Original

O-Methyltransferase as a Tool to Evaluate the Lignin Evolution

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Abstract——The ratios of sinapate (SA)- to ferulate (FA)- formation (SA/FA ratio) by *O*-methyltransferases (OMTs) were surveyed over fifty plant species of 43 families. The OMTs are roughly classified into three groups by using the SA/FA ratio, *i.e.* gymnosperm-, angiosperm- and grass-types. OMTs in gymnosperm only catalyze FA-formation whereas angiosperm ones catalyze both FA- and SA-formation. Monocotyledons and herbaceous plants showed the apparent ratios which lay between typical gymnosperm- and angiosperm-ones. These substrate specificities well explain why gymnosperm lignin contains almost entirely guaiacyl lignin whereas angiosperm one contains both guaiacyl and syringyl lignins. A few exceptional cases were found in Cupressaceae-, *Trochodendron*-, and grass-OMTs, which were discussed in relation to lignin biosynthesis. The ratios of the OMT activities are discussed with respect to the usefulness as an evolutional marker for analyzing lignin evolution.

1. Introduction

Mäule color reaction has generally been used as a tool to distinguish syringyl lignin from guaiacyl one in angiosperms and gymnosperms[1~3]. Many workers followed it up mainly by surveying lignin degradation products[4~10]. For example, alkaline nitrobenzene oxidation has been frequently applied and aromatic aldehydes yielded were analyzed[1,4,5]. In general, gymnosperms containing mostly guaiacyl lignin yield vanillin, but angiosperms containing guaiacyl and syringyl lignins, produce vanillin (V) and syringaldehyde (S) on nitrobenzene oxidation. Grass lignin yields *p*-hydroxybenzaldehyde in addition to the both aldehydes. These aldehydes are derived from the lignin structural units corresponding to guaiacyl-, syringyl- and *p*-hydroxyphenyl-nuclei, and they are considered to be related to lignin evolution[1,4~10].

A few *O*-methyltransferases (OMTs) have been characterized and purified[11~19,23], and it was found that the substrate specificity is closely related to the corresponding plant classes. Gymnosperm OMTs only catalyze ferulate-formation whereas angiosperm ones catalyze both ferulate (FA)- and sinapate (SA)-formation[20]. Thus, the formation ratio of SA to FA might be a good indicator to evaluate lignin evolution.

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The SA/FA ratios of the two different enzyme activities are more directly involved in genetic expression of lignin biosynthesis than the S/V ratio convensionally used for classification of lignin containing plants. Another advantage is that the ratio is essentially constant during plant differentiation, which means that respective plant species contain some amounts of OMT having both FA- and SA-activities in the ratio of a definite value[11,23]. This paper reports the SA/FA ratio of OMT activities from various plants in relation to lignin classification.

2. Experimental

2.1. Plant Materials

For assay of the SA/FA ratios, young shoots of various plants were used and in case of *Pinus, Oriza* and *Triticum,* seedlings were used. Many plants used for this experiment were kindly provided by the Botanical Garden of Osaka City University at Kisaichi, Osaka. Most plant materials were harvested in May. When the OMT activities were too small to detect in this time, the plant shoots were harvested in May or June, again. The experiments were done in 1978, 1979 and 1980.

2.2. Extraction of OMTs

All procedures were performed at 4°C. Plant materials (1 g fresh weight) were homogenized with Potassium (K)-phosphate buffer (1.5 ml of; 0.25 M, pH 7.5) containing 0.1 M each cysteine and NaN_3 in the presence of polyclar AT (0.1 g) with bovine serum albumin, using an ice-cooled mortar. Then the homogenates were squeezed with four-layered cheese-cloth and the juice obtained was centrifuged 17000 g for 30 minutes. The supernatant obtained was used for the assay.

2.3. Enzyme Assay

The assay is based on the transfer of $^{14}CH_3$ groups from *S*-adenosyl-L-methionine to caffeate or 5-hydroxyferulate forming FA- or SA-O$^{14}CH_3$, respectively. The products formed were extracted with ether (twice, 10 ml and 5 ml). The reaction mixture contained the following components; the enzyme solution (0.2 ml), the phenolic substrate (0.5 μmole), *S*-adenosyl-L-methionine-O$^{14}CH_3$ (0.05 μCi/0.5 μmole) K-phosphate buffer (pH 7.5, 20 μmole), NaN_3 (10 μmole), $MgCl_2$ (1 μmole), cysteine (10 μmole), 2-mercaptoethanol (10 μmole) and isoascorbate (10 μmole): total volume 1.3 ml. After 30, 60, 90 minutes incubation at 30°C, the enzyme reaction was terminated by the addition of 5% HCl (0.5 ml). The methylated product was extracted with ether, and the ether was evaporated under reduced pressure. The residue dissolved in 0.5 ml of dioxane was transfered in a vial with 5 ml of scintillator which contained 2,5-diphenyloxazole (4 g) and 1,4-Bis[2-(4-methyl-5-phenyloxazole-2-yl] benzene (0.1 g) in toluene (1.0 *l*). Then, the amounts of the reaction products were

calculated from the radioactivities determined with a Beckmann LS100 scintillation counter.

2.4. Mäule Color Reaction

The color test was carried out by the method of Browning[25].

3. Results and Discussion

3.1. General Remarks

The plants surveyed are listed in Table 1～3. 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 lower levels were difficult to evaluate especially in Pteridophyta and gymnosperm (Table 1). The difficulty might be ascribed to high terpene contents, and/or presence

Table 1. 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)[12]	−
	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)[17]	−
	Thuja standishii (seedlings)	(0.03)[17]	−
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 24. 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.

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 routine procedure, the condition of which may not always be optimal for the enzyme extraction and assay.

Table 2. 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[14]	+
Betulaceae*	*Betula nigra*	3.1	+
Fagaceae*	*Quercus myrsinaefolia*	2.5	+
Ulmaceae	*Ulmus americana*	3.2	+
Loranthaceae	*Viscum album*	2.2[13]	+
Leguminosae	*Robinia pseudo-acacia*	2.5	+
	Erythrina crista-galli	3.3[21]	−
	Glycine max (single cells)	1.9[18]	
Solanaceae	*Nicotiana tabacum* (single cells)	1.2[16]	
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[26]. Also see Table 1.

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 1), while angiosperm ones do both FA- and SA-formation (Table 2 and 3). This well explains why gymnosperms almost entirely contain guaiacyl unit and angiosperms contain both guaiacyl and syringyl units in lignin.

Table 3. 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[11,23]	+
Sparganiaceae	*Sparganium stoloniferum*	1.5	
Typhaceae	*Typha latifolia*	ND	+
Cyperaceae	*Eleocharis Kuroguwai*	ND	
	Scirpus triqueter	(2.2)	+

* See Table 1.

3.2. Gymnosperm OMTs

Most of the gymnosperm OMTs, as represented by Pinaceae enzymes, showed low SA/FA ratios with a few exceptions (Table 1). *Thuja, Podocarpus* and *Ephedra* OMTs showed rather high ratios in this experiment, although the latter two were not able to be confirmed 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*[17]. However, the ratio might also be evaluated from another point of view: Most of the species in Cupressaceae contain only guaiacyl lignin except *Tetraclinis* which contains guaiacyl-syringyl lignin[1,5,6]. 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 ratios seem to show a genetic variation in this family. Thus, the ratio might be a good tool to analyze evolution stage such species in Cupressaceae. The situation is probably the same in Podocarpaceae and Cycadaceae, because some species in the families contain both guaiacyl-syringyl lignin in spite of gymnosperm[1,5]. These groups are considered to be transition type between gymnosperm and angiosperm with respect to 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[1,5].

3.3. Angiosperm OMTs

Polycarpiidae and Amentifloriidae gave normal ratio (2～3.5; Table 2) in this experiment. These groups are considered to be primitive angiosperms from poly-

phyletic viewpoints in angiosperm phylogeny[26]. Winteraceae, which could not be surveyed, belongs to vessel-less Polycarpiidae, some of which give low S/V ratios[1,5]. *Trochodendron* OMT, however, showed rather a low SA/FA ratio, the plant of which lacks vessel elements but contains guaiacyl-syringyl lignin[4~6]. It is interesting to note that the primitive plant OMT gives the ratio between gymnosperm- and angiosperm-ones.

Unusual 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)[13]. The finding that FA-activity was almost completely inhibited in crude mistletoe preparation, strongly suggests the presence of an OMT-inhibitor in this plant. Another interesting example is *Erythrina*, secondary xylem except fibers of which contains almost entirely guaiacyl lignin[22], although the ratio SA/FA is normal angiosperm type[21]. Ferulate-5-hydroxylase was presumed to be absent in the plant, which may result low syringyl units[22].

Herbaceous angiosperm lignins contain rather small amounts of methoxyl group per C_9 unit, comparing with that of normal woody angiosperm lignins[6]. For example, swede *(Brassica napo-brassica)* roots the lignin of which comprises almost entirely guaiacyl unit showed a low SA-activity[15]. The low SA-activity might be resulted from 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 in the swede roots, lignin contents of which are usually very low. In such tissue as described above, the OMT activity which directly involved in lignin biosynthesis might be low comparing with other OMT levels, e.g. flavonoid specific one[19]. 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[19], while a lignin specific OMT in the same cells showed *ca.* 1.9 of the ratio[18]. If the lignin specific OMT presents in low level, apparent ratio would be affected by the presence of 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 3). 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[11,23]. In other words, crude bamboo OMT shows a net ratio, i.e. not such apparent ratios as discussed in herbaceous angiosperms. Grass lignins differ from other gymnosperm and angiosperm lignins because they carry *p*-hydroxyphenyl units. The SA/FA ratio in Gramineae OMTs showed to be around one. 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.

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