

Cloning and functional analysis of caffeic acid 3-*O*-methyltransferase from rice (*Oryza sativa*)

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A putative *O*-methyltransferase cDNA was cloned from UV-irradiated rice leaves based on an amino acid sequence reported as that of naringenin 7-*O*-methyltransferase, which is involved in the biosynthesis of a rice phytoalexin, sakuranetin. However, the recombinant protein (approximately 41 kDa) expressed in *Escherichia coli* showed not naringenin 7-*O*-methyltransferase activity but caffeic acid 3-*O*-methyltransferase activity. Semi-quantitative RT-PCR revealed that the mRNA was expressed in all tissues tested and was not affected by CuCl₂, jasmonic acid or UV treatment. The enzyme also methylated flavonoids which have two adjacent hydroxyl groups in the B ring. © Pesticide Science Society of Japan

Keywords: methyltransferase, rice, lignin, phytoalexin, caffeic acid.

Introduction

Rice plants produce diterpene and flavanone phytoalexins which are involved in the defense of the plant.^{1–4)} Rakwal *et al.* reported the purification and partial amino acid sequence of naringenin 7-*O*-methyltransferase (NOMT) which is involved in the biosynthesis of a flavanone phytoalexin, sakuranetin.⁵⁾ We have attempted to clone and characterize an NOMT gene from rice. However, we found that the NOMT protein reported by Rakwal *et al.* has not NOMT activity but caffeic acid 3-*O*-methyltransferase (COMT) activity.

COMT is widespread throughout the plant kingdom and is found in all lignin-producing plants.⁶⁾ COMT cDNAs or genes have been isolated from a number of species including aspen,⁷⁾ common zinnia,⁸⁾ wheat,⁹⁾ maize,¹⁰⁾ *etc.* Here, we report the cloning of a gene encoding COMT (*OsCOMT1*) from rice and the characterization of the recombinant protein, though our objective was the cloning of an NOMT gene. To our knowledge, this is the first report on the cloning and characterization of a rice COMT cDNA.

Materials and Methods

1. Plant materials

Rice plants (*Oryza sativa* L. cv. Nipponbare) were cultivated in a greenhouse.

2. Chemicals

Eriodictyol, homoeriodictyol, hesperetin, kaempferol, myricetin, isorhametin, luteolin and chrysoeriol were purchased from Extrasynthese. *p*-Coumaric acid, quercetin and apigenin were obtained from Sigma Chemical. Caffeic acid was from Katayama Chemical. (±)-Naringenin was from Aldrich Chemical. The configurations of eriodictyol, homoeriodictyol and hesperetin are not determined.

3. UV irradiation, CuCl₂ and jasmonic acid (JA) treatment

The second leaves of 5-week-old plants were used for all treatments. UV irradiation was applied as described previously.²⁾ The rice leaves on which brown spots appeared were harvested and stored at –80°C prior to use. The treatments with CuCl₂, jasmonic acid (JA) and H₂O (control) were carried out as follows. The rice leaves (0.5 g) were cut into 1–2 cm long sections. The sections were then floated in 100 ml of

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a 0.5 mM CuCl₂ or JA solution in plastic boxes. Milli Q water was used as a control. After incubation for 48 hr at 27°C under light, the leaves were harvested and stored at -80°C prior to use.

4. RNA extraction and cDNA preparation

RNA was isolated using TRIZOL Reagent (Invitrogen) from 0.5 g of UV-irradiated leaves following the manufacturer's instructions. First-strand cDNA was prepared using Superscript II (Invitrogen) according to the protocol recommended by the manufacturer. The cDNA was used as a template in the PCR.

5. Cloning of *OsCOMT1* cDNA

A tblastn search of the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) with the 40-amino acid sequence (KNHSVITKLLLDLYTGFDAASTVVDVGGG-VGATVAAVVS) of the NOMT given by Rakwal *et al.*⁵ was performed. The resulting EST sequence with accession No. XM_480185 was used to design the PCR primers for PCR.

A full-length sequence containing the open reading frame of *OsCOMT1* was obtained by PCR using a sense primer (5'-AACGGATCCATGGGTTCTACAGCCGCC-3'; the *Bam*HI site is indicated in italics) and an antisense primer (5'-CC-CAAGCTTCTACTTGGTGAAGCTCGATG-3'; the *Hind*III site is indicated in italics). The cDNA mixture was used as a template, and the PCRs were performed with GoTaq DNA polymerase (Promega) under standard conditions (1 cycle of 95°C for 2 min; 40 cycles of 95°C denaturation for 30 s, 55°C annealing for 30 s and 72°C extension for 1.1 min; and 1 cycle of 72°C for 8 min). Amplified DNA was digested with *Bam*HI and *Hind*III (Takara) and cloned into a pET-15b vector. The resulting plasmid was named pET-COMT. The inserted sequence was confirmed in fact by the dideoxy-chain termination method with a BigDye Terminator v3.1 cycle sequencing Kit (Applied Biosystems) using a PRISM 3100 DNA sequencer (Applied Biosystems).

6. Expression of *OsCOMT1* in *E. coli* and purification of the recombinant protein

E. coli BL21(DE3)pLysS cells harboring the pET-COMT plasmid were cultured until the OD₆₀₀ became 0.5 in LB medium (10 g of peptone, 5 g of yeast extract and 10 g of NaCl per liter of water, pH7.0-7.5) containing 50 µg/ml of ampicillin.

Isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) was then added to induce protein expression. After being cultured for 3 hr at 37°C, the cells were harvested by centrifugation and resuspended in binding buffer (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, and 5 mM imidazole). The cells were disrupted with an ultrasonic disruptor (Branson Sonifier 250D). The resulting extract was centrifuged at 17,000×g for 20 min, and the supernatant was purified by His-Bind column chromatography (His-Bind Kits, Novagen). The eluted protein was dia-

lyzed in 20 mM Tris-HCl buffer containing 10% glycerol and 14 mM 2-mercaptoethanol, pH 8.0, at 4°C for 1 hr and stored at -80°C. The molecular mass was estimated by SDS-PAGE using 10% gels and staining with Coomassie Brilliant Blue.¹¹ The protein concentration was determined by the Bradford method using bovine serum albumin as the standard.¹²

7. Enzyme assay for methyltransferase

The standard reaction mixture for the methyltransferase assay consisted of 5 ng/µl of purified enzyme, 0.3 mM substrate, 0.3 mM *S*-adenosyl-L-methionine (SAM), and 0.1 M Tris-HCl (pH 7.5) in a final volume of 50 µl. After a 20-min incubation at 30°C, the reaction was terminated by adding 50 µl of MeOH. The mixture was centrifuged at 17,000×g for 10 min. Five microliters of the supernatant was analyzed by LC-MS/MS.

To determine the NOMT activity, sakuranetin was detected by LC-MS/MS under conditions described previously.¹³ To determine the COMT activity, ferulic acid was detected by LC-MS/MS as follows. The analytes were injected into an 1100 HPLC instrument (Agilent Technologies) equipped with a Sciex API300 LC-MS/MS (Applied Biosystems) and were separated on an Inertsil ODS-2 column (2.1 mm, i.d.×150 mm, GL Science) by isocratic elution with acetonitrile/water (35/65, v/v) containing 0.1% (v/v) formic acid at a flow rate of 0.2 ml/min. The MS/MS analysis was carried out in the negative-ion mode with a turboionspray inlet system. Ferulic acid was detected in combination at *m/z* 193/134 in the multiple reaction monitoring mode.

To estimate the optimal pH for COMT activity, the enzyme activity was measured in a buffer at each pH (0.1 M MES-NaOH, pH 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5; 0.1 M Tris-HCl, pH 7.0, 7.5, 8.0, 8.5 and 9.0).

The effects of divalent cations, EDTA and dithiothreitol (DTT) on the COMT activity were examined by adding BaCl₂, MgCl₂, CaCl₂, MnCl₂, ZnCl₂, CoCl₂, HgCl₂, CuCl₂, Na₂EDTA or DTT to the standard reaction mixture at a final concentration of 5 mM.

8. Determination of K_m and V_{max} values

Steady-state kinetic data were determined under almost the same reaction conditions as the standard, except for the substrate (caffeic acid and SAM) concentrations. To determine the K_m for the substrate caffeic acid (or SAM), the final concentration of caffeic acid (or SAM) was varied between 15 µM and 300 µM, while SAM (or caffeic acid) was kept at a saturated level of 1 mM. All data shown are from two independent experiments. Apparent K_m and V_{max} values were calculated from Lineweaver-Burk plots.

9. Substrate specificity

Substrate specificity for various flavonoids was determined using [*methyl*-¹⁴C]SAM (1.8 GBq/mmol, Moravek Biochemicals) instead of an unlabelled SAM. After an incubation at

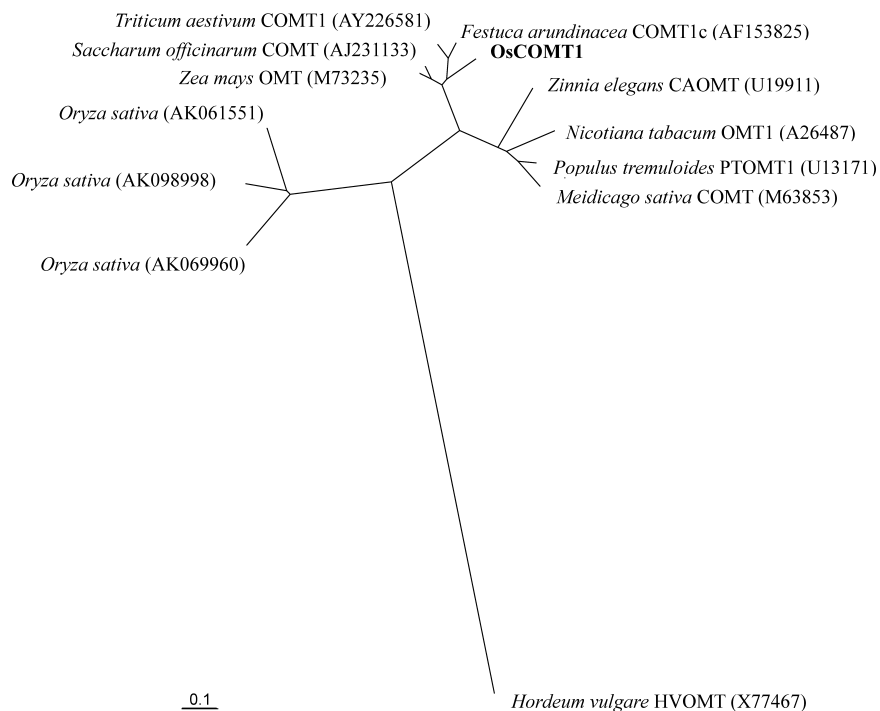


Fig. 1. A phylogenetic tree of OsCOMT1 and related OMTs based on amino acid sequences (<http://www.ddbj.nig.ac.jp/search/clustalw-j.html>). Related OMT data were obtained from GenBank with accession numbers given in brackets after each name.

30°C for 20 min, the reaction was terminated and the radioactivity of the nonpolar phase was measured as previously described.¹⁴⁾

10. Semi-quantitative analysis of mRNA transcripts

Total RNA was prepared from the following materials: the aerial parts and roots of 1-week-old to 5-week-old plants; the first (youngest) to the fourth (oldest) leaf of a 5-week-old plant; and UV-irradiated, CuCl₂-treated, JA-treated and H₂O (control)-treated rice leaves. The total RNA from each leaf sample was reverse transcribed to cDNA with Superscript II reverse transcriptase (Invitrogen). A gene-specific primer pair consisting of a sense primer (5'-GGGATGACGGCGTTC-GAG-3') and an antisense primer (5'-CACCTCCCGT-GCTCCGGCAG-3') was used to amplify parts of the *OsCOMT1* cDNA. PCR was carried out for 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 45 s. To ensure that the PCR products were undergoing logarithmic amplification related to the number of cycles, the amount of cDNA used as the template was first diluted to an appropriate concentration.

Actin was used as the control gene to adjust the cDNA concentration. The *syn-pimara-7,15-diene* synthase gene (*OsDTS2*)¹⁵⁾ was used as a positive control, which can be induced to express by UV-irradiation. PCR products were separated on a 1% agarose gel, which was then stained with ethidium bromide.

11. Preparation of crude enzyme from rice plants

Proteins were extracted from the aerial parts and roots of 1-week-old to 5-week-old plants; the first leaf to the fourth leaf of 5-week-old plants; and UV-irradiated, CuCl₂-treated, JA-treated and water (control)-treated leaves. The extraction was carried out at 4°C as described previously.¹⁴⁾

Results and Discussion

1. Cloning of *OsCOMT1* cDNA

A tblastn search of the GenBank database was performed, and 4 EST sequences with accession No. AK064768, AK061859, AB122056 and XM_480185 were obtained as perfect matches in the subject region. These 4 ESTs were identical except for their length and were considered to be a putative COMT. The full-length cDNA of XM_480185 was designated *OsCOMT1* and was obtained by PCR using a cDNA mixture prepared from UV-irradiated rice leaves as a template. It is 1425 bp in size and encodes an open reading frame with 368 amino acids and a calculated molecular mass of 42.3 KDa.

The amino acid sequences of 8 related COMTs, a barley OMT¹⁶⁾ and 3 unidentified rice genes (available in the GenBank database) were used to construct a phylogenetic tree together with *OsCOMT1* (Fig. 1). *OsCOMT1* shared 81.4%, 80.6% and 80.2% identity with *Triticum aestivum* (wheat) COMT, *Festuca arundinacea* (tall fescue) COMT1c, and *Zea mays* (maize) COMT, respectively. This result indicated the close relationship between *OsCOMT1* and the other 3 COMTs. A flavonoid, 7-*O*-methyltransferase from *Hordeum vulgare* (HVOMT),¹⁶⁾ which has a close functional relation-

ship with rice NOMT, shared only 19.8% identity with OsCOMT1.

The amino acid sequence of OsCOMT1 is highly homologous to the seven characteristic motifs of plant OMTs (data not shown).¹⁷⁾ Furthermore, OsCOMT1 shares 100%, 100%, 64% and 100% identity with 4 newly defined regions of the consensus sequence: motif S1, motif S2,¹⁷⁾ Motif 1 and Motif 2,¹⁸⁾ respectively. The 4 regions were proposed to be specific for COMTs and have a major influence on substrate specificity. The 100% identity in 3 of the regions strongly suggested that OsCOMT1 is a member of the plant COMT family.

Three unidentified cDNA sequences (accession No. AK098998, AK061551 and AK069960) were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) in a tblastn search with the OsCOMT1 sequence. They shared only 42.9%, 41.0% and 39.2% identity with OsCOMT1, respectively. On the tblastn search of the GenBank rice genome DNA database (<http://www.ncbi.nlm.nih.gov/BLAST/Genome/PlantBlast.shtml>) with the OsCOMT1 sequence, only one genome sequence was a hit, and no other closely related gene was found. These results indicated that rice plants contain only one copy of the COMT gene. *OsCOMT1* was found to be located at 27.1 cM on chromosome 8 (DDBJ FW-accession No. AU085932) by searching the rice EST map (<http://rgp.dna.affrc.go.jp/publicdata/estmap2001/index.html>).

2. Function of *OsCOMT1* recombinant protein

The OsCOMT1 recombinant protein was expressed in *E. coli*, and the protein was extracted and purified. A protein band of approximately 41 kDa was detected in the purified fraction (Fig. 2), which was consistent with the calculated value. Lane 1 indicated that the protein can be expressed even without IPTG induction, while the band was not found in the lysate of BL21 cells harboring only the pET-15b vector (data not shown).

NOMT activity was not detected under standard or other conditions, such as a higher protein concentration, longer reaction time, and different reaction buffer (0.1 M glycine-NaOH, pH 9.5, used in the previous report for NOMT assays¹⁴⁾). However, the protein showed strong COMT activity. The control experiments were performed with protein denatured by heating, with protein from *E. coli* harboring the vector without the *OsCOMT1* insert, or without SAM. The control experiments showed no COMT activity. These results indicated that *OsCOMT1* encodes not an NOMT but instead a COMT protein.

The optimal pH was found to be 7.5 in Tris-HCl buffer. The relative activity at pH 5.0 and 9.0 was 11% and 34%, respectively, relative to the activity at pH 7.5 (Tris-HCl).

Table 1 shows the effects of various metal ions, either with EDTA and DTT, on the activity of the enzyme by adding them to the reaction mixture at a final concentration of 5 mM.

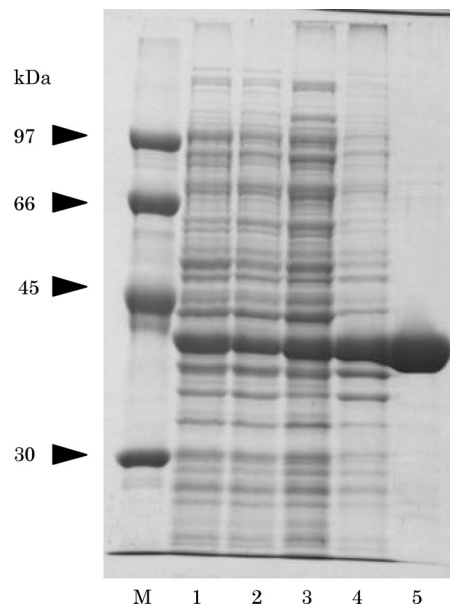


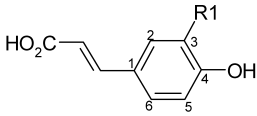
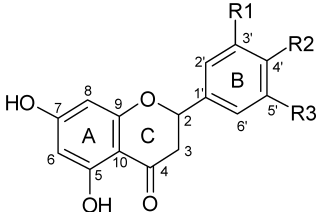
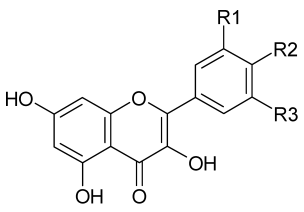
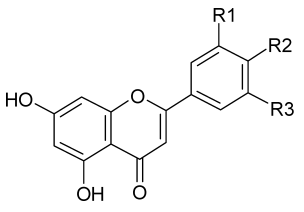
Fig. 2. SDS-PAGE analysis of the proteins during the purification of OsCOMT1. Lane M, the size of protein markers (number at the left indicates kDa); Lane 1, total lysate of BL21(DE3)pLysS containing pET-COMT without IPTG treatment; Lane 2, total lysate of BL21(DE3)pLysS containing pET-COMT with IPTG treatment (IPTG=1 mM); Lane 3, soluble lysate of BL21(DE3)pLysS containing pET-COMT with IPTG treatment (IPTG=1 mM); Lane 4, insoluble lysate of BL21(DE3)pLysS containing pET-COMT with IPTG treatment (IPTG=1 mM); Lane 5, column purified OsCOMT1 protein.

Table 1. Influence of divalent cations, EDTA and DTT on enzyme activity^{a)}

	Relative activity (% of control)
None	100
Ca ²⁺	58
Ba ²⁺	70
Mn ²⁺	24
Cu ²⁺	0
Zn ²⁺	9
Hg ²⁺	0
Co ²⁺	7
Mg ²⁺	64
EDTA	77
DTT	98

^{a)} Divalent cation, EDTA and DTT concentration is 5 mM. Enzyme was assayed in 0.1 mM Tris-HCl (pH 7.5) buffer with the indicated addition.

Table 2. Substrate specificity of purified COMT^{a)}

Structures	Substrates	R1	R2	R3	Relative activity (%)
	Caffeic acid	OH			100
	<i>p</i> -Coumaric acid	H			0
	Naringenin	H	OH	H	0
	Eriodictyol	OH	OH	H	131
	Homoeriodictyol	O-CH ₃	OH	H	0
	Hesperetin	OH	O-CH ₃	H	0
	Kaempferol	H	OH	H	0
	Quercetin	OH	OH	H	71
	Myricetin	OH	OH	OH	75
	Isorhametin	O-CH ₃	OH	H	0
	Apigenin	H	OH	H	0
	Luteolin	OH	OH	H	88
	Chrysoeriol	O-CH ₃	OH	H	0

^{a)} Relative activity was calculated with activity against caffeic acid as 100%.

This result does not agree completely with the finding that Rakwal *et al.*⁵⁾ reported for the NOMT, especially for the effect of Mn²⁺ and Co²⁺. In the previous report, NOMT activity was completely inhibited by Mn²⁺ (relative activity, 0%), while weak inhibition was obtained with Co²⁺ (relative activity, 61%). In the case of COMT, a relative activity of 24% was detected with the addition of Mn²⁺, and Co²⁺ was found to strongly inhibit the enzyme activity (relative activity, 7%).

Apparent K_m and V_{max} values were calculated from Lineweaver–Burk plots. The K_m and V_{max} values for caffeic acid were 69 μ M and 5.5 nkat/mg protein, respectively, when SAM was supplied at a constant concentration of 1 mM. The K_m and V_{max} values for SAM were 51 μ M and 5.2 nkat/mg protein, respectively, when caffeic acid was supplied at a constant concentration of 1 mM.

3. Substrate specificity

The results of the substrate specificity assay with various flavonoids, *p*-coumaric acid and caffeic acid are summarized in Table 2. Eriodictyol was the best substrate among the compounds tested. Flavonoid compounds which have two hydroxyl groups at adjacent positions on the B ring were toler-

ated as substrates. We assumed that the 3'-position of the B ring is methylated by OsCOMT1, considering that OsCOMT1 catalyzed the 3-*O*-methylation of caffeic acid to produce ferulic acid. It was also confirmed that naringenin is not accepted as a substrate in this experiment using radiolabelled SAM.

COMT demonstrates a greater degree of substrate promiscuity and activity at different hydroxyl sites, as exhibited by its strong activity towards 3- and 5-hydroxylated phenylpropanoids.⁶⁾ *In vitro* studies of alfalfa COMT have shown that the enzyme can methylate caffeic acid, 5-hydroxyferulic acid, caffeoyl aldehyde, caffeoyl alcohol, 5-hydroxyconiferyl aldehyde, and 5-hydroxyconiferyl alcohol.¹⁹⁾ In this study, we have not tested whether these compounds (except for caffeic acid) were tolerated as substrates, because the compounds are not easily available. However, it is inferable that OsCOMT1 also has OMT activity toward these compounds, because of its high homology to alfalfa COMT (56.9% identity at the amino acid level) and its substrate specificity for flavonoids.

It is unclear whether the flavonoids used in this experiment are actual substrates for OsCOMT1, because there is no information on the presence of flavonoids except for naringenin in

rice plants. Luteolin-6-*C*-glycosides and chrysoeriol-6-*C*-glycosides were reported to be isolated from rice plants.²⁰ It is likely that *OsCOMT1* catalyzes the 3'-*O*-methylation of luteolin-6-*C*-glycosides to produce chrysoeriol-6-*C*-glycosides, because luteolin was a good substrate for *OsCOMT1*. Tricin (3',5'-dimethoxy-4',5,7-trihydroxyflavone) was also reported to be isolated from rice plants.²¹ Although we have not tested whether its predicted precursors (3',4',5,5',7-pentahydroxyflavone and 3'-methoxy-4',5,5',7-tetrahydroxyflavone) were tolerated as substrates, it is likely that *OsCOMT1* catalyzes the conversion of the precursors into tricin by 3'- and 5'-*O*-methylation.

4. Semi-quantitative analysis of mRNA transcripts

Figures 3A and B show that *OsCOMT1* mRNA was expressed of all stages tested. No increase in the expression of *OsCOMT1* mRNA was detected in CuCl₂-, JA- and UV-treated leaves compared to the control (H₂O) (Fig. 3C). However, an obvious effect was found for the *OsDTS2* gene, which encodes *syn*-pimara-7,15-diene synthase involved in the biosynthesis of phytoalexin momilactones and was reported to be expressed in response to methyl jasmonate and UV.¹⁵

In general, phytoalexins are synthesized and accumulated only in tissues which are exposed to microorganisms, or chemical and physical stress, not in healthy tissues.²² Moreover, Rakwal *et al.* reported that NOMT activity was strongly induced by UV irradiation.¹⁴ However, the semi-quantitative RT-PCR revealed that *OsCOMT1* mRNA was constitutively expressed in the aerial parts and roots of plants up to 5 weeks after germination and that the expression was not induced by UV irradiation, CuCl₂ or JA. The physiological role of plant COMT is in the formation of lignin, which is a structural component of the cell wall that increases mechanical strength.²³ Therefore, COMT is anticipated to be expressed constitutively in plants. The expression pattern of *OsCOMT1* mRNA supported that this gene encodes COMT.

5. COMT activity of crude protein extract from rice plants

COMT activity was measured using a crude protein extract from rice plants. COMT activity was detected at almost a constant level in different growth stages (Fig. 4A) and different leaf positions (Fig. 4B); only a slight increase was detected with age. No induction was found upon UV, JA or CuCl₂ treatment (Fig. 4C). This result is consistent with the *OsCOMT1* mRNA expression.

6. Conclusions

In this report, we found that the NOMT reported by Rakwal *et al.*⁵ is actually a COMT. We speculated that their "purified" NOMT contains COMT as a major component and the "real" NOMT as a minor one and that the major component might be sequenced. However, no COMT activity was detected in

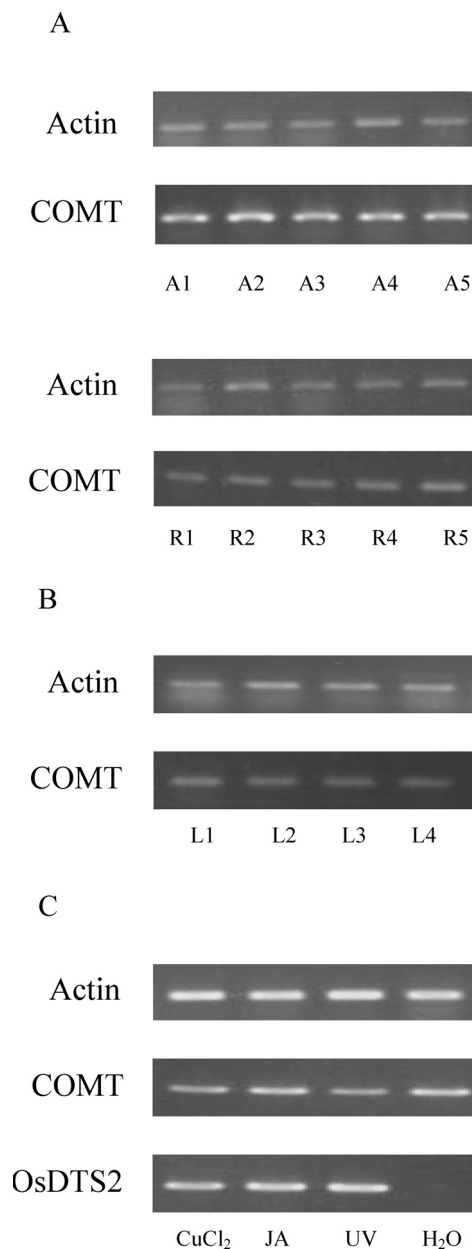


Fig. 3. Quantitative RT-PCR analysis of mRNA transcripts of COMT in different rice organs. Actin was analyzed as a control; A1 to A5, aerial parts of 1- to 5-week-old plants; R1 to R5, roots of 1- to 5-week-old plants; L1 to L4, first (youngest) to fourth (oldest) leaf of 5-week-old plants; *OsDTS2* was used as a positive control, which can be induced to express by UV and MeJA.

the previous report.⁵ The crude protein extract from UV-irradiated rice leaves showed both COMT and NOMT activity.¹⁴ Rakwal *et al.* purified their "NOMT" using an adenosine-agarose column, which possibly has affinity for various *S*-adenosyl-L-methionine-dependent methyltransferases. Therefore, it is possible that the purified protein contained both COMT and NOMT. We speculated that the COMT activity is lost during the purification, because it is more unstable than

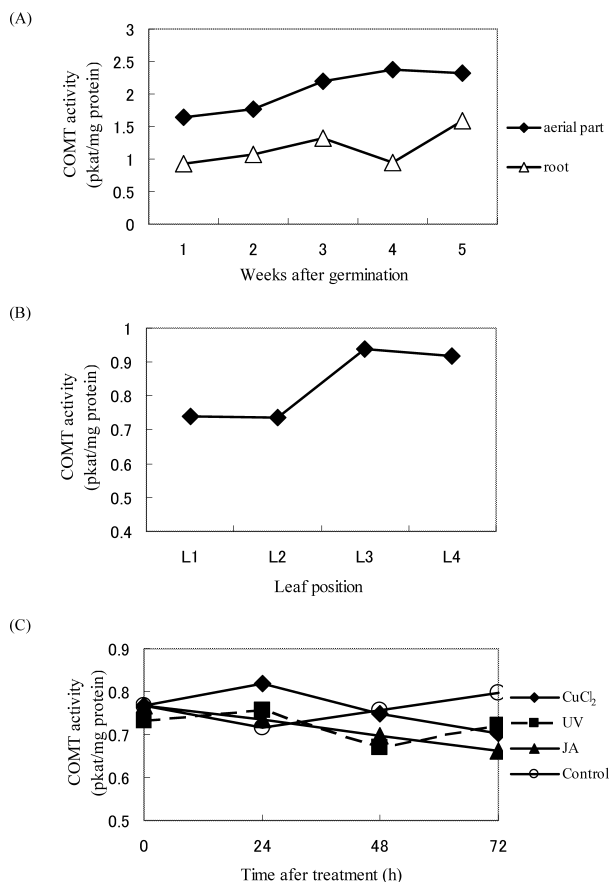


Fig. 4. COMT activities of proteins (A) from aerial parts and roots at different growth stages, (B) from different leaf positions of 5-week-old plants, and (C) from leaves treated with CuCl₂, UV and JA. L1 to L4, first (youngest) to fourth (oldest) leaf of 5-week-old plants.

the NOMT activity. Moreover, the activity of COMT in glycine-NaOH buffer, pH 9.5, which was used as an assay buffer in the previous report, was 21% relative to that in Tris-HCl, pH 7.5. The unsuitable conditions for the COMT reaction may partly explain why COMT activity was not detected in the “NOMT” fraction reported by Rakwal *et al.* We are now trying to identify the “real” NOMT involved in the biosynthesis of the rice phytoalexin sakuranetin.

The physiological role of OsCOMT1 in rice plants is still ambiguous. However, our results indicated that OsCOMT1 is involved in the biosynthesis of lignins and some flavonoids.

Acknowledgments

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