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Purification and Identification of Naringenin 7-*O*-Methyltransferase, a Key Enzyme in Biosynthesis of Flavonoid Phytoalexin Sakuranetin in Rice*[S]

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Background: Sakuranetin is a major rice phytoalexin and a potential pharmaceutical agent. Rice naringenin 7-*O*-methyltransferase (OsNOMT), the key enzyme for sakuranetin biosynthesis, was previously unknown.
Results: We isolated OsNOMT and identified Os12g0240900 as *OsNOMT*.
Conclusion: Stress-induced OsNOMT regulates sakuranetin biosynthesis in rice.
Significance: Identification of *OsNOMT* enables the production of large amounts of sakuranetin through transgenic rice and microorganisms.

Sakuranetin, the major flavonoid phytoalexin in rice, is induced by ultraviolet (UV) irradiation, $CuCl_2$ treatment, jasmonic acid treatment, and infection by phytopathogens. It was recently demonstrated that sakuranetin has anti-inflammatory activity, anti-mutagenic activity, anti-pathogenic activities against *Helicobacter pylori*, *Leishmania*, and *Trypanosoma* and contributes to the maintenance of glucose homeostasis in animals. Thus, sakuranetin is a useful compound as a plant antibiotic and a potential pharmaceutical agent. Sakuranetin is biosynthesized from naringenin by naringenin 7-*O*-methyltransferase (NOMT). In previous research, rice NOMT (OsNOMT) was purified to apparent homogeneity from UV-treated wild-type rice leaves, but the purified protein, named OsCOMT1, exhibited caffeic acid *O*-methyltransferase (COMT) activity and not NOMT activity. In this study, we found that OsCOMT1 does not contribute to sakuranetin production in rice *in vivo*, and we purified OsNOMT using the *oscomt1* mutant. A crude protein preparation from UV-treated *oscomt1* leaves was subjected to three sequential purification steps, resulting in a 400-fold purification from the crude enzyme preparation. Using SDS-PAGE, the purest enzyme preparation showed a minor band at an apparent molecular mass of 40 kDa. Two *O*-methyltransferase-like proteins, encoded by Os04g0175900 and Os12g0240900, were identified from the 40-kDa band by MALDI-TOF/TOF analysis. Recombinant Os12g0240900 protein showed NOMT activity, but the recombinant Os04g0175900 protein did not. Os12g0240900 expression was induced by jasmonic acid treatment in rice leaves prior to sakuranetin accumulation, and the Os12g0240900 protein showed reasonable kinetic properties to OsNOMT. On the basis of these results, we conclude that Os12g0240900 encodes an OsNOMT.

When plants are attacked by pathogenic microorganisms, they respond with a variety of defense reactions, including the production of secondary metabolites called phytoalexins (1), which serve as plant antibiotics. In rice, 15 phytoalexins have been isolated and characterized, including 14 diterpenes, namely momilactones A and B, phytocassanes A–E, oryzalexins A–F, and oryzalexin S (2–10), and one flavonoid phytoalexin, sakuranetin (Fig. 1) (11). Among them, sakuranetin is considered to be one of the most biologically important phytoalexins in terms of its high antimicrobial activity and high accumulation in rice leaves infected by *Magnaporthe oryzae*, one of the major photopathogenic fungi (11). In addition, it was recently reported that sakuranetin has anti-inflammatory activity by inhibiting 5-lipoxygenase, which is involved in arachidonic acid metabolism in animal cells (12), anti-mutagenic activity (13), anti-*Helicobacter pylori* activity by inhibiting β-hydroxyacyl-acyl carrier protein dehydratase (14), and antileishmanial and antitrypanosomal activities (15), and that sakuranetin can induce adipogenesis of 3T3-L1 cells through enhanced expression of peroxisome proliferator-activated receptor γ2 to contribute to the maintenance of glucose homeostasis in animals (16). Thus, sakuranetin is a useful compound as a plant antibiotic and a potential pharmaceutical agent.

Sakuranetin is biosynthesized from naringenin by *S*-adenosyl-L-methionine (AdoMet)[3]-dependent naringenin 7-*O*-methyltransferase (NOMT) (Fig. 1) (17). Naringenin is the first flavonoid transformed from naringenin chalcone by chalcone isomerase and is also a key biosynthetic intermediate to an isoflavone and a variety of flavones. Therefore, NOMT plays a key role in sakuranetin biosynthesis at a branching point from a common flavonoid biosynthetic pathway.

Sakuranetin was first identified from the cortex of the bark of a cherry tree (*Prunus* spp.) as an aglycone of sakuranin (18) and

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[3] The abbreviations used are: AdoMet, *S*-adenosyl-L-methionine; NOMT, naringenin 7-*O*-methyltransferase; COMT, caffeic acid *O*-methyltransferase; OMT, *O*-methyltransferase; JA, jasmonic acid; RACE, rapid amplification of cDNA ends; qRT, quantitative RT; Rubisco, ribulose-bisphosphate carboxylase/oxygenase.

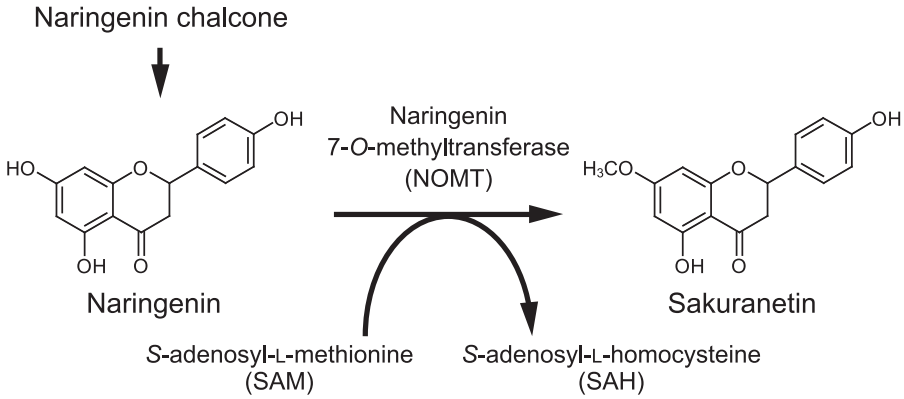


FIGURE 1. **Biosynthesis of sakuranetin from naringenin.**

then found in rice and several other plant species, including *Artemisia campestris*, *Prunus* spp., *Baccharis* spp., *Betula* spp., and *Juglans* spp. (19). However, NOMT has not been identified from such plant species. Although an *O*-methyltransferase (OMT) from barley (F1-OMT) that mainly methylates apigenin to form genkwanin has weak NOMT activity, sakuranetin has not been identified in barley (20). It was also reported that another OMT isolated from *Streptomyces avermitilis* (SaOMT-2) is able to catalyze the NOMT reaction, but SaOMT-2 has broad substrate specificity against isoflavones, flavones, and a flavanone, and its biological function remains unknown (21).

Although sakuranetin is not found in healthy rice leaves, its biosynthesis is rapidly induced by both biotic and abiotic stresses, such as infection with phytopathogens such as *M. oryzae, Xanthomonas oryzae,* and *Ditylenchus angustus*, infestation with *Sogatella furcifera*, ultraviolet (UV) irradiation, and treatment with $CuCl_2$ or jasmonic acid (JA) (11, 17, 22–25). Previously, it was reported that NOMT was purified to apparent homogeneity (~985-fold) from crude extracts of UV-treated wild-type rice leaves (22). However, the amino acid sequence of the purified protein was highly homologous to that of a caffeic acid 3-*O*-methyltransferase (COMT) from maize. In fact, the recombinant protein expressed in *Escherichia coli* showed COMT but not NOMT activity (26), and this enzyme was named OsCOMT1. These results suggest at least two possibilities as follows: one is that OsCOMT1 is a major component and the rice NOMT (OsNOMT) is a minor one in the purified fraction. If this is the case, isolation of OsNOMT might be quite difficult because of the masking effect by OsCOMT1. Another is that OsCOMT1 is involved in NOMT enzymatic activity in rice *in vivo* but that a post-translational modification and/or the presence of an interacting factor is needed to show NOMT activity. In the former case, if UV-irradiated leaves of the *OsCOMT1* tDNA insertion mutant *oscomt1* are used as plant material, OsNOMT is expected to be purified quite efficiently without masking by OsCOMT1. In the latter case, NOMT activity will not be induced in *oscomt1* mutant leaves even after elicitation.

In this study, we first confirmed that NOMT activity is induced by elicitor treatment in the *oscomt1* mutant leaves. We then purified and identified OsNOMT from UV-irradiated leaves of the *oscomt1* mutant. Kinetic analysis of recombinant OsNOMT and expression analysis of the *OsNOMT* gene in rice plants were also performed. This is the first report on the cloning and characterization of OsNOMT as a sakuranetin synthase in rice.

EXPERIMENTAL PROCEDURES

Chemicals—Racemic naringenin, luteolin, caffeic acid, and ferulic acid were purchased from Kanto Chemical, Co., Inc. (Tokyo, Japan). Racemic daidzein, biochanin A, and sinapic acid were purchased from Sigma. Apigenin, kaempferol, myricetin, and quercetin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Racemic liquiritigenin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). All chemicals used in matrix-assisted laser desorption/ionization two-stage time-of-flight mass spectrometry (MALDI-TOF/TOF) analyses were of analytical grade. In addition, 4-sulfophenyl isothiocyanate, α-cyano-4-hydroxycinnamic acid, sodium bicarbonate, and ammonium bicarbonate were purchased from Sigma.

Plant Materials, Growth Conditions, and Elicitation—The *oscomt1* mutant (PFG-2B-50240, *Oryza sativa* L. cv. Dongjin) was searched for in the Rice Functional Genomic Express Database as a tDNA insertion mutant of Os08g0157500 (TIGR ID, LOC_Os08g06100) and purchased from Postech Biotech Center. Because the seeds of the *oscomt1* mutant we obtained were from the F_1 generation, the F_1 generation plants were analyzed by genomic PCR using specific primers for *OsCOMT1* and tDNA to separate *oscomt1* homozygotes, heterozygotes, and wild-type rice plants, and the F_2 generation seeds were obtained from the respective plants. We used these F_2 generation seeds of homozygotes and wild type as "*oscomt1*" and "wild type" in the experiments on the characterization of the *oscomt1* mutant and purification of OsNOMT. Primers used for genomic PCR are described in Fig. 2*A* and supplemental Table S1. Wild-type rice (*O. sativa* L. cv. Nipponbare) plants were used for 5′- and 3′-RACE of *OsNOMT* mRNA and for analysis of the effects of JA treatment or inoculation of *M. oryzae* spores on the expression of *OsNOMT* and quantification of accumulated sakuranetin.

To grow rice plants, seeds were sterilized with 2.5% sodium hypochlorite solution (Kanto Chemical) for 30 min and then washed with sterilized water. Surface-sterilized seeds were incubated on 0.7% agar gel containing 0.1% of the liquid fertilizer Hanakoujou (Sumika-Takeda Garden Products, Tokyo, Japan) for 10 days at 28 °C in the light. Germinated seeds were

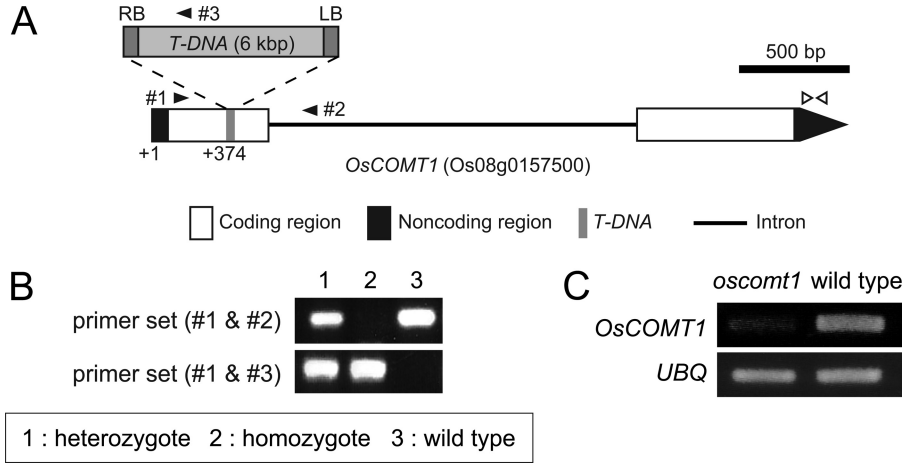


FIGURE 2. **Genome structure and gene expression of *OsCOMT1* in the *oscomt1* mutant.** *A,* tDNA sequence is inserted 374 bp downstream of the transcription initiation site of *OsCOMT1* in the *oscomt1* mutant. *Black arrowheads* (*#1, #2,* and *#3*) indicate the locations of primers used for genomic PCR, and *white arrowheads* indicate the locations of primers used for expression analysis. *B,* results of genomic PCR of the *oscomt1* mutant allele. *C,* transcriptional expression of *OsCOMT1* in wild-type rice and the *oscomt1* mutant. Ubiquitin expression was used as a control.

then transplanted into a mixture of vermiculite and artificial compost, Bonsol (Sumitomo Chemical, Tokyo, Japan), to grow in a greenhouse (12 h of light at 28 °C/12 h of dark at 25 °C). Three-month-old plants were used for subsequent experiments.

For UV irradiation, excised rice leaves were floated on distilled water and incubated for 20 min at 20 cm under a UV lamp in a biological safety cabinet. Then the irradiated leaves were incubated at 28 °C under continuous white light conditions. For JA or $CuCl_2$ treatment, a leaf disk (6 mm in diameter) from the uppermost leaf blades of rice plants was floated on 100 μl of an assay solution (500 μM of JA or $CuCl_2$) in each well of 96-well plastic plates.

Expression Analysis—RT-PCR and qRT-PCR were used to determine the level of gene expression. Total RNA extracted from plant tissue using Sepasol-RNA I Super (Nacalai Tesque, Inc., Tokyo, Japan) was used to synthesize cDNA by RT reaction using a Quantitect RT kit (Qiagen K.K., Tokyo, Japan). With the cDNA, RT-PCR and qRT-PCR were performed to determine the level of gene expression. For qRT-PCR, SYBR Green technology on an ABI PRISM 7300 real time PCR system (Applied Biosystems) was used. Raw data from qRT-PCR were analyzed using the standard curve method, and the results were expressed as relative mRNA values normalized to the expression level of ubiquitin (*OsUBQ*). Primers used for expression analysis are described in supplemental Table S1.

Extraction and Quantification of Naringenin and Sakuranetin from Rice Leaves—Plant tissue (40–200 mg fresh weight per sample) was homogenized by Multi Beads Shocker (Yasui Kikai, Osaka, Japan) and suspended in 2 ml of phytoalexin extraction solvent (ethanol/water/acetonitrile/acetic acid, 79:13.9:7:0.1, v/v). The sample was centrifuged at 8,000 × *g* for 15 min at 4 °C. The supernatant was collected to a vial and subjected to determination of phytoalexins by liquid chromatography two-stage mass spectrometry (LC-MS/MS), which was composed of API-3000 with an electrospray ion source (AB SCIEX) and an Agilent 1100 HPLC instrument (Agilent Technologies) equipped with a PEGASIL ODS SP100 column (150 mm long, 2.1 mm in diameter; Senshu Scientific, Tokyo, Japan). The analytical conditions were the same as the method described by Shimizu *et al.* (27). Naringenin and sakuranetin levels were determined with combinations of the precursor and product ions of *m/z* 273/153 for naringenin and *m/z* 287/167 for sakuranetin in the multiple reaction monitoring mode. The retention times of naringenin and sakuranetin were 2.5 and 3.3 min, respectively.

Preparation of Crude Protein Extract from Rice Leaves—All steps were carried out at 4 °C unless otherwise stated. Rice leaves were homogenized by using Polytron (Kinematica AG, Lucerne, Switzerland) with a 3-fold (v/w) volume of buffer A (0.2 M Tris-HCl (pH 8.5), containing 10% (v/v) glycerol). The homogenate was filtered through four layers of Miracloth (Calbiochem), and the filtrate was centrifuged at 10,000 × *g* for 30 min, with the resulting supernatant used as a crude protein extract.

NOMT and COMT Enzymatic Activity Assays—The standard assay mixture consisted of enzyme preparation (~500 ng of protein), 300 μM racemic naringenin or caffeic acid, 300 μM AdoMet, and 0.1 M Tris-HCl (pH 8.5) (containing 5 mM DTT and 1 mM EDTA) in a final volume of 50 μl. After an 18-h incubation at 28 °C, the reaction was terminated by adding 5 μl of 1 M HCl. The resulting products were extracted three times with 60 μl of ethyl acetate, evaporated to dryness, and finally dissolved in 100 μl of the phytoalexin extraction solvent. The content of sakuranetin or ferulic acid was quantified using an API-3000 LC-MS/MS system. Ferulic acid levels were determined with combinations of *m/z* 195/177 in the multiple reaction monitoring mode.

Purification of OsNOMT—All steps were carried out at 4 °C unless otherwise stated. A crude protein extract (620 ml) from 200 g, fresh weight, of *oscomt1* mutant rice plants 48 h after UV irradiation was applied to the sequential three-step purification described below.

Ammonium Sulfate Precipitation—Well ground ammonium sulfate powder (108.7 g; Kanto Chemical) was added to the crude protein extract, and the mixture was gently agitated for 2 h. The mixture was then centrifuged at 10,000 × *g* for 30 min. The supernatant was collected to a clean beaker; 77.5 g of ammonium sulfate powder was added, and the mixture was gently agitated for 2 h. After that, the mixture was centrifuged

at 10,000 × *g* for 30 min. The supernatant was removed, and 40 ml of buffer A was added to the pellet. Next, 48 ml of the obtained protein solution was ultrafiltered (10-kDa cutoff) and finally resolved in 12 ml of buffer A, and the resultant solution was subjected to the subsequent purification.

Anion Exchange on DEAE Column—The protein solution (10 ml) was loaded to an AKTA fast protein LC (FPLC) system (GE Healthcare) equipped with a pre-equilibrated Hi Prep™ 16/10 DEAE FF column (GE Healthcare). Buffer A was used as a pre-equilibration buffer. The applied proteins were washed with 50 ml of buffer A at a flow rate of 2 ml/min. After that, a 50-min linear gradient (0–60% buffer B (buffer A containing 1 M KCl)) was applied as an elution step with a flow rate of 2 ml/min and then the column was washed with 100 ml of 100% buffer B. The eluate was collected in steps of 5 ml each, checked for NOMT enzymatic activity, and the active fractions were pooled. The pooled samples were mixed together, ultrafiltered (10-kDa cutoff), and finally resolved in 6 ml of buffer A, with the product used for subsequent purification.

Affinity Chromatography—Affinity chromatography on adenosine-agarose was performed as described previously (22). First, 5′-AMP-agarose (5 ml; Sigma) was washed with distilled water and incubated with 800 units of calf intestinal alkaline phosphatase (Sigma) in calf intestinal alkaline phosphatase buffer (pH 9.0) in a total volume of 10 ml. The gel was then dephosphorylated for 24 h at 37 °C by gentle agitation, transferred to an open column (15 mm inner diameter), and washed with 50 ml of distilled water and then with 50 ml of buffer B. Finally, the column was equilibrated with 100 ml of buffer A. The DEAE-purified protein solution (1 ml, ~18.7 mg of protein) was directly applied onto the adenosine-agarose gel column. The column was washed first with 50 ml of buffer A and then with 50 ml of buffer B with gravity flow. Elution was done with 25 ml of 4 mM AdoMet in buffer B. Fractions (5 ml each) were collected, and the NOMT enzymatic activity of each fraction was checked. Active fractions were combined, ultrafiltered (10-kDa cutoff), and finally dissolved in 1.2 ml of buffer A. The resultant solution was subjected to SDS-PAGE.

In-gel Protein Digestion—Protein bands on SDS-polyacrylamide gels were enzymatically digested in-gel as described previously (28) using modified porcine trypsin (Promega). The resultant gel pieces were washed with 50% acetonitrile and vacuum dried, then rehydrated with trypsin solution (8–10 ng/μl 50 mM ammonium bicarbonate (pH 8.7)), and incubated for 8–10 h at 37 °C.

Identification of Purified Protein by MALDI-TOF and MALDI-TOF/TOF Analysis—For identification of components of the 40-kDa band in SDS-PAGE (Fig. 4*A*) by peptide mass fingerprinting with MALDI-TOF/TOF, in-gel digested protein samples were analyzed using the Applied Biosystems 4700 proteomics analyzer with TOF/TOF™ ion optics. Both MS and MS/MS data were acquired with a Nd:YAG laser with a 200 Hz repetition rate, and up to 4,000 shots were accumulated for each spectrum. The MS/MS mode was operated with 1 keV collision energy; air was used as the collision gas such that nominally single collision conditions were achieved. Both MS and MS/MS data were acquired using the instrument default calibration, without applying internal or external calibration. Sequence tag searches were performed with the program MASCOT.

For identification of components of the 45- and 52-kDa bands in SDS-PAGE (Fig. 4*A*) by peptide mass fingerprinting with MALDI-TOF, trypsin-digested proteins were mixed with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA and subjected to MALDI-TOF analysis (Ettan MALDI-ToF Pro, Amersham Biosciences) as described previously (29). Spectra were collected from 350 shots per spectrum over an *m/z* range of 600–3000 and calibrated by a two-point internal calibration using trypsin auto-digestion peaks (*m/z* 842.5099 and 2211.1046). A peak list was generated using the Ettan MALDI-TOF Pro Evaluation Module (version 2.0.16). The threshold used for peak picking was as follows: 5,000 for minimum resolution of monoisotopic mass, 2.5 for signal-to-noise ratio. The search program MASCOT, developed by Matrix Science, was used for protein identification by peptide mass fingerprinting. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. Peptide mass fingerprint acceptance criteria is probability scoring.

5′- and 3′-RACE Analysis of Os12g0240900—Because expressed mRNA of Os12g0240900 had not been previously identified, we used estimated ORF information in the rice genomic sequence of the locus for RACE analysis. Extraction of total RNA from UV-irradiated wild-type rice leaves (cv. Nipponbare) was conducted using Sepasol-RNA I Super (Nakalai Tesque). and the mRNA was then purified using the Absolutely mRNA™ purification kit (Agilent) from the total RNA. cDNA synthesis from the mRNA and the subsequent 5′- and 3′-RACE analyses were performed using the SMARTer™ RACE cDNA amplification kit (Clontech). Primers used in 5′- and 3′-RACE analyses are described in supplemental Table S1. The 1,548-bp sequence of the obtained cDNA of Os12g0240900 was deposited in the DNA Data Bank of Japan (DDBJ) under accession number AB692949.

Cloning, Expression, and Purification of Os04g0175900 and Os12g0240900—Full-length ORFs of Os04g0175900 and Os12g0240900 were amplified by RT-PCR using KOD-FX Neo (TOYOBO, Tokyo, Japan) with cDNA prepared from $CuCl_2$-treated wild-type rice leaves (cv. Nipponbare) as templates. To construct a directional TOPO cloning transfer vector, the 5′-CACC-3′ sequence was incorporated into the PCR upstream primer at its 5′ ends. Then the PCR products were inserted into the pENTR/D-TOPO vector (Invitrogen), and the ligated products were transformed into TOP10 chemically competent *E. coli*. The ORFs of Os04g0175900 and Os12g0240900 inserted into pENTR/D-TOPO vector were fused into pDEST15 (Invitrogen) using LR Clonase II enzyme mix (Invitrogen). Finally, we obtained expression vectors of N-terminal GST tag-fused OMTs, pDEST15-1759 and pDEST15-2409, respectively.

The resulting plasmids were transformed into *E. coli* Rosetta II (DE3) (Novagen). These strains were pre-cultured for 24 h at 37 °C in LB medium containing carbenicillin (100 μg/ml) and chloramphenicol (34 μg/ml) and then cultured for 48 h at 20 °C

in Overnight Express™ instant TB Medium (Novagen) containing carbenicillin (100 μg/ml) and chloramphenicol (34 μg/ml). The cells were collected by centrifugation, suspended in buffer A, and disrupted by mild sonication on ice. After centrifugation at 10,000 × *g* for 15 min, the supernatant was collected and loaded to the AKTA FPLC (GE Healthcare) equipped with a pre-equilibrated GSTrap HP column (bed volume 5 ml) (GE Healthcare). Buffer A was used as the pre-equilibration buffer. Applied protein was washed with 25 ml of buffer A with a flow rate of 2 ml/min. Next, 25 ml of buffer A containing 10 mM reduced glutathione was applied for the elution. The purified recombinant proteins were ultrafiltered (10-kDa cutoff) and finally resolved in 1 ml of buffer A, and that solution was used for kinetic analysis.

MS/MS Analysis of Sakuranetin Produced by NOMT Enzymatic Assays—To confirm that GST-2409 product was sakuranetin, we compared product ion spectrum of the product and authentic sakuranetin with LC-MS/MS system. MS/MS analysis was performed with AB SCIEX TripleTOF™ 5600 System (AB SCIEX) equipped with SHIMADZU Nexera UFLC system (Shimadzu, Kyoto, Japan). Four-min gradient elution with 10–90% acetonitrile containing 0.1% formic acid was performed with a CAPCELL PAK C18 IF S2 column (Shiseido Co., Tokyo, Japan). Ionization was performed with electrospray ionization and precursor ions (m/z = 100–1,000) and product ions (m/z = 50–1,000) were scanned with positive ion mode. The retention time of sakuranetin was 2.85 min. The product ion spectrum of precursor ion (m/z = 287.1) at the retention time of sakuranetin was observed.

Chiral Analysis of Sakuranetin Produced in the NOMT Enzymatic Assays—For chiral analysis of sakuranetin, 70 min of isocratic elution with 30% acetonitrile containing 0.1% acetic acid (v/v/v) was performed with a Sumichiral OA-7500 column (250 mm long, 2 mm in diameter; Sumika Chemical Analysis Service, Tokyo, Japan) and detected by using API-3000 LC-MS/MS system as described above. The retention times of natural and unnatural sakuranetin were 54 and 61 min, respectively.

Kinetic Analysis—The standard assay mixture without the enzyme was incubated at 30 °C for 5 min. The reaction was initiated by adding 1 pmol of the enzyme, and the reaction mixture was incubated at 30 °C for 5 min. The reaction was terminated by adding 5 μl of 1 M HCl. The resulting products were extracted three times with 60 μl of ethyl acetate, evaporated to dryness, and finally dissolved in 100 μl of phytoalexin extraction solvent. The content of sakuranetin was quantified using an API-3000 LC-MS/MS system. The kinetic parameters were derived from a Hanes-Woolf plot. The effect of substrate concentration on reaction velocity was examined at various concentrations (30, 24, 18, 12, 6, and 0 μM) of racemic naringenin, unnatural naringenin being not a substrate for OsNOMT. The concentration of natural naringenin was calculated as half of racemic naringenin.

Substrate Specificity Assay—The standard assay mixture without AdoMet was incubated at 30 °C for 5 min. The reaction was initiated by adding 45.9 pmol (1 μl) of *S*-[*methyl*-^{3}H] adenosyl-L-methionine (~444 GBq/mmol, 20.4 MBq/ml, PerkinElmer Life Sciences), and the reaction mixture was incubated at 30 °C for 10 min. The reaction was terminated by adding 5 μl of 1 M HCl. The resulting products were extracted with 150 μl of ethyl acetate, and 75 μl of the extract was applied to a liquid scintillation counter.

Preparation and Inoculation of Fungal Conidia—The rice blast fungus *M. oryzae* (race 001.0 P91-15B) was kindly supplied by Dr. E. Minami (National Institute of Agrobiological Sciences). The blast fungi were grown on oatmeal agar (60 g of homogenized oatmeal and 12 g of agar per liter of water) in Petri dishes incubated at 25 °C for 9 days. Conidial suspension was prepared as described previously (30) and then filtered through two layers of Miracloth (Calbiochem) to remove cell debris. Conidial suspension was subjected to an inoculation test within 2 h after preparation. For inoculation, the excised fourth leaf blades of rice plants at the 4.5-leaf stage were sprayed with conidial suspension at a concentration of 10^5 spores/ml. Then the inoculated leaf blades were incubated for 120 h at 25 °C under light conditions, and the samples were applied for gene expression analysis and quantitative analysis of sakuranetin.

RESULTS

Characterization of oscomt1 Mutant—We obtained PFG-2B-50240 (cv. Dongjin) as a tDNA insertion mutant in the first exon of Os08g0157500 (TIGR ID, LOC_Os08g06100, *OsCOMT1*) (Fig. 2*A*) from the Rice Functional Genomic Express Database and selected the *oscomt1* homozygote mutant and wild-type rice from the F_2 generation of seeds obtained by genomic PCR (Fig. 2*B*). After confirmation of impaired transcriptional expression of the *OsCOMT1* gene in leaves of the *oscomt1* mutant by RT-PCR (Fig. 2*C*), we examined the ability of the *oscomt1* mutant leaves to produce sakuranetin. Excised leaf blades of *oscomt1* and wild-type rice were treated with $CuCl_2$, and the accumulated amounts of sakuranetin in the leaf blades 72 h after treatment were determined by LC-MS/MS analysis. As shown in Fig. 3*A*, the accumulation of sakuranetin in *oscomt1* was comparable with that in the wild type. We also compared NOMT activity and COMT activity in leaf blades of *oscomt1* and the wild type. Crude enzyme extracts derived from rice leaves of *oscomt1* and the wild type 48 h after $CuCl_2$ treatment were subjected to NOMT and COMT enzymatic assays in which naringenin and caffeic acid were used as substrates, respectively. To determine NOMT and COMT activity, the reaction products of these assays, namely sakuranetin and ferulic acid, were measured by LC-MS/MS. As shown in Fig. 3, *B* and *C*, the specific NOMT activity of *oscomt1* was comparable with that of the wild type, whereas the specific COMT activity of *oscomt1* was severely suppressed compared with that of the wild type. This indicates that UV-irradiated *oscomt1* leaf blades are suitable plant materials for purification of OsNOMT.

Purification of OsNOMT from oscomt1 and MALDI-TOF-TOF Analysis of Purified Protein—Because NOMT activity in *oscomt1*, as already described, was comparable with that in the wild type, we performed purification of OsNOMT from *oscomt1* leaves using a series of chromatographic steps. A crude enzyme extract prepared from *oscomt1* leaves 48 h after UV treatment was purified through three steps of ammonium sul-

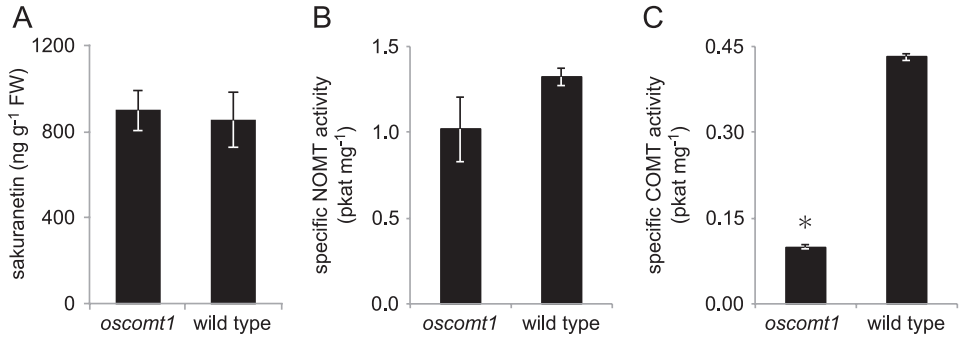


FIGURE 3. **Accumulation of sakuranetin and NOMT and COMT enzymatic activity in elicited *oscomt1* and wild-type rice leaves.** *A,* accumulated sakuranetin in *oscomt1* and wild-type rice leaves 48 h after $CuCl_2$ treatment was determined by LC-MS/MS. *B* and *C,* specific NOMT (*B*) and COMT (*C*) activities of crude enzyme preparations from *oscomt1* and wild-type rice leaves 48 h after $CuCl_2$ treatment. Amounts of sakuranetin and ferulic acid extracted from the enzymatic reaction mixture were measured by LC-MS/MS, and the specific activities were determined. The data are means ± S.D. of three replicates. An *asterisk* indicates significant differences compared with wild-type rice in a *t* test at $p < 0.05$.

TABLE 1
Purification of OsNOMT from UV-irradiated *oscomt1* rice leaves

Purification steps	Total activity	Total protein	Specific activity	Recovery	Purification
	picokatal	*mg*	*picokatal/mg*	*%*	*-fold*
Crude extract	104.0	695	0.150	100	1
Ammonium sulfate precipitation	43.6	288	0.152	42.0	1.01
Anion exchange (DEAE)	26.4	112.2	0.235	25.4	1.57
Affinity chromatography (adenosine-agarose)	5.8	0.096	60.629	5.6	405.35

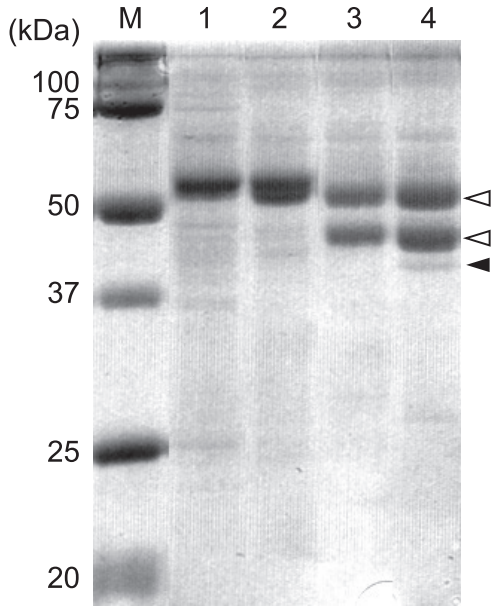
fate precipitation, DEAE anion exchange chromatography, and adenosine-agarose chromatography. The specific activity of OsNOMT and the results of SDS-PAGE analysis at each step are given in Table 1 and Fig. 4*A*, respectively. The specific activity of OsNOMT was dramatically concentrated by adenosine-agarose chromatography, and a 40-kDa band was detected on SDS-PAGE after adenosine-agarose chromatography. The 40-kDa band was excised from the gel and treated with trypsin, and the products of tryptic digestion were analyzed by MALDI-TOF/TOF MS. The obtained data were entered into the MASCOT Database. As a result, two putative OMTs encoded by loci Os04g0175900 (TIGR ID, LOC_Os04g09654) and Os12g0240900 (TIGR ID, LOC_Os12g13800) were identified as components of the 40-kDa band (Fig. 4, *B* and *C*). The mass spectra of these OMTs are shown in supplemental Fig. S1. Although proteins of 45 and 52 kDa were also detected as major bands in the fraction after adenosine-agarose chromatography (Fig. 4*A*), they were identified by MALDI-TOF MS as Rubisco-related proteins (45-kDa protein, Rubisco large chain precursor (AK058623, 40.2 kDa); 52-kDa protein, Rubisco large chain (AK105600, 52.8 kDa)).

cDNA Cloning and Functional Identification of Os04g0175900 and Os12g0240900—To investigate whether the Os04g0175900 and Os12g0240900 gene products have NOMT enzymatic activity, their cDNAs were expressed as recombinant proteins in *E. coli*. For preparation of the Os04g0175900 cDNA, sequence information of AK104764 in the RAP-DB was used. Because a full-length cDNA clone of Os12g0240900 had not been previously isolated, 5′- and 3′-RACE analysis was performed using predicted ORF information from rice genomic sequence of this gene locus. Total RNA was extracted from wild-type leaf blades 48 h after UV irradiation. mRNA was purified from the total RNA and subjected to cDNA synthesis followed by 5′- and 3′-RACE PCR. Finally, the full-length cDNA (1,548 bp) of Os12g0240900 was amplified by RT-PCR using primer sets constructed based on the sequences of the 5′ and 3′ ends and deposited in the DNA Data Bank of Japan under accession number AB692949.

The longest ORFs of Os04g0175900 (AK104764) and Os12g0240900 (AB692949) were amplified by RT-PCR, cloned using pENTR/D-TOPO vector (Invitrogen), incorporated into the expression vector pDEST15 (Invitrogen), and overexpressed in *E. coli* as N-terminal GST-tagged proteins, designated as GST-1759 and GST-2409, respectively. The crude extracts containing the recombinant proteins were subjected to FPLC with a glutathione-Sepharose column (GSTrap HP, Amersham Biosciences) to obtain the pure GST-1759 and GST-2409. NOMT enzymatic activities of the purified recombinant proteins (Fig. 5*A*) were examined, and the Michaelis-Menten kinetic parameters (K_m and k_{cat} values) were determined. As shown in Fig. 5*B* and supplemental Fig. S2, sakuranetin was detected as a product of the enzymatic reaction catalyzed by GST-2409 but not by GST-1759. As we used racemic naringenin as a substrate, we confirmed by using chiral column chromatography that sakuranetin produced by the NOMT enzymatic assay with GST-2409 was a natural form (supplemental Fig. S3). K_m and k_{cat} values of GST-2409 for natural naringenin determined by the Hanes-Woolf plot (Fig. 5*C*) were 1.9 ± 0.1 μM and 25 ± 3/s, respectively. These results indicate that the gene product of Os12g0240900, but not that of Os04g0175900, can catalyze 7-*O*-methylation of naringenin. We therefore designated Os12g0240900 as *OsNOMT*.

Substrate Specificity of GST-OsNOMT—It was reported that F1-OMT and SaOMT-2 catalyze the methylation of several flavonoids, including naringenin (20, 21). Therefore, we determined the substrate specificity of OsNOMT against several types of phenolic compounds, including flavonoids and isoflavonoids. The relative activity of purified GST-OsNOMT was

A



B

>Os04g0175900

```
  1 MASGISRTPATGVTAGGGDDEEAAWLHALELISGFTVSMTLK***AAIQLGLI***
 51 ***DALTAAADGRALTAGELVAQLPAVDDAEAATSVDR***MLR***LLASFNVVR***CST
101 EAGPGGDPLRRYSPAPVCRWFTAGDNHQGSLAPRLMLDVDEDNLSTWHQM
151 AAAVVSGGPSAFERAHGMPLFEYMGTNHRFNMLFNQAMSQQSMMVMNKLL
201 DRFHGFDGISVLVDVGGGTGVTLKMIISRYKHITGVNFDLPHVISQAPSL
251 PGVNHVAGNMFESVPKGDAIFLKSMLLRNDEECIKILKNCHYALSDNGK***V***
301 ***IVVDIVLPETPKPVPEAQNPLR***MDVMMLNNLRGGKIRTEQEYAKLAMDSG
351 FSGSFRTTYIFANFMAIELCK
```

C

>Os12g0240900

```
  1 MGDMVSPVVHRHAAGGGSGGDDDDQACMYALELLGGSVVSMTLK***AAIELG***
 51 ***LVDELLAAAGAAVTAEELAAR***LRLPAAVAAAAAVDRMLRLLASYGVVRCA
101 TEAGPDGKARRSYAAAPVCKWLAAGSSSGEGSMAPLGLLNLDKVFMENWY
151 YLKEAVSEGGTAFDKAYGTSLFQYLGQDGNEPSNTLFNQAMASHSVVITN
201 K***LLQFFRGFDAGAGVDVLVDVGGGVGATLR***MITARHPHLRGVNYDLPHVI
251 AQAPPVEGVEHIGGSMFDHVPSGSAILLKWILHLWGDEECVKILKNCYKA
301 LPAKGKVILVEYVLPASPEATLAAQEAFRLDVMMLNRLAGGKERTQQEFT
351 DLAVDAGFSGDCKPTYIFTNVWALEFTK
```

FIGURE 4. **Identification of *O*-methyltransferase-like proteins by MALDI-TOF/TOF analysis.** *A,* Coomassie-stained SDS-PAGE of purified enzyme preparations is shown. *Lane 1*, crude enzyme; *lanes 2–4*, ammonium sulfate precipitation-, DEAE-, and adenosine-agarose affinity-purified enzymes; *lane M*, protein marker. *Black arrowhead* indicates the 40-kDa proteins, which include OsNOMT. *White arrowheads* indicate the major components of Rubisco-related proteins. *B* and *C,* amino acid sequences of *O*-methyltransferase-like proteins encoded by gene loci Os04g0175900 (*B*) and Os12g0240900 (*C*) obtained from the 40-kDa band by MALDI-TOF/TOF analysis are shown. *Bold italic* characters indicate the fragments identified by MALDI-TOF/TOF analysis.

tested on the methylation of two flavanones (naringenin and liquiritigenin), five flavones (apigenin, luteolin, kaempferol, quercetin, and myricetin), an isoflavanone (daidzein), an isoflavone (biochanin A), caffeic acid, and sinapic acid. A mixture of a substrate and the purified OsNOMT was incubated with ^{3}H-labeled AdoMet for methylation quantification. The reaction product was extracted with ethyl acetate, and its ^{3}H radioactivity was determined by liquid scintillation counter to evaluate the substrate specificity of OsNOMT. As shown in Fig. 6, OsNOMT preferentially methylated flavanones and flavones, showing the highest methylation activity with racemic naringenin (100%), followed by kaempferol (73%), apigenin (61%), luteolin (44%), racemic liquiritigenin (34%), and quercetin (30%). The methylation activity of OsNOMT with myricetin, isoflavones, and other phenolics was similar to that of the negative control.

Relationship between Expression of OsNOMT and Accumulation of Sakuranetin in Rice Leaves after JA Treatment and M. oryzae Infection—Accumulation of sakuranetin is induced by treatment with elicitors, such as JA and $CuCl_2$ in rice leaves, and $CuCl_2$ treatment induced JA prior to accumulation of

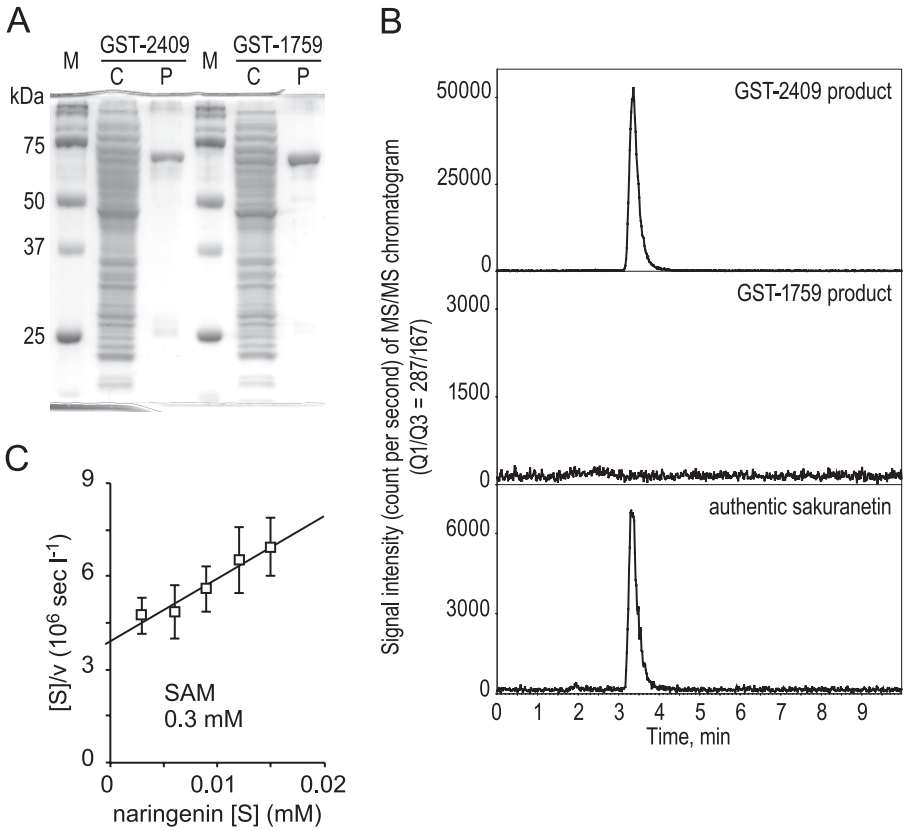


FIGURE 5. **NOMT enzymatic activity of GST-fused recombinant proteins.** Crude enzyme preparations from *E. coli* expressing either GST-2409 or GST-1759 were purified by affinity chromatography with GSTrap HP column, and the purified preparations were applied to SDS-PAGE and NOMT enzymatic activity assays. *A,* Coomassie-stained SDS-PAGE of affinity purified GST-1759 and GST-2409. (*C*, crude; *P*, purified). *B,* LC-MS/MS chromatograms of reaction products in the NOMT enzymatic activity assay using either GST-2409 or GST-1759 and an authentic standard of sakuranetin. *C,* Hanes-Woolf plots for GST-2409 with natural naringenin. The concentration of natural naringenin was calculated as the half of racemic naringenin. The data are means ± S.E. of three replicates.

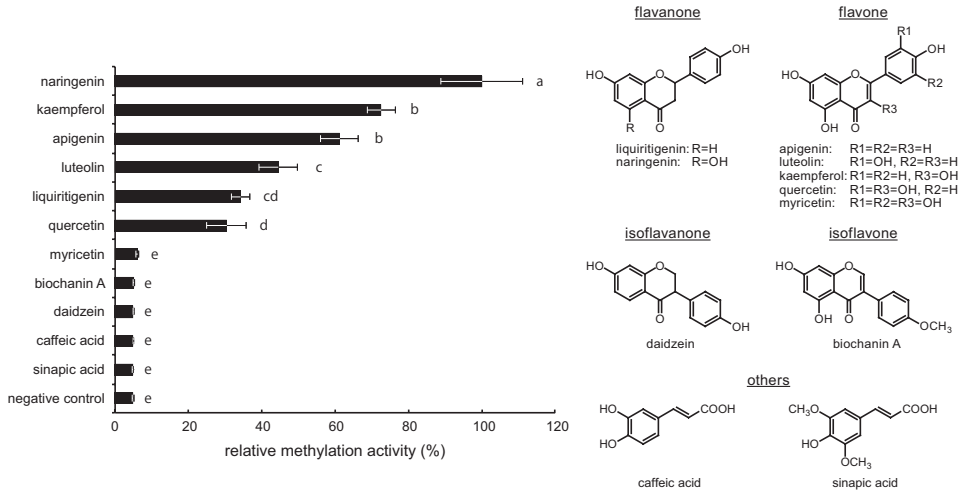


FIGURE 6. **Relative methylation activities of OsNOMT against flavonoids and other phenolic compounds.** Purified GST-OsNOMT was incubated with 0.3 mM of phenolic substrates and a trace amount of *S*-[*methyl*-^{3}H]adenosyl-L-methionine. The reaction was terminated by adding 1 M HCl. The reaction products were extracted with ethyl acetate and the incorporated *methyl*-^{3}H was measured. The data are means ± S.E. of three replicates. Different characters indicate significant differences in a Tukey's test ($p < 0.05$) among the activities against the substrates.

sakuranetin (17). Therefore, we performed time course analyses of expression levels of *OsNOMT* and accumulation of sakuranetin to further support the involvement of OsNOMT in sakuranetin production in rice leaves after JA treatment.

For JA treatment, rice leaf disks were floated on 100 μM JA. Then the expression levels of *OsNOMT* in the leaf disks were determined by qRT-PCR, and the endogenous levels of sakuranetin were quantitated by LC-MS/MS. The mRNA level of *OsNOMT* was transiently induced at 6 h and then gradually decreased until 72 h after JA treatment (Fig. 7*A*). Accumulation of sakuranetin started at 6 h and continuously increased until at least 72 h after JA treatment, when the accumulation level of sakuranetin reached ~4 μg/g FW (Fig. 7*B*).

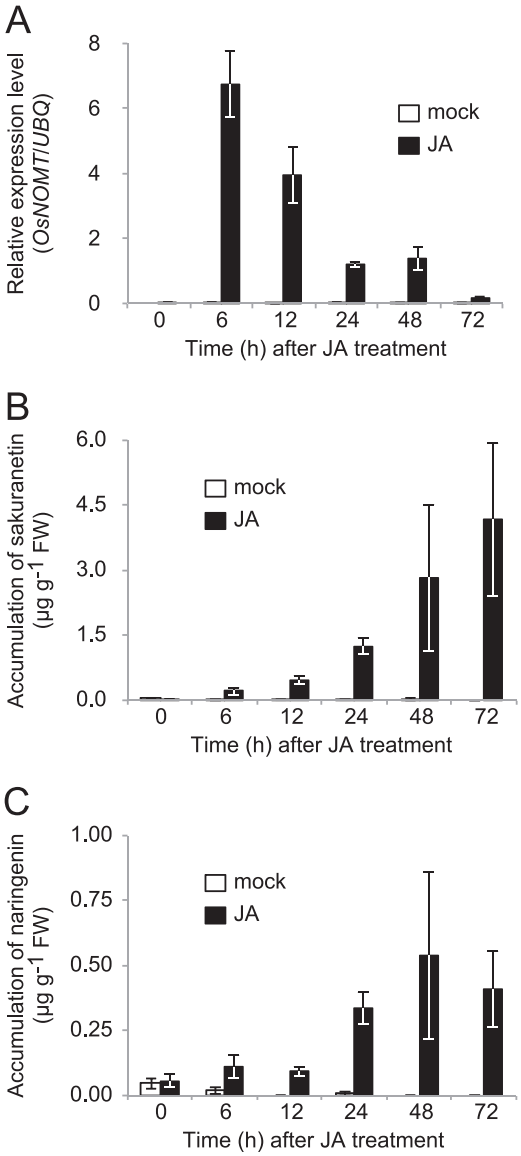


FIGURE 7. **Time course analysis of *OsNOMT* mRNA and accumulation of sakuranetin and naringenin in JA-treated wild-type rice leaves.** *A,* relative expression levels of *OsNOMT* in rice leaves from 0 to 72 h after treatment with 100 μM JA. The mRNA levels were determined using qRT-PCR. Each value was normalized to the *OsUBQ* mRNA level. The data are means ± S.D. of three experiments. *B* and *C,* accumulated amounts of sakuranetin (*B*) and naringenin (*C*) from 0 to 72 h after treatment with 100 μM JA were quantified using LC-MS/MS. The data are means ± S.E. of three experiments.

In addition, we found that accumulation of naringenin, a direct precursor of sakuranetin, was transiently induced slightly prior to accumulation of sakuranetin, peaking at ~0.5 μg/g FW 48 h after JA treatment (Fig. 7*C*). Thus, it was indicated that when rice produces sakuranetin, both the expression of the *OsNOMT* gene and the supply of the substrate naringenin are induced.

Besides, accumulation of sakuranetin is also induced by *M. oryzae* infection. We confirmed up-regulation of *OsNOMT* expression and increase in accumulation of both naringenin and sakuranetin in rice leaves at 5 days post-inoculation with *M. oryzae* spores (supplemental Fig. S4).

DISCUSSION

Here, we present details of the purification of the rice naringenin 7-*O*-methyltransferase OsNOMT from rice *oscomt1* mutant leaves and its detailed characterization. Although a putative OsNOMT was previously partially purified from UV-irradiated wild-type rice leaves, as described above, a major protein in the purified fraction showed high homology to maize COMT, and its recombinant protein expressed in *E. coli* showed COMT, but not NOMT, activity and was named OsCOMT1 (26).

Our study indicated that sakuranetin accumulation and NOMT enzymatic activity were similarly induced after elicitation in the *oscomt1* mutant and wild-type rice leaves. This strongly suggests that OsCOMT1 is not involved in sakuranetin production in rice. A possible reason why OsNOMT was not detected from the purified fraction with NOMT enzymatic activity from crude extracts of UV-treated wild-type rice leaves in the previous study (17) may be that the more abundantly generated OsCOMT1 exhibited similar behavior to OsNOMT in a series of purification steps and masked the presence of OsNOMT. In this study, purification of OsNOMT was successfully performed without masking by OsCOMT1 by using elicited *oscomt1* leaves. Among the three purification steps of OsNOMT, adenosine-agarose chromatography is most effective, leading to a more than 400-fold enrichment of the enzyme. This chromatography is known to be very effective in purifying AdoMet-dependent methyltransferases (31). After this purification step, a 40-kDa band on SDS-PAGE was detected. It was shown that molecular masses of type 1 OMTs, whose substrates are caffeic acid, flavonoids, coumarin, and alkaloids, are about 38–43 kDa (32), and OsNOMT and OsCOMT1 were suggested to have molecular masses of around 41 kDa (22). The 40-kDa band was excised from the gel and subjected to MALDI-TOF/TOF MS analysis after treatment with trypsin, resulting in identification of the two OMTs as candidate proteins for OsNOMT. Preparation of GST-tagged gene products of Os04g0175900 (AK104764) and Os12g0240900 (AB692949) in *E. coli* followed by *in vitro* NOMT enzymatic assays clearly demonstrated that Os12g0240900 encodes OsNOMT.

Kinetic analysis of recombinant GST-OsNOMT revealed that the K_m of GST-OsNOMT for naringenin was ~1.9 μM. Conversely, the endogenous level of naringenin accumulation was ~1.8 μM in rice leaves 48 h after JA treatment (Fig. 7*C*). Taken together, the K_m value was quite reasonable in understanding the function of OsNOMT in sakuranetin production in rice leaves.

Substrate specificity of OsNOMT was also determined in this study as described by Christensen *et al.* (20), and the results revealed that GST-OsNOMT showed higher methylation activity on naringenin than the flavanone liquiritigenin and flavones and no methylation activity on other phenolics, including isoflavonoids (Fig. 6). In the case of F1-OMT in barley, the methylation activity on apigenin was three times higher than that on naringenin (20). In contrast, SaOMT-2 has broad substrate specificity and can catalyze the methylation of isoflavonoids as well as naringenin (21). Sakuranetin has not been identified in either barley or *S. avermitilis*. These results indi-

cate that the actual biological functions of F1-OMT and SaOMT-2 are still unknown.

Time course analyses of *OsNOMT* expression and of accumulation levels of sakuranetin and its direct precursor naringenin in rice leaves treated with JA indicate that *OsNOMT* expression was induced transiently prior to accumulation of sakuranetin, and accumulation of naringenin was also transiently induced slightly prior to that of sakuranetin (Fig. 7*C*). Up-regulation of *OsNOMT* was confirmed in the pathogen *M. oryzae*-infected rice leaves in which accumulation of sakuranetin and its precursor naringenin was increased (supplemental Fig. S3). These results further support that OsNOMT functions as a sakuranetin synthase and is involved in defense responses through elicitor-induced production of sakuranetin in rice. The success of identification of OsNOMT as a sakuranetin synthase in this study will enable enhancement of pathogen resistance through regulation of the endogenous content of sakuranetin in rice. In addition, as described in Introduction, sakuranetin is a useful compound showing various pharmaceutical activities (12–16). Because the fermentative production of naringenin by microorganisms carrying an artificially assembled phenylpropanoid pathway has been established (33), our success in cloning OsNOMT will also enable the production of a large amount of sakuranetin by microorganisms for medical research.

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FIGURE S1. MALDI-TOF/TOF analysis of triptic peptides from the 40-kDa band. The MS spectrum of precursor ions generated by MALDI (A) and MS/MS spectra of product ions that were assigned by MASCOT to peptide fragments from the gene products of Os04g0175900 (B, C, D, E) and Os12g0240900 (F, G, H) are shown.

FIGURE S2. MS/MS analysis of produced compound by NOMT enzymatic assay using GST-2409. Chromatograms of precursor ions detected on m/z = 287.1 (A, upper: authentic sakuranetin, lower: produced compound by NOMT enzymatic assay using GST-2409) and mass spectrums of product ions detected from the major peaks (B, upper: authentic sakuranetin, lower: produced compound by NOMT enzymatic assay using GST-2409) are shown.

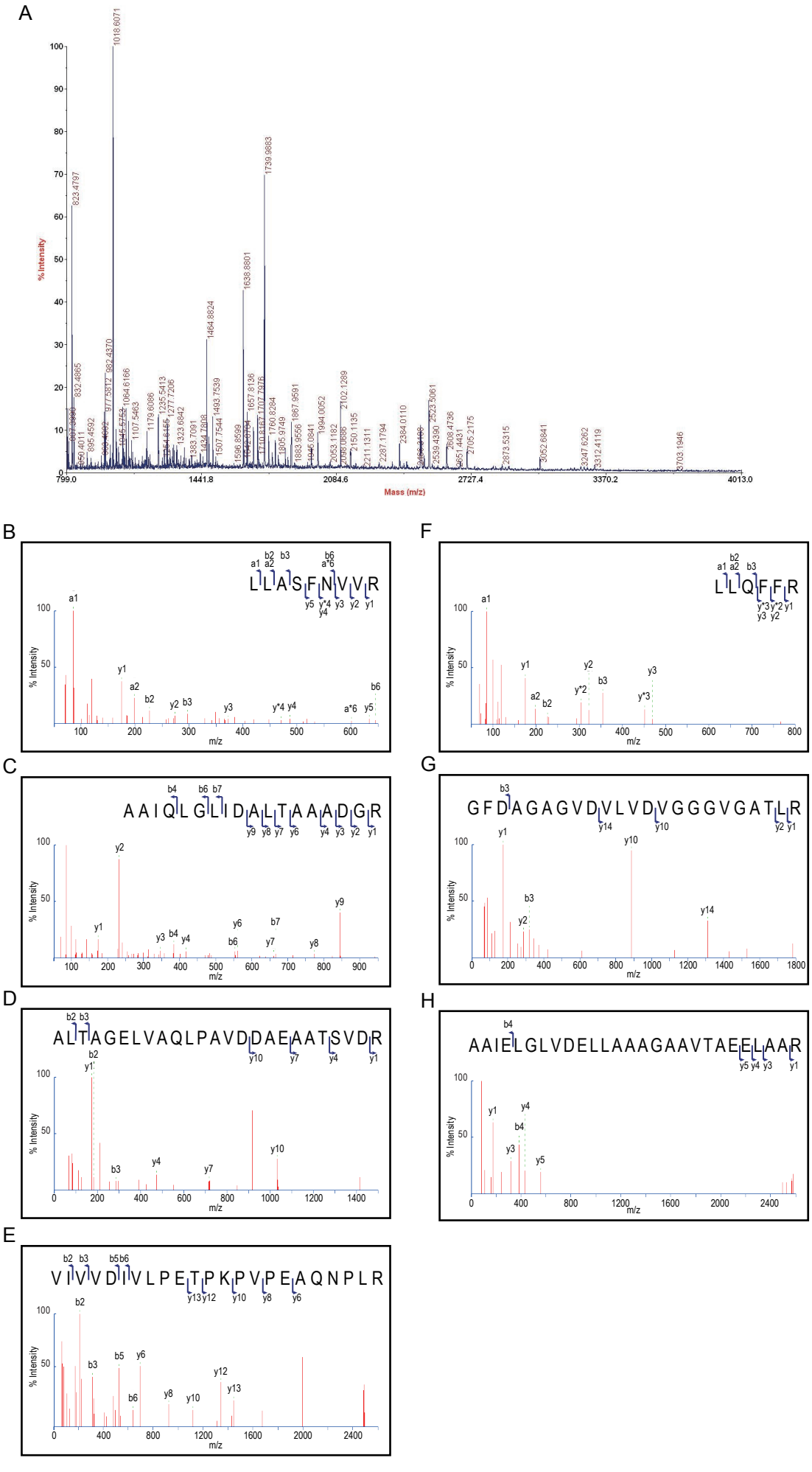
FIGURE S3. Chiral analysis of natural and unnatural sakuranetin. LC-MS/MS chromatograms of sakuranetin produced by the NOMT enzymatic activity assay using GST-2409, sakuranetin extracted from *M. oryzae* infected rice leaves and an authentic standard of racemic sakuranetin are shown.

FIGURE S4. OsNOMT mRNA levels and sakuranetin accumulation in *M. oryzae*-infected wild-type rice leaves. (A) The relative mRNA levels of *OsNOMT* in rice leaves 5 days post-inoculation (dpi) with *M. oryzae*. The mRNA levels were determined using qRT-PCR. Each value was normalized to the *OsUBQ* mRNA level. The data are means ± standard deviations of three experiments. (B, C) The accumulated amounts of sakuranetin (B) and naringenin (C) in rice leaves 5 dpi with *M. oryzae* were quantified using LC-MS/MS. The data are means ± standard errors of three experiments.

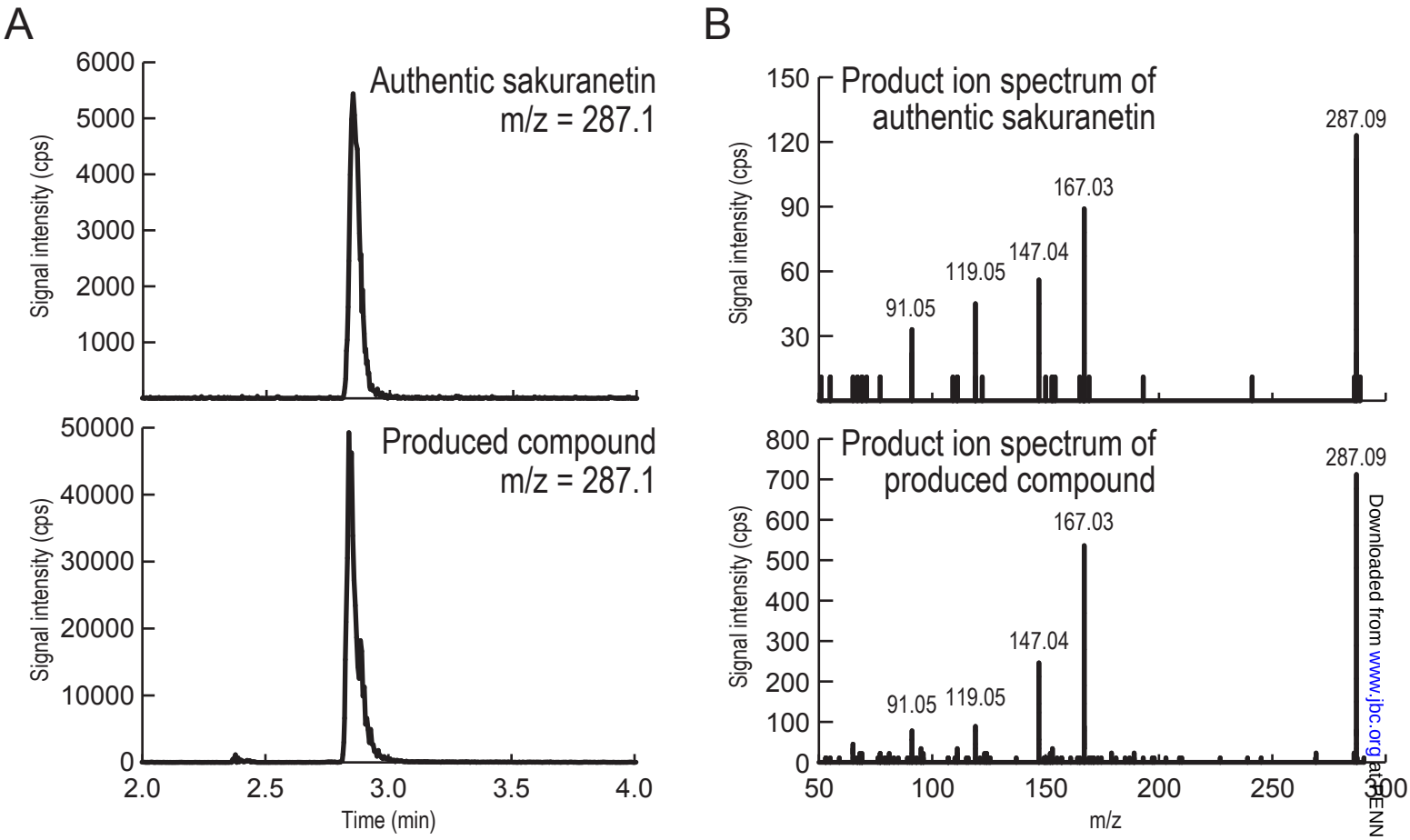
TABLE S1

Primers used in this paper

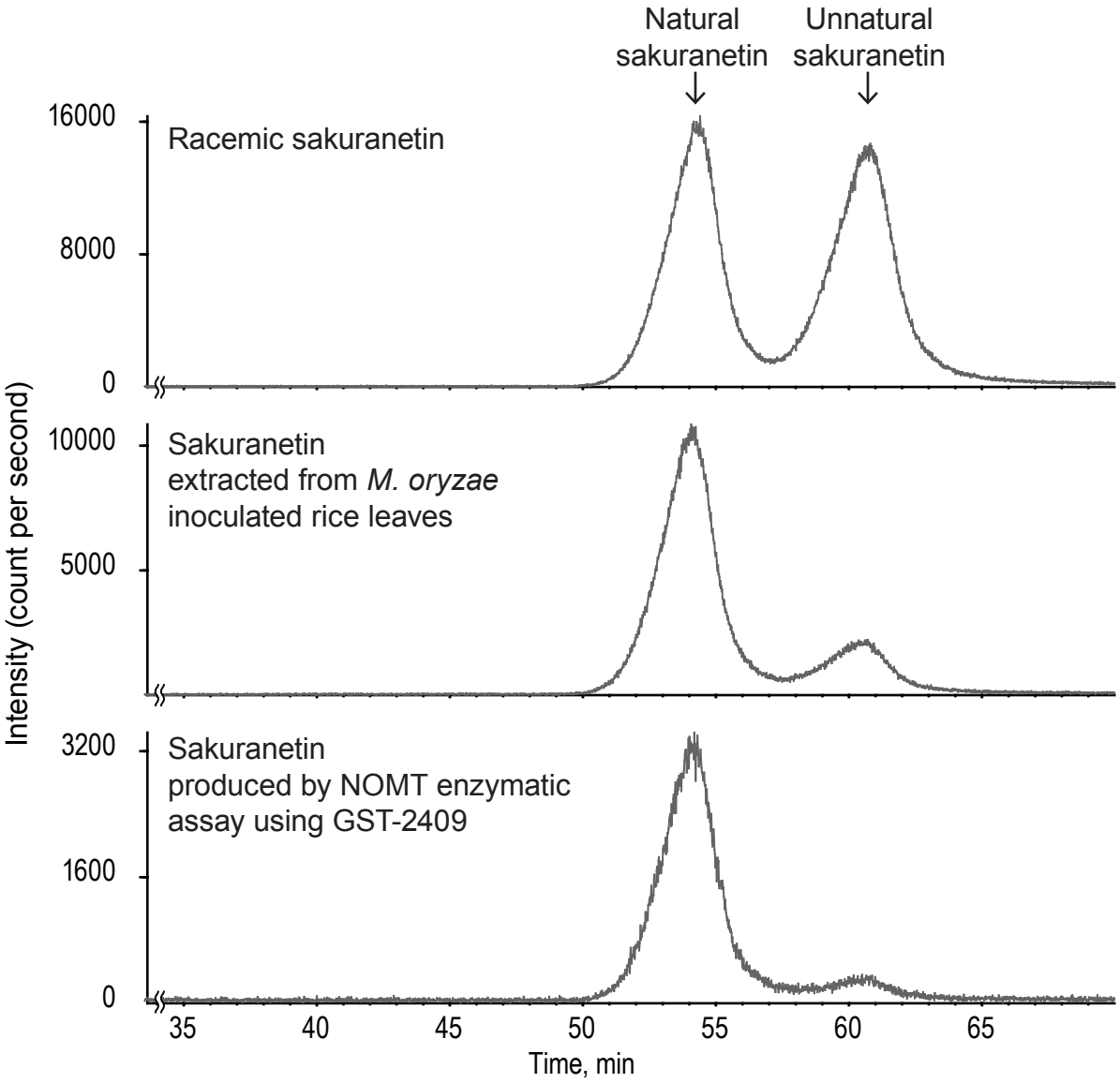
Supplemental FIGURE S1



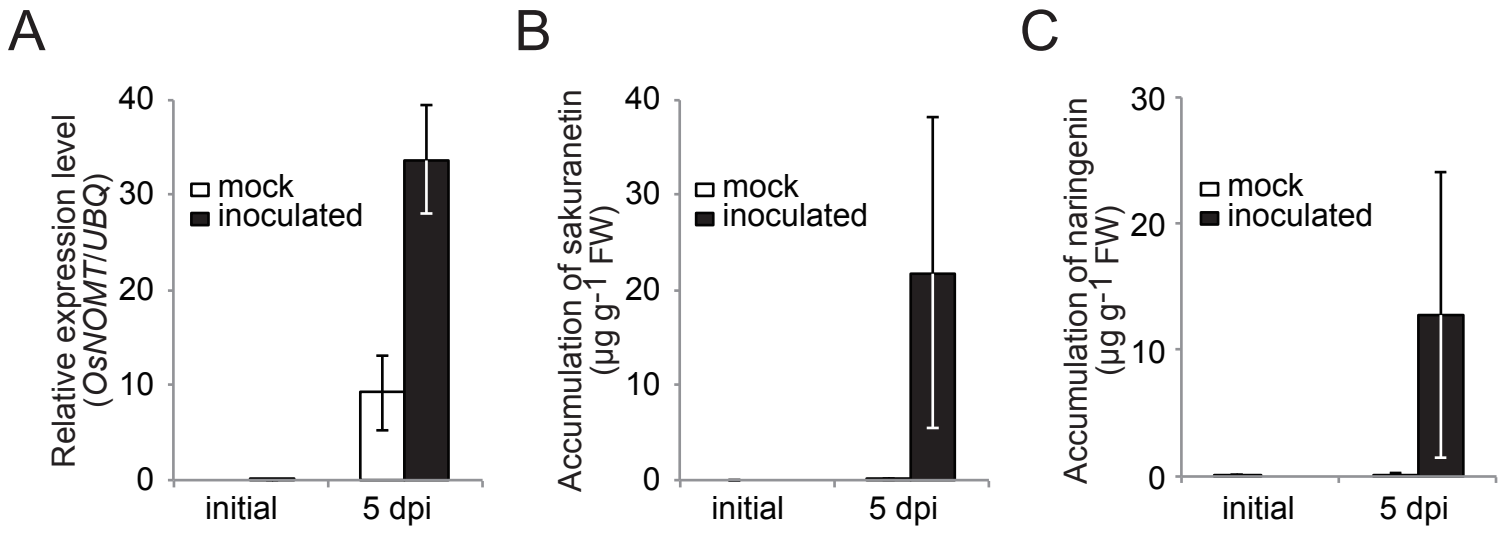
Supplemental FIGURE S2



Supplemental FIGURE S3



Supplemental FIGURE S4



Supplemental TABLE S1

Used for	Primer name	Primer sequence
Genomic PCR	COMT1T-DNA check F	5'-GACAAGCTGCCGTCCAAG-3'
	COMT1T-DNA check R	5'-ATGTCAGCGAGAGAGGAGGA-3'
	T-DNA Seq2 RB outer R	5'-TTGGGGTTTCTACAGGACGTAAC-3'
Gene expression assay	OsCOMT1 F	5'-AGGTGTTCGACCATCGTCTT-3'
	OsCOMT1 R	5'-CACCGGAATTGAACATCAAA-3'
	OsNOMT F	5'-CTAGCCGGATGCATGAAAGT-3'
	OsNOMT R	5'-TGCACGTATAGGCACACACA-3'
	OsUBQ F	5'-GGACTGGTTAAATCAATCGTCA-3'
	OsUBQ R	5'-CCATATACCACGACCGTCAAAA-3'
5'- and 3'-RACE	2409-3RACE-F	5'-CATGGAGAACTGGTACTACCTGAAG-3'
	2409-5RACE-R	5'-GGTAGTACCAGTTCTCCATGAACAC-3'
DNA cloning	1759 pENTR ORF F	5'-CACCATGGCTTCGGGCATTAGCA-3'
	1759 ORF R	5'-CTACTTGCATAGTTCAATTG-3'
	2409 pENTR ORF F	5'-CACCATGGGAGACATGGTGAGCCC-3'
	2409 ORF R	5'-TTACTTTGTGAACTCGAGAG-3'