

***O*-methyltransferase (OMT) cDNA Clones from *Pinus densiflora* Seedlings*¹**

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Introduction

S-adenosyl-L-methionine (SAM) dependent *O*-methyltransferase (SAM-OMTs: EC 2.1.1.6x) is ubiquitous, and distributed in bacteria, fungi, plants and animals. Because the methylation is a critical step in secondary metabolism of plant kingdom, the numbers of the registered plant (PI-OMT) genes are rapidly growing. PI-OMTs consist of PI-OMT I and PI-OMT II. The former catalyzes only caffeoyl-CoA as a substrate with divalent cation, i.e. Mg²⁺, while the latter catalyzes various substrates without divalent cation.

Pinus taeda OMTs I and II have been discussed in relation to lignin biosynthesis^{1,2)}. However, there are still ambiguous aspects in the conifer OMTs, especially in the latter OMT type. The specific activity for caffeate in the OMT II was remarkably smaller than the case of angiosperm³⁾ although they did not show the kinetic constants. They had used a low concentration of the substrates for the assay that may not saturate the OMT II¹⁾. Using the assay system, they had reported that the formation ratio of syringyl to guaiacyl nuclei was ca. one in both crude xylem and recombinant extracts. They designated the OMT as AEOMT because it catalyzed methylation of both corresponding acids and CoA esters.

Polymorphism is one of the aspects to be solved before understanding their respective function. Distinct OMT II clones from AEOMT have also been suggested in the conifer as following reasons. First, the xylem extract remained OMT activity when an antibody for AEOMT had added¹⁾. This is quite reasonable because the conifer carries high gene duplication. Secondary, crude pine extract had resolved into two OMT fractions after electrophoresis⁴⁾. Third, another formation ratio of syringyl to guaiacyl nuclei by the enzyme had been reported as ca. 0.1 in Japanese black pine seedlings⁴⁾. Finally, pinosylvin methylating OMT with broad substrate specificity has also reported from *Pinus sylvestris*^{5,6)}. Here, we confirmed pine OMT polymorphism by isolating five OMT clones with full respective coding sequences from Japanese red pine (*Pinus densiflora*) elicited with sodium salicylate.

Experimental

Total RNA from the roots of Japanese red pine seedlings, cut off at 16 hours after elicitation with 10 mM of sodium salicylate, was extracted by using modified guanidium extraction method. Then cDNA was synthesized by using SuperScriptTM Plasmid System (Life Technologies). The cDNA was fractionated on 1% agarose gel to enrich the portion larger than 1.0 Kb. They were inserted into plasmid vector pSPORT1 (Life Technologies). The recombinant plasmid DNA was transformed into *Escherichia coli* DH10B Cells (Life Technologies) by using Cell-PoraterTM Electroporation System (Life Technologies). The library contains the cDNA larger than 1.0 Kb was used for isolation of full-length OMT cDNA (ca. 1.2 Kb) clone.

The 629 bp probe for OMT cDNA was prepared from a cDNA library of *P. densiflora* by polymerase chain reaction (PCR). The 21 mer primer pairs were designed from the corresponding nucleotides sequence of *Pinus radiata* (Accession No. U70873 in GenBank). This probe contains a conserved SAM binding domain in plant OMT. The probe was fractionated on 1% agarose gel electrophoresis. After purification, the probe was labeled by ECLTM direct nucleic acid labeling and detection systems (Amersham Pharmacia). Out of the cDNA library (5×10⁵ clones), 2×10⁴ clones were screened by using the probe describes above. The nucleotides of the OMT clones were sequenced for both strands by the dideoxy chain termination method, by using Big DyeTM terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems), Big DyeTM terminator v3.0 Ready Reaction Cycle Sequencing Kit (PE Applied Biosystems), and an ABI PRISMTM 377 DNA sequencer (PE Applied Biosystems).

For recombinant OMT expression, the wheat germ cell-free protein synthesis core kit Proteios (Toyobo) was used after mRNA was synthesized *in vitro* by Thermo T7 RNA polymerase (Toyobo). After the translated reaction mixture was applied to a spin column, OMT activities were monitored with ¹⁴C-SAM and phenolic substrates (caffeic acid, 5-hydroxy ferulic acid, pinosylvin, pinocembrin) as described before with slight modified reaction mixture⁴⁾.

Results and Discussion

Eleven *O*-methyltransferase cDNA clones (PDOMT) with full-coding lengths were fully sequenced in both

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directions, which had been cloned from an elicited *P. densiflora* seedlings root cDNA library. All of the PDOMT belonged to OMT II and, had conserved sequence motifs that could possibly act as SAM-binding domains. Five distinct PDOMTs were recognized by the sequence analyses. Seven PDOMT cDNA clones showed identical sequences while the other 4 clones are distinct ones. Phylogenetic analyses showed that the 11 clones fall into two distinct groups. They are distinct group from *P. taeda* AEOMT, which was fallen into the same group of *P. sylvestris* PSOMT by the phylogenetic classification. Here, we concluded that pine OMT shows polymorphism.

The characterization of each PDOMT enzyme was now going on. Preliminary, PDOMT cDNAs were transcribed *in vitro* and corresponding PDOMT were expressed in the wheat germ cell-free protein synthesis system. The translation products showed methylation activities for caffeic acids. Because of those isolated OMT cDNA clones and the successful OMT assay system in hand, further research will gain insight into the OMT II function.

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