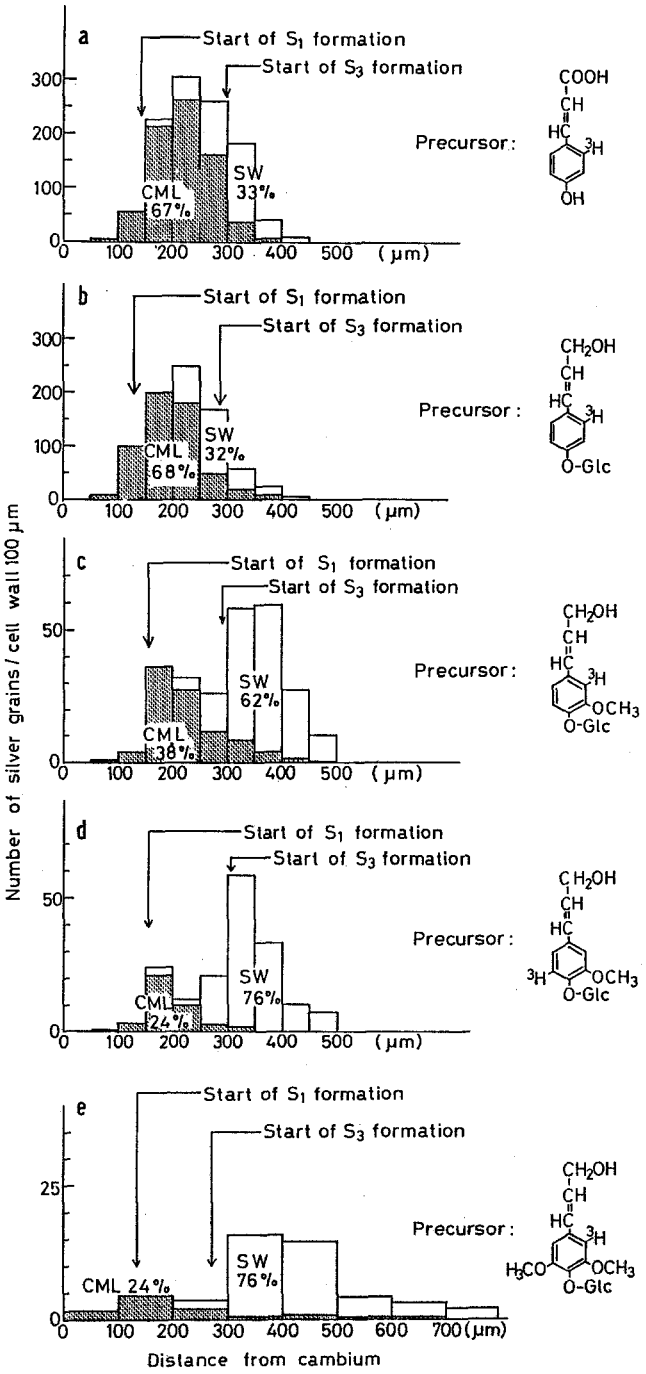

Fig. 3. **a** Distribution of silver grains in microautoradiograms of pine xylem administered with p-coumaric acid-[ring-2-^{3}H]: **b** p-coumaryl-β-D-glucoside-[arom.ring-2-^{3}H]: **c** coniferin-[arom.ring-2-^{3}H]: **d** coniferin-[arom.ring-5-^{3}H]: **e** and syringin-[arom.ring-2-^{3}H]

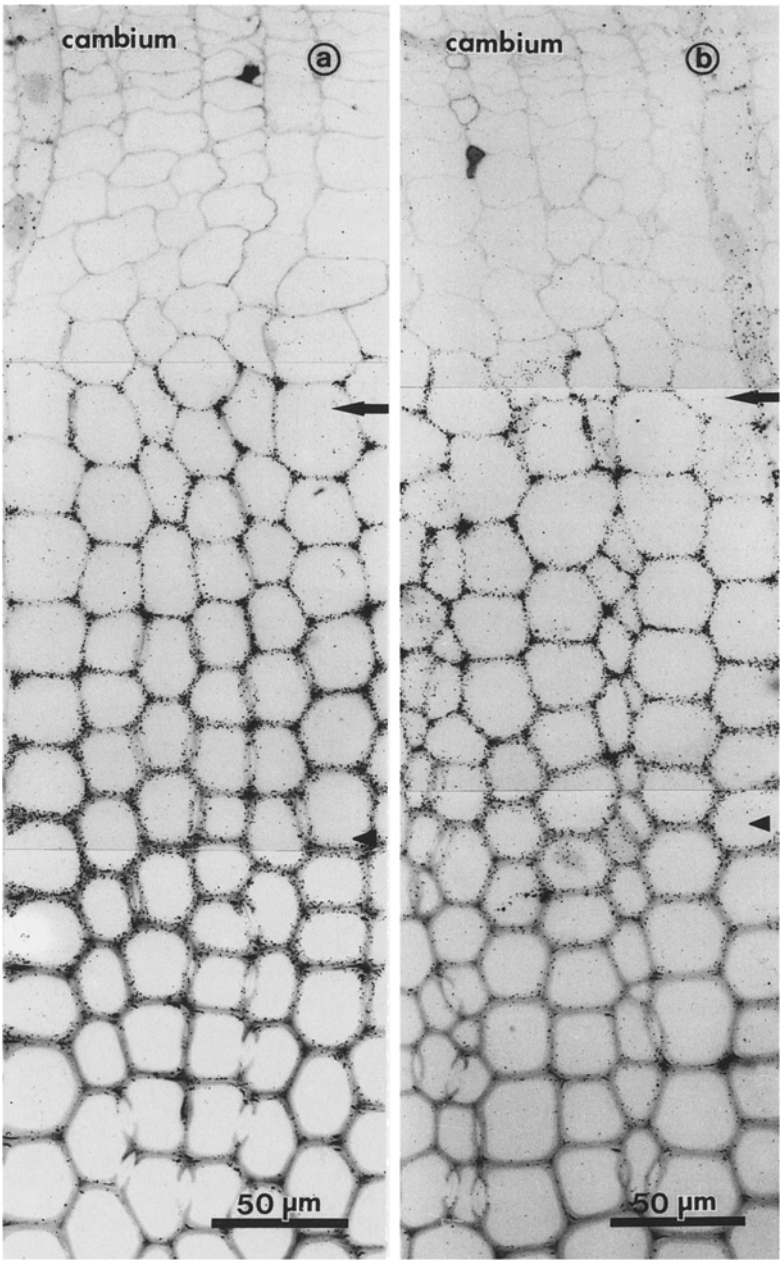

Fig. 4a and b. Microautoradiograms of differentiating xylem of pine administered with p-coumaric acid-[ring-2-^{3}H]: **a,** p-coumaryl-β-D-glucoside-[arom.ring-2-^{3}H]: **b.** Arrow and arrowhead indicate start of S_1 and S_3 formation respectively

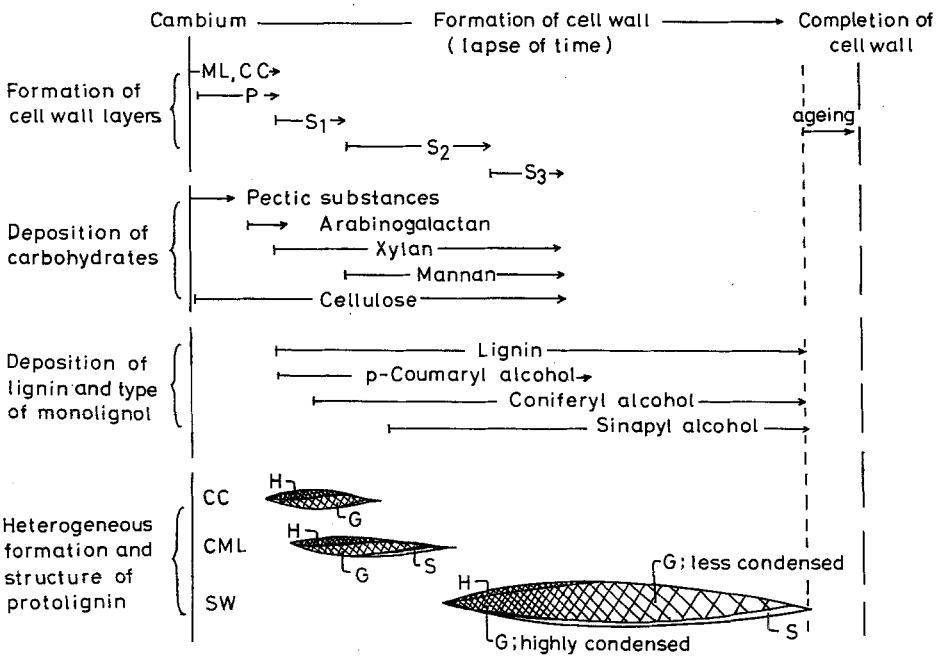

Fig. 5. A schematic representation to explain that the structural heterogeneity of protolignin originates from the heterogeneous process of lignin formation in the differentiating cell wall. ML: middle lamella, CC: cell corner, P: primary wall, CML: compound middle lamella, S_1, S_2, S_3: outer, middle and inner layers of secondary wall, SW: secondary wall, H, G, S: p-hydroxyphenyl-, guaiacyl- and syringylpropane units

According to the chemical characterization of tissue fractions from the middle lamella and secondary wall of black spruce tracheids by Whiting and Goring (1982), the secondary wall lignin contains 1.7 times as much methoxyl per C_9 as the middle lamella lignin, indicating a substantial proportion of p-hydroxyphenylpropane residues in the middle lamella. On the other hand, Westermark (1985) reported that the content of p-hydroxyphenylpropane units in the middle lamella of normal wood tracheids estimated by nitrobenzene oxidation and acidolysis was very low, while that in compression wood tracheids was high.

The present results suggest that the p-hydroxyphenylpropane unit is one of the normal building stones and may exist in significant amounts in the middle lamella and at the cell corner of pine. Because the molecular weight and degree of condensation of the middle lamella lignin is not yet high enough in the beginning of cell wall differentiaton, it is possible to detect substantial amounts of p-hydroxybenzaldehyde by nitrobenzene oxidation of the lignin at this stage, as shown in Table 1. However, in a mature cell wall, the p-hydroxyphenyl lignin may be highly condensed with bonds at 3- and/or 5-positions, resulting in only a small amounts of monomeric degradation products by oxidation or acidolysis.

The radioactivity from p-coumaric acid was incorporated during a longer period than that from p-coumaryl-β-D-glucoside (Figs. 3a, 3b, 4a, and 4b). This is due to the fact that p-coumaric acid is a common precursor of p-hydroxyphenyl,

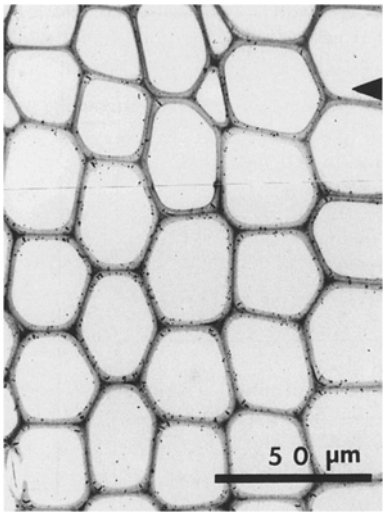

Fig. 6. Microautoradiogram of differentiating xylem of pine administered with syringin-[arom.ring-2-^{3}H]. Arrowhead indicates start of S_3 formation

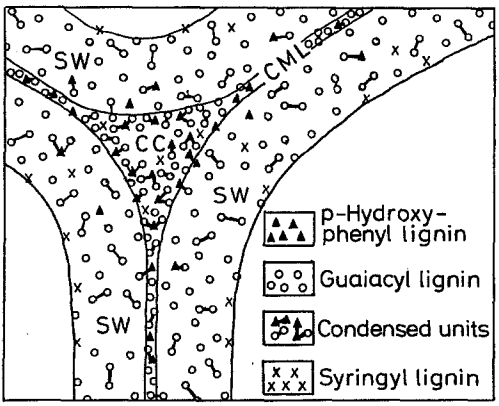

Fig. 7. A schematic representation of morphological distribution of various structural moieties in mature cell wall of pine tracheid. CC: cell corner, CML: compound middle lamella, SW: secondary wall

guaiacyl and syringyl lignins, and a part of the p-coumaric acid is converted into guayacyl and syringyl units (Table 1 and Fig. 3). A few syringyl units were incorporated mainly at the end of the differentiation, and most of it was located in the inner layer of secondary wall (Fig. 6 and 3e).

The morphological distribution of three kinds of lignin moieties in mature cell wall was estimated by integration of their incorporation patterns, as shown in Fig. 7.

It is noted that p-coumaric acid and the three kinds of monolignol glucosides are incorporated into the differentiating cell wall in the same order as that of their biosynthesis. It seems natural that the kind of monolignol biosynthesized by the cell changes with its age, because lignin is a kind of secondary metabolites. These results indicate that the precursors administered are metabolized under normal biochemical conditions. One of the regulating factors may be a substrate specificity of

β-glucosidase which catalyzes the hydrolysis of monolignol glucosides to corresponding alcohols. It has been shown that the cell-wall-bound β-glucosidase in spruce seedlings has different substrate specificity towards the three kinds of monolignol glucosides (Marcinowski and Griesebach 1978).

Conclusions

The heterogeneous formation process of the pine lignin macromolecule was visually demonstrated by a combination of high-resolution microautoradiography and the technique of selective labeling of specific structural moieties in the lignin.

Lignin is a kind of secondary metabolite, and lignification is controled fundamentally by the individual cell, and hence the mode of its biosynthesis changes with the age of the cell. It is noteworthy that the deposition of p-hydroxyphenyl, guaiacyl and syringyl lignins occurs in the same order as in the biosynthesis of their monolignols. Dehydrogenative polymerization of lignols proceeds in carbohydrate gels which affect the formation of a condensed structure, and the gel also changes with the progress of cell wall differentiation. Participation of p-coumaryl alcohol in polymerization increases the content of condensed structures. As a result, a highly condensed p-hydroxyphenyl lignin and a condensed guaiacyl lignin are formed mainly at the cell corners and in the compound middle lamella at the early stage of cell wall differentiation, while the syringyl lignin is formed at the late stage and mainly in the inner layer of the secondary wall.

These observations indicate that lignin develops in the differentiating cell wall under definite biochemical controls to a macromolecule which is heterogeneous in structure and specific to different morphological regions. The origin of the heterogeneity is proposed to be based on the biogenesis of the protolignin in the cell wall, as summarized in Fig. 5.

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