Comparative Studies on *O*-Methyltransferases involved in Lignin Biosynthesis

Hiroyuki KURODA*

Contents

Introduction

A FOREST TREE is often compared to a building which is made of reinforced concrete. Just like concrete in the building, lignin (reviews cited in references[1~5]) plays a role to fix cellulose and hemicelluloses in plant cell walls, supporting trees against intensive stress. The lignin can be readily classified by investigating their degradation products and by the color reaction such as Mäule and Cl_2-Na_2SO_3 reactions.[6] Comparative studies of lignin structural units have provided an interesting phylogenic difference in plant kingdom.[7~11] The lower land plants such as club moss, ferns, and gymnosperms contain guaiacyl lignins whereas plants of more recent origin such as angiosperms, contain guaiacyl-syringyl lignins. Additional lignin structural unit, *p*-hydroxyphenylpropane is found in Gramineae lignins, although there are some exceptions to these generalization as shown in Table 1.

* Research Section of Wood Biology

Lignin is widely distributed as a cell wall constituent in terrestrial vascular plants, except Tracheophyta plants grown in buoyant aqueous solution. Bacteria, fungi, red and green algae contain no flavonoids[12] and lignins. On the other hand, mosses and liverworts generally contain a few flavonoids[12] but no lignin. Most of the aquatic angiosperms which degenerate their vessel elements, contain a trace amount of (or no) lignin. It may be ascribed to that the plants need neither the accumulation of waste products nor strengthen the mechanical elements with lignin because of the aquatic life type. Vessel elements[13~15] are widely distributed in angiosperms. Exceptional angiosperms which lack vessel elements are concentrated in Polycarpiidae in addition to the aquatic plants described above. Polycarpiidae is considered to be one of the most primitive groups in angiosperms,[16] the lignin of which demonstrated to contain syringyl units except *Sarcandra* lignin.[17] On the other hand, the vessel elements are observed in several genera of Pteridophyta and Gymnospermae, the lignins of which are known to contain syringyl units in some species. These facts indicate that the presence of vessel elements is not directly related to the phylogenic distribution of syringyl units. These are summarized in Table 1 and 2.

Most of the lignin biosynthetic pathway was elucidated by tracer experiments

Table 1 Exceptional distribution of syringyl lignins

genus or (family)	vessel elements	Mäule reaction	syring-aldehyde	remarks (class)
Selaginella	+	+	+	Glossopsida
Equisetum	+	−	−	Sphenopsida
Pteridium	+	±		Pteropsida
Dennstaedtia		±		
Colysis		+		
Elaphoglossum		+		
Plagiogyria		±		
Ceratopteris		+		
Zamia		+		Cycadopsida
Tetraclinis		+	+	Coniferopsida
Podocarpus		±	±	
(Welwitschiaceae)	+	+	+	Chlamydospermopsida
(Ephedraceae)	+	+	+	
(Gnetaceae)	+	+	+	
(Winteraceae)	−	+	+	Angiospermopsida
Trochodendron	−	+	+	
Tetracentron	−	+	+	
Amborella	−			
Sarcandra	−	−	+	

Table 2 Exceptional distribution of vessel elements

The Pteridophyta and Gymnospermae which carry vessel elements	
Glossopsida	*Selaginella*
Sphenopsida	*Equisetales*
Pteropsida	*Pteridium*
Chlamydospermopsida	*Welwitschia*
	Ephedra
	Gnetum
The Angiospermae which lack vessel elements	
Dicotyledoneae*	Winteraceae
	Trochodendron
	Tetracentron
	Amborella
	Sarcandra
	Nymphaeaceae
The Angiospermae which degenerate vessel elements	
Dicotyledoneae	*Myriophyllum*
	Podostemonaceae
	Monotropastrum
Monocotyledoneae	Hydrocharitaceae
	Lemnaceae
	Najadaceae
	Ruppiaceae
	Zannichelliaceae
	Zosteraceae

* all these plants belong to Polycarpiidae,
the scientific names are essentially based on ref. 131 & 132.

in 1950's and 1960's, although the pathway was mainly studied in grass plants.[1~3] During the last decade, attentions are focused on the cell free system in lignin biosynthetic pathway, and the regulatory mechanisms in the biosynthesis have been investigated. Therefore, following paragraphs outline some enzymes which control the formation of guaiacyl and syringyl units in lignins. Other important enzymes involved in lignin biosynthesis are well documented in the literatures cited.[18~30]

Cinnamate derivatives are activated to the corresponding CoA esters by *p*-hydroxycinnamate: CoA ligase (EC 6.2.1.12) prior to the lignin, flavonoid and ester formations. The enzyme catalyzes the formation of cinnamoyl-CoA derivatives from the corresponding acids, with ATP, CoA and Mg^{2+}.[28,31] The ligase is inhibited by cinnamate, CoA and ATP,[32] which are the substrates and a products, respectively. Sinapate: CoA ligase could not be detected in gymnosperms and in most of the angiosperms,[28,32~37] although the enzyme was reported in Leguminosae,[33,38,40] and some other species.[41~43] In soybean culture cells, two ligase isoenzymes were found:

one was specific for *p*-coumarate, ferulate, 5-hydroxyferulate and sinapate, while the other catalyzed the same esterification except sinapoyl-CoA formation.[38] The former enzyme assumed to be involved in lignin biosynthesis, and the latter in flavonoid biosynthesis, although the evidence was insufficient.[25] Thus, these findings cast a doubt whether sinapate is an universal intermediate of syringyl lignin formation. The details in this point will be discussed later in relation to other viewpoints.

The cinnamoyl-CoA derivatives are reduced to the corresponding alcohols in lignin biosynthesis,[28] the reaction of which are catalyzed by cinnamoyl-CoA: NADPH oxidoreductase (EC 1.1.1.-)[44,46] and *p*-hydroxycinnamyl alcohol: NADP oxidoreductase (EC 1.1.1.-).[45,47] The former and the latter enzymes belong to B-[44] and A-groups[45] of NAD(P)-specific dehydrogenase, respectively. The best substrate for the former enzyme was feruloyl-CoA among the cinnamoyl-CoA derivatives in soybean cultured cells[46] and *Forsythia* young shoots.[44] In the soybean enzyme, sinapoyl-CoA was partially reduced into sinapaldehyde, while the spruce enzyme scarcely reduced this CoA ester.[48] In the second reduction step from the cinnamaldehyde to the corresponding alcohols, the enzyme was strongyl inhibited by the cinnamaldehydes and the two isoenzymes were found in soybean cultured cells.[47] One of them was specific for coniferyl alcohol and other specific for both coniferyl and sinapyl alcohols. The gymnosperm enzymes were rather specific for the reduction of coniferaldehyde, while angiosperm ones catalyzed equally both conifer- and sinap-aldehydes to the corresponding alcohols.[49]

Peroxidase (EC 1.11.1.7) was classified into land type and marine type.[18] The former enzyme is distributed in green algae and the terrestrial vascular plants including aquatic angiosperm, which catalyzes the oxidation of guaiacyl compounds. On the other hand, the latter enzyme which is distributed in red and brown algae, is not able to catalyze the oxidation of guaiacyl compounds. Polymorphism of this enzyme is well known and each multiple form showed different substrate specificities for guaiacyl and syringyl compounds.[50] Another interesting aspect of the enzyme is the finding that H_2O_2 was produced by the peroxidase coupled with malate dehydrogenase in the presence of oxygen.[51]

The methoxyl content is often examined as an essential criterion to characterize lignins. The methoxyl groups are derived from the methyl group of *S*-adenosyl-L-methionine, which is transfered to the catechol moieties of lignin precursors by *S*-adenosyl-L-methionine: caffeate 3-*O*-methyltransferase (EC 2.1.1.-; OMT). Several plant OMTs participating in various biosynthetic pathway have been characterized since the OMTs in apple (*Malus* sp.) and *Nerine bowdenii* were extracted.[52~54] Characteristics of the reported enzymes will be described in the latter sections of

this text. Shimada *et al.* first pointed out that the OMT controls the formation of guaiacyl or guaiacyl-syringyl lignin.[55] They used the ratio of sinapate (SA)- to ferulate (FA)-formation (SA/FA ratio) by OMTs in order to estimate the substrate specificities.

In these circumstances, this text focuses on the elucidation of plant OMTs which participate in lignin biosynthesis in taxonomically different plants and of the ambiguous parts in the enzyme properties. In section 1., pine (*Pinus thunbergii*) OMT is described in details as a typical gymnosperm type one. Gymnosperm enzymes have never been purified until this enzyme was isolated from the pine seedlings and characterized.[61]

Aspen (*Populus euramericana*) OMT is discussed as a typical dicotyledon type one in section 2. The matured secondary xylems of woody plants are desirable to use for studying lignin biosynthesis. However, heavily lignified tissues are usually unsuitable as enzyme sources because of its toughness. The OMT was extracted from the tree trunks, which was an attempt to characterize enzyme from such heavily lignified tissues.[39]

In section 3., bamboo (*Phyllostachys pubescens*) OMT is discussed as a typical grass type one. In this section, it is clearly demonstrated that the FA- and SA-activities are originated from the same enzyme. This finding indicates that the ratios of FA- to SA-formation keep a constant value during the differentiation.[76,80]

Mistletoe (*Viscum album*) lignin was a typical angiosperm type as it is demonstrated in section 4. Mistletoe OMT is discussed in this section, because the crude enzyme was found to show apparently no ferulate formation which may remind us of heterotrophism of the parasitic plants. The unusual substrate specificities are discussed in relation to the question whether the parasitic plant has a lignin biosynthetic pathway.[92]

In section 5., the three types of OMT are summarized by estimating SA/FA ratios in various plants, and the relationship between OMTs and lignin evolution is discussed.[130]

1. Roles of pine *O*-methyltransferase in the biosynthesis of gymnosperm lignin

Conifer lignins are known to be mainly composed of guaiacyl units, and usually lack syringyl ones.[7,8,11,56~58] A few enzymes were reported in relation to lignin biosynthesis in conifers,[33,49,59~62] although the enzymes are generally difficult to extract and characterize as it will be discussed in section 5. The gymnosperm lignin is produced via reactions mediated by a series of enzymes with less activities for syringyl

unit formation. For example, ferulate is preferentially converted to feruloyl CoA by the gymnosperm CoA ligase, and the CoA ester is preferentially reduced to the corresponding alcohol, while sinapate is hardly transformed to the corresponding alcohol *in vitro* in the gymnosperm.[33,48,49,63]

Finding that *O*-methyltransferase (OMT) catalyzes ferulate (FA)-formation but scarcely does sinapate (SA)-formation, first explained the reason why gymnosperm almost entirely contains guaiacyl lignin.[55] Although a little amount of syringyl lignin in gymnosperms can be explained by the substrate specificity, an OMT-inhibitor might be present and apparently masking sinapate formation in the crude preparation. Such possibility, as shown in section 4., is not negligible when the OMT is investigated without purification. Thus, pine (*Pinus thunbergii*) OMT was purified and characterized to confirm whether the OMT is specific for the formation of guaiacyl nucleus.

1.1 Partial purification of the OMT from pine seedlings

Sixteen-day-old seedlings, which contained lignin in the primary xylem, were used for the starting materials. The OMT was purified about 90-fold in a yield of 22% of the starting crude homogenate as shown in Table 3. The OMT was specific for the guaiacyl unit formation, but not for the syringyl unit formation through the purification. The ratio of SA- to FA-(SA/FA ratio) became smaller during the enzyme purification (from 0.065 to 0.037), suggesting the removal of SA-activity during the purification. However, chromatographic profiles apparently showed a single peak with FA- and SA-activities, e.g. as shown in Figure 1, probably because of small amounts of the minor fraction which carries rather high SA/FA ratio.

Table 3 Purification of the OMT in the pine seedlings

Purification procedure	Total protein (mg)	Specific activity (units/mg) FA	SA	Recovery (%) FA	Purification FA	SA/FA ratio
Homogenate	5330	0·18	0·012	100	1·0	0·065
0–60% $(NH_4)_2SO_4$	1079	0·62	0·041	70	3·5	0·066
DEAE-cellulose	321	1·82	0·093	61	10	0·051
Sephadex G100	23	14·6	0·609	36	82	0·042
DEAE-cellulose	13	15·9	0·588	22	89	0·037

Therefore, ammonium sulfate precipitate (step 2 in the Table 3) was examined by polyacrylamide gel electrophoresis, and compared with the result of the electrophoresis at final purification step. Under sensitive assay,[61] minor fraction was clearly resolved from the major one (Figure 3), but no minor fraction in the final step preparation (Figure 2). The minor fraction was more negatively charged on the gel in the electrophoresis and eluted faster than the major one in gel filtration chromatography as shown in Figure 4. Table 4 summarized the electrophoretic

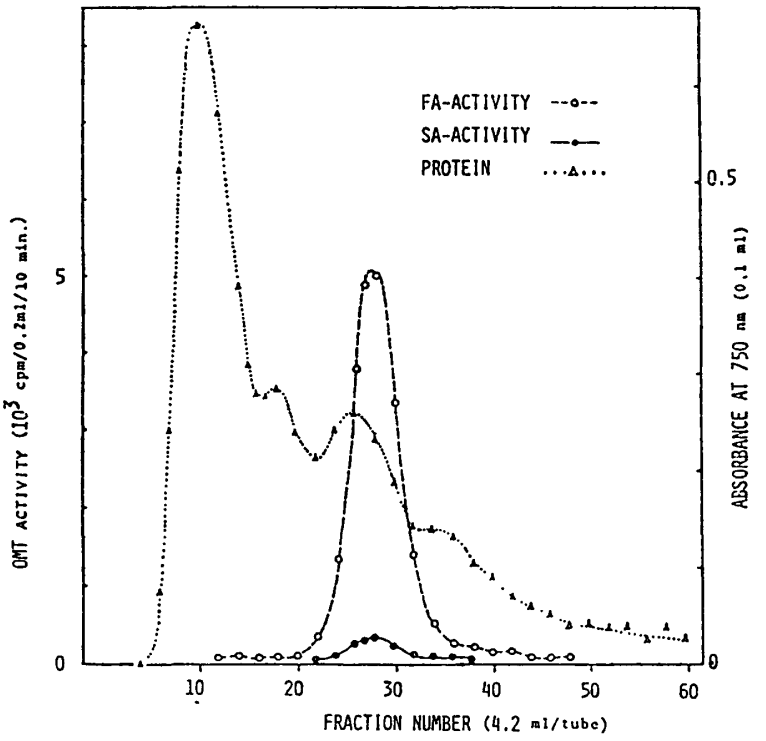


Figure 1 Gel filtration pattern of the pine OMT on Sephadex G100 at the purification step 4

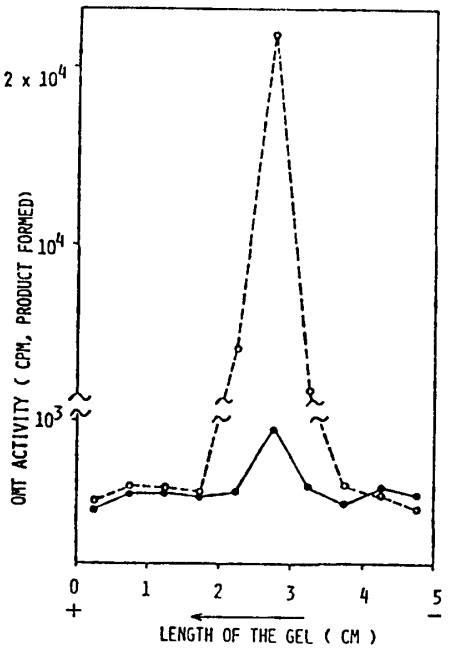


Figure 2 Eletrophoretic patterns of the pine OMT on polyacrylamide gel at the purification step 5

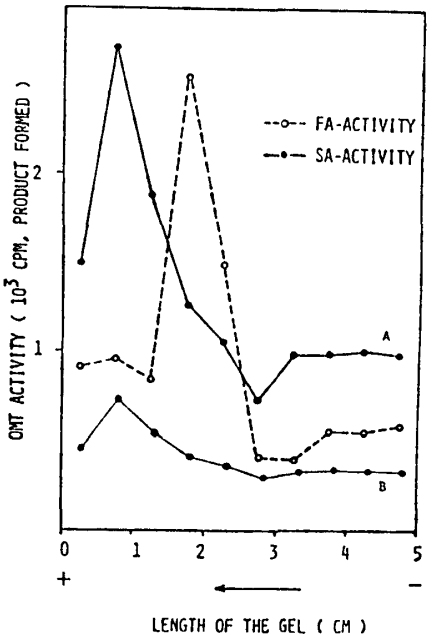


Figure 3 Electrophoretic patterns of the pine OMT on polyacrylamide gel at the purification step 2. A: Found activity. B: Calculated activity. See ref. 61

Table 4 SA/FA ratio of the major and minor fractions of the pine OMT after disc electrophoresis on polyacrylamide gel

OMT fraction	$(NH_4)_2SO_4$ Precipitate (step 2) Relative activity FA	SA	Ratio SA/FA	Final step ratio SA/FA
Major	1	0·05	0·05	0·03
Minor	0·30	0·18	0·6	*

* Minor fraction was not observed.

data described above. The overall SA/FA ratio at step 2 was calculated to be 0.17 by using the data shown in Table 4, and the calculated ratio was higher than that found in the purification as shown in Table 3. A similar discrepancy between the calculated and found ratios was also observed in the fractions separated by gel filtration chromatography, although the causes were not elucidated. In any event, the changes of the ratio during the purification is at least partially explained by the removal of the minor fraction which catalyzes SA-formation.

The pine OMT showed very poor SA-activity, which is good contrast to the angiosperm OMTs as summarized in section 5. No inhibitors or activators were observed in the pine OMT during the purification, and hence the low SA/FA ratio belongs to the OMT itself. This well explains very low amounts of syringyl nuclei in the pine lignin.

Concerning the OMT-polymorphism, it is necessary to ascertain whether or not the minor fraction in the crude enzyme preparation is a naturally occurring enzyme. In this point, it is interesting to consider the OMT activity in *Pinus strobus* and *Pinus taeda* which gave relatively high SA/FA ratios (section 5). *Thuja* OMT was also reported to show higher SA-activity among gymnosperm OMTs, although the enzyme characterized was concluded to be related to lignan biosynthesis.[62] The SA/FA ratio gradually decreased during the purification and the minor fraction of the enzyme was found in this experiment. However, conformational changes of the binding site, being known in a number of other enzymes,[75] might also explain the elimination of the SA-activity during the purification. Such possibility will be discussed in section 2.

1.2 Some enzymic properties of the pine OMT

The crude pine OMT (ammonium sulfate precipitate) was almost completely inhibited by 5 mM of EDTA, and that Mg^{2+} might stimulate the activity.[59] In contrast with the previous results, Table 5 shows that the finally purified enzyme was not inhibited but activated by 0.5 mM EDTA or NaF in the absence of Mg^{2+}. Therefore, Mg^{2+} seems to be no absolute requirement for the enzymic activity, although Mg^{2+}, Ca^{2+} and Mn^{2+} were useful for keeping high enzyme activity. A

Table 5 Effect of metal ions on the purified pine OMT

Additions	Concn. (mM)	Methylation rate of CA (10^3 cpm/0·1 ml/hr)	Relative rate (%)
None*		7·8	80·1
NaF	0·5	9·8	99·8
EDTA	0·5	9·6	97·9
$MgCl_2$	1·0	9·8	100·0
$MgCl_2$	0·5	9·3	95·0
$CaCl_2$	0·5	9·5	96·9
$MnCl_2$	0·5	9·3	94·4
(Less NaN_3)†		9·9	100·1

The total volume of reaction mixture was 1·0 ml, and the methylation rate was evaluated by comparison of that of the complete reaction mixture as described in the text.
* None consists of the complete system without $MgCl_2$.
† Removal of NaN_3 from the complete system.

similar result was lately reported on *Thuja* OMT.[62] Such properties of the pine OMT were similar to those of purified catechol-*O*-methyltransferase from human liver, the enzyme of which was stabilized by 0.2 mM $MgCl_2$ and EDTA.[64]

Addition of glycerol was found to be effective against denaturation of the OMT during freeze-thawing, and the presence of 10 mM of iso-ascorbate, cystein and 2-mercaptoethanol was necessary for the full enzymic methylation. The provisional molecular weight of the major fraction at step 2 was estimated to be 6.7×10^4 by the gel filtration on Sephadex G100 (Figure 4). The value was comparable to that of *Thuja* OMT.[62]

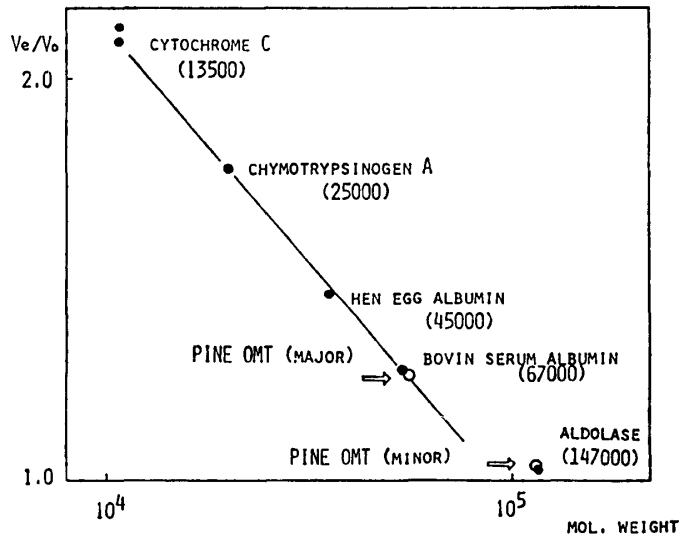


Figure 4 Molecular weight of the pine OMT determined by gel filtration chromatography on a calibrated Sephadex G100 column

Regulation of OMT has been studied. *S*-Adenosyl-L-homocystein is a potent inhibitor of this enzyme in plants[67~70] and animals.[71] Phenylalanine and cinnamate were examined their inhibitory effect on OMT, but no significant effect was observed.[66] The effect of illumination on OMT activity has also been investigated.[72~74] On the other hand, OMT might control phenolase activity by converting caffeate to ferulate.[18] In anion exchange chromatography, the gymnosperm OMT was eluted by lower KCl concentration than bamboo OMT's (section 3). Major fraction of the pine OMT seems to be more positively charged comparing with aspen OMT (section 2), bamboo OMT (section 3) and the minor fraction of the pine OMT on the polyacrylamide gel. Such positive charge of the major fraction might reflect some OMT-conformation which restricts the substrate specificity, and some enzymic properties.

1.3 Substrate specificities of the pine OMT

The crude OMT methylated caffeate most efficiently among catechols followed by protochatechuic aldehyde, 3,4-dihydroxyphenylacetic acid, 3,4,5-trihydroxycinnamic acid, protocatechuic acid, and chlorogenic acid. 5-Hydroxyferulic acid was hardly methylated by the enzyme. Km value for caffeate was 51.1 μM in the purified OMT, the value of which is hundred times lower than that of parsley OMT.[65] The difference seems to be ascribed to the fact that the parsley OMT is a flavonoid specific one whereas the pine OMT is only responsible for the biosynthesis of guaiacyl lignin. The kinetic constants summarized in Table 6 show that the affinity of the enzyme for caffeate is higher than 5-hydroxyferulate (5-HFA) in contrast with angiosperm OMTs which will be discussed in the following section.

Table 6 Kinetic constants of the pine OMT

Substrate	K_m (μM)	Relative value of $1/K_m$	V_{max} (cpm/ng/hr)	Relative value of V_{max}
Caffeic acid	51·1	5·4	66·1	24·8
5-Hydroxyferulic acid	277	1·0	2·7	1·0
S-adenosyl-L-methionine	40·6	6·8	69·9	26·2

The Lineweaver-Burk plots for the methylation of 5-HFA to SA in the presence or absence of caffeate gave a typical competitive inhibition pattern indicating that the SA-formation was greatly inhibited by caffeate (Figure 5). The Ki value for SA-formation, which should be equal to the Km value for 5-HFA, and other kinetic constants obtained by these plots were in good agreement with the values shown in Table 6. Further experiments indicated that SA-formation from 5-HFA (2.5×10^{-4}M) is completely inhibited by 2.3×10^{-5}M of caffeate added (Figure 6).

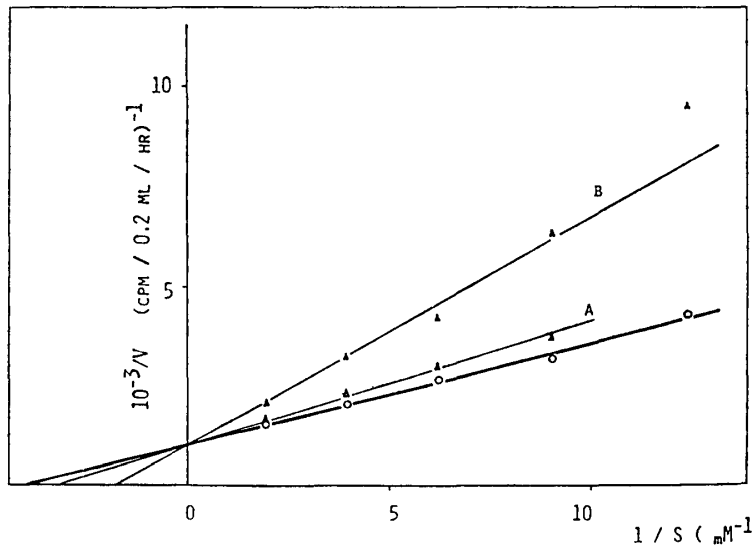


Figure 5 Reciprocal plot of velocity against 5-hydroxyferulate in the presence and absence of caffeate (A: 10^{-5} M, B: 5×10^{-5} M)

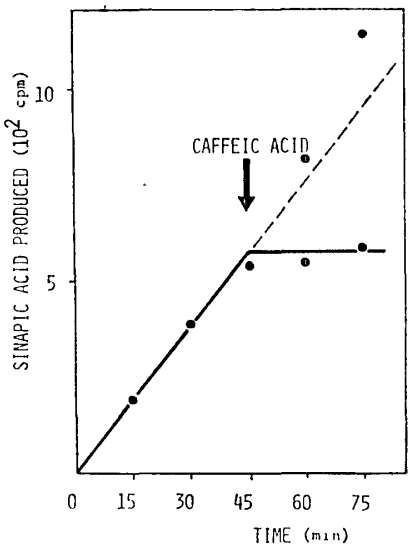


Figure 6 Effect of caffeate on the formation of sinapate. The concentration of caffeic and 5-hydroxyferulic acids were 2.77×10^{-4} M and 5×10^{-4} M, respectively

Km values of the OMT showed that guaiacyl units would be formed smoothly in the conifer. These findings indicate that no sinapate formation in the presence of caffeate *in vivo*. Sinapate formation would be practically impossible in the conifer because of the high Km value for 5-HFA and of the "feed-fore" inhibition by the presence of caffeate. This clearly explains the phylogenic difference between gymnosperm and angiosperm lignins, the details of which will be discussed in section 5. Thus, the OMT is one of the most important enzyme which determines the phylogenic

difference of lignin structural units.

2. Roles of aspen *O*-methyltransferase in the biosynthesis of angiosperm lignin

Shimada *et al.*[55] pointed out that plant OMTs are classified into two groups: one is almost entirely specific for guaiacyl unit formation, and the other specific for both guaiacyl- and syringyl unit formations. This finding well explains the reason why gymnosperm lignins are composed of only guaiacyl unit while angiosperm lignins are composed of both guaiacyl and syringyl units.

Several OMTs were characterized and their functions were discussed in relation to the biosynthesis of lignin,[43,55,61,69,76~79] flavonoids,[65,67,70,81,82] furanocoumarin[83] and *o*-, *p*-methylation.[81,84~86] The source used for the extraction and purification of OMTs were buds,[81] leaves,[68] roots,[79] young shoots,[76,82] seedlings,[61] and callus.[65, 67,69,70,77,78,83,86] Various methylated phenolics in addition to lignin precursors often occur in the same tissues and multiple forms of the OMT were, in fact, reported in plant tissues.[78,81~85] In order to characterize the OMT preferentially responsible for the biosynthesis of guaiacyl and syringyl lignin, the tissues which exclusively produce lignified cells are desirable for the OMT characterization. OMTs in such tissues are considered to be more directly involved in lignin biosynthesis. Trunks of big trees are suitable for this purpose, because young shoots often produce guaiacyl rich lignin even in angiosperm.[90] The isolation of cell free enzymes from tree trunks has, however, scarcely been reported[87] because of the difficulty of the large scale extraction.

This section focuses on the properties of OMTs from differentiating xylem tissues of ten-year-old aspen trunks. The role of the angiosperm-type OMT is discussed in relation to the formation of guaiacyl and syringyl lignin precursors in dicotyledonous plants.

2.1 Evaluation of aspen trunks as an enzyme source

OMT was extracted from the differentiating xylem of aspen (*Populus euramericana*) trunks: two ten-year-old aspen trees were chopped down in June and the trunks free of the bark were scraped by a scraper, then the chips were immediately frozen in liquid nitrogen; the wood frozen was milled with continuous addition of liquid nitrogen in a Wiley mill which was previously stored at −20°C. The homogenate from the trunks was clear, while the homogenate from the phloem and young shoots of the aspen was rapidly turned brown even in the addition of polyclar AT. This might be ascribed to a high level of polyphenol oxidase activity in the crude extract of the phloem and shoots. Luca and Ibrahim recently reported that such trouble

was prevented by mixing frozen starting materials with polyclar AT before being homogenized, and by other additives.[82]

The specific activity of the OMT extracted from the homogenate was reasonably high (10 pKat/mg protein for 5-HFA) comparing with the activity reported on other plant materials for purification. The specific activities for caffeate of the starting OMTs usually lie between one or two pKat and several tens pKat per mg protein.[61, 69,76,84] Although it was possible to improve the recovery of the enzyme by means of grinding the differentiating xylem with sea sand in a cold mortar, an alternative procedure using a Wiley mill for frozen wood meal with liquid nitrogen was employed to extract the enzyme in a large scale. It was found that the differentiating xylem of aspen trunk was a good enzyme source for the large scale extraction.

Lisosome-like vesicles and vacuoles were found to be developed in differentiating xylem tissues of an aspen trunks, suggesting high levels of proteolytic enzyme activities in the starting materials. In fact, the OMT was rather unstable during the purification, as described below, although a clear homogenate was obtained.

2.2 Partial purification of the OMT from aspen trunks

The apparent OMT polymorphism was observed when the crude OMT, fractionated by ammonium sulfate, was subjected to DEAE-cellulose chromatograph. Figure 7 shows elution profiles of the DEAE-cellulose column chromatograms, where two peaks were observed. The OMT eluted first from the column was observed to remain slightly in subsequent purification steps. The second peak was found to increase (peak I) and the other relatively to decrease during the purification. The enzyme was considerably unstable for time consuming purification. It was unsuccessful to increase purification fold as a result of repeating experiments of the purification. Addition of cystein or EDTA was no effect on the stabilization of the enzyme, and a thorough purification was not achieved because of the instability of the enzyme.

Table 7 summarizes one of the purification experiments of the aspen OMT. FA- and SA-activities show the formation of ferulate from caffeate and of sinapate from 5-hydroxyferulate, respectively. It was found that both activities in each peak of DEAE-cellulose chromatography were not resolved. The both activities were also unseparable by polyacrylamide gel electrophoresis at the final preparation (Figure 8), in which OMT was located to the peak I. The ratio of SA- to FA-activities, which is an indicator for the ability of sinapate formation to ferulate one by the enzyme,[55] remained constant (ca.3) during the purification. It was found that two peaks on the DEAE-cellulose chromatogram showed almost same SA/FA ratio (peak I 3.2; peak II 3.0): SA activities were not resolved from FA-activities in the both peaks.

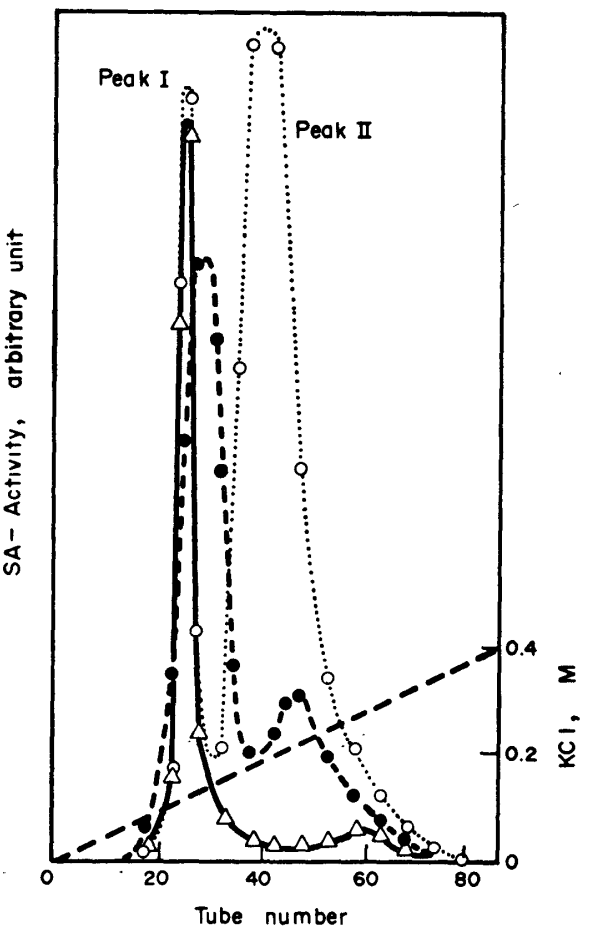


Figure 7 Elution profiles of aspen OMTs from DEAE-cellulose column chromatography. ···○··· A crude juice corresponding to step 1, - -●- - step 3 in the Table 7, —△— re-chromatogram after step 3 (kept in a refrigerator more than 2 weeks)

Multiple forms of plant OMTs were reported by several authors.[78,81~85] It was shown that *Populus* glandular tissue contained a few OMTs.[81] We also observed polymorphism of the OMT in the differentiating xylem of an aspen trunk. It is interesting to consider this point in relation to the finding that the differentiating xylem cell contains many lysosome-like vesicles and developing vacuoles. It seems likely that the proteases occur in such organelles in the differentiating xylem, to redistribute nitrogen from lignified xylem to cambium in tree trunks. Such phenomenon was reported during grain growth in wheat.[88] Therefore, proteases of the organelles might cause lability and modification of aspen OMT when the tissue was ground and subjected to a rather time consuming purification. Such assumption is in accord with the result that the molecular weight of the peak II was slightly smaller than that of the peak I. It must be kept in mind that multiple forms of the reported OMT might be produced during the extraction and purification.

Table 7 Parial purification of aspen OMT

Purification step*	Protein (mg)	Specific activity (nkat/mg) SA	FA	Ratio SA/FA	Recovery (%)
Step 1	461	10.6	3.38	3.14	100
Step 2	159	21.5	6.68	3.22	69.9
Step 3	31.3	82.0	25.1	3.27	52.5
Peak I	12.9	175	53.0	3.30	
Peak II	18.4	16.8	5.56	3.02	
Step 4	12.4	135	42.2	3.20	34.1
Peak I	5.80	224	68.9	3.25	
Peak II	6.60	56.1	18.5	3.04	
Step 5	3.71	183	57.9	3.16	13.9

* Step 1: Crude extract; step 2: $(NH_4)_2SO_4$ fractionation; step 3: DEAE-cellulose chromatography; step 4: Sephadex G200 chromatography; step 5: hydroxyapatite chromatography (see also ref. 39)

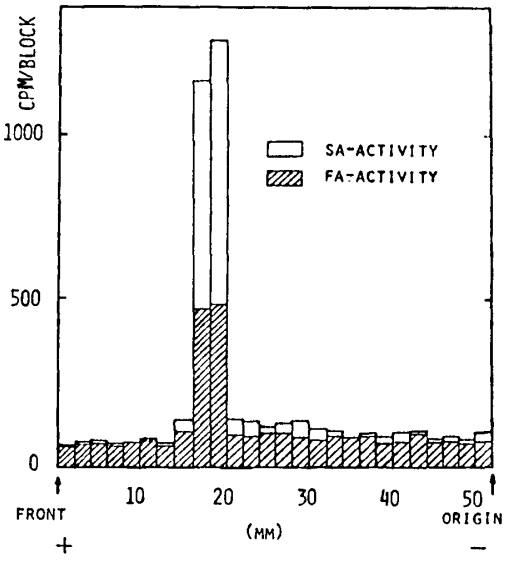


Figure 8 Disc electrophetic pattern of aspen OMT at the purification step 5. FA- and SA-activities in an OMT were not separated by the gel electrophoresis at the final purification step. The control (reaction mixture minus substrate) was not subtracted from the both activities illustrated

However, it is also assumed that the polymorphism observed is not an artifact but reflects some physiological role *in vivo*. At present, there is no direct evidence to show multiplicity of the aspen OMT, and further research is needed to clarify these problems.

2.3 Some enzymic properties of the aspen OMT

The molecular weight of the peak I and II were almost same, and were estimated

to be 72000 and 75000, respectively, by gel filtration chromatography. Optimal pH of the enzymes for FA- and SA-activities at step 2 was ca. 8.0, although the preparation was a mixture of peak I and II. Optimal pH for the activities at the final preparation was not measured, because the amount of the enzyme preparation was insufficient for other characterization and its instability. The ratio of SA/FA activities were almost kept constant in these experiments.

Table 8 shows the effect of metal ions on the FA- and SA-activities of the final step preparation. Addition of Mg^{2+} (1 and 10 mM) was no effect on the enzyme activities. Other two valent metal ions (1 mM, respectively) were also no requisite on the full methylation rate. Addition of EDTA, NaF and NaN_3 (1, 0.1, 10 mM, respectively) did not inhibit the enzyme activities, but moderately activated. The finding that the aspen OMT was not inhibited by EDTA and NaF, supports no requirement for Mg^{2+} ion in the enzyme activities. Heavy metal ions (1 mM, respectively) inhibited the methylation rates and the ions caused more than 90% inhibition when cystein in the reaction mixture was omitted.

Table 8 Effect of metal ions on aspen OMT

System*	Relative FA	Relative SA	Ratio SA/FA
Standard	100	100	3.15
$MgCl_2$	100.2	100.6	3.16
$CaCl_2$	101.4	96.8	3.01
$MnCl_2$	88.8	83.0	2.95
$BaCl_2$	104.8	102.1	3.07
$NiCl_2$	83.4	77.2	2.91
$ZnCl_2$	63.7	63.1	3.12
$CoCl_2$	72.8	67.0	2.90
$HgCl_2$	73.9	72.0	3.07
NaN_3	113.2	113.4	3.16
NaF	117.8	117.3	3.14
EDTA	114.5	107.8	2.97

*The system contained a standard reaction mixture (see ref. 39) except that the added chemicals substituted for Mg^{2+}. When NaF was added to the system, KPi buffer was used instead of Tris. All salts were added at 1 mM except NaN_3 (10 mM) and NaF (0.1 mM).

Table 9 shows that SH group is essential for the optimal catalytic activities. Thiourea and monoiodoacetate (0.1 mM, respectively) inhibited the activities, while cystein and mercaptoethanol were effective for the optimal methylation rates.

It is difficult for plant OMTs to find the general correlation between their biosynthetic role and properties. The properties of an OMT in different plants were

Table 9 Participation of SH-group in the enzymic methylation

System*	SA (pkat/mg)	FA	Ratio SA/FA	Relative SA
Standard	171	54.8	3.12	100
Minus $MgCl_2$ MESH CySH	36.4	11.8	3.08	21.3
Minus MESH CySH	37.4	11.9	3.14	21.9
Minus $MgCl_2$ MESH	172	55.3	3.11	100.6
Minus $MgCl_2$ CySH	146	47.9	3.05	85.4
Plus *p*CMB (0.1 mM)	3.59	2.79	1.29	2.10
Plus thiourea (0.1 mM)	32.1	11.2	2.87	18.8

* MESH = 2-mercaptoethanol, CySH = cysteine, *p*CMB = *p*-mercuric benzoate.

not always consistent even if the enzyme plays the same biosynthetic role. Purified preparation of an OMT does not always show the same properties as the crude one in an enzyme source. These make it difficult to describe the general properties of an OMT accurately, but plant OMTs might be roughly characterized as follows. The optimal pH of caffeate-specific OMTs was ranged from pH ca. 6.5 to 8.0.[59,51,69,76,79] Lignin-specific OMTs generally do not require Mg^{2+} ion[61,69] except the case of bamboo.[89] This is in good contrast to that of flavonoid-specific OMTs which absolutely required Mg^{2+} ion.[65,70,82] Sulfhydryl group inhibitors generally inhibit plant OMTs in various extent[61,67,83,84,89] except flavonoid-specific parsley OMT which was not affected by the inhibitors.[65] Effect of EDTA on the OMTs is not always the same: Some OMTs showed no effect (e.g. lignin-[69] and flano-coumarin-[83] specific OMTs) but others were moderately activated (lignin-specific OMT[61]) or inhibited (flavonoid-specific OMT[70]). Molecular weight of plant OMTs reported lie between 40000 and 110000.[61,65,67,82~84] The aspen OMT characterized in this section fills the general properties of lignin specific OMT as described above.

Table 10 shows the kinetic data of the OMT of the final step preparation. Km values for protocatechualdehyde, 5-hydroxyvanillin, caffeate and 5-hydroxyferulate were found to be of the order of 10^{-4}M. Vmax/Km for 5-hydroxyferulate was 5.4 times greater than that for caffeate. Protocatechualdehyde was a better substrate than caffeate. The kinetic data indicate that the formation of syringyl unit is preferential than that of guaiacyl unit. The other substrates examined were quercetin,

Table 10 K_m, relative V_{max} and V_{max}/K_m values of aspen OMT

Substrates	K_m (10^{-4} M)	Relative V_{max}	Relative V_{max}/K_m
Caffeate	3.8	1.0	1.0
Protocatechualdehyde	2.6	2.6	3.8
5-Hydroxyvanillyl alcohol	3.4	3.7	4.1
5-Hydroxyvanillin	4.8	3.5	2.8
5-Hydroxyferulate	3.1	4.4	5.4

cyanin, catechin, protocatechuic acid and pyrocatechol. The methylation rate for these substrates was less than 2% of that for caffeate. Almost no methylation was observed in the case of other phenols, i.e., apigenin, kaempferol, 3,5-, 2,4-, 2,6-, 2,5-dihydroxybenzoates, saligenin, salicin, salicylaldehyde, arbutin, *o*-, *p*-coumarate, ferulate, *iso*-ferulate and sinapate.

It is noteworthy that catechols with C_1-side chains of aldehyde and alcohol were as good substrates as caffeate. The finding indicates that the enzyme might be involved in the methylation of C_6–C_1 diphenols. However, almost no methylation of protocatechuic acid was observed although vanillic and syringic acids are widely distributed in this genus. Such substrate specificity of the OMT seems to be affected by the functional groups of the C_1-side chains. Thus, the C_6–C_3 *o*-diphenolic substrates carrying these side chains might be also methylated by a lignin-specific OMT. In fact, Kutsuki *et al.* recently found that 5-hydroxyconiferylaldehyde was methylated to sinapaldehyde by angiosperm OMTs (unpublished results), while caffeoyl-CoA was not methylated by the OMT.[69] The cell free system involved in the reduction of sinapate to sinapyl alcohol was demonstrated, but the universal process of syringyl lignin formation is still obscure.[33] Further survey of the substrate specificity for the OMT is desired to clarify the lignin biosynthetic pathway, especially the formation of syringyl lignins.

The results in this section is concluded as follows. First, the characterized aspen OMT is related to lignin biosynthesis, because of lignin specific phenolic metabolism in the xylem tissues and of substrate specificities of the enzyme. The cambium of aspen trunks differentiates secondary xylem, where lignin polymers are present with small amounts of lignin precursors. Since phenolic metabolism in the differentiating xylem tissues considered to be exclusively directed to the formation of lignin precursors. The substrate specificities of plant OMTs have been discussed from the standpoint of lignification, flavonoid biosynhestis and *o*-, *p*-*O*-methylation. It was found that the aspen OMT can methylate neither flavonoids nor *p*-position in the phenolic substrates surveyed. These substrate specificities

strongly suggest that the OMT is related to lignin biosynthesis in the xylem of aspen trunks. Second, the aspen OMT probably operates as a "fine adjustment" enzyme in the regulation of guaiacyl and syringyl lignin formation. This is concluded from the findings that the same OMT catalyzes the formation of both guaiacyl and syringyl nuclei with preferential formation of the latter nuclei. If caffeate and 5-hydroxyferulate are formed in the same site of a xylem cell, the latter substrate will be preferentially methylated by the OMT until the substrate concentration is decreased to the level which does not interfere the former methylation. The aspen OMT may control guaiacyl and syringyl lignin formation in this way, i.e., fine adjustment which is capable of very minute alteration of the both units. Both fine and coarse adjustments seem to participate in the increasing ratio of syringyl to guaiacyl nuclei during the development of the xylem in dicotyledonous plants.[43,90] The latter adjustment might be also conducted by the enzymes participating in the reduction of *p*-hydroxycinnamates to corresponding alcohols.[33,49] Last, the substrate specificities of aspen OMT explain a part of the reason why lignin contains much syringyl nuclei comparing with gymnosperm one. This property is generally applicable to the angiosperm OMTs which are related to lignin biosynthesis. Interesting examples were reported in swede root,[79] *Erythrina*[91] and mistletoe[92] (section 4). The swede root OMT was found to show a low SA-activity. These points will be discussed in section 5.

3. Roles of bamboo *O*-methyltransferase in the biosynthesis of grass lignin

The *O*-methyltransferases (OMTs) involved in lignin biosynthesis could be roughly classified into gymnosperm- and angiosperm types.[55] The former catalyzes the formation of guaiacyl-unit but scarcely does that of syringyl unit (section 1). On the other hand, the latter type OMT catalyzes both guaiacyl and syringyl unit formation (section 2 and 4).

In the angiosperm described above, not only ferulate-5-hydroxylase has not been isolated, but also sinapate: CoA ligase is not universally distributed. Such situation cast a doubt on the universal participation of sinapate in angiosperm lignin biosynthesis (section 2). Gramineae, however, seems to utilize sinapate as an intermediate, because of the presence of sinapate: CoA ligase,[33] efficient incorporation of sinapate into lignins,[1,3] and universal distribution of free and esterified sinapate. Therefore, the formation of syringyl to guaiacyl lignin in bamboo is probably regulated by the ratio of sinapate to ferulate formation (SA/FA ratio). Bamboo OMT belongs to angiosperm type and catalyzes the formation of both guaiacyl and syringyl units at nearly same rates.[55,76,89,93,95] This section focuses on the control mechanism of the both unit formations by bamboo OMT.

3.1 The ratio of SA- to FA-activities of the bamboo OMT

The ratio SA- to FA-activities (SA/FA ratio) was kept constant during denaturation by heat and purification of the OMT. Bamboo OMT showed the ratio ca. one, which seems to be characteristic for Gramineae and allied species as described in section 5. Considering the ratio of syringaldehyde to vanillin, produced by nitrobenzene oxidation of lignin, Gramineae OMT seems to form a group other than gymnosperm and angiosperm OMTs.

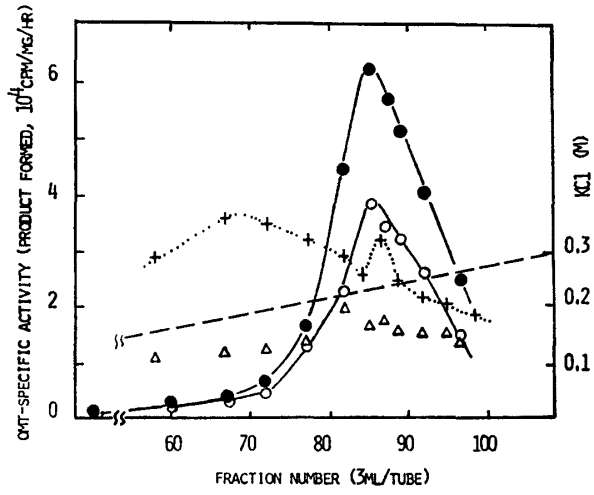


Figure 9 Elution pattern of bamboo OMT on a DEAE-cellulose column. —○— FA-activity, —●— SA-activity, △ SA/FA ratio, ···+··· protein concentration

The SA/FA ratio, however, varied a little, when the crude OMT was assayed in different hydrogen ion concentrations. The fractions by ammonium sulfate precipitation also showed the ratio-fluctuation. These observations may imply possible presence of some OMT isoenzymes. Thus, the crude bamboo OMT concentrated by ammonium sulfate precipitation was preliminary examined by chromatography on the OMT polymorphism. Figure 9 and 10 showed the profile of crude enzyme on DEAE-cellulose and Sephadex G200 chromatography, where neither OMT-polymorphism nor resolution of the FA- and SA-activities were observed. The apparent SA/FA ratio, however, tended to decrease from OMT peak to its base on the chromatograms.

These variation of SA/FA ratio possibly due to the following reasons. When the OMT activity is as low as the control value on the chromatogram, the SA/FA ratio decreases and closes to one, and such a low activity causes experimental error to result in the ratio-flactuation. Another reason is due to low protein concentration. When a crude preparation contained a small amount of protein (less than ca. 80 ng), the apparent ratio also varied.

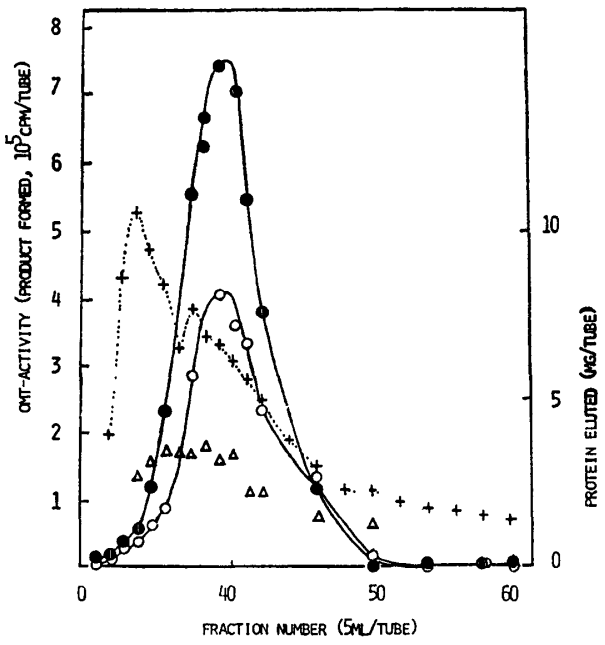


Figure 10 Gel filtration pattern of bamboo OMT on Sephadex G100. —○— FA-activity, —●— SA-activity, △ SA/FA ratio, ···+··· protein concentration

FA- and SA-activities similarly decreased by 10 minute incubation at various temperature (Figure 11C & D). The deactivation time-courses of both activities were almost identical at 50°C, and a small difference was found at 45°C in both presence and absence of the phenolic substrates (Table 11). The heat treatment at 45°C changed SA/FA ratio which was higher in the presence of substrate than in its absence. The half-life period at 50°C was found to be six times longer in the presence of substrate than in its absence as shown in Figure 11A & B. Protecting effect of 5-hydroxyferulate on the heat treatment was remarkable comparing with that of caffeate although the former substrate was not effective on the enzyme stabilization when incubated at 4°C for 5 days. The OMT-stability at 4°C was improved by adding glycerol which kept 96% of the activity during the 5-day-incubation at 4°C, while no glycerol kept 74% of the activity during the incubation.

These experiments essentially supported that FA- and SA-activities belong to the same OMT with higher affinity for the latter substrate. The finding that the heat treatment at 45°C changed SA/FA ratio is probably due to the different conformational stability for the FA- and SA-activities, because the ratio flactuation is larger in the presence of substrate than its absence, and furthermore, the denaturation rate was faster in the presence of caffeate than the presence of 5-hydroxyferuate. Thus, the flactuation of the ratio seems to be derived by partial denaturation of the enzyme by heat, acid or alkali, and ammonium sulfate, which cause conformational

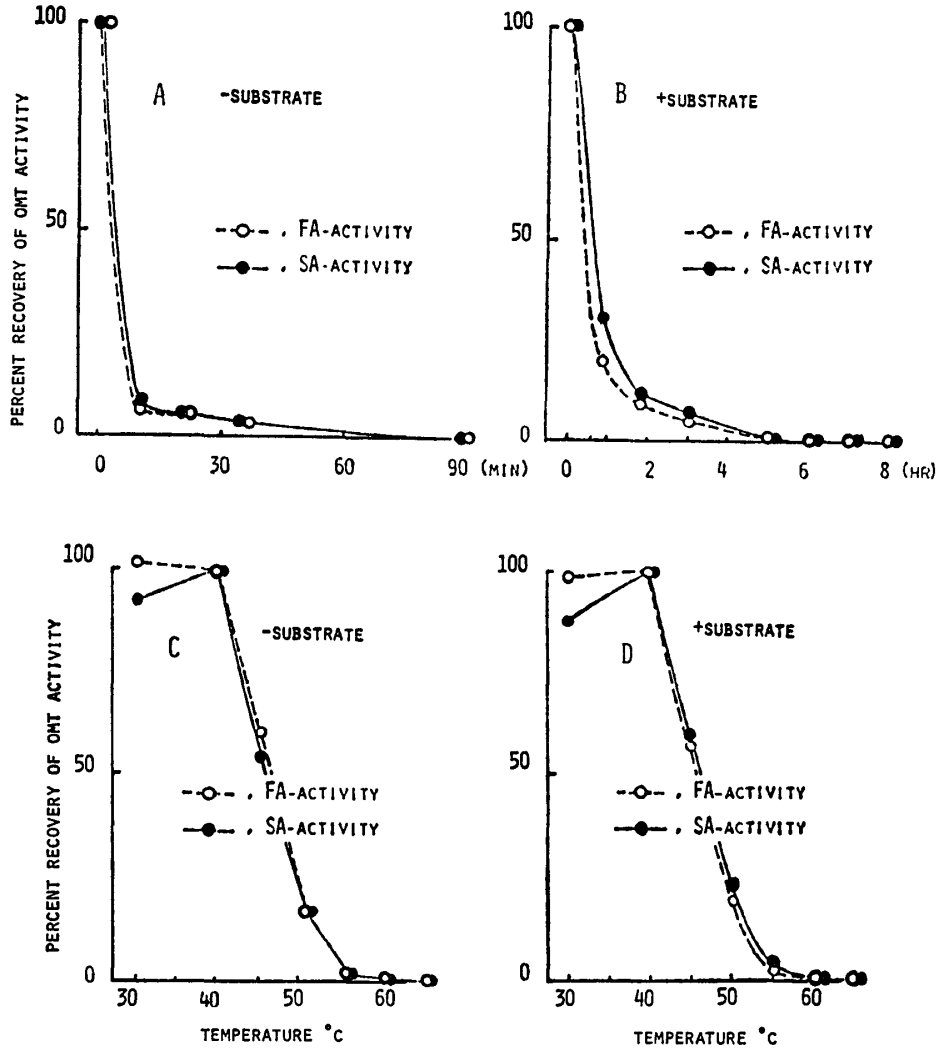


Figure 11 Heat treatment of bamboo OMT. A & B: effect of caffeate or 5-hydoxyferulate on the thermostabilty of bamboo OMT during heat treatment at 50°C. C & D: denaturation patterns of bamboo OMT on 10 minutes heat tretment in the presence and absence of caffeate and 5-hydroxyferulate.

Table 11 Thermostability of bamboo OMT at 45°C in the presence or absence of phenolic substrates

Time min.	Minus substrates (cpm)			Plus substrates (cpm)		
	FA	SA	SA/FA	FA	SA	SA/FA
0	22,630	31,530	1.39	25,130	30,930	1.23
10	10,510	13,310	1.27	13,510	18,010	1.33
20	7,300	11,430	1.57	9,530	19,030	2.00
30	8,030	8,630	1.07	10,830	16,030	1.48
40	8,340	9,640	1.16	11,240	17,940	1.60
50	7,170	8,570	1.20	10,670	15,870	1.49
100	4,090	5,090	1.24	6,190	7,390	1.19

change. Such modification of the OMT derived by the cytoplasmic alterations *in vivo*, i.e. temperature, pH changes, and protease attack etc., may cause the variation of SA/FA ratio. In other words, the substrate specificity of OMT might be finely changed via cytoplasmic alterations to adjust guaiacyl- and syringyl unit formations in lignin biosynthesis.

3.2 Some enzymic properties of bamboo OMT

The properties of the crude OMT are summarized as follows.[89] The crude enzyme showed optimal pH ca. 8.0 for both FA- and SA-activities with half maximal activities at pH 8.6±0.2 and 6.4±0.2. PCMB (3 mM) and EDTA (10 mM) moderately inhibited the enzyme activity (10% and 20% inhibition, respectively), but monoiodoacetate (0.3 and 3 mM) showed no significant inhibition. EDTA and SH reagents prevented its deactivation when the enzyme was precipitated by ammonium sulfate. Mg^{2+} ion and SH group seem to be involved in the enzymic methylation. In addition, purified OMT was found to be an acidic protein with an isoelectric point at pH 4.61 at 4°C as shown in Figure 12 and 13.

Methylation by the bamboo OMT was found in caffeate, 5-hydroxyferulate, 3,4,5-trihydroxycinnamate, 5-hydroxyvanillin, protocatechuate, but no or little methylation in chlorogenate, isoferulate, *m*-, *p*-coumarate, 3,4-dihydroxyphenylacetate

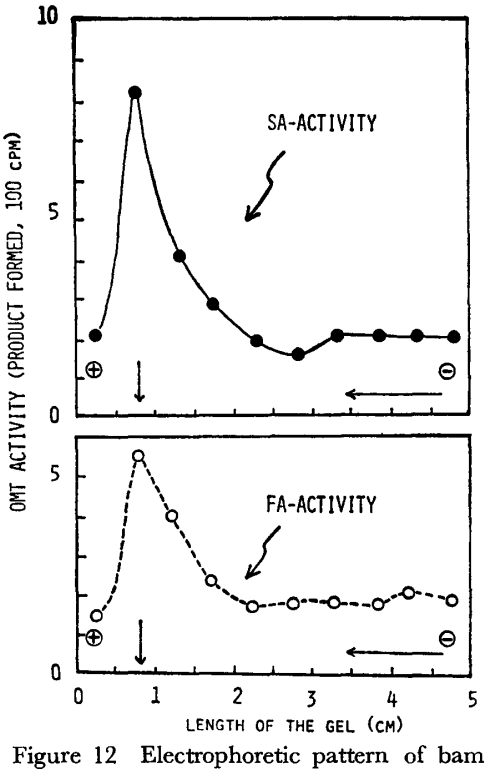


Figure 12 Electrophoretic pattern of bamboo OMT on polyacrylamide gel

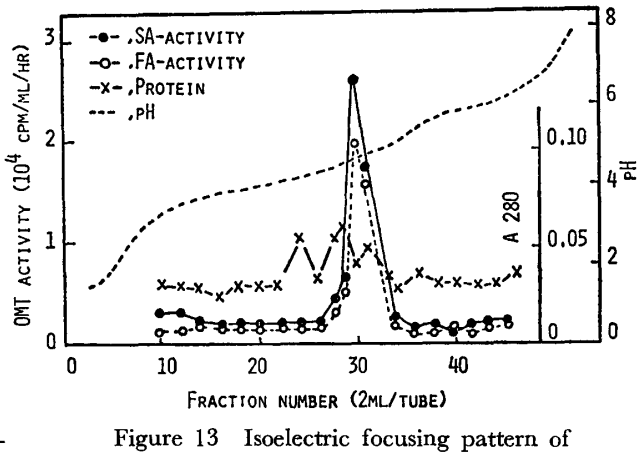


Figure 13 Isoelectric focusing pattern of bamboo OMT

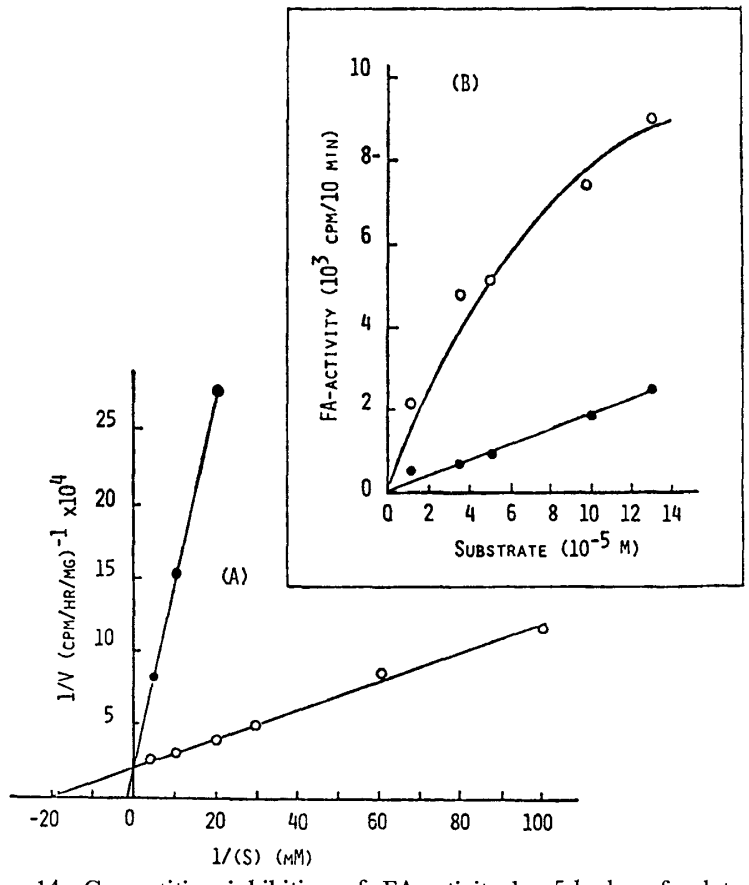


Figure 14 Competitive inhibition of FA-activity by 5-hydroxyferulate. A : reciprocal plot of methylation-velocity against caffeate concentration in the presence and absence of 5-hydroxyferulate (10^{-4} M), 70% ammonium sulfate ppt, passed through Sephadex G100. B: effect of 5-hydroxyferulate (4×10^{-5} M) on the methylation-velocity for caffeate, (32-fold purified enzyme was used).

3,4-dihydroxyphenylmanderate, gallate, pyrocatechol, pyrocatechol phthalein and *d*-catechin. Km values for caffeate and 5-hydroxyferulate were 5×10^{-5} and 10^{-5}M, respectively, with the excess amount of *S*-adenosyl-L-methionine. Figure 14 shows that the latter phenolic substrate competitively inhibited FA-formation, and Ki for FA-formation was identical to the Km for 5-hydroxyferulate. The findings demonstrated that both FA- and SA-formations were competitively controlled, suggesting that *in vivo*, a feedback inhibition by the latter substrate might operate on the former formation.

Syringyl lignin is formed later than guaiacyl lignin during xylem differenciation, [43,90] which seems to be ascribed to the inducible enzymes responsible for syringyl lignin formation. For example, two multiple forms were found in soybean *p*-coumarate: CoA ligase[38] and cinnamyl alcohol dehydrogenase,[47] which respectively

catalyze only guaiacyl group, or guaiacyl and syringyl groups. Thus, these enzymes may be involved in the control as a coarse adjustment in the syringyl lignin biosynthesis. On the other hand, the OMT is probably involved in the control as a fine adjustment, i.e., by means of the conformational change and the feedback inhibition which are capable of very minute alterations as described above.

3.3 Partial purification of the OMT from bamboo shoots

Bamboo OMT was purified and demonstrated it to be a single enzyme which is specific for both guaiacyl and syringyl nuclei. Young bamboo shoots were good enzyme source for investigating lignin biosynthesis. Because the shoots are not only suitable for enzyme extraction (soft, no browning of the homogenate), but also producing lignins. The OMT extracted from the young shoots showed specific activity of ca. 3 pKat per mg protein for FA-activity and was rather stable.

Table 12 Purification of bamboo OMT

Purification of *O*-methyltransferase from bamboo (1)

Purification procedure	Total protein (mg)	Total activity (units)		Specific activity (units/mg)		Recovery(%)		SA/FA ratio	Fold
		FA	SA	FA	SA	FA	SA		
1. 0-70% Am_2SO_4	2900	5980	7300	2.06	252	100	100	1 22	1
2 20-55% Am_2SO_4	1300	6890	8640	53	6.8	118	118	1 26	3
3. DEAE-cellulose	118	5480	6870	465	583	92	93	1.25	23
4. Sephadex G-200	65	3720	4180	57.5	64.0	63	57	1.11	28
5. DEAE-cellulose	15	300	368	200.0	244.0	5	5	1 22	97

Purification of *O*-methyltransferase from bamboo (2)

Purification procedure	Total protein (mg)	Total activity (units)		Specific activity (units/mg)		Recovery(%)		SA/FA ratio	Fold
		FA	SA	FA	SA	FA	SA		
1. 0-70% Am_2SO_4	1590	2600	3190	1.6	2.0	100	100	1.22	1
2. 20-55% Am_2SO_4	695	2360	2980	3.4	4 3	91	93	1.26	2
3 DEAE-cellulose	92	1210	1630	13 2	17.7	47	51	1.34	9
4. Sephadex G-100	13	396	520	30.0	39.5	15	16	1.31	20
5 DEAE-cellulose	14	165	195	117.0	138.0	6	6	1.18	69

Table 12 summarizes two series of purification achieved. Bamboo OMT was rather stable, in contrast to the enzyme of aspen cambial zone and developing xylem as described in section 2. Ammonium sulfate fraction at step 2, precipitated by 55%- to 20%-saturation of ammonium sulfate, contained ca. 90% of the OMT activity. Further profiles of the chromatograms during purification were almost identical to the crude ones. The bamboo OMT was finally purified 97-fold with 5% recovery, specific activities of which were 2.50 and 3.05 nKat per mg protein for FA- and SA-activities, respectively. The ratio SA/FA kept constant during

the purification, and was found to be 1.25±0.1 at pH 8.0. The final preparation was further examined by electrophoresis and isoelectric focusing, whether the FA- and SA-activities were resolted, and/or showing multiple forms. FA- and SA-activities showed a single peak at the same place as shown in Figure 12 and 13, and these accurate methods conclusively demonstrated no resolution of the both activities. Thus, the two methylation steps in the lignin biosynthesis, i.e., caffeate to ferulate, 5-hydroxy-ferulate to sinapate, were found to be catalyzed by the same OMT. On the contrary, three methylation sequence in a flavonoid biosynthesis are known to be catalyzed by three different OMTs, respectively.[82]

Bamboo OMT was chromatographically demonstrated to be a single enzyme which catalyzes FA- and SA-formations with preferential latter formation. This is supported by remaining a constant SA/FA ratio during the purification, and by deactivation pattern on heat treatment. The finding that caffeate and 5-hydroxy-ferulate were competitively methylated also suggests that the bamboo OMT is a single enzyme which has one active methylating-center for FA- and SA-formation. Although apparent polymorphism had been reported on a previous work,[93] no OMT-polymorphism was observed in the present experiments.

4. Roles of mistletoe *O*-methyltransferase in the biosynthesis of parasite lignin

Mistletoe (*Viscum album*) is an evergreen dicotyledon which is usually parasitic on *Celtis sinensis* and occasionally on cherry trees in Japan. Several characteristic compounds such as physiologically active proteins,[94~96] viscotoxine,[97,98] acetylcholine,[99] verazine,[100] a cyclic peptide[101] and flavonoids[102~104] were isolated from mistletoes.

On the other hand, a few studies have been reported on the nutrition dependence of mistletoe on the hosts.[105~107] Mistletoe usually gets water from the host, and some species are known to be depend carbon sources as well on the host.[105~107] Freudenberg reported that European mistletoe grown on either gymnosperm or angiosperm trees gave the corresponding type lignins.[108] However, the lignins in European mistletoe lately demonstrated to be angiosperm type one independent on the host trees.[109~111] It has been further reported that flavnoids in a parasitic plant are quite different from those of the host plants.[102~104] These observations may indicate that the biosynthesis of mistletoe lignin is generally controlled by the parasitic plant itself and not by host trees.

However, the direct demonstration about such nutrition dependency of the lignin biosynthesis was still missing. Apparently, mistletoe *O*-methyltransferase did not catalyze ferulate-formation, suggesting no lignin biosynthesis in the plant. The

mistletoe grown on *Pinus silvestris* tree was found to be rather less abundant in syringyl lignin.[110,111] This section describes chemical properties of parasitic and host lignins, and elucidates if the mistletoe plant produces lignins independently from the host. The enzyme in cinnamate pathway, especially *O*-methyltransferase is also discussed.

4.1 Characterization of mistletoe lignin

Analytical results of mistletoe (*Viscum album*) lignin was compared with the host (*Celtis sinensis*) ones, and the data obtained are summarized in Table 13.

Table 13 Chemical properties of mistletoe lignin

	Viscum	*Celtis*
Lignin content	22.0%	21.6%
Empirical formulae of MWL*	$C_9H_{5.77}O_2(H_2O)_{1.44}(OMe)_{1.33}$	
	$C_9H_{5.44}O_2(H_2O)_{1.45}(OMe)_{1.29}$	
UV λ_{max} (nm)	277.5	278.0
A (1/g cm)	15.3	14.4
IR	Typical angiosperm type	
^{13}C-NMR	Typical angiosperm type	
Nitrobenzene oxidation†	1.7	—
Acidolysis‡	0.92	0.73

* Upper: *Viscum*, lower: *Celits*
† The ratio, syringaldehyde/vanillin
‡ The ratio, syringyl acetone/guaiacyl acetone

Lignin contents in the mistletoe and host woods were estimated to be 22.1 and 21.6% by Klason method, respectively. The values showed typical angiosperm ones in normal woods. Emperical formulae of the milled wood lignins (MWLs) showed rather high oxygen contents in comparison with Freudenberg's results.[108] Since the MWLs in this experiment were purified by the Björkman's standard method,[112] the lignins probably contained some contaminated materials such as sugars which may cause high oxygen contents in the formulae.

Infrared spectra of the mistletoe and host MWLs gave almost identical absorption bands. The main bands observed were 1325, 1235, 1130 cm^{-1} (syringyl ring); 1275, 1145 1030 cm^{-1} (guaiacyl ring); 1595, 1505, 1425 cm^{-1} (aromatic skeletal bands) and the bands assigned to aliphatic and aromatic bonds.[113] Mistletoe MWL showed higher relative absorptivities than the host one at the band ratios 1665/1505, 1325/1505, 1230/1275, and 1130/1030 except the ratio 1735/1505 which shows the amount of unconjugated ester carbonyl groups.[114] The relative ratio (1600/1505) is known to be increased by the presence of syringyl component, *p*-hydroxyphenyl esters, carboxylate ions in carbohydrates and condensed tannin impurities.[113] However, the higher value at 1600/1505 in mistletoe MWL seems to be mainly ascribed to

the predominance of syringyl component considering the methoxyl contents and the ratios (1325/1505, 1230/1275, 1130/1030). Furthermore, the presence of conjugated ester groups, such as *p*-coumaryl esters observed in UV spectrum of bamboo lignin,[115] was negligible in the MWLs. The UV spectra in the MWLs of the mistletoe and the host showed maxima at 277.5 nm (absorptivity 15.3 l/g-cm) and 278.0 nm (absorptivity 14.4 l/g-cm), respectively. No absorption was observed in the region around 350 nm, the absorption at which is due to the presence of the conjugated ester groups, and the spectra in this region showed a general absorption curve. These observations thus indicated that both mistletoe and host lignins are typical angiosperm type ones, although some variation was observed in the intensities of IR region.

The MWLs were degraded by acidolysis, and then the monomeric products were analyzed to confirm the given information in the spectral analyses. The yields of acidolysis oils were 58 and 61% of the mistletoe and host MWLs, respectively. The ratios of syringyl acetone, (vanilloyl methyl ketone and syringoyl methyl ketone) to guaiacyl acetone in the acidolysis of mistletoe and host lignins were 0.92 (0.38 and 0.18) and (0.27 and 0.11), respectively. These values showed that both lignins are comparable to a typical angiosperm one: i.e. the corresponding values of beech wood were 0.71, 0.38 and 0.15. The following compounds were identified by GC-MASS analyses as TMS-derivatives of the acidolysis products of the MWLs and a beech wood (control); guaiacyl acetone, vanilloyl methyl ketone, syringyl acetone and syringoyl methyl ketone. A few other peaks which appeared faster than guaiacyl and syringyl derivatives were observed on gas chromatograms in all cases. These peaks were very small in their areas except rather large one, M^+ 220 which was identical with M^+ of trimethyl silylated *p*-coumaryl aldehyde but showed a little different fragment ions from those of the aldehyde. *p*-Hydroxyphenyl derivatives may not be ascribed to these peaks, because they were observed not only in the acidolysis products of beech wood, but also mass spectra of the *p*-hydroxyphenyl monomers,[116] such as *p*-hydroxybenzaldehyde, *p*-hydroxyphenylacetone, 1-hydroxy-1-(4-hydroxyphenyl)-2-propanone and *p*-hydroxybenzoyl methyl ketone, were different from those of these peaks. The analytical results indicated that both mistletoe and host lignins are typical angiosperm type, although the mistletoe lignin gave some degradation products which were in small amount and could not be identified.

Recently, FT-^{13}C-NMR was introduced into the structural studies of lignins and showed to give many useful informations.[111,117~121] Thus, the method was applied to the mistletoe lignin and the MWLs, which gave typical angiosperm-type spectra in aromatic and aliphatic carbon regions. Ester carbonyl carbon (172.4 ppm from TMS)[118] which was not observed in the mistletoe lignin, was observed in the host MWL in accordance with the absorption ratio (1735/1505) in IR region. The

absorption peaks of guaiacyl and syringyl ring carbons were observed in both mistletoe and host MWLs. The peaks at 154.3, 139.3, 135.9, 107.7 and 105.7 ppm from TMS internal standard observed in these MWLs may be assigned to the absorption of syringyl ring carbons on the position 3 and 5, 1 and 4, 4, 2 and 6, 2 and 6, respectively.[118,119] No distinct peaks appeared in the region of *p*-hydroxyphenyl ring carbons (160.9, 131.3 and 117.0 ppm from TMS, control: bamboo MWL) in these MWLs.

Thus, the mistletoe lignin as well as the host lignin were confirmed to be typical normal angiosperm type ones.

4.2 Mistletoe OMT in the biosynthesis of parasite lignin

O-Methyltransferases (OMTs) involving in the biosynthesis of angiosperm lignin catalyze the formation of ferulate (FA) and sinapate (SA) from caffeate and 5-hydroxyferulate, respectively,[76] while the gymnosperm ones hardly catalyze the formation of SA.[61] The ratio of SA- to FA-activities (SA/FA) is very useful as an indicator for the ability of syringyl lignin formation.[55] As it will be discussed in section 5, the phylogenic distribution of gymnosperm and angiosperm lignins is partially ascribed to the ratio SA/FA of these OMTs.

The SA/FA ratio of the mistletoe homogenate was quite different from these generalization and showed remarkably high value as a result of negligible FA-formation. On the basis of the biosynthesis of lignin monomers in mistletoe, no FA-formation contradicts the results which showed the mistletoe lignin as a typical angiosperm-type irrespective of the host species.[110,111] Hence, the cause of the no detection of FA-formation should be considered as follows; i) the presence of a particular OMT which is unable to catalyze the FA-formation. ii) normal angiosperm-type OMT is present but FA-activity is inhibited by some causes. Each case above mentioned may be understood in the following way. In the case of i), a. Mistletoe has no ability to synthesize lignin itself. b. Mistletoe is able to synthesize lignin monomers, but the biosynthetic pathway is different from normal angiosperm one. In the case of ii), a. Mistletoe has no ability to synthesize lignin itself because FA-activity is inhibited. b. Normal angiosperm-type pathway in lignin biosynthesis is present in mistletoe, but the FA-activity is inhibited by some inhibitors which may be liberated by the breakage of the compartmentation in the cell during the OMT extraction.

Thus, the mistletoe OMT was purified to elucidate the cause of the no FA-activity in relation to the ability of lignin biosynthesis. The SA-activity of the mistletoe OMT in crude homogenate showed optimal pH around 7.2, FA-activity at which was not detected. However, a weak FA-activity optimal pH of which is 5.5 was observed in the lower pH. The FA-activity was detected even at pH 7.5 after ammonium sulfate precipitation followed by dialysis of the crude extract. The

Table 14 Recovery of mistletoe FA-activity during purification

Purification procedure	Protein (%)	Recovery (%)		Fold		SA:FA
		FA	SA	FA	SA	Ratio
1. Crude juice	100.0	100	100.0	1.0	1.0	46.7
2. Centrifugation	71.8	86	92.0	1.2	1.3	50.0
3. Ion-exchange	10.8	1020	56.9	94.4	5.3	2.6
4. $(NH_4)_2SO_4$ ppt.	7.5	1060	51.2	141.0	6.9	2.3
5. Gel filtration	3.8	944	44.1	247.0	11.5	2.2

purification results of the mistletoe OMT are shown in Table 14. The FA-activity was remarkably activated by dialysis followed by the treatment of DEAE-cellulose, and the ratio SA/FA (2.6) became small as in common angiosperm one (the ratio of the host, 2.7).

Since the presence of specific substrate(s) inhibiting FA activity was suggested, the DEAE-cellulose after elution of the OMT was treated with 0.05 M K-phosphate buffer (pH 5.2) and then the eluate was adjusted to pH 7.0. This fraction inhibited not only the FA-activity of mistletoe enzyme but also that of gymnosperm (*Pinus thunbergii*) and angiosperm (*Pueraria thunbergiana*) OMTs, although SA-activities in these plants were gradually inhibited by aging of the fraction. The aged fraction was able to inhibit the both activities even by treatment of heating (100°C, 5 minute) or by pronase treatment. No inhibition was observed in the inhibitor fraction after dialysis against 0.025 M K-phosphate buffer (pH 7.4) for 8 hours. It is noteworthy that β-glucosidase[122] and glucan phosphorylase[123,114] activities were inhibited by the addition of crude mistletoe juice, and the latter inhibition was suggested to be caused by some phenols.[124] The inhibition of OMT may not be caused by an *ortho*-phenol which is a methyl acceptor of *S*-adenosyl-L-methionine and compete with caffeate or 5-hydroxyferualte, because no methylation was found in control assay (minus substrate) in these experiments. Although the characterization of the inhibitor(s) is incomplete, it seems that the mistletoe OMT is a normal angiosperm one and that the methylation of caffeate to ferulate is not blocked *in vivo*. Thus, the assumption i) a. and i) b. described above were denied.

4.3 Biosynthesis of mistletoe lignin

The ability of mistletoe to synthesize lignin was evaluated by the other enzyme reactions relating to lignin biosynthesis. Phenylalanine ammonia-lyase (PAL) was not detected in the intact mistletoe plant even in early July in which the high activity was expected. Figure 15 shows the development of enzyme activities during the incubation of the sliced mistletoe tissues under illumination. PAL activity, the

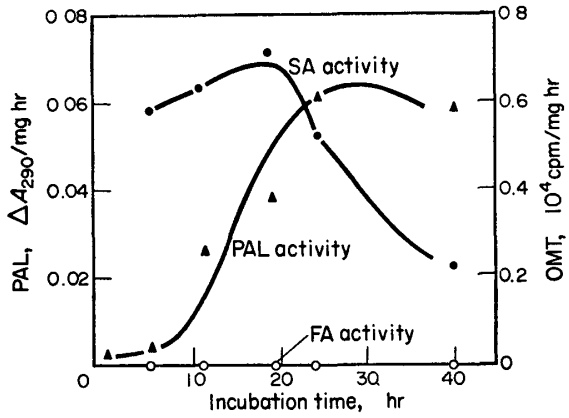


Figure 15 Development of enzyme activities during incubation of mistletoe tissues

product of which was identified to be *trans*-cinnamate by GC-MASS spectrometry, was gradually activated but the FA-activity of the OMT was not detected during the incubation, showing that the inhibitor(s) of OMT did not affect PAL activity. In addition, it was suggested that the OMT and PAL may be under different genetic control because of the different activation pattern similar to those of parsley cell suspension culture.[72]

Cinnamate-4-hydroxylase activity, which was very low in this plant, was detected and the product formed was also identified by mass spectrometry, and the *p*-coumarate was not detected in control assay (minus substrate).

Overall enzyme reactions in lignin biosynthesis were examined by tracer experiment using phenylalanine-U-^{14}C. The plant fed was allowed to metabolize for 24 hours and then the plant tissues were subjected to alkaline nitrobenzene oxidation after extraction with 80% hot ethanol and ethanol-benzene (1:2), successively. The results shown in Table 15 indicate that mistletoe is able to synthesize both guaiacyl and syringyl nuclei, and that the ratio of the oxidation products (syringaldehyde/vanillin) is in accord with that of the products of normal angiosperm lignin. The high yield of *p*-hydroxybenzaldehyde as an oxidation product was found in the mistletoe. However, it is suggested that the aldehyde may not be derived from the mistletoe

Table 15 Incorporation of L-phenylalanine-U-^{14}C into mistletoe lignin*

Nitrobenzene oxidation products†	Yield % of KL‡	Yield (ratio)	Specific activity cpm/μmol	Dilution value
p-Hydroxybenzaldehyde	3.0	(0.7)	570	1070
Vanillin	4.4	(1.0)	1580	386
Syringaldehyde	8.1	(1.7)	1090	560

* *Viscum album* 24.5 g (shoot 13.4 g): L-Phenylalanine 6.67 μCi/24.2 μmole
† *m*-Nitrobenzhydrazone derivatives. ‡ Klason lignin

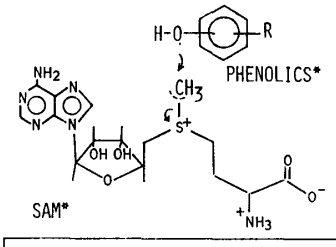
lignin, because of its low specific activity and of no evidence for the presence of *p*-hydroxyphenyl nuclei in the lignin. The origin of *p*-hydroxybenzaldehyde in the oxidation products of the mistletoe remains obscure at the present time.

All the results obtained concluded that the mistletoe has, at least, enzyme systems from phenylalanine to lignin and is able to synthesize the angiosperm-type lignin.

5. Comparative evaluation of plant *O*-methyltransferases in lignin biosynthesis

Mäule color reaction has been generally used as a tool to distinguish syringyl lignin from guaiacyl lignin in angiosperm and gymnosperm.[8~10] Many workers followed it up mainly by surveying lignin degradation products.[7,11,64,125~128] For example, alkaline nitrobenzene oxidation has been frequently applied and the aromatic aldehydes yielded were analyzed.[7,8,11] Gymnosperm lignins almost entirely yield vanillin, but angiosperm ones generally produce vanillin (V) and syringaldehyde (S) in the nitrobenzene oxidation. Grass lignins yield *p*-hydroxybenzaldehyde in addition to the both aldehydes. These aldehydes show the lignin structural units corresponding to guaiacyl, syringyl and *p*-hydroxyphenyl nuclei, and they are considered to be related to lignin evolution.[7,8,11,58,125~128]

A few *O*-methyltransferases (OMTs) have been characterized and purified,[39, 61,62,69,70,76,79,80,82,84,92] and it was found that the substrate specificity is closely related to the corresponding plant classes. Gymnosperm OMTs only catalyze



REACTION MIXTURE (TOTAL VOL. 1.3 ML)	
ENZYME SOLUTION	0.2 ML
PHENOLIC SUBSTRATE*	0.5 μMOLE
SAM* (0.05 μCi)	0.5 μMOLE
K-PHOSPHATE(PH 7.5)	20 μMOLE
NaN_3	10 μMOLE
$MgCl_2$	1 μMOLE
CYSTEIN	10 μMOLE
2-MERCAPTOETHANOL	10 μMOLE
ISOASCORBATE	10 μMOLE

Figure 16 Standard reaction mixture for the assay of *O*-mthyltransferases in plant kingdom. See also references No. 39, 61, 80, 92 for the assay of pine, aspen, bamboo and mistletoe OMTs, respectively.

ferulate- (FA) formation (section 1), while angiosperm ones catalyze both FA- and sinapate (SA)-formation[55] (section 2–4). Thus, the formation ratio of SA to FA might be a good indicator to evaluate lignin evolution. The SA/FA ratio more directly reflects genetic information comparing to the results obtained by chemical analyses of lignins, e.g. S/V ratio. Another advantage is that the ratio essentially keeps constant values during plant differentiation, which is ascribed to the fact that an OMT carries both FA- and SA-activities, the ratio of which shows a definite value.[76,80] This section will be discussed on plant OMTs whether the SA/FA ratios are generally available to investigate lignin evolution. Standard reaction mixture for the assay of various OMTs was shown in Figure 16.

General Remarks: The plants surveyed are listed in Table 16–18. OMTs were found to be distributed widely in the plants. The tables show SA/FA ratio, values of which were listed only when the enzyme activities were more than 10^3 cpm/hr. Enzyme activities in such levels were difficult to detect, especially in Pteridophyta and gymnosperm (Table 16). The difficulty might be ascribed to high terpene contents, and/or presence of some inhibitors in the tissues, although the young shoots were carefully selected and homogenized with additives to prevent such trouble. The low enzyme levels are also partially due to the less activity by routine procedure, condition of which is not always optimal for the enzyme extraction and assay.

Generally the SA/FA ratio in various plants showed a good contrast between gymnosperm and angiosperm. As postulated, gymnosperm OMTs catalyze almost only FA-formation (Table 16), while angiosperm ones do both FA- and SA-formation (Table 17 and 18). This well explains why gymnosperms almost entirely contain guaiacyl unit and angiosperms contain both guaiacyl and syringyl units in lignins.

Gymnosperm OMTs: Most of the gymnosperm OMTs, as represented by the Pinaceae enzymes, showed low SA/FA ratios with a few exceptions (Table 16). *Thuja, Podocarpus* and *Ephedra* OMTs showed rather high ratios in this experiment, although the latter two were not able to confirm because of the low enzyme activity. The ratio in *Thuja orientalis* seedlings was found to be 0.83, the value of which was discussed in relation to lignan biosynthesis in *Thuja*.[62] However, the ratio might be also evaluated from another point of view.

Most of the species in Cupressaceae contain only guaiacyl lignin except *Tetraclinis* which contains guaiacyl-syringyl lignin.[7,8,58] In addition to this fact, the finding of a high SA/FA ratio in *Thuja* indicates that the lignin biosynthetic pathway might be tinged with characteristics of angiosperm type in Cupressaceae. The ratio SA/FA more directly reflects genetic codes comparing with lignins as a phenotype, and the

Table 16 SA/FA ratio in pteridophyta and gymnosperm OMTs

Family	Scientific name	SA/FA	Mäule
Psilotaceae	*Psilotum nudum*	ND	−
Lycopodiaceae	*Lycopodium clavatum*	ND	−
Selaginellaceae	*Selaginella tamariscina*	ND	+
Equisetaceae	*Equisetum arvense*	ND	−
Marsileaceae	*Marsilea quadrifolia*	ND	−
Ginkgoaceae	*Ginkgo biloba*	0.1	−
Cephalotaxaceae	*Cephalotaxus drupacea var. koraiana*	ND	−
Taxaceae	*Taxus cuspidata*	0.1	−
Pinaceae	*Pinus densiflora* (seedlings)	0.1	−
	Pinus thunbergii (seedlings)	0.1[61]	−
	Pinus taeda (seedlings)	0.3	−
	Pinus strobus (seedlings)	0.4	−
Taxodiaceae	*Sciadopitys verticillata*	ND	−
	Cryptomeria japonica	0.1	−
	Sequoia sempervirens	ND	−
Cupressaceae	*Thuja orientalis* (seedlings)	0.83[62]	−
	Thuja standishii (seedlings)	0.03[62]	−
Araucariaceae	*Araucaria brasiliana*	ND	−
Podocarpaceae	*Podocarpus macrophylla*	ND	−
Ephedraceae	*Ephedra sinica*	ND	+

Young shoots were used in this experiment except the plants of which scientific names are followed by the explanatory notes. The scientific names are essentially based on reference 131. ND: OMT activity was too low to evaluate the ratio SA/FA (less than 500 cpm/hr in the both FA- and SA-activities). ( ): 500～1000 cpm/hr in the higher value of FA- and SA-activities. + or −: Positive or negative Mäule color reaction. Suffix numbers on the SA/FA ratio: reference numbers.

ratios seem to show a genetic variation in this family. Thus, the ratio might be a good tool in order to analyze evolution stage of such species in Cupressaceae. The situation is probably the same in Podocarpaceae and Cycadaceae, because some species in the families contain guaiacyl-syringyl lignin in spite of gymnosperm.[7,8] These groups are considered to be a transition type between gymnosperm and angiosperm in the point of lignin biosynthesis. On the other hand, the lignin biosynthetic pathway in Gnetales is considered to be completely transformed into angiosperm one, because they contain guaiacyl-syringyl lignin.[7,8]

Angiosperm OMTs: Polycarpiidae and Amentifloriidae gave normal ratio (2.0–3.5; Table 17) in this experiment. These groups are considered to be primitive angiosperms in the viewpoint of polyphyletic theories in angiosperm phylogeny.[16] Winteraceae, which could not be surveyed, belongs to vessel-less Polycarpiidae,

some of which give low S/V ratios.[7,8] *Trochodendron* OMT, however, showed rather a low SA/FA ratio, the plant of which lacks vessel elements but contains guaiacyl-syringyl lignin.[7,11,58] It is interesting to note that the primitive plant OMT gives the ratio inbetween gymno sperm and angiosperm.

Usual ratios were observed in a few plants. Crude mistletoe (*Viscum album*) OMT apparently showed a very high ratio, but the ratio by the purified enzyme was found to be normal (SA/FA=2.2).[92] The finding that FA-activity was almost completely inhibited in crude mistletoe preparation, strongly suggests the presence of OMT-inhibitor in this plant. Another interesting example is *Erythrina,* secondary xylem except fibers of which contains almost entirely guaiacyl lignin, although the

Table 17 SA/FA ratio in dicotyledon OMTs

Family	Scientific name	SA/FA	Mäule
Trochodendraceae*	*Trochodendron aralioides*	1.6	+
Magnoliaceae*	*Magnolia grandiflora*	3.0	+
	Liriodendron tulipifera	2.5	+
Cercidiphyllaceae*	*Cercidiphyllum japonicum*	3.2	+
Eupteleaceae*	*Euptelea polyandra*	(3.6)	+
Illiciaceae*	*Illicium religiosum*	ND	+
Nelumbonaceae*	*Nelumbo nucifera*	ND	
Nymphaeaceae*	*Nymphaea tetragona*	ND	
	Nuphar japonicum	(2.3)	
Ranunculaceae*	*Ranunculus acris*	3.6	+
Paeoniaceae	*Paeonia suffruticosa*	ND	+
Casuarinaceae*	*Casuarina* sp.	ND	+
Juglandaceae*	*Juglans mandshurica*	2.8	+
Salicaceae*	*Populus euramericana*	3.2[39]	+
Betulaceae*	*Betula nigra*	3.1	+
Fagaceae*	*Quercus myrsinaefolia*	2.5	+
Ulmaceae	*Ulmus americana*	3.2	+
Loranthaceae	*Viscum album*	2.2[92]	+
Leguminosae	*Robinia pseudo-acacia*	2.5	+
	Erythrina crista-galli	3.3[91]	−
	Glycine max (single cells)	1.9[69]	
Solanaceae	*Nicotiana tabacum* (single cells)	1.2[84]	
Tiliaceae	*Tilia japonica*	(2.3)	+
Scrophulariaceae	*Paulownia tomentosa*	2.9	+
Oleaceae	*Forsythia suspensa*	(6.4)	+
	Syringa reticulata	ND	+

* Polycarpiidae and Amentifloriidae, the plants of which are considered to be primitive groups in the viewpoint of polyphyletic theories in angiosperm phylogeny[16]. Also see Table 1.

Table 18 SA/FA ratio in monocotyledon OMTs

Family	Scientific name	SA/FA	Mäule
Alismataceae	*Alisma canaliculatum*	ND	
	Sagittaria trifolia	ND	
Liliaceae	*Aloë arborescens*	(1.1)	+
Juncaceae	*Juncus effusus*	ND	
Commelinaceae	*Tradescantia virginiana*	1.6	
Gramineae	*Oryza sativa*	0.9	+
	Triticum aestivum	1.0	+
	Zizania latifolia	0.97	+
	Phyllostachys pubescens	1.3[76,80]	+
Sparganiaceae	*Sparganium stoloniferum*	1.5	
Typhaceae	*Typha latifolia*	ND	+
Cyperaceae	*Eleocharis Kuroguwai*	ND	
	Scirpus triqueter	(2.2)	+

* See Table 1.

ratio SA/FA was normal angiosperm type.[91] Ferulate-5-hydroxylase was presumed to be absent in the plant, which may result in low syringyl units.[129]

Herbaceous angiosperm lignins contain rather small amounts of methoxyl group per C_9 unit, comparing with that of normal woody angiosperm lignins.[58] For example, swede (*Brassica napobrassica*) roots the lignin of which comprise almost entirely guaiacyl unit showed a low SA-activity.[79] The low SA-activity might be resulted from a regressive evolution of the OMT because OMTs are usually considered to give a constant ratio during differentiation. The low ratio might be also ascribed to the poor vascular bundle tissues in the swede roots, lignin contents of which are usually very low. In such tissue as described above, the OMT activity which is directly involved in lignin biosynthesis might be low comparing with other OMT levels, e.g. flavonoid specific one.[70] When such OMT coexists in the herbaceous tissues surveyed, the apparent SA/FA ratio will be affected. For instance, a flavonoid specific OMT in soybean (*Glycine max*) suspension cells showed ca. 1.4 of the ratio,[70] while a lignin specific OMT in the same cells showed ca. 1.9 of the ratio.[69] If the lignin specific OMT presents in low level, apparent ratio would be lower by the flavonoid specific OMT.

Gramineae OMTs showed a SA/FA ratio around 1.0 and other OMTs in monocotyledon showed rather a small ratio comparing with normal angiosperm OMTs (Table 18). The SA/FA ratio in crude bamboo (*Phyllostachys pubescens*) OMT was demonstrated to be the lignin specific one and no other OMTs were detected in the crude preparation.[76,95] In other words, crude bamboo OMT showed a net ratio, i.e. not apparent ratios discussed in herbaceous angiosperms. Grass lignins

Footnotes

(Table 16)	(Table 17)	(Table 18)
matsubaran	yamaguruma	hera-omodaka
hikagenokazura	taisanboku	kuwai
iwahiba	yurinoki	aroe
sugina	katsura	i (igusa)
denjisoh	husazakura	murasaki-tsuyukusa
	shikimi	ine
ichoh	hasu	komugi
chosen-maki	hitsujigusa	makomo
ichii	kohhone	mohso-chiku
akamatsu	umanoashigata	mikuri
kuromatsu	botan	gama
tehda-matsu	mokumaoh	kuro-guwai
sutorohbu-matsu	oni-gurumi	sankaku-i
kohyamaki	popura	
sugi	kaba-zoku, sp.	
sekoia	shira-kashi	
konotegashiwa	nire-zoku, sp.	
kurobe (nezuko)	yadorigi	
burajiru-matsu	nise-akashia	
inumaki	amerika-deigo	
maoh	daizu	
	tabako	
	shinanoki	
	kiri	
	rengyo	
	hashidoi	

differ from other gymnosperm and angiosperm lignins because they contain *p*-hydroxyphenyl units. Since Gramineae is thought to be more evolved comparing to gymnosperm and dicotyledon, it is recognized that the ratio does not simply increase with the phylogenical evolution.

Although some unusual ratios were found in this experiment, it is concluded that OMTs are roughly classified into three groups, i.e., gymnosperm-, angiosperm- and grass-type OMTs, which are related to the lignin evolution.

Conclusion

In section 1, characterization of a gymnosperm *O*-methyltransferase (OMT) was described. An OMT was extracted from Japanese black pine (*Pinus thunbergii*) seedlings with differentiated primary xylem which was undergoing lignification.

The enzyme was purified 90-fold by ammonium sulfate fractionation, and by chromatography on DEAE-cellulose and Sephadex G100. The purified enzyme catalyzed caffeate-methylation to give ferulate, but scarcely did 5-hydroxyferulate to sinapate. The latter methylation was completely inhibited by the presence of caffeate. The Vmax for caffeate was 25 times higher than that for 5-hydroxyferulate, and corresponding Km values were 5.11×10^{-5} and 2.77×10^{-4}M, respectively. These findings indicate that syringyl nuclei are not practically formed in this conifer. The provisional molecular weight of the enzyme was estimated to be 6.7×10^{4} by gel filtration chromatography. Magnesium ions were not absolute requirement but increased enzymic activity. Thus, the OMT well explains the preponderance of guaiacyl units in the conifer lignin.

In section 2, characterization of an angiosperm OMT was described. OMTs were extracted from the differentiating xylem of ten-year-old aspen (*Populus euramericana*) trunks. The enzymes were partially purified by ammonium sulfate precipitation, and column chromatography on DEAE-cellulose, Sephadex G200 and hydroxyapatite. The enzymes were resolved into two peaks by DEAE-cellulose chromatography, and the molecular weights of the respective enzymes were estimated to be 72000 and 75000 by gel filtration chromatography. The enzyme corresponding to the latter peak was unstable and the former peak enzyme was characterized. Magnesium ion showed no effect on the methylation rate. EDTA moderately stimulated the methylation rate, whereas heavy metals and SH group inhibitors strongly inhibited the enzyme activity. Km values for caffeate and 5-hydroxyferulate were estimated to be 3.8×10^{-4} and 3.1×10^{-4}M, respectively. Vmax/Km for 5-hydroxyferulate was 5.4 times greater than that for caffeate. FA- and SA-activities, which show the formation of ferulate from caffeate and the formation of sinapate from 5-hydroxyferulate respectively, were not separated during the purification and by the disc electrophoresis using polyacrylamide gel. Quercetin, cyanin and catechin were not methylated by this enzyme. The OMT in aspen trunks, where the phenolic metabolism is exclusively directed to lignin biosynthesis, catalyzes the methylation of both guaiacyl and syringyl lignin precursors, with preferential utilization of the latter substrate. These findings lead to the conclusion that the enzyme is a typical angiosperm-type OMT which participates in guaiacyl and syringyl lignin biosynthesis in aspen trunks. The polymorphism observed was discussed in relation to the ability of this enzyme.

In section 4, lignin biosynthesis and characterization of OMT in a parasite plant was described. The crude OMT in mistletoe (*Viscum album*) apparently catalyzed only 5-hydroxyferulate-methylation, but scarcely did caffeate-methylation. However, latter methylation was recovered by purification, and the occurrence of

specific inhibitor(s) was suggested in the homogenate. Thus, the OMT was concluded to be a normal angiosperm-type enzyme. The mistletoe lignin was found to be a typical angiosperm one based on the spectral (UV, IR ^{13}C-NMR) and functional group analyses, and on degradation products (nitrobenzene oxidation and acidolysis), the analytical results of which were compared with those of the host lignin. A tracer experiment showed that L-phenylalanine was efficiently incorporated into the mistletoe lignin. Phenylalanine ammonia-lyase and cinnamate 4-hydroxylase were detected in the mistletoe by incubation of the tissue slices under illumination. and the products formed in the enzyme reactions were found to be identical with authentic samples by mass spectrometry. These results indicated that the mistletoe lignin is synthesized independent of its host, in other words, the mistletoe itself has an ability of lignin biosynthesis.

In section 3, characterization of a grass OMT was described. An OMT was extracted from bamboo (*Phyllostachys pubescens*) young shoots. The enzyme was purified 97-fold by ammonium sulfate fractionation, and by chromatography on DEAE-cellulose, Sephadex G200 and G100. The purified enzyme was further analyzed by polyacrylamide gel electrophoresis and isoelectric focusing. Thus, two methylation in bamboo lignin biosynthesis, i.e., caffeate to ferulate and 5-hydroxyferulate to sinapate, was demonstrated to be catalyzed by the same enzyme. This is also supported by the ratio of sinapate-formation to ferulate-formation (SA/FA) which was kept constant during the purification, and by denaturation pattern on heat treatment. Methylation by bamboo OMT was found in caffeate, 5-hydroxyferulate, 3,4,5-trihydroxycinnamate, 5-hydroxyvanillin, protocatechuate, but no or less methylation in chlorogenate, *iso*-ferulate, *m*-, *p*-coumarate, 3,4-dihydroxyphenylacetate, 3,4-dihydroxyphenylmanderate, gallate, pyrocatechol, pyrocatecholphthalein and *d*-catechin. Km values for caffeate and 5-hydroxyferulate were 5×10^{-5} and 10^{-5}M, respectively. The former methylation, i.e., guaiacyl unit formation, was competitively inhibited by the latter phenolic substrate. The enzyme was an acidic protein with pI 4.61 at 4°C, showing optimal pH 8.0 with half maximal activities at pH 8.6 ± 0.2 and 6.4 ± 0.2. Possible fine control mechanism in the formation of guaiacyl and syringyl lignins, by means of feedback inhibition and OMT-conformational change, was also discussed.

In section 5, the ratio of sinapate (SA)- to ferulate (FA)-formation (SA/FA ratio) were examined over fifty plant species of 43 families. Generally, the OMTs were found to be roughly classified into three groups by the SA/FA ratio, i.e., gymnosperm-, angiosperm- and grass-types. OMTs in gymnosperm only catalyze FA-formation while angiosperm ones catalyze both FA- and SA-formation. Monocotyledons and herbaceous plants apparently showed the ratios inbetween typical

gymnosperm and angiosperm ones. These substrate specificities well explain why gymnosperm lignins contain almost entirely guaiacyl lignin while angiosperm ones contain both guaiacyl and syringyl lignins. A few exceptional ratios were found in Cupressaceae, *Trochodendron,* and grass OMTs, and discussed in relation to lignin biosynthesis. The significance of the ratio as a tool for analyzing lignin evolution was also discussed.

Roles of OMT in lignin biosynthesis are summarized as follows. In phylogenic point of view, OMT is an key enzyme responsible for the guaiacyl unit formation in gymnosperm lignins (section 1) and for both guaiacyl and syringyl unit formations in angiosperm lignins (section 2–4) as illustrated in Figure 17. Some of the exceptional cases were also discussed in section 5. In ontogenic point of view, the role of angiosperm OMTs seems to regulate syringyl lignin formation by means of fine adjustment, i.e., feedback inhibition (Figure 17) and the conformational change of the enzyme (section 3.1 & 3.2). However, rate limiting step, i.e. coarse adjustment, for the syringyl lignin formation is probably controlled by the reduction and hydroxylation in the biosynthesis of syringyl lignin. Figure 18 shows possible biosynthetic pathway of the syringyl lignin. As discussed in section 2.3, ubiquitous intermediate of the syringyl lignin is not established yet, although the formation of syringyl lignins usually explained by the following sequence: ferulate→5-hydroxyferulate→sinapate→ → → sinapyl alcohol→syringyl lignin.

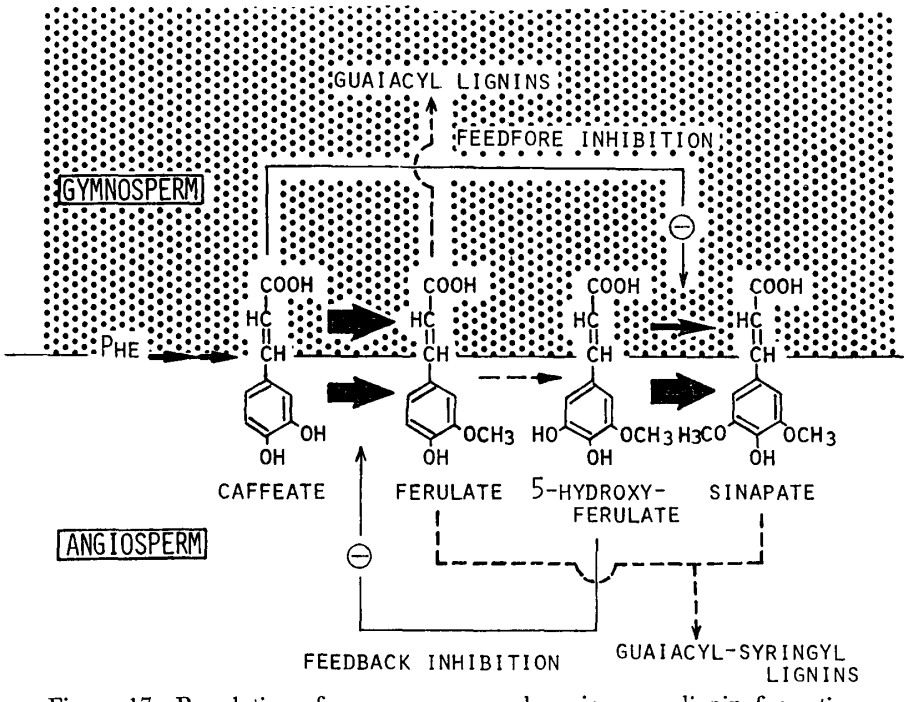


Figure 17 Regulation of gymnosperm and angiosperm lignin formations by the respective *O*-methyltransferases

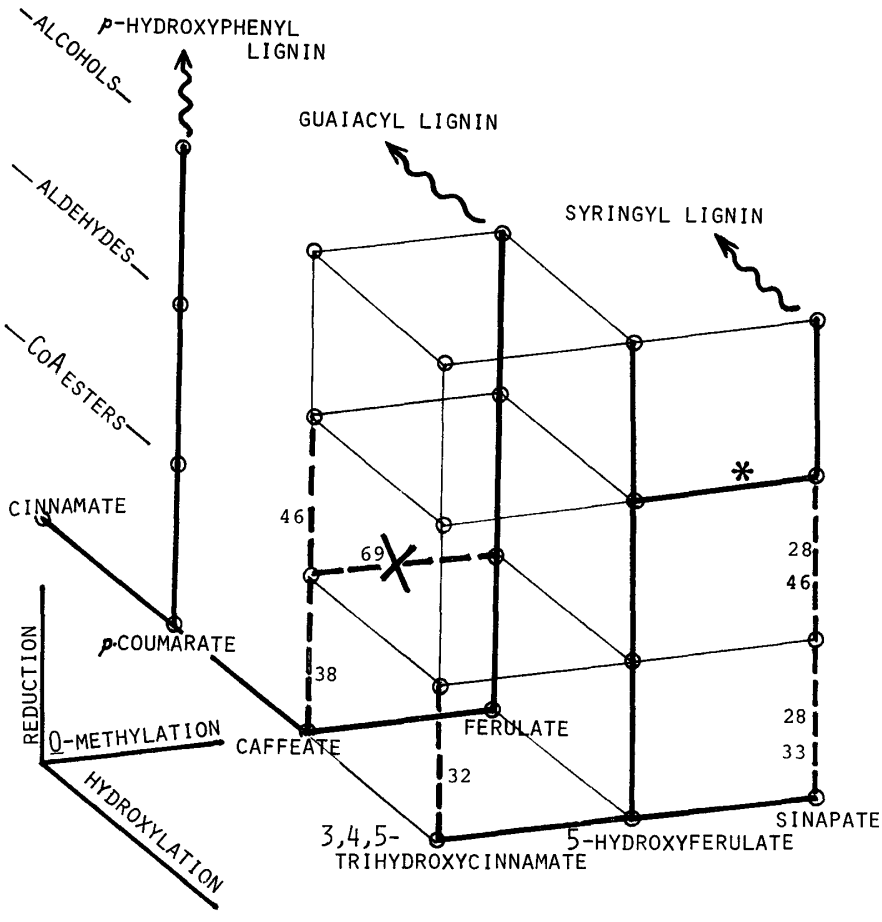


Figure 18 Possible biosynthetic pathways in the formation of syringyl lignin. ○——○ convertible reactions *in vitro*, ○- - -○ low reaction rate or no reaction *in vitro*, ○——○ possible pathway for syrinhyl lignin biosynthesis. Numbers in this figure indicate reference numbers. ※ Kutsuki *et al.* unpublished result.

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