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RESEARCH ARTICLE

Functional characterization of caffeic acid *O*-methyltransferase in internode lignification of switchgrass (*Panicum virgatum*)

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Abstract Caffeic acid *O*-methyltransferase (COMT) is a crucial enzyme that mainly methylates phenylpropanoid *meta*-hydroxyl of C_5 in the biosynthesis of syringyl lignin in angiosperms. A putative *COMT*, named as *PvCOMT1*, was isolated from switchgrass (*Panicum virgatum*), a C_4 warm-season dual-purpose forage and bioenergy crop. Our results showed that recombinant PvCOMT1 enzyme protein catalyzed the methylation of 5-OH coniferyl alcohol, 5-OH coniferaldehyde (CAld5H) and 5-OH ferulic acid. Further *in vitro* studies indicate that CAld5H can dominate COMT-mediated reactions by inhibiting the methylation of the other substrates. Transgenic switchgrass plants generated by an RNAi approach were further employed to study the function of *COMT* in internode lignification. A dramatic decrease in syringyl lignin units coupled with an obvious incorporation in 5-OH guaiacyl lignin units were observed in the COMT-RNAi transgenic plants. However, the constitutive suppression of COMT in switchgrass plants altered neither the pattern of lignin deposition along the stem nor the anatomical structure of internodes. Consistent with the biochemical characterization of PvCOMT1, a significant decrease in sinapaldehyde was found in the COMT-RNAi transgenic switchgrass plants, suggesting that CAld5H could be the optimal intermediate in the biosynthesis syringyl lignin.

Keywords biofuel crop, caffeic acid *O*-methyltransferase, forage, lignin, *Panicum virgatum*, switchgrass, transgenic plant

1 Introduction

Lignin present in all vascular plants is a complex phenolic polymer that provides mechanical support, assists in water transport, and protects against abiotic and biotic stress during growth and development[1]. *p*-hydroxyphenyl, guaiacyl (G) and syringyl (S) units are three principal lignin monomers most commonly observed in lignin polymers, which undergo various hydroxylation and methoxylation of their aromatic ring[2]. Over recent decades, the pathway of monolignol biosynthesis has been comprehensively investigated in dicot species[3–5]. However, the lignin biosynthetic pathway has yet to be well characterized in monocots.

Caffeic acid *O*-methyltransferase (COMT), initially designated as a caffeic acid/5-OH ferulate *O*-methyltransferase, is involved in the methoxylation of C_3 and C_5 positions of monolignol precursors in angiosperms[6,7]. However, the substrate specificity of COMT is broad. Recent *in vitro* studies have indicated that the well-characterized COMT can use 5-OH coniferaldehyde (CAld5H), 5-OH coniferyl alcohol (CAlc5H) and 5-OH ferulate (FAc5H) as substrates, but not caffeic acid[8,9]. Moreover, CAld5H has been recognized as an inhibitor of the methylation of caffeic acid and 5-OH ferulate in aspen[10]. Thus, according to the currently accepted model of lignin biosynthesis, COMT efficiently catalyzes the 5-*O*-methylation of CAld5H/CAlc5H into sinapaldehyde/sinapyl alcohol leading to the biosynthesis of S monolignol.

Although another *O*-methyltransferase subfamily member, namely caffeoyl CoA *O*-methyltransferase, is involved in the methoxylation of C_3 of monolignols, individual suppression of COMT can sufficiently reduce the S lignin proportion in plant species, which dramatically affects

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forage quality, bioethanol production and pulping efficiency[9,11,12]. Furthermore, a severe decrease in S/G lignin ratio caused by *COMT* downregulation has been described in transgenic corn and poplar[13,14]. Incorporation of unusual 5-OH G lignins and aberrantly pigmented xylem were observed as other obvious consequences of *COMT* downregulation[13,14].

Disruption of the monolignol biosynthetic pathway can lead to some hydroxycinnamic derivatives, such as *p*-coumaric acid and ferulic acid, being incorporated into lignin and polysaccharides via ester/ether conjugates that make cell walls more resistant to enzymatic hydrolysis[15]. The downregulation of *COMT* in transgenic alfalfa and tall fescue did not reduce the yield of wall-bound hydroxycinnamates[9,16]. In contrast, a significant decrease in wall-bound *p*-coumarate was obtained in *COMT*-deficient mutants of maize (*bm3*)[14,17]. Most strikingly, the wall-bound ferulate levels were elevated in these mutants compared with their corresponding wild-type plants[14,17]. Since little is known about the synthetic pathway of these wall-bound phenolics, it is unclear as to the role of COMT in the conversion of hydroxycinnamates into lignin.

Switchgrass (*Panicum virgatum*), a perennial C_4 warm-season tall grass native throughout North America, has been developed into a dual-purpose forage and bioenergy crop[18]. Lignin is cross-linked to hemicellulose and has a strong negative impact on switchgrass biomass conversion[19]. Previously, we have achieved effective lignin modification by an RNAi-mediated *COMT* downregulation in switchgrass and thereby reduced the resistance to saccharification for conversion of lignocellulosic biomass into ethanol[12]. However, the biochemical characterization of COMT from switchgrass has yet to be comprehensively studied. Moreover, the effects of COMT suppression on the gradual lignification of internodes remains largely elusive in switchgrass. In addition, abundant wall-bound *p*-coumaric and ferulic acids are cross-linked with lignin and hemicellulose, which is a characteristic of grass cell walls[20,21]. These compounds are important for cell wall rigidity and plant defenses[21]. They share the intermediates with monolignol in phenylpropanoid biosynthetic pathway. Thus, it remains to be investigated whether COMT could be involved in their biosynthesis, particularly for ferulic acid.

In the current research, we identified two COMTs, *PvCOMT1* and *PvCOMT2*, from switchgrass genome. Although they shared high amino acid identity, the expression levels of *PvCOMT2* was much lower than those of *PvCOMT1*. Thus, we further studied the biochemical characterization of PvCOMT1 and its function in the process of gradual internode lignification. Our results suggest that CAld5H could be a more efficient substrate of PvCOMT1 in switchgrass. Moreover, downregulation of *COMT* significantly reduced the concentrations of S lignins and wall-bound *p*-coumaric acid in transgenic switchgrass plants; however, the gradual deposition pattern of lignins in internodes was not changed. The comprehensive analysis of COMT from switchgrass will provide the basic information for lignin engineering aimed at improvement of cell wall digestibility of forage and biofuel crops in future.

2 Materials and methods

2.1 Plant materials and transformation

We used a lowland type switchgrass cultivar Alamo for genetic transformation and lignin modification. The development of switchgrass in our greenhouse was divided into four elongation stages (E1 to E4) and reproductive stages (R1 to R5) according to the criteria described by Hardin et al.[22].

A highly embryogenic callus line with single genotype generated by large scale screening of switchgrass Alamo seed-derived calli were used for *Agrobacterium*-mediated transformation following the procedure described by Xi et al.[23]. T1 generation plants were obtained by crossing T0 transgenics with a wild-type Alamo plant. Transgenic plants were grown in the greenhouse at 26°C with 16 h light (390 $\mu E \cdot m^{-2} \cdot S^{-1}$).

2.2 Cloning and molecular characterization COMTs from switchgrass

Two COMT isoforms, *PvCOMT1* and *PvCOMT2*, were found by blasting the maize *COMT* sequence against the Switchgrass Genome Database v1.1. The expression pattern of *PvCOMT1* and *PvCOMT2* were retrieved using the Switchgrass Gene Expression Server. The transcript abundances of *PvCOMT1* were analyzed by quantitative RT-PCR (qRT-PCR) as described by Fu et al.[12]. The primers used for qRT-PCR were listed in Table S1. *PvCOMT1* was isolated from switchgrass stem tissues by RT-PCR and was subjected to sequencing. Alignment of multiple sequences and phylogenetic tree analysis were performed using the MEGA 5 software suite.

2.3 Expression of recombinant PvCOMT1 proteins

The open reading frame of *PvCOMT1* was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from cDNA samples of switchgrass internodes. An *Eco*R I enzyme site before the start codon and a *Hin*d III site after the stop codon were introduced by primers. The amplified sequence was cloned into pMAL-C2X vector downstream from a *malE* gene, which encodes maltose binding protein as a purification tag (New England Biolabs, Ipswich, MA, USA). The accuracy of insertion was confirmed by sequencing. The engineered construct was transferred

into host bacterial stain TB1. The induction, expression and purification of recombinant PvCOMT1 proteins were conducted as described in the manual of pMAL™ protein fusion and purification system (New England Biolabs, #E8000S).

2.4 Enzyme activity assay

The concentration of purified recombinant PvCOMT1 protein was measured by the Bradford method[24]. COMT activity assay was performed as described by Liu et al.[25]. According to previous studies[10,12], COMT can catalyze hydroxycinnamyl alcohol, hydroxycinnamyl aldehyde, and hydroxycinnamate into their corresponding methylated products. Thus, CAlc5H, CAld5H and FAc5H were employed for enzyme activity assay of recombinant PvCOMT1. The corresponding methylated products were analyzed by high-performance liquid chromatography photodiode array (HPLC-PDA) and monitored at 265, 345 and 325 nm for sinapyl alcohol, sinapaldehyde and sinapic acid, respectively.

COMT activity in crude plant extracts was measured using CAlc5H, CAld5H and FAc5H as described by Liu et al.[25]. Hydroxycinnamyl aldehyde-induced inhibition in COMT-mediated reaction was conducted as described by Li et al.[10]. CAld5H and FAc5H were sourced from the University of North Texas, Denton, USA. The reference standard of CAlc5H was synthesized by the Chemistry Research Solution LLC (Bristol, PA, USA). Sinapyl alcohol, sinapaldehyde, and sinapic acid were obtained from Sigma-Aldrich company (St. Louis, MO, USA).

2.5 Microarray analysis

The tillers at R1 stage were collected from three COMT RNAi-positive and-negative (null sergeant) progeny. The internodes (I2 to I4) were separated by removing leaf, sheath and node. RNA extraction and purification, probe labeling, hybridization, and scanning for microarray analysis were conducted as previously described by Fu et al.[26]. The most likely monolignol biosynthetic genes were selected[27]. Their expression patterns were clustered using Spotfire software (TIBCO Software Inc., Palo Alto, CA, USA). The transcript abundances of *COMT* and other monolignol related genes were validated by qRT-PCR as described by Fu et al.[12]. The data were normalized using the levels of switchgrass *Ubq1* was used as the reference for normalization.

2.6 Histochemical assay

The internodes collected from the same stem at the R1 stage were cut into 0.5 cm sections with a razor blade and photographed immediately with an Olympus *SZX*12-Fluorescent Stereo Microscope system (Olympus, Tokyo, Japan) for phenotypic characterization of transgenic plants. For histochemical characterization, the Mäule staining of lignin was performed as described previously[28]. The micrographs were taken under a Nikon Microphot-FX system with a Nikon DXM 1200 color camera.

2.7 Determination of S and 5-OH G lignin units

The individual internodes (basal to distal, I1 to I4) were harvested at the R1 stage. The samples were ground in liquid nitrogen and lyophilized. Lyophilized extractive-free material was used for lignin analysis. The thioacidolysis method was used to determine lignin composition[29]. S and 5-OH G lignin units were identified and quantified by gas chromatography-mass spectrometry using a Hewlett-Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column HP-1, 60 m × 0.25 mm, film thickness 0.25 μm).

2.8 Determination of wall-bound and soluble phenolics

An alkaline hydrolysis procedure described by Shen et al.[28] was used to release wall-bound phenolics from switchgrass cell walls. The ester- and ether-linked phenolics were recovered by high-temperature hydrolysis (4.0 mol·L^{-1} NaOH, 121°C, 4 h). Standard solutions of *p*-coumaric and ferulic acids were prepared, and analyzed together with the above samples by HPLC-PAD. The UV-absorbing metabolites were monitored at 325 nm. Total soluble phenolics content was determined by using the Folin-Ciocalteu method[30]. The methanolic extracts was further analyzed by LC-PDA/ESI-MS/MS, and sinapaldehyde was identified by comparing its retention time, UV-visible and mass spectra with the corresponding standard compounds[31].

2.9 Statistical analysis

Samples were collected from three biological replicates of each transgenic line. The mean values were used for statistical analyses. Data from each trait were subjected to Student's *t*-test. The significance of treatments was tested at the $P = 0.05$ level. Standard errors were provided in all tables and figures as appropriate.

3 Results

3.1 Switchgrass *COMT* encodes a CAld5HOMT

Two COMT isoforms, *PvCOMT1* and *PvCOMT2*, occur on switchgrass chromosomes 2 and 6, respectively. Sequence alignment revealed that PvCOMT1 protein (Pavir.Fa01907) shared 99% sequence identities with previously isolated switchgrass COMT (ADX98508) and

84% identities with PvCOMT2 (Pavir.Ba00498). Phylogenetic tree analysis showed that PvCOMT1 and PvCOMT2 clustered together in a group containing the typical functional COMTs (Fig. 1a). Gene atlas analysis indicated that *PvCOMT1* signal intensity was about 200-fold higher in some tissues than that of *PvCOMT2* (Fig. 1b). Therefore, we isolated the full length cDNA sequences of *PvCOMT1* from switchgrass for further functional investigation.

3.2 CAld5H inhibits the methylation of CAlc5H and FAc5H

Enzyme activity assay showed that PvCOMT1 had capacity to use CAlc5H, CAld5H, and FAc5H as substrates. To distinguish these three putative 5-*O*-methylation pathways, enzymatic activities of recombinant PvCOMT1 against a mixture of equal molar CAld5H to CAlc5H were measured by HPLC. Our results revealed that the recombinant PvCOMT1 exhibited comparable turnover efficiency for individual CAlc5H or CAld5H (Fig. 2a). The enzymatic methylation of CAlc5H, however, was dramatically reduced, whereas methylation of CAld5H was not affected in reactions of PvCOMT1 with mixed substrates (Fig. 2a). In contrast, sinapaldehyde did not inhibit the methylation of CAlc5H (data not shown), suggesting that the inhibition of the methylation of CAlc5H resulted from CAld5H, rather than its corresponding product. Moreover, both CAld5H and CAlc5H strongly inhibited the methylation of FAc5H (Fig. 2a).

To test whether the CAld5H/AldOMT modulation observed for recombinant PvCOMT is a part of a regulation mechanism in switchgrass, the raw plant protein extracts from the stems at E4 stage were tested for COMT activity with mixed substrates, CAld5H, CAlc5H and FAc5H. These soluble plant protein extracts showed competitive inhibition of methylation activity with CAlc5H and FAc5H when CAld5H was present (Fig. 2b). In both cases, methylation of CAld5H was still the dominant reaction.

3.3 *COMT* downregulation in switchgrass did not change lignification pattern and tissue structures of internodes

The previously generated transgenic switchgrass line, TCOMT2, with strongly downregulated *COMT* was outcrossed with a wild-type plant[12]. To study the detailed effects of *COMT* downregulation on lignin deposition, the internodes (I1 to I4) of R1 stage representing the gradual process of cell wall lignification were collected from both COMT RNAi positive and negative (null sergeant) plants identified among the resultant progeny. Cross sections of the internodes were stained with Mäule reagent for histochemical observation (Fig. 3). In the control internode, an apparent red coloration, diagnostic for S lignins,

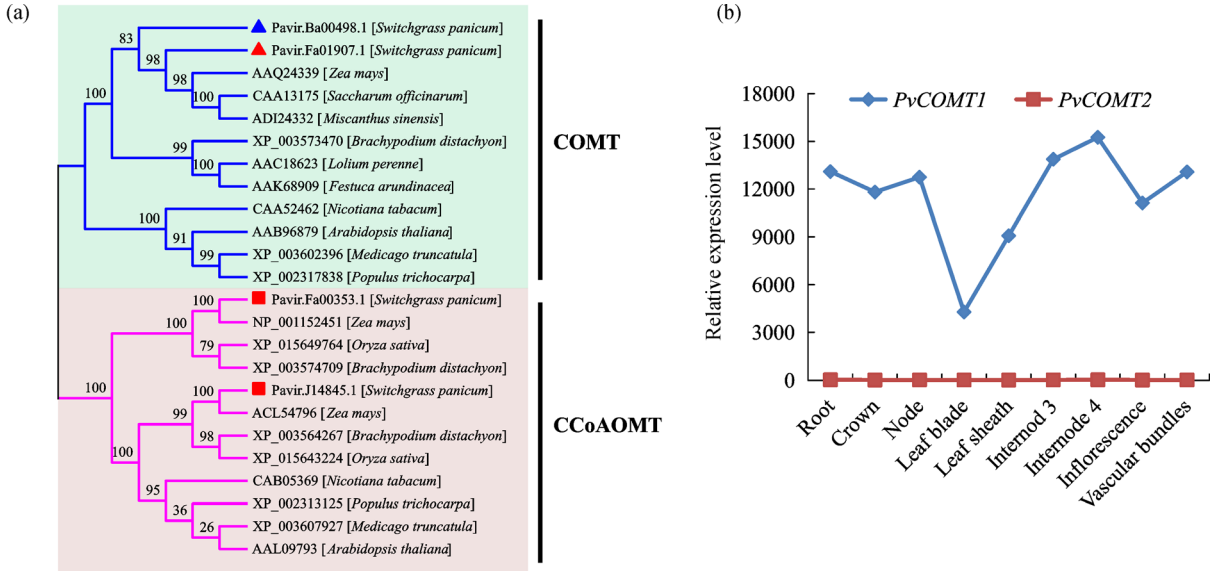

Fig. 1 Identification of caffeic acid *O*-methyltransferases from the switchgrass genome. (a) Phylogenetic tree analysis of plant COMT (caffeic acid *O*-methyltransferase) and CCoAOMT (caffeoyl CoA *O*-methyltransferase) protein sequences. Switchgrass PvCOMTs and other members of the *O*-methyltransferases (OMT) family in *Arabidopsis thaliana*, *Medicago truncatula*, *Nicotiana tabacum*, *Populus trichocarpa*, *Brachypodium distachyon*, *Festuca arundinacea*, *Festuca tabacum*, *Lolium perenne*, *Miscanthus sinensis*, *Oryza sativa*, *Saccharum officinarum* and *Zea mays*. GenBank accession numbers are shown after species names. Phylogenetic tree of deduced OMT amino acid sequences constructed by using the neighbor-joining method. Bootstrap values (%) based on 1000 replications are indicated at nodes; (b) gene expression analysis of *PvCOMT1* and *PvCOMT2*. The data was downloaded from Switchgrass Functional Genomics Server.

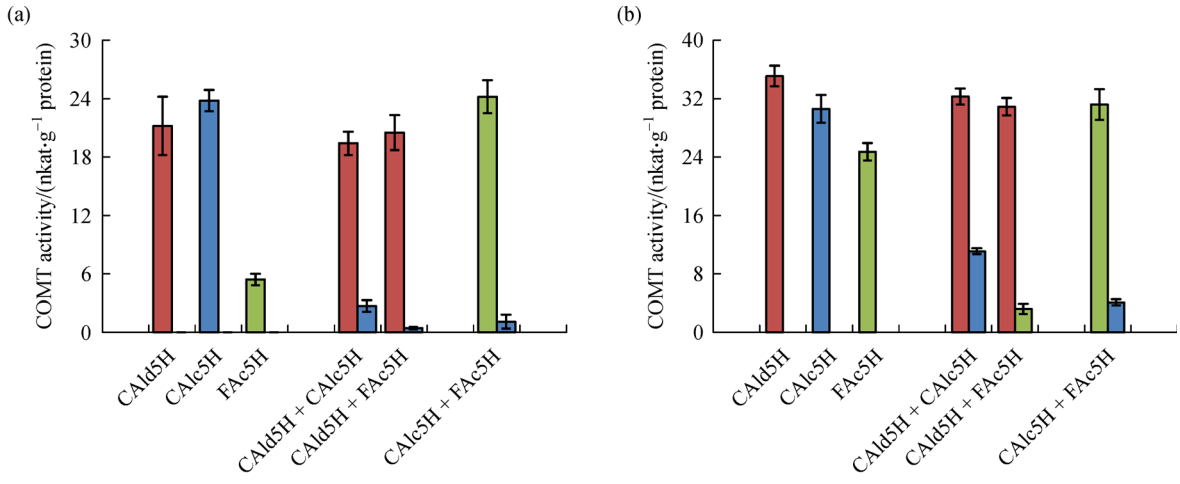

Fig. 2 Effects of 5-OH coniferaldehyde on the *O*-methyltransferase activity of recombinant PvCOMT (a) and extractable protein from switchgrass internodes (b). CAld5H, 5-OH coniferaldehyde; CAlc5H, 5-OH coniferyl alcohol; FAc5H, 5-OH ferulic acid. Values are means±SE (n = 3).

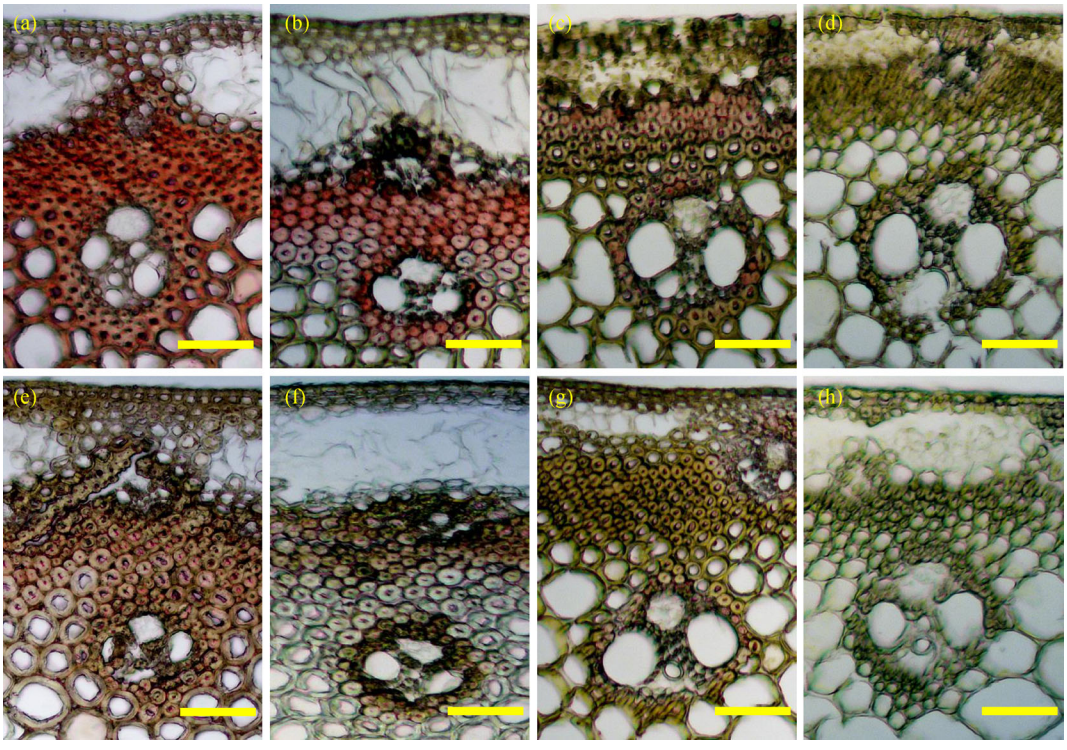

Fig. 3 Mäule staining of internodes cross sections (basal to distal) of the stems of control (a–d) and transgenic (e–h) switchgrass plants. Bar = 0.1 mm.

was especially evident in the well-lignified cells, such as fiber, sclerenchyma and vascular bundle (Fig. 3a–3d). In contrast, the staining was substantially reduced in the internodes of transgenic plants. The structure of vascular bundle in transgenic plants, however, resembled that of the control plants (Fig. 3e–3g). In addition, the staining of S lignins in both the control and transgenic plants was gradually enhanced from the upper internode (I4) to the basal one (I2).

Due to the low sensitivity of Mäule staining, the consequences of *COMT* downregulation on the biosynthesis of S lignin were evaluated further by chemical analysis. The gradual increase in S and 5-OH G lignin deposition in control plants indicated a strictly regulated

process of cell wall lignification during internode development (Fig. 4). In contrast, the internodes of transgenic plants had a strong reduction in S lignin units and a significant increase in 5-OH G lignin units; however, a similar lignification pattern along the internodes occurred in these plants (Fig. 4).

3.4 Effects of *COMT* downregulation on the expression of other lignin genes

As expected, the transcript levels of *COMT* gene were heavily reduced in the transgenic progeny. Compared with control plants, the transcript levels of target gene were reduced by up to 91% in well-lignified internodes (I2 and I3) of the transgenic plants. More *COMT* transcripts (23% of control plants) remained in the immature internode (I4) (Fig. 5a). This suggests that a relative low efficacy of RNAi-mediated transcript degradation exists in the tissue under active lignification. Since *COMT* downregulation differed in the internodes, there could be different effects on the expression patterns of other genes in the monolignol biosynthetic pathway. Thus, the genes transcript abundances in TCOMT2 transgenic switchgrass progeny were investigated for all internodes by microarray analysis. Transcript abundance of 1040 probe sets were altered on the chip of all internodes; 308 were upregulated and 732 downregulated in transgenic plants (Fig. 5b). The expression patterns of monolignol-related genes clustered into five groups. All genes, except *CCR2* and four *ZRP4*_like genes, showed little effects from the downregulation of *COMT*. Notably, *CCR2* (*Ap13CTG15044*) and one *ZRP4*_like (*KanlowSLT49902*) gene had a similar expression pattern and showed significantly increased transcript abundance in the mature internodes (Fig. 5c).

3.5 Downregulation of *COMT* alters wall-bound phenolics incorporation

Besides lignin polymers, switchgrass secondary cell walls contain wall-bound *p*-coumaric and ferulic acids. These hydroxycinnamates cross-link to polysaccharides and/or lignin by ester/ether bonds in grass cell walls. The concentrations of wall-bound (ester and ether) *p*-coumaric acid in control plants exhibited a maturation-related increase along the internodes (Table 1). It is coincident with the process of lignin deposition in the internodes that suggests some underlying correlations between them. In contrast, no correlation between lignin and wall-bound ferulate was observed in the internodes in the wild-type plants. Compared with control plants, a significant reduction of wall-bound *p*-coumaric acid was obtained in the internodes of transgenic plants (Table 1). Notably, similar quantities of esterifies/etherified ferulate were released from the internodes of control and transgenic plants.

3.6 Downregulation of *COMT* alters soluble phenolics accumulation

To determine whether *COMT* downregulation had any effect on soluble phenolics other than lignin and wall-bound phenolics, total phenolics were extracted with 50% methanol from the internodes, and estimated using Folin-Ciocalteu reagent. A significant increase in total phenolics concentration was revealed in the methanolic extracts of transgenic plants (Table 1). To obtain a better insight into the levels and composition of phenolics, a liquid chromatography integrated with tandem mass spectrometer and photo diode array (LC-MS/MS-PDA) analysis was performed to profile metabolites in the extracts previously analyzed. Two peaks detected in the methanolic extracts of internodes were characterized as chlorogenic acid and sinapaldehyde with maximum absorptions at 325 and 330 nm, respectively. In control and transgenic plants, chlorogenic acid biosynthesis was gradually enhanced from I4 to I1, while sinapaldehyde exhibited an inverse pattern. However, transgenic plants accumulated higher amounts of chlorogenic acid and sinapaldehyde than control plants (Table 1).

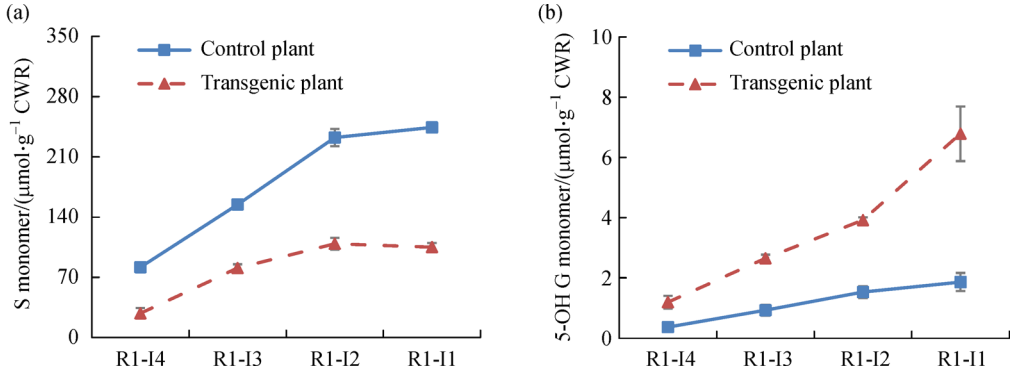

Fig. 4 Lignification pattern of internodes in control and transgenic switchgrass plants. Yields of syringyl (S) lignin (a) and 5-OH guaiacyl (G) lignin (b) units determined by gas chromatography-mass spectrometry. Values are means±SE (n = 3).

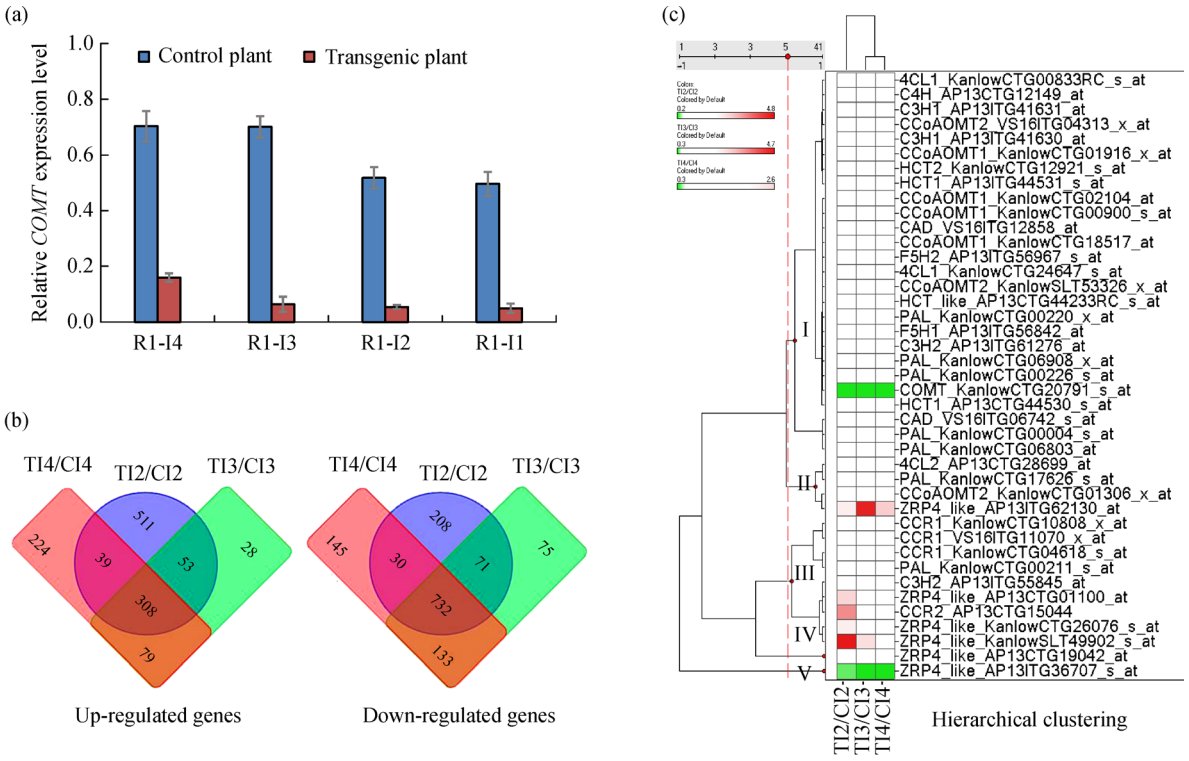

Fig. 5 Microarray analysis of caffeic acid *O*-methyltransferase (COMT)-RNAi transgenic switchgrass plants. (a) Transcript abundance of *COMT* gene in different internodes of transgenic plants revealed by qRT-PCR. Switchgrass *Ubq1* was used as the reference for normalization. Values are means±SE ($n = 3$); (b) the number of altered probe sets in microarray chips; (c) hierarchical cluster analysis of differentially expressed lignin genes in different internodes of control and transgenic switchgrass.

Table 1 Wall-bound and soluble phenolics accumulation (dry matter) in transgenic switchgrass plants (mg·g^{-1})

Plant sample	Wall-bound phenolics				Soluble phenolics		
	Ester-linked		Ether-linked		Total phenolics	Chlorogenic acid	Sinapaldehyde
	p-CA	FA	*p*-CA	FA			
Control plants							
R1-I4	7.1±0.3^{c}	4.2±0.7^{a}	1.7±0.2^{c}	2.7±0.5^{a}	2.11±0.16^{a}	0.306±0.020^{a}	0.0040±0.0007^{c}
R1-I3	13.8±0.3^{b}	5.3±0.9^{a}	3.9±0.1^{b}	3.7±0.2^{a}	1.53±0.03^{b}	0.150±0.009^{b}	0.0074±0.0011^{b}
R1-I2	17.2±0.4^{ab}	4.2±0.3^{a}	5.4±0.3^{a}	3.6±0.3^{a}	1.56±0.07^{b}	0.095±0.006^{c}	0.0086±0.0007^{b}
R1-I1	20.0±0.9^{a}	4.4±0.9^{a}	5.8±0.2^{a}	3.4±0.2^{a}	1.32±0.06^{c}	0.075±0.003^{d}	0.0137±0.0005^{a}
Transgenic plants							
R1-I4	4.8±0.2^{c}	4.2±0.5^{a}	1.0±0.1^{c}	2.6±0.3^{a}	3.14±0.32^{a}	0.424±0.023^{a}	0.0021±0.0002^{c}
R1-I3	11.3±0.1^{b}	5.1±0.5^{a}	1.6±0.1^{b}	2.6±0.3^{a}	1.93±0.07^{c}	0.189±0.013^{b}	0.0049±0.0003^{b}
R1-I2	12.5±0.2^{a}	4.0±0.4^{a}	1.7±0.3^{b}	2.8±0.2^{a}	2.34±0.03^{b}	0.118±0.003^{c}	0.0052±0.0002^{b}
R1-I1	12.3±0.5^{a}	4.4±0.9^{a}	2.5±0.2^{a}	2.9±0.4^{a}	3.11±0.15^{a}	0.129±0.019^{c}	0.0074±0.0004^{a}

Note: Stems at the R1 stage were collected and the different internodes were separated. Ester-linked phenolic compounds were released with 2 mol·L^{-1} NaOH at room temperature. Ether-linked phenolic compounds were released with 4 mol·L^{-1} NaOH under pressure and high temperature. Values are means±SE ($n = 3$). Means with the different letter are significantly different (one-way ANOVA, Duncan's test, $P < 0.05$). *p*-CA, *p*-coumaric acid; FA, ferulic acid.

4 Discussion

Previous studies on lignin modification have demonstrated that COMT is a key enzyme involved in the biosynthesis of S lignin precursors[6,7,9,11]. COMT suppression can substantially increase forage digestibility and bioethanol production with less alteration in cell wall size and plant biomass[5]. Manipulation of lignin biosynthesis in switchgrass was successfully performed by RNAi-mediated *COMT* downregulation, and improves neutral detergent

fiber digestibility by up to 11% and increases ethanol production by up to 38% compared with control plants. Moreover, the transgenic switchgrass materials require three to four times less cellulase loading for cellulose conversion than control plants for equivalent ethanol yields[12]. To further understand the function of the switchgrass COMT gene, the kinetic parameters were determined for recombinant PvCOMT, and the overall effects of COMT suppression on relative phenylpropanoid metabolites, such as lignin, wall-bound and soluble phenolics, were investigated in internodes at different development stages along individual stems.

Switchgrass COMT more efficiently used CAl5H as an *in vitro* substrate compared to CAlc5H and FAc5H. This supports the 5-*O*-methylation function of plant COMTs previously reported[8,9]. Moreover, CAld5H was both a superior substrate for COMT as well as an inhibitor of FAc5H methylation. Notably, CAlc5H gave similar inhibition for FAlc5H methylation. Furthermore, down-regulation of *COMT* in switchgrass resulted in a significant decrease in sinapaldehyde, suggesting that CAl5H is a superior *in vivo* substrate for PvCOMT1. This is consistent with the fact that hydroxycinnamyl aldehyde can be converted into hydroxycinnamic acid and hydroxycinnamyl alcohol by aldehyde dehydrogenase and cinnamyl alcohol dehydrogenase, respectively, in the cytoplasm of plant cells[32].

Constitutive suppression of COMT in transgenic switchgrass plants led to a substantial decrease in S lignin units and a dramatic increase in 5-OH G lignin units. Similar observations have been reported in *Arabidopsis*[33], poplar[12] and maize[13]. However, our results further reveal that constitutive suppression of COMT in switchgrass was not able to alter the lignification pattern of internodes, suggesting that lignin biosynthesis is regulated by a complex network. In addition, alkaline hydrolyzed *p*-coumaric and ferulic acids were the major wall-bound phenolic acids in grass cell walls[14]. Downregulation of *COMT* had effects on the ester and ether-linked *p*-coumaric acid, but not the wall-bound ferulic acid. The decrease in esterified/etherified *p*-coumaric acid yield in transgenic plants reflects an indirect effect of the reduced amount of S lignin units[22]. Similar results were reported for maize *bm3* mutants[13]. In contrast, slight variation in concentrations of wall-bound ferulic acid suggests that *COMT* downregulation had little effect on the biosynthesis of ferulic acid in transgenic switchgrass. Although the biosynthetic pathway of ferulic acid has not been elucidated, other *O*-methyltransferases or flexible phenylpropanoid biosynthetic pathways could be supposed to contribute to its biosynthesis in switchgrass. Beside wall-bound phenolics, the impaired lignin biosynthesis was also associated with increased total phenolic extractives from COMT-RNAi transgenic switchgrass plants. The enhanced soluble phenolics yield implicated a probable shift from monolignol biosynthesis to other phenylpropanoid metabolism. Similar alterations in soluble phenolics have been described in *COMT*-deficient *Arabidopsis*[33], poplar[34] and *Leucaena*[35].

5 Conclusions

The biochemical characterization of switchgrass COMT was investigated by measuring the kinetic properties of recombinant PvCOMT1, and the effects of COMT suppression on internode structure and lignification pattern were studied in COMT-RNAi transgenic switchgrass plants. Our experimental evidences indicate that constitutive suppression of COMT in switchgrass can dramatically reduce the biosynthesis of S lignins, but does not change lignification pattern of internodes. Besides impaired lignin biosynthesis, a shunt in the phenylpropanoid pathway led to altered wall-bound and soluble phenolics accumulation in transgenic switchgrass plants. The basic information established in this research provides an understanding of the comprehensive effects of *COMT* downregulation on the derivatives from the phenylpropanoid pathway including lignin, wall-bound and soluble phenolics. Thus, our work will facilitate evaluation and inspection of the impacts of lignin gene regulation on cell wall lignification and digestibility of genetic modified forage and biofuel crops in future.

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