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ORIGINAL ARTICLE

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At5g54160 gene encodes *Arabidopsis thaliana* 5-hydroxyconiferaldehyde *O*-methyltransferase

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Abstract The function of an *Arabidopsis thaliana* gene, At5g54160 annotated as a caffeic acid *O*-methyltransferase CAOMT gene was characterized. The recombinant enzyme of this gene (*AtOMT1*) catalyzed the *O*-methylation of phenylpropanoid and flavonoid substrates. The specificity constants (k_{cat}/K_m) for 5-hydroxyconiferaldehyde (5-HCAld) and quercetin were both 0.11 $\mu M^{-1} \cdot min^{-1}$. On the other hand, lignins of At5g54160-knockout *Arabidopsis* mutants lacked syringyl units. In addition, we showed that the gene silencing also resulted in significant accumulation of caffeyl alcohol (CaAlc). These results strongly suggested that At5g54160 gene is involved in syringyl lignin synthesis for the methylation of both 5-hydroxyconiferaldehyde and 3,4-dihydroxyphenyl compound(s).

Key words At5g54160 · 5-Hydroxyconiferaldehyde *O*-methyltransferase · Lignin

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Introduction

Caffeic acid *O*-methyltransferase (CAOMT) was originally reported to be the methyltransferase responsible for the methylation of the 3-hydroxyl group of caffeic acid (CA) and the 5-hydroxyl group of 5-hydroxylferulic acid (5-HFA). This notion had long been widely accepted.[1] However, this was later challenged by the reports on the biochemical analysis of its recombinant protein.[2,3] Thus, *bona fide* substrate of this enzyme was identified as 5-HCAld, indicating its specific role in sinapyl alcohol (SAlc) and syringyl lignin biosyntheses (Fig. 1).[2,3] Therefore, the enzyme was renamed as 5-hydroxyconiferaldehyde OMT (CAldOMT).[3]

Following the completion of the *Arabidopsis thaliana* genome sequence, the identification of the *bona fide* functions of annotated genes has become a central subject in plant bioscience. At5g54160 was annotated as a *CAOMT* gene based on its high sequence homology to *Populus tremuloides CAOMT*.[4] Interestingly, however, it was reported that a recombinant protein of the gene expressed in *Escherichia coli* methylated the flavonoids, quercetin, myricetin, and luteolin, but not CA.[5] Later, an *Arabidopsis* knockout mutant of this gene was subjected to lignin characterization.[6] In this mutant, lignins lacked syringyl units, and contained more 5-hydroxyguaiacyl units.[6] Therefore, the expression product of At5g54160 gene appeared to be involved in syringyl lignin formation. This result suggested that the gene encoded AtCAldOMT, which did not totally agree with the previous report.[5]

A. thaliana has been the subject of intense plant functional genomics, and firm identification of the gene encoding CAldOMT involved in lignin biosynthesis is a prerequisite for network analysis of plant metabolisms including cell wall formation and lignification. In this article, we report that At5g54160 gene encodes an OMT with dual functions, CAldOMT and flavonoid OMT activities. We also show that the gene is involved in syringyl lignin biosynthesis in *A. thaliana*.

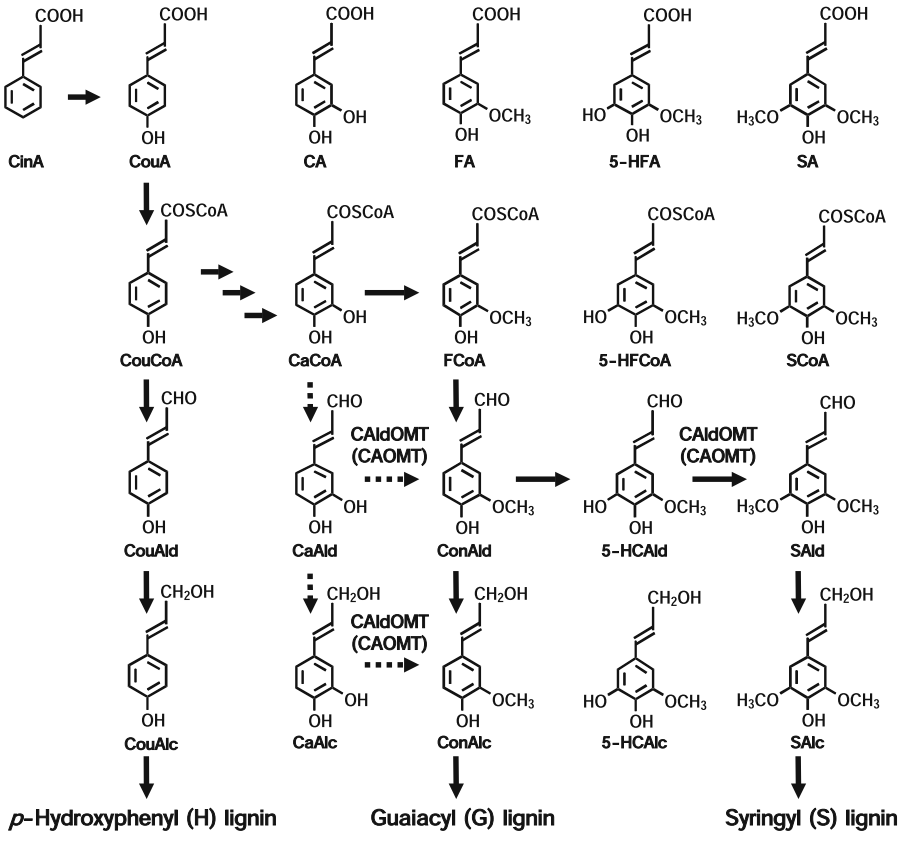

Fig. 1. The cinnamate/monolignol pathway. *Bold arrows* indicate the lignin biosynthetic pathway that has been accepted and *dotted arrows* indicate steps suggested by this work. *CinA*, cinnamic acid; *CouA*, *p*-coumaric acid; *CA*, caffeic acid; *FA*, ferulic acid; *5-HFA*, 5-hydroxyferulic acid; *SA*, sinapic acid; *CouCoA*, *p*-coumaroyl CoA; *CaCoA*, caffeoyl CoA; *FCoA*, feruloyl CoA; *5-HFCoA*, 5-hydroxyferuloyl CoA; *SCoA*, sinapoyl CoA; *CouAld*, *p*-coumaraldehyde; *CaAld*, caffealdehyde; *ConAld*, coniferaldehyde; *5-HCAld*, 5-hydroxyconiferaldehyde; *SAld*, sinapaldehyde; *CouAlc*, *p*-coumaryl alcohol; *CaAlc*, caffeyl alcohol; *ConAlc*, coniferyl alcohol; *5-HCAlc*, 5-hydroxyconiferyl alcohol; *SAlc*, sinapyl alcohol

Experimental

Instrumentation

Gas chromatography-mass spectrometry (GC-MS) was performed with a Shimadzu QP-5050A GC-MS system [electron-impact mode (70 eV); column, Shimadzu Hicap CBP10-M25-025 column (20 m × 0.22 mm); carrier gas, helium; injection temperature, 240°C; column temperature (for enzyme reaction products, 40°C at t = 0 to 2 min, then to 230°C at 25°C/min; for lignin degradation products, 40°C at t = 0 to 2 min, then to 230°C at 40°C/min)]. Direct-inlet mass spectrometry (DI-MS) was conducted with a JMS-DX303HF mass spectrometer (JEOL) equipped with a JMA-DA 5000 mass data system (JEOL) (electron impact mode, 70 eV) ^{1}H Nuclear magnetic resonance (NMR) spectra were obtained with a JNM-LA400MK FT NMR system (JEOL). Chemical shifts and coupling constants (J) were reported in δ and hertz, respectively. Liquid chromatography-mass spectrometry (LC-MS) was carried out with a Shimadzu LC/MS-2010A single quadrupole mass spectrometer using electrospray ionization (ESI). LC separation was achieved using a hydrosphere column C18 (10 cm × 2 mm, particle size 3 μm, YMC) maintained at 40°C. The mobile phase consisted of 0.1% HCOOH (A) and methyl alcohol (MeOH) (B) with a linear gradient elution from 90% A/10% B at t = 0 to 3 min, and then to 10% A/90% B at t = 23 min, this latter composition being held for an additional 5 min. The flow rate was 0.25 ml/min. The curve dissolution line (CDL) temperature and the heat block temperature were maintained at 230°C and 200°C, respectively. The CDL voltage and detector voltage were fixed at 4.5 kV, –30 V, and 1.2 kV, respectively.

Chemicals

Quercetin, 7-*O*-methylquercetin (rhamnetin), 3′-*O*-methylquercetin (isorhamnetin), and 4′-*O*-methylquercetin (tamarixetin) were purchased from Tokyo Chemical and Extrasynthese. 3-*O*-Methylquercetin and 5-*O*-methylquercetin (azaleatin) were synthesized from quercetin according to the method reported by Bouktaib et al.[7]

3-*O*-Methylquercetin: ^{1}H NMR (400 MHz, acetone-d_6) δ 3.79 (3H, s, OCH_3), 6.18 (1H, d, J = 1.7, C_6H), 6.41 (1H, d, J = 1.7, C_8H), 6.92 (1H, d, J = 8.5, $C_{5'}H$), 7.51 (1H, dd, J = 8.4, 1.8, $C_{6'}H$), 7.62 (1H, d, J = 2.2, $C_{2'}H$). MS m/z 316 (M^+) 315, 301, 287, 273, 203, 187, 153, 144, 137.

5-*O*-Methylquercetin: ^{1}H NMR (400 MHz, acetone-d_6) δ 3.82 (3H, s, OCH_3), 6.37 (1H, d, J = 2.2, C_6H), 6.53 (1H, d, J = 2.2, C_8H), 6.90 (1H, d, J = 8.5, $C_{5'}H$), 7.57 (1H, dd, J = 8.5, 2.2, $C_{6'}H$), 7.70 (1H, d, J = 2.2, $C_{2'}H$). MS m/z 316 (M^+) 315, 298, 287, 270, 137.

Ferulic acid-d_3 (FA-d_3), sinapic acid-d_3 (SA-d_3), coniferaldehyde-d_3 (ConAld-d_3), sinapaldehyde-d_3 (SAld-d_3), coniferyl alcohol-d_3 (ConAlc-d_3), and SAlc-d_3 were prepared previously.[8] Caffeoyl CoA (CaCoA) and 5-hydroxyferuloyl CoA (5-HFCoA) were prepared by the method of Stöckigt and Zenk.[9]

Expression of recombinant AtOMT1 in *E. coli*

Polymerase chain reaction (PCR) was used to introduce the *Nde*I site at the 5′-end and *Not*I site at the 3′-end of the coding sequence of At5g54160 (Accession no. U70424) encoding the AtOMT1 polypeptide using a sense primer (5′-TCATATGGGTTCAACGGCAGAG-3′) and an antisense primer (5′-TGCGGCCGCGAGCTTCTTGAGTA ACTCAA-3′). The PCR product was first cloned into a pCR2.1 vector (Invitrogen). After confirming sequence accuracy, the *AtOMT1* coding region was then cloned into a pET-23 (Novagen) expression vector to fuse a His-tag at the C-terminal of the cloned sequence. The construct was transferred into *E. coli* BL21 (DE3) cells (Novagen). The induction and expression of recombinant AtOMT1 were conducted according to Li et al.[3] The BL21 (DE3) cell strain containing pET-23 vector without an *AtOMT1* cDNA insert was used as the control. After harvesting by centrifugation (2000 *g* for 10 min), the cell pellet was processed for affinity purification of AtOMT1 protein using the His·Bind Resin affinity purification system (Novagen) according to manufacturer's protocol. Protein concentration was determined using the Bradford method[10] with bovine serum albumin as a standard protein.

CAldOMT activity of recombinant AtOMT1

The activity of the recombinant AtOMT1 was investigated in vitro using the following phenylpropanoid substrates: CA, 5-HFA, CaCoA, 5-HFCoA, caffealdehyde (CaAld), 5-HCAld, CaAlc, and 5-hydroxyconiferyl alcohol (5-HCAlc). For the determination of the pH optimum of purified AtOMT1, assays were conducted in 50 mM potassium phosphate (pH 6.0–8.5) and 50 mM Tris-HCl (pH 7.5–9.0) buffers, each containing 2 mM $MgCl_2$, 200 μM *S*-adenosyl-L-methionine (SAM), 2.0 μg of purified recombinant AtOMT1 protein, and 100 μM of substrate. Each reaction mixture (200 μl) was incubated at 30°C for 1 h. When CA and 5-HFA were incubated individually, the reaction was stopped by the addition of 200 μl 2 N HCl, and the product was extracted with 500 μl ethyl acetate (EtOAc) containing an internal standard (1.25 μg, each of FA-d_3 and SA-d_3 for CA and 5-HFA, respectively). In the individual incubation of CaCoA and 5-HFCoA, the reaction was stopped by the addition of 15 μl of 5 N NaOH, and CoA esters were hydrolyzed by incubating the reaction mixture at 40°C for 15 min. Then the reaction mixture was acidified by the addition of 240 μl 2 N HCl, and extracted with 500 μl EtOAc containing an internal standard (1.25 μg, each of FA-d_3 and SA-d_3 for CaCoA and 5-HFCoA, respectively). When CaAld, 5-HCAld, CaAlc, and 5-HCAlc were incubated individually, the reaction was stopped by adding 500 μl EtOAc containing an internal standard (1.25 μg, deuterium-labeled compound corresponding to the reaction product). The EtOAc extracts were dried and dissolved in *N,O*-bis(trimethylsilyl) acetamide (BSA) (6 μl). After standing at 60°C for 45 min, an aliquot of the solution was subjected to GC-MS analysis, and the products were identified and quantified.

Flavonoid OMT activity of recombinant AtOMT1

The activity of the recombinant AtOMT1 was investigated in vitro using the flavonoid, quercetin, as a substrate. For the determination of the pH optimum of purified AtOMT1, assays were conducted in 50 mM potassium phosphate (pH 6.0–8.5) and 50 mM Tris-HCl (pH 7.5–9.0) buffers, each containing 2 mM $MgCl_2$, 200 μM SAM, 2.0 μg purified recombinant AtOMT1 protein, and 100 μM substrate. The reaction mixtures (200 μl) were incubated at 30°C for 1 h. The reactions were stopped by the addition of 500 μl EtOAc. The EtOAc extracts were dried and dissolved in 50 μl MeOH. An aliquot of the solution was injected into the LC-MS system.

Kinetic properties of recombinant AtOMT1

For the determination of K_m values for 5-HCAld, 5-HCAlc, and quercetin, assays were conducted in 50 mM Tris-HCl (pH 8.5) for 5-HCAld, 50 mM potassium phosphate buffer (pH 8.5) for 5-HCAlc, and 50 mM potassium phosphate buffer (pH 7.5) for quercetin. Each reaction mixture contained 0.5–4 μM 5-HCAld, 2.5–100 μM 5-HCAlc, or 4–100 μM quercetin, 2 mM $MgCl_2$, 200 μM SAM, and 2.0 μg AtOMT1. K_m and V_{max} values were determined from Lineweaver-Burk plots.

For inhibition kinetic analysis, 5-HCAld (0–30 μM) was used to assay the 5-HCAld-induced inhibition of AtOMT1-mediated methylation of 5-HCAlc, while 5-HCAlc (0–150 μM) was used to assay the 5-HCAlc-induced inhibition of AtOMT1-mediated methylation of 5-HCAld. Each reaction mixture (200 μl) was incubated at 30°C for 20 min in the conditions of each optimum pH.

Lignin analysis of the At5g54160-knockout *Arabidopsis* line

The seeds of the *A. thaliana* T-DNA insertion mutant lines of At5g54160 (SALK_002373 and SALK_135290) were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus). T-DNAs of SALK_002373 and SALK_135290 were inserted in the second intron and third exon, respectively. After selection of the homozygous T-DNA insertion mutants, total RNA extracted from the leaves was submitted to reverse-transcription (RT) PCR using the gene specific primers (AtOMT-F and AtOMT-R) according to the method reported by Kai et al.,[11] so that the absence of functional transcripts was confirmed. Nucleotide sequences of the primers were as follows: AtOMT-F, 5′-CGGCAGAGACACAATTAAC TCCGG-3′ and AtOMT-R, 5′-TCCTCTGGAAGTGAC TCGTAGCAG-3′. Thioacidolysis was carried out according to the method reported by Hamada et al.[12] Briefly, freeze-dried *A. thaliana* stems were cut with scissors, and then extracted with hot MeOH. The extract-free samples were treated with 3 ml dioxane/ethanethiol (9:1, v/v) containing 92 mM BF_3 etherate, at 100°C for 4 h. The reactions were stopped by the addition of 0.4 N $NaHCO_3$. Then the

reaction mixtures were adjusted to pH 3 by adding HCl and extracted with diethyl ether. The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The sample was trimethylsilylated with BSA (60°C, 45 min) and subjected to GC-MS.

Quantitative analysis of caffeyl alcohol in *A. thaliana*

Freeze-dried *A. thaliana* stems and roots were cut with scissors, and extracted with hot MeOH. Then deuterium-labeled internal standard (FA-d_3) was added to the MeOH solution. Solvents of the resultant MeOH solution were evaporated off, and the MeOH extracts thus obtained were treated with β-glucosidase (from almonds, Sigma G-0395, 16 units in 1.0 ml of sodium acetate buffer at pH 5.0) for 24 h at 37°C. The reaction mixture was extracted with EtOAc, and the solvent was evaporated off. The extracts were trimethylsilylated as above and subjected to GC-MS.

Results and discussion

CAldOMT (formerly known as CAOMT) is a key enzyme for syringyl lignin biosynthesis.[3,13] However, this enzyme has not yet been fully identified in *A. thaliana*. Namely, At5g54160 was initially annotated as the gene encoding a CAOMT (AtOMT1);[4] then, it was reported that its recombinant protein expressed in *E. coli* catalyzed the methylation of CA and 5-HFA based on product identification only by thin-layer chromatography.[14] However, this finding was challenged by Muzac et al.,[5] reporting that a recombinant protein of the gene expressed in *E. coli* did not methylate CA. Therefore, in the present study, a recombinant enzyme for this gene was expressed in *E. coli* and purified to be electrophoretically homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 2). Next, we tested whether the recombinant AtOMT1 exhibited CAldOMT activity using the following eight possible substrates: CA, 5-HFA, CaCoA, 5-HFCoA, CaAld, 5-HCAld, CaAlc, and 5-HCAlc. GC-MS analysis of the assay products unequivocally indicated that recombinant AtOMT1 catalyzed the *O*-methylation of these eight substrates (Table 1). These results clearly showed that At5g54160 encodes an OMT having CAldOMT activity, which sharply contrasted with the previous report.[5]

With the evidence that *AtOMT1* (At5g54160) encodes an OMT with CAldOMT activity, we next investigated the kinetic properties of this enzyme. Because 5-HCAld and 5-HCAlc showed almost equal specific activities (Table 1), the kinetic constants of recombinant AtOMT1 toward 5-HCAld and 5-HCAlc were examined (Table 2). These substrates gave similar values of k_{cat}/K_m, whereas the K_m for 5-HCAld showed a smaller value than 5-HCAlc (Table 2). In contrast, 5-HCAld inhibited the methylation of 5-HCAlc with a K_i of 0.5 μM, and the K_i value of the inhibition of 5-HCAld methylation by 5-HCAlc was 73.4 μM. Thus, the *O*-methylation of 5-HCAlc by AtOMT1 was significantly inhibited in the presence of 5-HCAld; that is, AtOMT1 catalyzes the *O*-methylation of 5-HCAld preferentially in the presence of the two substrates. This accorded well with the previous results of Li et al.,[3] showing that *O*-methylations of CA and 5-HFA by *P. tremuloides* CAldOMT were effectively inhibited in the presence of 5-HCAld. Taken together, these results strongly suggest that 5-HCAld is the physiologically important substrate of AtOMT1.

In order to further confirm the role of *AtOMT1* as *AtCAldOMT*, we analyzed the lignins present in the At5g54160-knockout *Arabidopsis* mutant lines (SALK_002373 and SALK_135290). The functional deficiency of

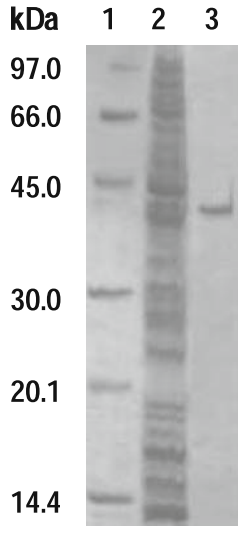

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of the recombinant AtOMT1 protein during affinity purification. *Lane 1*, molecular weight markers (*numbers* at the left indicate kDa); *Lane 2*, *Escherichia coli* lysate; *Lane 3*, column-purified AtOMT1 protein

Table 1. Substrate specificities of recombinant AtOMT1

Substrate	Specific activity (nmol min^{-1} μg^{-1} proteins)	Relative activity (%)
CA	3.2 ± 1.0	8
5-HFA	9.3 ± 1.5	23
CaCoA	3.5 ± 1.2	9
5-HFCoA	2.9 ± 0.8	7
CaAld	10.5 ± 5.7	26
5-HCAld	40.1 ± 6.1	100
CaAlc	24.4 ± 6.9	61
5-HCAlc	39.1 ± 9.1	98

Data given as mean ± standard deviation (n = two to five independent assays). No products were detected when the extracts of *Escherichia coli* containing pET-23 vector without the *AtOMT1* insert were used instead of purified recombinant AtOMT1

CA, Caffeic acid; 5-HFA, 5-hydroxyferulic acid; CaCoA, caffeoyl CoA; 5-HFCoA, 5-hydroxyferuloyl CoA; CaAld, caffealdehyde; 5-HCAld, 5-hydroxyconiferaldehyde; CaAlc, caffeyl alcohol; 5-HCAlc, 5-hydroxyconiferyl alcohol

Table 2. Kinetic parameters of the methylation of various substrates by purified recombinant AtOMT1

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M \cdot min^{-1}$)
5-HCAld	12.7	1.41	0.11
5-HCAlc	54.4	8.01	0.15
Quercetin	63.0	7.17	0.11

AtOMT1 in the mutant lines was confirmed by RT-PCR. The lignin-derived monomers released by thioacidolysis were analyzed by GC-MS for the wild type and the mutants. GC-MS analysis of the reaction products indicated that both guaiacyl and syringyl units were detected in the wild type (Fig. 3). In contrast, the syringyl units were not detected in the At5g54160-knockout lines (Fig. 3). Instead, small amounts of 5-hydroxyconiferyl compounds identified tentatively by their mass spectra were detected (Fig. 3). This is in good agreement with the syringyl-deficient lignin of another At5g54160-knockout line.[6] Thus, the present results indicated that At5g54160 gene is involved in syringyl lignin synthesis and the gene encodes AtCAldOMT.

In addition, we analyzed the MeOH extracts of the stem and root of the At5g54160-knockout line. GC-MS analysis showed that the content of CaAlc in stem extract of the mutant line was 6 times higher than that of wild type, while CaAlc content in root extracts of the mutant line was 44 times higher than that of wild type (Fig. 4). This strongly suggested that AtOMT1 may be involved in the *O*-methylation of the C3 position of 3,4-dihydroxyphenyl in vivo. Although this does not agree with the currently accepted lignin biosynthetic pathway, where CAldOMT catalyzes the methylation of 5-HCAld to SAld,[3] the in vitro assay (Table 1) demonstrated that recombinant AtOMT1 can catalyze the *O*-methylation of the four 3,4-dihydroxyphenyl compounds, and the specific activities for CaAld and CaAlc were comparable with those for 5-HCAld and 5-HCAlc. Similar results were reported by Parvathi et al.[15] Thus, alfalfa (*Medicago sativa*) CAldOMT exhibited high catalytic efficiency not only with 5-HCAld and 5-HCAlc, but also CaAld and CaAlc. The present results are also in good accordance with the recent report of mutant analysis,[16] suggesting that AtCAldOMT can methylate not only the 5-hydroxyl group of 5-HCAld but also the 3-hydroxyl group of 3,4-dihydroxyphenyl moiety. Thus, an At4g34050 (the gene encoding *A. thaliana* caffeoyl CoA OMT1, *AtCCoAOMT1*)-knockout mutant produced both guaiacyl and syringyl lignins with slightly lower total lignin contents, while the syringyl lignin content of an At5g54160 (*AtOMT1* = *AtCAldOMT*)-knockout mutant decreased drastically. In addition, lignin content of the double-knockout mutant of At4g34050 and At5g54160 was very low, and the development of the mutant was arrested at the plantlet stage.[16] These results indicated that the functions of *AtCCoAOMT1* (At4g34050) and *AtCAldOMT* (At5g54160) cannot be compensated by other gene family members of *AtCCoAOMT* and *AtCAldOMT*, while importantly, the methylation of 3,4-dihydroxyphenyl performed by AtCCoAOMT1 was replaced by AtCAldOMT. In addition, using a radio-tracer method, Matsui et al.[17] suggested the conversion of CaAlc to SAlc in dicotyledonous angiosperms.

As for the flavonoid OMT activity of AtOMT1, Muzac et al.[5] reported that recombinant AtOMT1 efficiently methylated the flavonoid, quercetin, to give rise to isorhamnetin (3′-*O*-methylquercetin), which was identified by LC-MS with an authentic sample of isorhamnetin. In the present study, we prepared the other regioisomers and used authentic samples in LC-MS analysis, which clearly indicated that

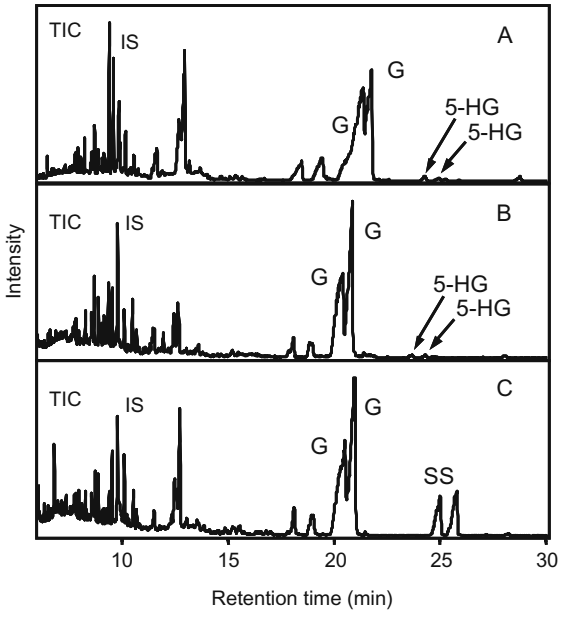

Fig. 3A–C. Gas chromatography-mass spectrometry (GC-MS) total ion chromatograms (*TIC*) of thioacidolysis products. **A** At5g54160-knockout (SALK_002373); **B** At5g54160-knockout (SALK_135290); **C** wild type. *G*, Thioacidolysis monomer from guaiacyl lignin; *S*, thioacidolysis monomer from syringyl lignin; *5-HG*, thioacidolysis monomer from 5-hydroxyguaiacyl lignin; *IS*, internal standard (docosane)

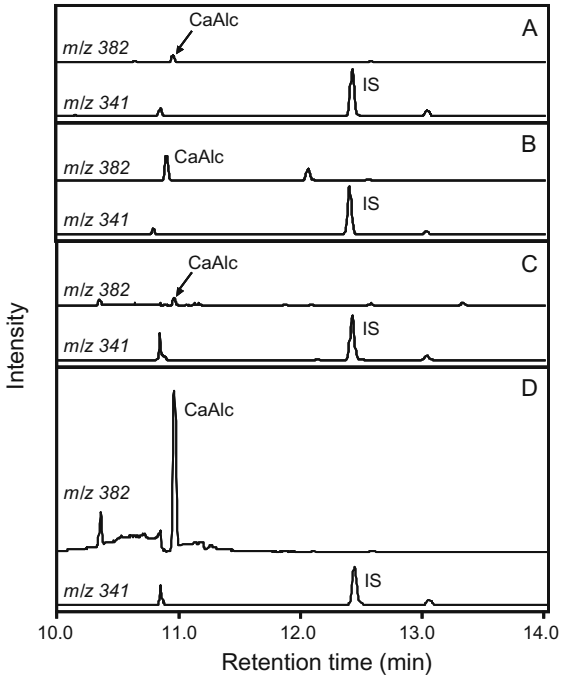

Fig. 4A–D. Mass chromatograms of molecular ions of the trimethylsilyl ethers of CaAlc (*m/z* 382) and FA-d_3 (*m/z* 341) as an internal standard. **A** Stem extracts of wild type; **B** stem extracts of At5g54160-knockout (SALK_135290) line; **C** root extracts of wild type; **D** root extracts of the At5g54160-knockout (SALK_135290) line

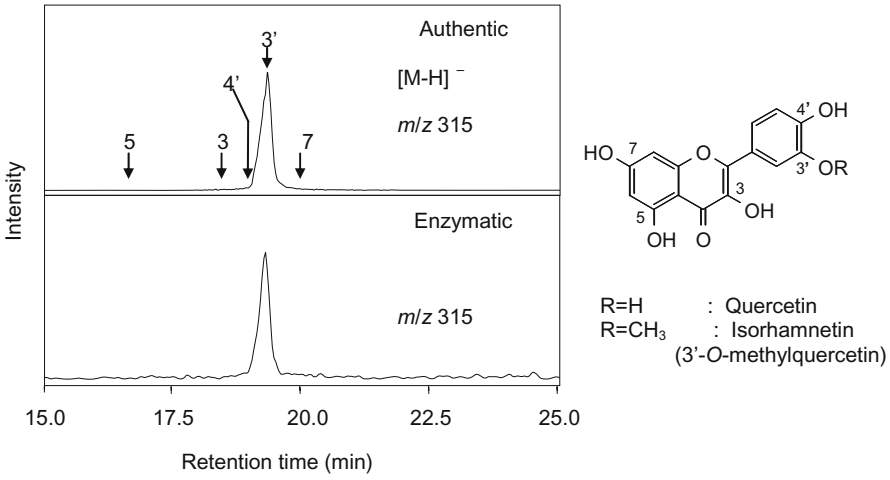

Fig. 5. Liquid chromatography-selected ion monitoring chromatograms of products obtained following incubation of quercetin with AtOMT1 and authentic 3′-*O*-methylquercetin. *3*, *5*, *7*, *3′*, and *4′* indicate the retention time of 3, 5, 7, 3′, and 4′-*O*-methylquercetins, respectively

the product was isorhamnetin, by comparing its retention time and mass spectrum with those of authentic samples (Fig. 5). It was found that the other regioisomers were not formed, confirming the previous conclusion by Muzac et al.[5] (Fig. 5). The K_m value of this reaction was determined as 63 μM (Table 2).

Taken together, the present study demonstrated that the At5g54160 gene has dual functions; that is, CAldOMT and flavonoid OMT activities, and this gene is involved in lignin biosynthesis, not only at the step of methylation of 5-HCAld but also of that of the 3,4-dihydroxyphenyl moiety.

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