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**Original Paper**

# Characterization of 5-Hydroxyconiferaldehyde *O*-Methyltransferase in *Oryza sativa*

Taichi Koshiba[1], Norie Hirose[1], Mai Mukai[1], Masaomi Yamamura[1], Takefumi Hattori[1,a], Shiro Suzuki[1], Masahiro Sakamoto[2], Toshiaki Umezawa[1,3,*]

[1] Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan; [2] Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan; [3] Institute of Sustainability Science, Kyoto University, Uji, Kyoto 611-0011, Japan
* E-mail: tumezawa@rish.kyoto-u.ac.jp Tel: +81-774-38-3625 Fax: +81-774-38-3682

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**Abstract** Little is known regarding syringyl lignin biosynthesis in rice (*Oryza sativa* L. cv. Nipponbare). In the present study, the role of rice caffeic acid *O*-methyltransferase (OsCOMT1, Q6ZD89), was examined. The recombinant OsCOMT1 catalyzed the 5-*O*methylation of 5-hydroxyferulate (5-HFA) and 5-hydroxyconiferaldehyde (5-HCAld). 5-HCAld inhibited 5-HFA methylation by this *O*-methyltransferase (OMT), while 5-HFA mitigated self-inhibition in 5-HCAld methylation. A rice plant in which *OsCOMT1* expression was downregulated exhibited weakened cell wall staining with Wiesner reagent in vascular bundle cells and sclerenchyma tissue, compared with wild-type plants. The lignin content of transgenic rice plants was decreased and the syringyl lignin content reduced largely compared with that of the wild type. Taken together, these data indicated that OsCOMT1 functioned as a 5-HCAld OMT (OsCAldOMT1) in the biosynthetic pathway to syringyl lignin.

**Key words:** *O*-methyltransferase, *Oryza sativa*, syringyl lignin, biosynthetic pathway.

Recently, because of the increased demand for biobased materials as alternatives to fossil carbon resources, gramineous plants that produce large amounts of inedible lignocellulosic biomass have been drawing attention as potential materials for biofuel and industrial feedstock production (Yamamura et al. 2013). Lignocellulose is composed mainly of lignin and polysaccharides. Lignin is a complex phenylpropanoid polymer, and fills the spaces between cell wall polysaccharides and confers mechanical strength and imperviousness to the cell wall (Boerjan et al. 2003). The inherent robust characteristics of lignin present obstacles to enzymatic hydrolysis of plant cell wall polysaccharides for biorefining, chemical pulping, and forage digestion. On the other hand, lignin is a promising raw material for aromatic feedstock production.

Lignins are generally classified into three major groups: guaiacyl (4-hydroxy-3-methoxyphenyl), syringyl (3,5-dimethoxy-4-hydroxyphenyl), and *p*-hydroxyphenyl lignins. Lignin structures affect their reactivity and thermal properties. For example, plants with high syringyl lignin content are more easily delignified in kraft pulping than those with low syringyl lignin content (Chiang and Funaoka 1990; Lourenço et al. 2012; Shimizu et al. 2012). Condensed lignin structures reduce the thermal mobility of lignin (Kubo et al. 1997), indicating that syringyl lignin is beneficial for use in plastics, compared with guaiacyl lignin, because syringyl lignin lacks the condensed structures. Characterization of lignin and its biosynthetic mechanisms is a basis for the practical use of lignocelluloses.

Syringyl lignin had long been proposed to be formed from *p*-coumaric acid (CouA) via caffeic acid (CA), ferulic acid (FA), 5-hydroxyferulic acid (5-HFA), sinapic acid (SA), sinapoyl CoA (SCoA), sinapaldehyde (SAld), and sinapyl alcohol (SAlc) (Figure 1), based on tracer experiments with isotope-labeled phenylpropanoid monomers and associated enzymatic experiments

Abbreviations: 4CL, 4-hydroxycinnamate CoA ligase; 5-HCAlc, 5-hydroxyconiferyl alcohol; 5-HCAld, 5-hydroxyconiferaldehyde; 5-HFA, 5-hydroxyferulic acid; 5-HFCoA, 5-hydroxyferuloyl CoA; C4H, cinnamate 4-hydroxylase; CA, caffeic acid; CaAlc, caffeyl alcohol; CaAld, caffealdehyde; CaCoA, caffeoyl CoA; CAD, cinnamyl alcohol dehydrogenase; CADL, cinnamyl alcohol dehydrogenase-like; CAld, coniferaldehyde; CAld5H, coniferaldehyde 5-hydroxylase (=F5H, ferulate 5-hydroxylase); CAldOMT, 5-hydroxyconiferaldehyde *O*-methyltransferase; CAOMT (=COMT), caffeic acid *O*-methyltransferase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CouA, *p*-coumaric acid; F5HL, ferulate 5-hydroxylase-like; FA, ferulic acid; H, *p*-Hydroxybenzaldehyde; H/V, *p*-hydroxybenzaldehyde/vanillin; OMT, *O*-methyltransferase; S, syringaldehyde; SA, sinapic acid; SAlc, sinapyl alcohol; SAld, sinapaldehyde; S/V, syringaldehyde/vanillin; V, vanillin.

[a] Present address: Institute of Socio-Arts and Sciences, The University of Tokushima, 1-1 Minamijosanjima-cho, Tokushima 770-8502, Japan.

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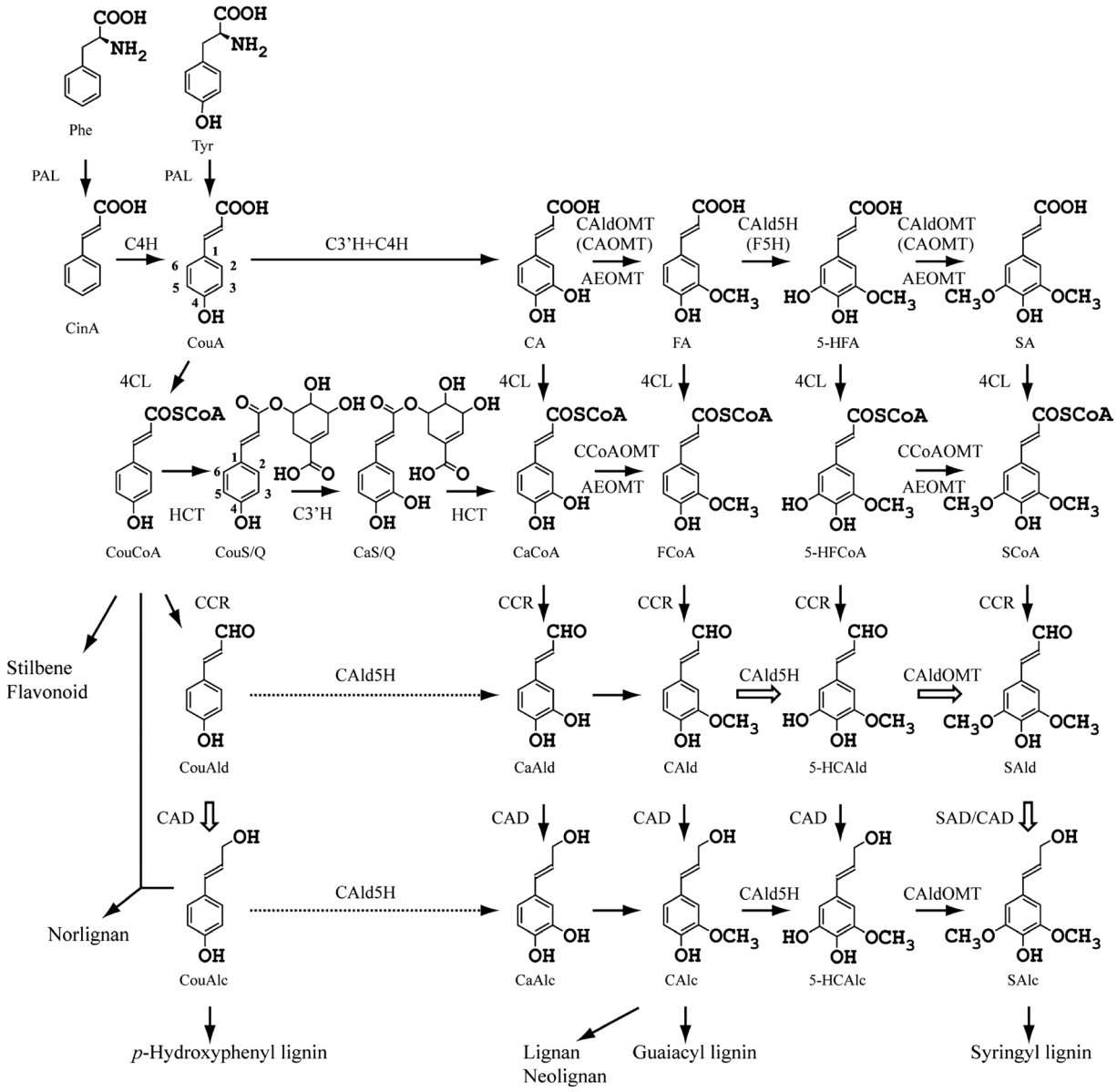


Figure 1. The cinnamate/monolignol pathway. Thick open arrow represents the major routes for lignin biosynthesis. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3'H, *p*-coumaroyl shikimate/quinate 3-hydroxylase; CAldOMT, 5-hydroxyconiferaldehyde *O*-methyltransferase; CAOMT, caffeic acid *O*-methyltransferase; AEOMT, hydroxycinnamic acid/hydroxycinnamoyl CoA esters *O*-methyltransferase; CAld5H, coniferaldehyde 5-hydroxylase; F5H, ferulate 5-hydroxylase; 4CL, 4-hydroxycinnamate CoA ligase; HCT, hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase; Phe, phenylalanine; Tyr, tyrosine; CinA, cinnamic acid; CouA, *p*-coumaric acid; CouCoA, *p*-coumaroyl CoA; CouS/Q, *p*-coumaroyl shikimate/quinate*; CaS/Q, caffeoyl shikimate/quinate*; CA, caffeic acid; FA, ferulic acid; 5-HFA, 5-hydroxyferulic acid; SA, sinapic acid; CaCoA, caffeoyl CoA; FCoA, feruloyl CoA; 5-HFCoA, 5-hydroxyferuloyl CoA; SCoA, sinapoyl CoA; CouAld, *p*-coumaraldehyde; CaAld, caffealdehyde; CAld, coniferaldehyde; 5-HCAld, 5-hydroxyconiferaldehyde; SAld, sinapaldehyde; CouAlc, *p*-coumaryl alcohol; CaAlc, caffeyl alcohol; CAlc, coniferyl alcohol; 5-HCAlc, 5-hydroxyconiferyl alcohol; SAlc, sinapyl alcohol. *: Only the structure of shikimate ester is shown.

(Higuchi 1985, 1997; Sarkanen 1971). In the pathway from CouA to SAlc, caffeic acid *O*-methyltransferase (CAOMT or COMT) was assigned to the conversion of CA and 5-HFA to FA and SA, respectively, while ferulate 5-hydroxylase (F5H) catalyzed the hydroxylation of FA to 5-HFA. Subsequently, this pathway has been revised based on kinetic analyses of recombinant and plant enzymes. First, a new *O*-methyltransferase (OMT), caffeoyl CoA *O*-methyltransferases (CCoAOMT), was found to be involved in lignification (Umezawa 2010; Ye et al. 2001). In addition, the true function of CAOMT and F5H was found to be methylation of 5-HCAld to give SAld and hydroxylation of coniferaldehyde (CAld) to give 5-HCAld, respectively. Therefore, they were renamed as 5-hydroxyconiferaldehyde OMT (CAldOMT) and coniferaldehyde 5-hydroxylase

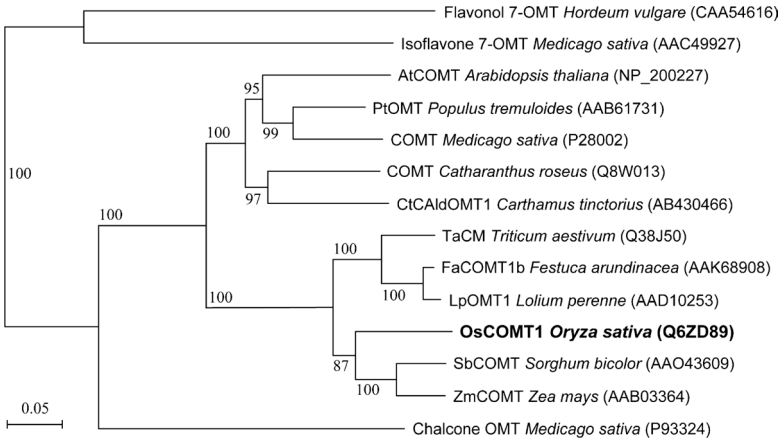


Figure 2. Phylogenetic analysis of OsCOMT1 (=OsCAldOMT1) and selected OMT proteins. Phylogenetic tree of deduced amino acid sequences constructed using neighbor-joining method; scale bar, 0.05 amino acid substitutions per position in the sequence; bootstrap values obtained by 1,000 bootstrap replicates; and numbers above branches refer to bootstrap values. Accession nembers are given in parentheses.

(CAld5H) (Li et al. 2000, 2001; Osakabe et al. 1999; Umezawa 2010). From these experiments together with characterization of transgenic plant chemotypes in which genes encoding these enzymes were downregulated, the 5-HCAld pathway from CAld to SAlc via 5-HCAld and SAld is considered a major pathway for syringyl lignin biosynthesis (Figure 1; Li et al. 2000; Umezawa 2010). In addition, caffealdehyde (CaAld) conversion to CAld has also been proposed for syringyl lignin biosynthesis in medicago (Figure 1) (family *Fabaceae*, Zhou et al. 2010).

As the pathways to syringyl lignin formation were established mainly in dicotyledonous angiosperms, much remains to be done before its useful applicability to grasses. Recombinant CAOMTs from a few grasses display disparate substrate specificities. A recombinant tall fescue (*Festuca arundinacea* Schreb. cv. Kentucky-31) CAOMT (FaCOMT1b) showed the highest $V_{max}$ $K_m^{-1}$ for 5-HFA (relative activity, 100), followed by CaAld, caffeyl alcohol (CaAlc), 5-HCAld, CA, and 5-hydroxyconiferyl alcohol (5-HCAlc) with relative activity of 68.49, 84.85, 27.74, 5.48, and 3.41, respectively (Chen et al. 2004). The enzyme activity for 5-HCAld was rather low, which was in sharp contrast to those of dicotyledonous CAOMTs whose function is in fact CAldOMT (Li et al. 2000; Umezawa 2010). In contrast, perennial ryegrass (*Lolium perenne*) CAOMT (LpOMT1) showed the highest $V_{max}$ $K_m^{-1}$ for 5-HCAlc (relative activity, 100) followed by 5-HFA and CA (5.4 and 3.5, respectively; Louie et al. 2010). For wheat (*Triticum aestivum* L. cv. H4564) CAOMT (TaCM), 5-HCAld showed the highest $k_{cat}$ $K_m^{-1}$ (relative activity, 100), followed by CaAld, caffeyl alcohol (CaAlc), CA, and 5-HCAlc (65.1, 46.9, 38.4, and 29.5, respectively). In addition, the OMT shows significant activity against caffeoyl CoA (CaCoA) and 5-hydroxyferuloyl CoA (5-HFCoA; 16.8 and 14.9, respectively; Ma and Xu 2008).

These results indicate that there is a substantial difference among CAOMTs from different gramineous plants with respect to substrate preference. A rice (*Oryza sativa* L. cv. Nipponbare) CAOMT (OsCOMT1) (Figure 2) that methylates caffeic acid as well as a number of flavonoid compounds was reported (Lin et al. 2006). However its substrate specificities against other phenylpropanoid monomers were not reported. In this context, we characterized here OsCOMT1-catalyzed reactions and a chemotype of transgenic *O. sativa* in which the *OsCOMT1* was downregulated, and found indications that OsCOMT1 was in fact a 5-HCAld OMT (OsCAldOMT1).

## Materials and methods

### *Expression of recombinant OsCOMT1*

The pFLCI plasmid containing a cDNA sequence from *O. sativa* L. cv. Nipponbare (accession no. AK064768) that corresponds to caffeic acid 3-*O*-methyltransferase (OsCOMT1, Figure 2, Lin et al. 2006) was obtained from National Institute of Agrobiological Sciences (Tsukuba, Japan). PCR was used to introduce an *Nde*I site and a *Blp*I site at the N and C-terminal ends, respectively, of the OMT gene coding sequence, using a sense primer 5′-AAC CAT ATG GGT TCT ACA GCC GCC GAC-3′ and an antisense primer 5′-CCC GCT CAG CCT ACT TTG TGA ACT CGA TGG-3′. A polymerase chain reaction (PCR) mixture (50 $\mu$l) was composed of 0.2 mM dNTPs, 1.5 mM $MgSO_4$, 15 pmol each of primers, 44 ng of plasmid DNA, 1×buffer for KOD-Plus- Ver.2, and 1 U of KOD-Plus-DNA polymerase (Toyobo Co., Ltd., Osaka, Japan). PCR cycling conditions was as follows: one cycle at 94°C for 2 min, five cycles at 98°C for 10 s, 53°C for 30 s and 68°C for 1.5 min, thirty cycles at 98°C for 10 s, 60°C for 30 s and 68°C for 1.5 min followed by one cycle at 68°C for 10 min. The PCR product was then digested with *Nde*I and *Blp*I (New England Biolabs, Inc., Beverly, MA, USA), cloned into the pET15b vector (Novagen, Inc., Madison, WI, USA) to fuse a His-tag at the cDNA

expression product N-terminal. After sequencing to confirm the accuracy of the resulting *OsCOMT1*-pET15b construct, it was introduced into *E. coli* BL21 (DE3) for protein expression. The bacteria were cultured at 37°C and 250 rpm until the $OD_{600}$ reached 0.7–0.9, collected, resuspended in 100 ml of LB medium containing 100 mg $l^{-1}$ of ampicillin, and grown at 37°C. Upon the culture reaching an $OD_{600}$ of 0.5, isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) was added to the culture at the final concentration of 1 mM to induce protein expression, and incubated for 3 h at 37°C. His-tagged protein purification from the cells was carried out as previously described (Nakatsubo et al. 2007) and protein concentration was determined using a Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### Enzyme assay

Enzymatic reactions with purified recombinant OsCOMT1 were carried out according to Nakatsubo et al. (2007), using CA, 5-HFA, CaAld, 5-HCAld, CaAlc, and 5-HCAlc, which were prepared previously (Sakakibara et al. 2007). The typical reaction mixture (200 $\mu$l) contained 50 mM Tris-HCl (pH 7.5), 200 $\mu$M *S*-adenosyl-L-methionine (SAM), 20 $\mu$l of 2 mM substrate solution in dimethylsulfoxide (DMSO), and 5 $\mu$l of 1 g $l^{-1}$ enzyme solution. After incubation for 10 or 20 min, the reaction was terminated by adding 0.5 ml of ethyl acetate (EtOAc) and 10 $\mu$l of 0.1 mM chemically synthesized deuterium-labeled phenylpropanoid monomers (Sakakibara et al. 2007) in DMSO, as internal standards. The solvent was then evaporated using a Svant® SPD 111V SpeedVac® Concentrator (Thermo Fisher Scientific, Inc., Rockford, IL, USA). GC-MS analysis was performed with a Shimadzu QP-2010 Plus GC-MS system (Shimadzu Corp., Kyoto, Japan) with a Shimadzu Hicap CBP10-M25-025 (10 m×0.22 mm) column, helium carrier gas, 250°C injection temperature, and a programmed column temperature regime from 80 to 250°C at 10°C $min^{-1}$ followed by 250°C for 15 min. Quantitative analysis of the reaction products was conducted by a stable-isotope dilution method according to Sakakibara et al. (2007).

### Determination of kinetic parameters

The optimum reaction time for each substrate in kinetic analysis was determined by assaying at 30°C for 2, 5, 10, 20, 30, and 60 min. Then, varying concentrations of CA, 5-HFA, CaAld, 5-HCAld, CaAlc, and 5-HCAlc (10–150 $\mu$M) and 200 $\mu$M SAM were used to measure $K_m$, $V_{max}$, and the enzyme turnover number, $k_{cat}$. The reactions of 5-HCAld and CaAld were performed at 30°C for 10 min, and all others at 30°C for 20 min. Reactions were terminated by EtOAc addition and the reaction products analyzed by GC-MS as described above.

For inhibition kinetics analyses of OsCOMT1 on 5-HFA methylation, 5-HCAld (0–10 $\mu$M) was used as an inhibitor, while 5-HFA (0–50 $\mu$M) was used as an inhibitor of 5-HCAld methylation. These reactions were carried out similarly to the above typical reaction mixtures except that the substrate concentration was varied and the inhibitor also added. 5-HFA methylation was performed at 30°C for 10 min and that of 5-HCAld at 30°C for 20 min. $K_i$ values were determined based on Dixon plots.

### Plant growth and harvest

$T_1$ seeds were harvested from $T_0$ rice plants (*O. sativa* L. cv. Nipponbare) of *OsCOMT1* RNA-interference (RNAi) lines (Hattori et al. 2012). Dehusked seeds from wild type and the $T_1$ *OsCOMT1* RNAi line were surface sterilized with 2.5% NaClO and germinated on a nylon mesh floated on 1 l of distilled water in a plastic container. On day 7, twenty seedlings were transplanted to a 3-l plastic container containing a nutrient solution and grown in a growth chamber at 27°C with a 12 h daily photoperiod. The nutrient solution contained 1 mM $NH_4NO_3$, 0.6 mM $NaH_2PO_4$, 0.3 mM $K_2SO_4$, 0.3 mM $CaCl_2$, 0.6 mM $MgCl_2$, and micronutrients at 46 $\mu$M Fe-EDTA, 50 $\mu$M $H_3BO_3$, 9 $\mu$M $MnSO_4$, 0.3 $\mu$M $CuSO_4$, 0.7 $\mu$M $ZnSO_4$, and 0.1 $\mu$M $Na_2MoO_4$, at pH 5.8; the nutrient solution was renewed every 3–4 days. Plants reaching the mature stage were harvested and separated into leaves, leaf sheaths, culms, and panicles and the parts, except for panicles, dried at 70°C for 48 h, pulverized twice with a TissueLyser (Qiagen, Hilden, Germany) for 1.5 min at 25 Hz, and stored at room temperature over desiccant (silica gel) until use.

### qRT-PCR analysis

Total RNA was isolated individually from leaf, leaf sheath, and culm by a phenol/chloroform extraction and LiCl precipitation method (Shirzadegan et al. 1991). Genomic DNA contamination was eliminated by treatment with RNase-free DNase (Promega Corp., Madison, WI, USA). First-strand cDNA was synthesized using in a 20 $\mu$l reaction mixture containing 1 $\mu$l of SuperScript II Reverse Transcriptase (Life Technologies, Carlsbad CA, USA), 1 $\mu$g of total RNA, 50 pmol $Oligo(dT)_{20}$ primer (Life Technologies), 10 nmol dNTPs, 0.2 $\mu$M DTT, and 1 $\mu$l of RNaseOUT (Life Technologies) at 42°C for 50 min. The reaction mixture was diluted 8-fold with TE buffer (pH 8.0).

Quantitative real-time PCR (qRT-PCR) was conducted using an Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, Forester City, CA, USA). For each reaction, a 20 $\mu$l mixture containing 1 $\mu$l of first-strand cDNA, 12.5 $\mu$l of Power SYBR Green PCR Master Mix (Applied Biosystems), and 2 pmol each of the forward and reverse primer sets described in Table 1. The amplification program was 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min (Suzuki et al. 2006). A single fluorescence reading was performed during each cycle immediately following the elongation period at 60°C to ensure single product amplification. Cycle threshold (Ct) values were determined automatically using Sequence Detection Software ver. 1.2 (Applied Biosystems). Each reaction was performed in duplicate. A ubiquitin gene (*OsUBQ5*; accession no. AK061988) was amplified using specific primers (Table 1) and used as an internal control. The ΔΔCt method was adopted for calculation

Table 1. Primers for qRT-PCR used in this study.

| Gene name | TIGR ID | Accession no. | Primers used |
|---|---|---|---|
| *COMT2* | LOC_Os02g57760 | AK105029 | 5′-GCATCAATTTGCTGCTGAAATC-3′<br>5′-TTGACAGTGTCACGAGACCAAAA-3′ |
| *OsCOMT1* | LOC_Os08g06100 | AK064768 | 5′-CCTCGCCCTCATGAACCA-3′<br>5′-CGTCCAGGACTGCGTCCTTA-3′ |
| *CADL* | LOC_Os01g34480 | AK068824 | 5′-CGTCCAAATCATTCGTGAGATG-3′<br>5′-TGGCACTGATGGCTTGTCAT-3′ |
| *CAD2* | LOC_Os02g09490 | AK105011 | 5′-TGTGTGAGACTCTGACGACTTGTC-3′<br>5′-CATATATTGCGAGGCCGAATTT-3′ |
| *CAD8c* | LOC_Os09g23550 | AK067085 | 5′-TGCGGCTAATCTGTCTAGTGGAT-3′<br>5′-CGTCCTTCTGAGCCCTGAAA-3′ |
| *4CL2* | LOC_Os02g08100 | AK070083 | 5′-GGACAGGGTTACGGTATGACTGA-3′<br>5′-TTAACCTTGAAGGGCTCCTTGGCA-3′ |
| *4CL3* | LOC_Os08g34790 | AK120964 | 5′-ATCATGAAGGGATACCTGAACAAC-3′<br>5′-CAGCCACCCGTCCTTGTC-3′ |
| *4CL4* | LOC_Os04g24530 | J080083H24 | 5′-CGACGCCATCCCCAAGT-3′<br>5′-CGGTTTCATCCTCTTGATGAACT-3′ |
| *HCT1* | LOC_Os04g42250 | AK072528 | 5′-TGAAGGGCTGGCGTTTGT-3′<br>5′-CCTGCAATGAGATGGCTATGG-3′ |
| *HCT2* | LOC_Os02g39850 | AK060705 | 5′-CCTGCAAATTTTTCCCTTCCT-3′<br>5′-CAAATACAGTTGGCACAAAATGTG-3′ |
| *HCT3* | LOC_Os09g25460 | AK101900 | 5′-AGTGTATGCTGGTACTGTGCAACA-3′<br>5′-TGTAGAGAGAGCTGCAACCAACA-3′ |
| *C3H1* | LOC_Os05g41440 | AK099695 | 5′-TTGGGCTTCTATGGGACATGA-3′<br>5′-CCATTGCCCACTCGACTGAT-3′ |
| *COA1* | LOC_Os06g06980 | AK065744 | 5′-AACCTGCTGCTGAAGCTCATC-3′<br>5′-GGTGGCGAGGAGGGAGTAG-3′ |
| *COA20* | LOC_Os08g38900 | AK071482 | 5′-GCTCAAACGGAGGAAGATCTCA-3′<br>5′-CGCTGTGGGCGTTCTTG-3′ |
| *CCoAOMT1* | LOC_Os08g38910 | AK061757 | 5′-GCTGTCACCGAGGAGCATATG-3′<br>5′-GGGTGTGTACATCGTGCTTCTC-3′ |
| *CCR19* | LOC_Os09g25150 | AK067949 | 5′-CAAGCTCTTCCCCGAGTATCC-3′<br>5′-GCTGCTTCCGTGGGTTCAT-3′ |
| *CCR20* | LOC_Os08g34280 | AK072872 | 5′-GCGTCGTGGCTCGTGAA-3′<br>5′-CGCGTTCTTCGGGTCATCT-3′ |
| *CCR21* | LOC_Os02g08420 | AK070746 | 5′-AGCACCAAGCACATTCTCAAGTAC-3′<br>5′-GGCGTGGGACTCGTTGAC-3′ |
| *F5HL2* | LOC_Os03g02180 | AB207253 | 5′-CGTGGCAGAGTACTTGCTGATG-3′<br>5′-AATGCGAACGCCATGGA-3′ |
| *F5H1* | LOC_Os10g36848 | AK067847 | 5′-GGTGACAGTGTGATATATAGAC-3′<br>5′-AAATTCAGAGGCTGTGCTTATGTG-3′ |
| *PAL1* | LOC_Os02g41630 | AK102817 | 5′-AGGAGCTCGGCTGCGTATT-3′<br>5′-ATGCCGAGGAACACCTTGTT-3′ |
| *PAL2* | LOC_Os02g41650 | NM_001054095 | 5′-TGGCTCGGCCCACAGAT-3′<br>5′-GTTGTCGTTGACGGAGTTGATC-3′ |
| *C4H1* | LOC_Os02g26810 | | 5′-AAGAGAAGGAAGGTGATGGACACT-3′<br>5′-CGCCTCAAGGATATGGTCGAT-3′ |
| *C4H2* | LOC_Os05g25640 | AK100598 | 5′-TGCAGCTGATGATGTACAACGA-3′<br>5′-TCGTCCACGCTGTCGAAA-3′ |
| *OsUBQ5* | LOC_Os01g22490 | AK061988 | 5′-ACCACTTCGACCGCCACTACT-3′<br>5′-ACGCCTAAGCCTGCTGGTT-3′ |

of gene expression (User Bulletin #2, Applied Biosystems).

### *Histochemical analysis*

Fresh hand-cut sections (ca. 100 $\mu$m-thick) were prepared from the third internode of rice plants at the heading stage. For Wiesner (phloroglucinol-HCl) staining, sections were incubated for 10 min in phloroglucinol solution (1% in 70% ethanol, v/v), and treated with 18% HCl for 5 min. The sections were then photographed under light microscopy (Olympus BX-51, Olympus Corp., Tokyo, Japan; Li et al. 2009; Zhang et al. 2006).

### *Lignin analysis*

Thioglycolic acid lignin was determined according to Suzuki et al. (2009). Thioacidolysis and nitrobenzene oxidation were carried out as previously described (Nakatsubo et al. 2008;

Table 2. Kinetic properties of recombinant OsCOMT1 protein.

| Substrate | $K_m$ ($\mu$M) | $V_{max}$ (nM $s^{-1}$) | $k_{cat}$ ($s^{-1}$) | $k_{cat}$ $K_m^{-1}$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|
| CA | 201.8 | 12.2 | $9.2\times10^{-3}$ | 45.6 |
| 5-HFA | 22.2 | 47.0 | $35.4\times10^{-3}$ | 1597.3 |
| CaAld | 65.8 | 6.5 | $4.9\times10^{-3}$ | 73.9 |
| 5-HCAld | 26.4 | 34.4 | $25.9\times10^{-3}$ | 980.7 |
| CaAlc | 50.0 | 39.8 | $30.0\times10^{-3}$ | 600.0 |
| 5-HCAlc | 132.3 | 111.5 | $84.0\times10^{-3}$ | 634.6 |

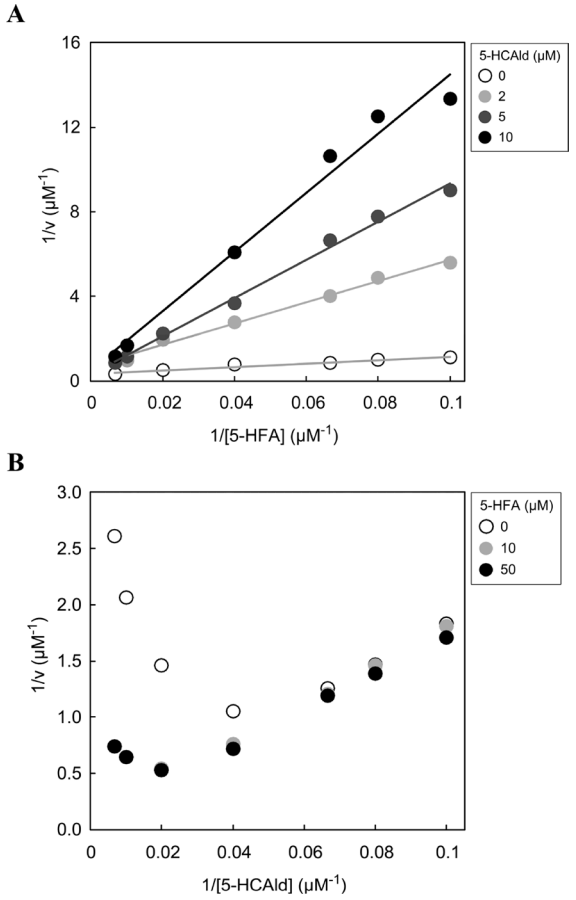


Figure 3. Inhibition kinetic assay of OsCOMT1-catalyzed methylation of 5-HFA and 5-HCAld. A, 5-HCAld as inhibitor added to 5-HFA reaction mixture at 0, 2, 5, and 10 $\mu$M. B, 5-HFA as inhibitor added to 5-HCAld reaction mixture at 0, 10, and 50 $\mu$M.

Yamamura et al. 2010, 2011).

### *Enzymatic saccharification*

Enzymatic saccharification was performed according to Hattori et al. (2012).

## Results

### *Enzyme kinetics of the recombinant OsCOMT1*

The OMT activity of the purified recombinant OsCOMT1 was assayed for activity toward CA, 5-HFA, CaAld, 5-HCAld, CaAlc, and 5-HCAlc. This GC-MS-based assay indicated that OsCOMT1 exhibited *O*-methylation activity for all substrates (Table 2). Among them, 5-HFA and 5-HCAld showed smaller $K_m$ (22.2 and 26.4 $\mu$M, respectively) and larger $k_{cat}$ $K_m^{-1}$ values (1597.3 and 980.7 $M^{-1}$ $s^{-1}$, respectively) than those of the other substrates (Table 2). CA showed the highest $K_m$ and smallest $k_{cat}$ $K_m^{-1}$. These results implicated that 5-HFA was the best substrate *in vitro* and suggested that it might be a physiological substrate. To test this speculation, we performed inhibition kinetics for 5-HCAld and 5-HFA. The *O*-methylation activity of OsCOMT1 on 5-HFA was strongly inhibited by 5-HCAld (competitive inhibition, a $K_i$ for 5-HCAld was 2 $\mu$M, Figure 3A), while 5-HCAld was not inhibited by 5-HFA (Figure 3B). When higher 5-HCAld concentrations were used in the assay, substrate inhibition was observed in the range of 0.005–0.04 as the expressed values of 1/[5-HCAld]. Interestingly, it was mitigated by adding at least 10 $\mu$M of 5-HFA (Figure 3B). Together, these results indicated 5-HCAld to be the best substrate for OsCOMT1 in terms of experiments *in vitro*.

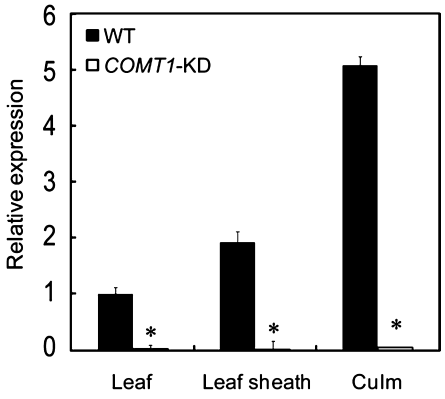


Figure 4. *OsCOMT1* expression in wild type (WT) and *COMT1*-KD lines. Quantitative RT-PCR analysis of *OsCOMT1* expression performed in each tissue of WT and *COMT1*-KD plants at heading stage; *OsCOMT1* expression normalized using *OsUBQ5* expression; and * significant differences (Student's *t*-test; $p<0.05$).

### *Effects of* OsCOMT1 *downregulation on lignin content, deposition, and composition*

The function of OsCOMT1 *in planta* was characterized by cultivation and analysis of a $T_1$ population of *OsCOMT1*-downregulated transgenic rice (*COMT1*-KD) plants, generated by an RNAi technique (Hattori et al. 2012). First, total RNA was extracted from leaf, leaf sheath, and culm from these plants in the heading stage and subjected to qRT-PCR analysis. *OsCOMT1*

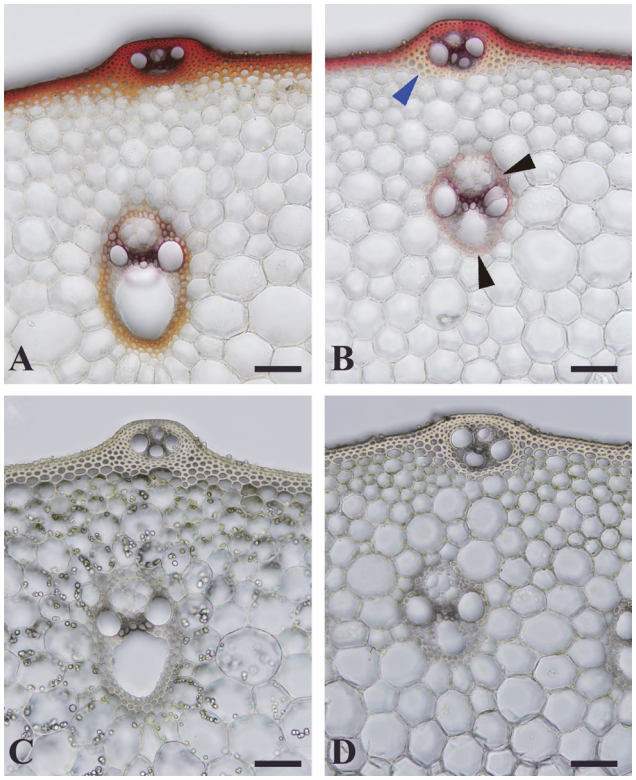


Figure 5. Histochemical analysis in wild-type (WT) and *COMT1*-KD lines. Transverse sections of third internode of WT (A, C) and *COMT1*-KD (B, D) plants stained with Wiesner reagent; A and B stained with Wiesner reagent; C and D, bright field; blue and black arrowheads, cell walls of sclerenchyma and vascular bundle cells, respectively; and bars, 100 $\mu$m.

expression in *COMT1*-KD plants was found to be strongly suppressed in all parts compared with wild-type plants (WT) (Figure 4). Histochemical analyses of transverse sections of *COMT1*-KD plant third internodes treated with Wiesner reagent revealed no cell wall staining in the sclerenchyma (Figure 5, blue arrowhead), while vascular bundle cell walls exhibited weakened staining compared with WT (Figure 5, black arrowhead). Further histochemical analyses indicated reduced lignin in this transgenic rice, which was further confirmed by lignin quantitation. The lignin content in leaf, leaf sheath, and culm of *COMT1*-KD plants measured by the thioglycolic acid method was significantly reduced by 30.8, 29.3, and 51.2%, respectively, compared with WT (Figure 6).

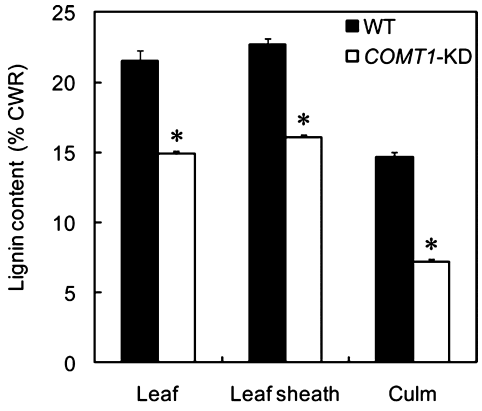


Figure 6. Lignin content in wild-type (WT) and *COMT1*-KD plants. Lignin content in each tissue between WT and *COMT1*-KD plants; * significant differences (Student's *t*-test; $p<0.05$); and CWR, cell wall residues.

Aromatic compositions of lignins in *COMT1*-KD and WT plants were examined by thioacidolysis and nitrobenzene oxidation. The GC-MS analysis of the thioacidolysis products (Figure 7) showed the presence of phenyltrithioethylpropane compounds, which are derived specifically from $\beta$-*O*-4 substructures of lignin polymers (Nakatsubo et al. 2008; Yamamura et al. 2012). In addition, the products from all three organs of WT contained guaiacyl, syringyl, and *p*-hydroxyphenyl compounds, indicating that lignins of all these organs consisted of guaiacyl, syringyl, and *p*-hydroxyphenyl units (Figure 7). In contrast, the syringyltrithioethylpropane yields from transgenic plants were drastically decreased, which was compensated for by the occurrence of increased 5-hydroxyguaiacyltrithioethylpropane (Figure 7).

The lignin aromatic components of other substructures as well as of $\beta$-*O*-4 substructures, determined by analysis of each organ by a microscale nitrobenzene oxidation method (Yamamura et al. 2010, 2011) and GC-MS analysis of the nitrobenzene oxidation products, indicated that syringaldehyde

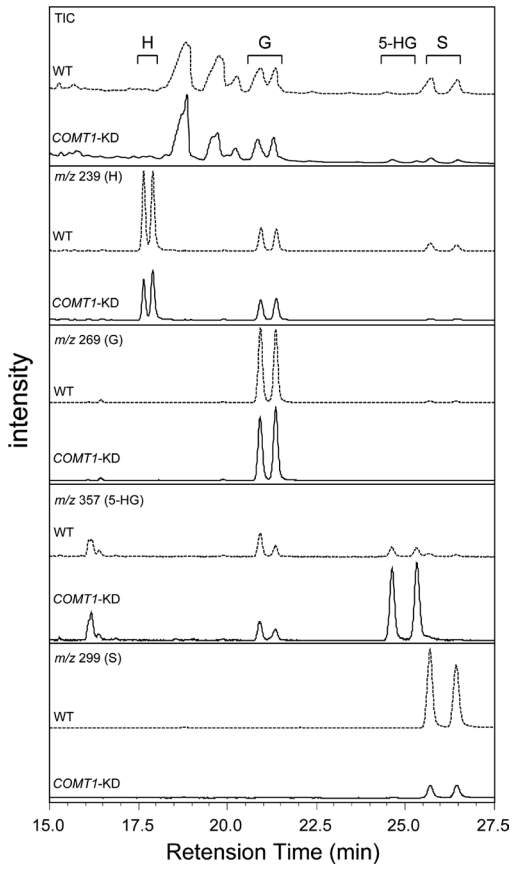


Figure 7. GC-MS chromatograms of thioacidolysis products from whole culm lignin samples of wild-type (WT) and *COMT1*-KD plants. H, G, 5-HG, and S represent *p*-hydroxy-, guaiacyl-, 5-hydroxyguaiacyl-, and syringyl-trithioethylpropanes, respectively; ions at *m/z* 239, 269, 357, and 299, base ions of H, G, 5-HG, and S, respectively; TIC, total ion chromatogram.

(S) yields from *COMT1*-KD plants were significantly decreased to 15.2, 13.4, and 25.7% for leaf, leaf sheath, and culm, respectively, compared with WT (Table 3). *p*-Hydroxybenzaldehyde (H) yields were also reduced to 70.1, 53.3, and 80.3% for leaf, leaf sheath, and culm, respectively (Table 3), whereas vanillin (V) yields from *COMT1*-KD plants were not significantly altered except for the leaf sheath (Table 3). As a result, the S/V ratios of *COMT1*-KD plants were 0.05, 0.05, and 0.10 for leaf, leaf sheath, and culm, respectively. These values were drastically decreased compared with WT tissues, at 0.30, 0.27, and 0.36 for leaf, leaf sheath, and culm, respectively (Table 3). In addition, the syringic acid yields from *COMT1*-KD plants were decreased compared with those from WT, while the vanillic acid and *p*-hydroxybenzoic acid yields were not changed (Table 3). The H/V ratios of *COMT1*-KD plants for all tissues tested were slightly decreased compared to WT (Table 3). These results clearly indicated the specific reduction of syringyl units, which were in agreement with the thioacidolysis analyses.

### *Comprehensive gene expression analysis in the cinnamate/monolignol pathway of* COMT1-*KD plant*

The effect of *OsCOMT1* downregulation on the expression of other genes involved in lignin biosynthesis was examined by subjecting 23 genes, annotated as cinnamate/monolignol pathway enzymes, to qRT-PCR analysis. Among the genes tested, seven genes, caffeic acid *O*-methyltransferase 2 (*COMT2*), cinnamyl alcohol dehydrogenase-like (*CADL*), cinnamyl alcohol dehydrogenase 8c (*CAD8c*), 4-hydroxycinnamate CoA ligase 3 (*4CL3*), ferulate 5-hydroxylase 1 (*F5H1*), and

Table 3. Changes of lignin aromatic ring compositions in *COMT1*-KD plants.

| | | Leaf | Leaf sheath | Culm |
|---|---|---|---|---|
| | | $\mu$mol g$^{-1}$ cell wall residue (CWR) | | |
| Vanillin (V) | Wild type | 228.27±10.95 | 323.11±38.59 | 128.74±18.43 |
| | *COMT1*-KD | 199.36±24.47 | 228.44±11.34* | 123.68±10.65 |
| Syringaldehyde (S) | Wild type | 67.95±5.59 | 87.29±11.59 | 46.96±11.21 |
| | *COMT1*-KD | 10.33±0.02* | 11.69±1.15* | 12.08±0.66* |
| *p*-Hydroxybenzaldehyde (H) | Wild type | 19.98±0.48 | 30.86±3.23 | 21.37±4.03 |
| | *COMT1*-KD | 14.01±1.34* | 16.46±0.34* | 17.16±1.27* |
| Vanillic acid | Wild type | 12.33±0.91 | 13.14±0.46 | 12.49±0.15 |
| | *COMT1*-KD | 13.16±0.38 | 13.42±0.20 | 12.28±0.29 |
| Syringic acid | Wild type | 11.92±0.89 | 10.38±0.81 | 8.25±0.56 |
| | *COMT1*-KD | 7.01±0.49* | 6.46±0.15* | 5.84±0.06* |
| *p*-Hydroxybenzoic acid | Wild type | 13.51±1.12 | 14.95±0.34 | 14.58±0.51 |
| | *COMT1*-KD | 14.83±0.74 | 15.23±0.31 | 15.02±0.11 |
| S/V | Wild type | 0.30±0.02 | 0.27±0.01 | 0.36±0.03 |
| | *COMT1*-KD | 0.05±0.01* | 0.05±0.00* | 0.10±0.01* |
| H/V | Wild type | 0.09±0.00 | 0.10±0.00 | 0.17±0.01 |
| | *COMT1*-KD | 0.07±0.00* | 0.07±0.00* | 0.14±0.00* |

Data, average±SD ($n$=3) and * significant differences (Student's $t$-test; $p$<0.05). S/V and H/V: Molar ratios of S to V and H to V, respectively.

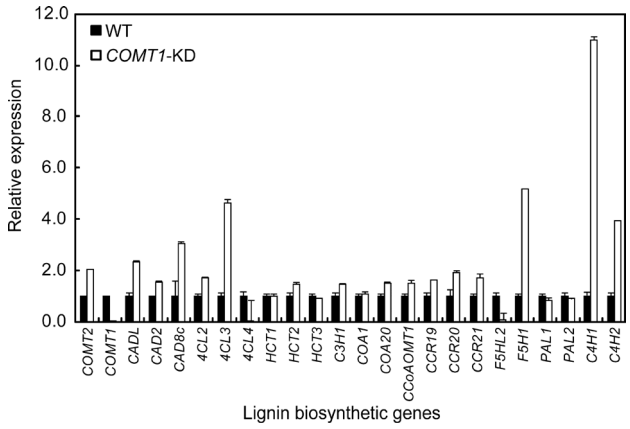


Figure 8. Gene expression analysis of lignin biosynthesis in *COMT1*-KD plant and wild type (WT). Caffeic acid *O*-methyltransferase (*COMT1*, *COMT2*), cinnamyl alcohol dehydrogenase-like (*CADL*), cinnamyl alcohol dehydrogenase (*CAD2*, *CAD8c*), 4-hydroxycinnamate CoA ligase (*4CL2*, *4CL3*, *4CL4*), hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase (*HCT1*, *HCT2*, *HCT3*), *p*-coumaroyl shikimate/quinate 3-hydroxylase (*C3H1*), caffeoyl CoA *O*-methyltransferase (*COA1*, *COA20*, *CCoAOMT1*), cinnamoyl CoA reductase (*CCR19*, *CCR20*, *CCR21*), ferulate 5-hydroxylase-like (*F5HL2*), ferulate 5-hydroxylase (*F5H1*), phenylalanine ammonia-lyase (*PAL1*, *PAL2*), cinnamate 4-hydroxylase (*C4H1*, *C4H2*). TIGR IDs and accession numbers were listed in Table 1.

Table 4. Saccharification efficiency improvement in *COMT1*-KD plants.

| | Leaf | Leaf sheath | Culm |
|---|---|---|---|
| | % Total glucan | | |
| WT | 42.3±1.2 | 31.4±0.2 | 52.9±0.6 |
| *COMT1*-KD | 45.9±0.4* | 33.3±0.3* | 65.1±0.8* |

Data, average ±SD ($n=3$) and * significant differences (Student's *t*-test; $p<0.05$).

cinnamate 4-hydroxylase 1 and 2 (*C4H1*, *C4H2*), were upregulated in *COMT1*-KD plants by more than double compared with WT (Figure 8).

### *Saccharification efficiency*

Table 4 shows the saccharification efficiency of *COMT1*-KD plants having lower lignin content than the WT. All three organs showed higher enzymatic saccharification efficiency, and in *COMT1*-KD culm, the efficiency increased up to 123.1% compared with WT. This result was in accordance with the fact that lignin generally inhibits the enzymatic saccharification process (Li et al. 2008), although not only lignin but also other factors affect enzymatic saccharification of gramineae lignocellulose (Yamamura et al. 2013).

## Discussion

In dicotyledonous plants, CAOMT (CAldOMT) is a key enzyme for syringyl lignin biosynthesis (Umezawa 2010). Detailed kinetic characterization including inhibition experiments was first reported for aspen CAOMT (Li et al. 2000). Methylation of 5-HFA and CA by the OMT was inhibited by 5-HCAld, whereas inhibition of 5-HCAld methylation by CA and 5-HFA was insignificant. Therefore, the real function of the OMT was found to be 5-*O*-methylation of 5-HCAld; as such, it was renamed 5-HCAld OMT (CAldOMT). Later, safflower (*Carthamus tinctorius* L. cv. Round-leaved White) CAOMT was examined by detailed inhibition experiments, which showed that 5-HCAld inhibited methylation of other substrates, including CA, 5-HFA, CaAld, CaAlc, and 5-HCAlc (Nakatsubo et al. 2007). Other dicot CAOMTs examined thus far have also shown high specific activity against 5-HCAld (Maury et al. 1999; Nakatsubo et al. 2008; Parvathi et al. 2001; Zubieta et al. 2002). Furthermore, these dicot CAOMTs show rather high specific activity against 5-HCAlc and CaAld. Together, these results indicated the *bona fide* nature of 5-HCAld OMT (CAldOMT) for dicot CAOMTs.

In contrast, recombinant grass CAOMTs showed diverse substrate specificities. Tall fescue (*F. arundinacea*) CAOMT (FaCOMT1b) showed the highest specific activity towards 5-HFA, followed by CaAld, CaAlc, and 5-HCAld. The enzyme's activities for CA and 5-HCAlc are very weak (Chen et al. 2004). In comparison, perennial ryegrass (*L. perenne*) CAOMT (LpOMT1) showed ca. 7-fold greater activity against CaAlc compared with 5-HFA (Louie et al. 2010). However, wheat (*T. aestivum*) CAOMT (TaCM) has a strong activity only towards 5-HCAld, but only small activities against other substrates, such as CA, CaAld, CaAlc, and 5-HCAlc (Ma and Xu 2008). For rice CAOMT, Lin et al. (2006) have reported that OsCOMT1 has both CA and flavonoid OMT activities and methylates CA, eriodictyol, quercetin, myricetin, and luteolin. They suggested that OsCOMT1 was involved in lignification, although the other phenylpropanoid monomers in the cinnamate/monolignol pathway were not tested.

In the present research, investigation of the kinetic

properties possessed by OsCOMT1 showed that OsCOMT1 exhibited the highest specific activity against 5-HFA, while it retained rather high activity towards 5-HCAld, 5-HCAlc, and CaAlc (at 61.4, 39.8, and 37.8%, respectively compared with 5-HFA in terms of $k_{cat}$ $K_m^{-1}$ values). However, this enzyme showed only weak activity for CaAld and CA (4.6 and 2.9%, respectively). This specific activity spectrum is similar to those of several dicot CAldOMTs (*Populus tremuloides* CAldOMT, Li et al. 2000; *Arabidopsis thaliana* CAldOMT, Nakatsubo et al. 2008; and *C. tinctorius* CAldOMT, Nakatsubo et al. 2007) and a grass CAOMT (TaCM) from *T. aestivum* (Ma and Xu 2008), but not another grass CAOMT (FaCOMT1b) from tall fescue (*F. arundinacea*, Chen et al. 2004).

The similarity of OsCOMT1 to dicot CAldOMTs was also observed in the inhibition kinetics. Although 5-HFA showed the highest specific activity, OsCOMT1-catalyzed methylation of the compound was inhibited by 5-HCAld. On the other hand, the OsCOMT1-catalyzed methylation of 5-HCAld was not inhibited by 5-HFA. Similar inhibition of 5-HFA by 5-HCAld has also been observed for PtreCAldOMT (Li et al. 2000) and CtCAldOMT1 (Nakatsubo et al. 2007). In addition, the acid form mitigated the self-inhibition in 5-HCAld methylation. Taken together, these results indicated that this OMT had the character of a 5-HCAld OMT, and therefore it was referred to as OsCAldOMT1 in this report.

The nature of OsCAldOMT1 correlated with the modified lignin structures and gene expression perturbation in the *COMT1*-KD line. The lignin content of this transgenic rice was lower than that of WT (Figure 6), which was mainly ascribed to a reduction in syringyl lignin (Table 3), which was partly compensated for by a significant increase in 5-hydroxyguaiacyl moieties (Figure 7). The observed modification of lignin structures in the *COMT1*-KD line was consistent with the conclusion that OsCAldOMT1 served the role of 5-HCAld methylation *in vivo*. In the *COMT1*-KD line, *F5H1* gene expression as well as *C4H1*, *C4H2*, and *4CL3* expressions were upregulated more than 4-fold (Figure 8), suggesting that homeostatic responses restore the syringyl lignin biosynthesis against the downregulation of the *OsCAldOMT1* gene. These results differ significantly from the case of *AtCAldOMT* (*AtCAOMT*, At5g54160)-downregulation in *A. thaliana*, which resulted in a significant decrease in *AtF5H* (*AtCAld5H*) gene expression together with many genes encoding enzymes in the cinnamate/monolignol pathway (Vanholme et al. 2012).

In conclusion, the function of OsCOMT1 was demonstrated here to be that of a CAldOMT (OsCAldOMT1), and its physiological function to be to catalyze 5-methylation in the biosynthetic pathway to syringyl lignin.

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