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Molecular and biochemical characterization of the jasmonic acid methyltransferase gene from black cottonwood (*Populus trichocarpa*)

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ABSTRACT

Methyl jasmonate is a metabolite known to be produced by many plants and has roles in diverse biological processes. It is biosynthesized by the action of *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT), which belongs to the SABATH family of methyltransferases. Herein is reported the isolation and biochemical characterization of a *JMT* gene from black cottonwood (*Populus trichocarpa*). The genome of *P. trichocarpa* contains 28 *SABATH* genes (*PtSABATH1* to *PtSABATH28*). Recombinant PtSABATH3 expressed in *Escherichia coli* showed the highest level of activity with jasmonic acid (JA) among carboxylic acids tested. It was therefore renamed PtJMT1. PtJMT1 also displayed activity with benzoic acid (BA), with which the activity was about 22% of that with JA. PtSABATH2 and PtSABATH4 were most similar to PtJMT1 among all PtSABATHs. However, neither of them had activity with JA. The apparent *Km* values of PtJMT1 using JA and BA as substrate were 175 μM and 341 μM, respectively. Mutation of Ser-153 and Asn-361, two residues in the active site of PtJMT1, to Tyr and Ser respectively, led to higher specific activity with BA than with JA. Homology-based structural modeling indicated that substrate alignment, in which Asn-361 is involved, plays a role in determining the substrate specificity of PtJMT1. In the leaves of young seedlings of black cottonwood, the expression of *PtJMT1* was induced by plant defense signal molecules methyl jasmonate and salicylic acid and a fungal elicitor alamethicin, suggesting that *PtJMT1* may have a role in plant defense against biotic stresses. Phylogenetic analysis suggests that *PtJMT1* shares a common ancestor with the Arabidopsis *JMT*, and functional divergence of these two apparent *JMT* orthologs has occurred since the split of poplar and Arabidopsis lineages.

1. Introduction

Methyl jasmonate (2) (MeJA) (Fig. 1) is a naturally occurring compound known to be produced by many plants; it is involved in diverse biological processes (Cheong and Choi, 2003). It has also been shown to play important roles in regulating plant defenses against insect herbivory (Creelman et al., 1992; Howe, 2005; Koo et al., 2009). When released as an insect-induced airborne signal, MeJA (2) can prime the defense of neighboring plants of insect-infected plants for inter-plant communications (Reymond and Farmer, 1998; Kessler and Baldwin, 2001). As a constituent of floral scents of many plants (Knudsen et al., 1993), MeJA (2) may be involved in attracting pollinators. Furthermore, it has been shown to be involved in regulating plant growth and reproductive development (Creelman and Mullet, 1995; Nojavan-Asghari and Ishizawa, 1998).

MeJA (2) is biosynthesized by the action of *S*-adenosyl-L-methionine (SAM):jasmonic acid carboxyl methyltransferase (JMT), whereby a methyl group from SAM is transferred to the carboxyl group of jasmonic acid (1) (JA) to form methyl jasmonate (2) and *S*-adenosyl-L-homocysteine (SAH). JMTs belong to the plant protein family called SABATH (D'Auria et al., 2003). Other known members of the SABATH family related to JMTs include salicylic acid MT (SAMT) (Ross et al., 1999; Chen et al., 2003a; Zhao et al., 2010), benzoic acid MT (BAMT) (Murfitt et al., 2000), salicylic acid and benzoic acid MT (BSMT) (Chen et al., 2003a), indole-3-acetic acid MT (IAMT) (Qin et al., 2005; Zhao et al., 2007, 2008), and gibberellic acid MT (GAMT) (Varbanova et al., 2007). Because most SABATH proteins exhibit strict substrate specificities, it has been an interesting question to ask how such substrate specificities evolved. Structural, biochemical and phylogenetic analysis suggested that IAMT is an evolutionarily ancient member of the SABATH family conserved in seed plants. Other members, such as SAMTs, may have evolved from IAMT (Zhao et al., 2008). The evolutionary rela-

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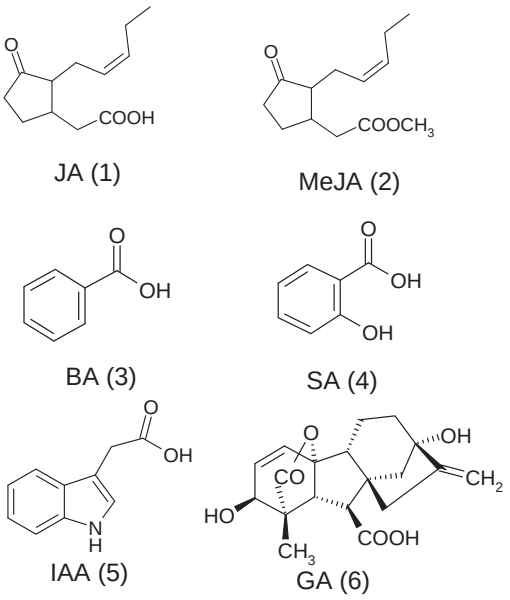

Fig. 1. Structures of compounds: JA (1), MeJA (2), BA (3), SA (4), IAA (5), and GA (6).

tionship between JMT and other members of the SABATH family, however, has not been extensively studied.

While *SAMT*/*BAMT* and *IAMT* genes have been isolated and characterized from multiple plant species, *JMT* genes have been isolated from only Arabidopsis (Seo et al., 2001) and tomato (Tieman et al., 2010). Overexpression of the Arabidopsis *JMT* gene (*AtJMT*) led to the elevated level of MeJA (2), and the transgenic Arabidopsis plants exhibited a higher level of resistance to a virulent fungus *Botrytis cinerea* than wild type plants (Seo et al., 2001). The biological function of the tomato *JMT* gene (cLEI3O14) has not been studied. AtJMT and the tomato JMT are not apparent orthologs, with the tomato JMT showing closer relatedness to BSMT than AtJMT (Tieman et al., 2010). AtJMT and tomato JMT also displayed differences in substrate specificity, with AtJMT being highly specific with JA (1) (Seo et al., 2001) while tomato JMT showing activity with BA (3) in addition to JA (1) (Tieman et al., 2010). It is also intriguing to ask how JMTs with different degrees of promiscuity have evolved. In order to gain insight into these questions, it is important to isolate and characterize *JMT* genes from other plant species.

In this study, black cottonwood (*Populus trichocarpa*) was chosen as a model plant species for the isolation and functional characterization of *JMT*. Due to the relative ease of genetic manipulation and the availability of a fully-sequenced genome (Bradshaw et al., 2000; Tuskan et al., 2006), black cottonwood has become an important model for studying tree physiology and ecology. It has also been used as a model plant for the study of the plant SABATH family of methyltransferasese: The *IAMT* gene has been isolated and characterized from this plant (Zhao et al., 2007). Herein is reported the molecular cloning and biochemical functional characterization of the *JMT* gene from black cottonwood (*PtJMT1*), which is the apparent ortholog of *AtJMT*.

2. Results

2.1. Identification of the jasmonic acid carboxyl methyltransferase gene from poplar

Using the protein sequence of *C. breweri* SAMT (CbSAMT), the prototype SABATH (Ross et al., 1999) as query, the blast search analysis of the genome of *P. trichocarpa* led to the identification of 28 SABATH proteins designated PtSABATH1 to PtSABATH28 (Supplemental Table 1). PtSABATH3 was most similar to Arabidopsis JMT (AtJMT) with a 57% sequence identity. To determine whether PtSABATH3 function as JMT, a full-length cDNA of PtSABATAH3 was amplified from poplar tissues using RT-PCR, cloned into a protein expression vector, and expressed in *Escherichia coli* to produce recombinant enzyme. Recombinant PtSABATH3 was tested for methyltransferase activity with JA (1), BA (3), salicylic acid (4) (SA), indole-3-acetic acid (5) and gibberellic acid (6) (structures 3–6 not shown). PtSABATH3 showed the highest level of specific activity with JA (1). Methyl transfer catalyzed by PtSABATH3 using JA (1) as substrate was demonstrated to occur to the carboxyl group (Supplementary Fig. 1). PtSABATH3 also showed activity with BA (3), which was about 22% of that with JA (1). It did not show activity with other carboxylic acids tested. Therefore, PtSABATH3 was renamed as PtJMT1.

To obtain information about whether the close homologs of PtJMT1 have JMT activity, PtSABATH2 and PtSABATH4 were analyzed. These two genes were selected for analysis because among all PtSABATHs these two were most similar to PtJMT1. Full-length cDNAs for PtSABATH2 and PtSABATH4 were cloned from poplar tissues and expressed in *E. coli*. *E. coli*-expressed PtSABATH2 and PtSABATH4 were tested for methyltransferase activity with JA (1), BA (3), SA (4), indole-3-acetic acid (5) and gibberellic acid (6). PtSABATH2 did not show activity with any of the substrates tested. PtSABATH4 also did not show activity with JA (1). However, PtSABATH4 displayed the highest level of catalytic activity with SA (4) and a relatively low level of activity with BA (3).

2.2. Biochemical properties of PtJMT1

Escherichia coli-expressed PtJMT1 was purified and then subject to detailed biochemical characterization. Under pseudo Michaelis–Menten conditions, PtJMT1 exhibited apparent *Km* values 175.7 ± 15.8 μM and 341.3 ± 31.2 μM for JA (1) and BA (3), respectively (Fig. 2A and B). The *Kcat* for JA (1) and BA (3) was 0.013 S^{-1} and 0.003 S^{-1}, respectively. For thermostability, PtJMT1 was 100% stable for 30 min at 4 °C. It lost about 10%, 20% and 40% of its maximal activity when incubated at 37 °C, 42 °C and 50 °C, respectively for 30 min. When incubated at 60 °C for 30 min, PtJMT1 lost all of its activity (Fig. 2C). The optimum pH of PtJMT1 was 6.5. At pH 6.0, pH 7.0, pH 8.0 and pH 8.5, the enzyme displayed about 80%, 50%, 20% and 5% of its maximal activity, respectively (Fig. 2D). Certain ions can affect PtJMT1 activity. K^+ significantly stimulated PtJMT1 activity. NH^{4+} and Na^+ had a mild stimulation on PtJMT1 activity. Ca^{2+} and Mg^{2+} had a mild inhibitory effect. Mn^{2+} decreased the activity of PtJMT1 by 40%, and Cu^{2+}, Fe^{2+}, Fe^{3+} and Zn^{2+} all had a strong inhibitory effect on PtJMT1 activity (Fig. 2E).

2.3. Identification of residues of PtJMT1 important for different activities for JA (1) and BA (3)

To understand the structural basis underlying the different specific activities of PtJMT1 with JA (1) and BA (3), in particular why PtJMT1 showed higher specific activity with JA (1) the former, (3), two amino acids, Ser-153 and Asn-361, of PtJMT1 were selected for site-directed mutagenesis studies. As an active site residue, Ser-153 was selected because previous studies have shown that the residue at this position is important for determining the activity of SABATH proteins in methylating JA (1) or SA/BA (4/3). For example, a Tyr to Ser mutation at this position of CbSAMT showed increased activity with JA (1) (Zubieta et al., 2003). For the S153Y mutant of PtJMT1, its activities with JA (1) and BA (3) were reduced to about 3% and 38% of its activities with the wild type enzyme (Fig. 3). Asn-361 is another active site residue. It was noticed that several enzymes using BA (3) as the most preferred substrate, but which have no activity with JA (1), have

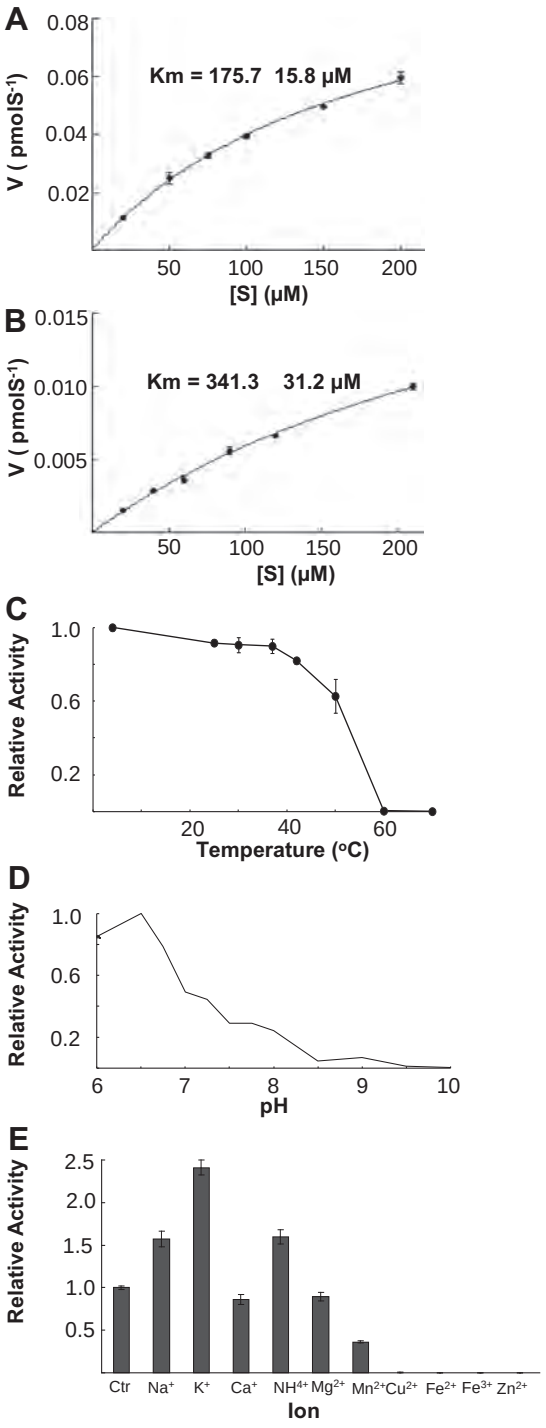

Fig. 2. Biochemical properties of PtJMT1. (A) Steady-state kinetic measurements of PtJMT1 using jasmonic acid (1) as substrate. (B) Steady-state kinetic measurements of PtJMT1 using benzoic acid (3) as substrate. (C) Thermostability of PtJMT1. The activity of PtJMT1 incubated at 4 °C for 30 min (0.71 ± 0.01 pmol/s) was arbitrarily set at 1.0. (D) pH effect on PtJMT1 activity. Level of PtJMT1 activity in the buffer of pH 6.5 (0.84 ± 0.04 pmol/s) was arbitrarily set at 1.0. (E) Effects of metal ions on activity of PtJMT1. Metal ions were added to reactions in the form of chloride salts at a final concentration of 5 mM. Level of PtJMT1 activity without any metal ion added as control (0.74 ± 0.04 pmol/s) was arbitrarily set at 1.0. Jasmonic acid (1) was used as the substrate for enzyme assays in (C) to (E). Each value is an average of three independent measurements.

a Ser at this position (Supplementary Fig. 2). To test the importance of this variation, a N361S mutant was generated. The activities of the N361S mutant using JA (1) and BA (3) as substrate were about

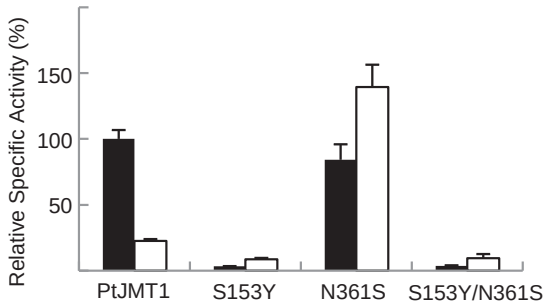

Fig. 3. Substrate specificities of PtJMT1 and its mutants. The relative specific activities of two mutants of PtJMT1, S153Y and N361S, and double mutant S153Y/N361S for using jasmonic acid (1) (black bars) and benzoic acid (3) (white bars), were compared to those of PtJMT1. The PtJMT1 activity with jasmonic acid (1) (0.72 ± 0.02 pmol/s) was arbitrarily set at 1.0. Each value is an average of three independent measurements.

84% and 140% of the activities of the wild type enzyme (Fig. 3). In contrast, the S153Y/N361S double mutant showed activities that were very similar to those of the S153Y single mutant (Fig. 3). Because the N361S mutant exhibited specific activities that were comparable to those of the wild type enzyme, it was further examined for its kinetic parameters. The apparent *Km* values of the N361S mutant using JA (1) and BA (3) as substrate were determined to be 310.3 ± 12.6 μM and 63.1 ± 13.6 μM, respectively. Its corresponding *Kcat* values for using JA (1) and BA (3) as substrates were determined to be 0.015 S^{-1} and 0.028 S^{-1}, respectively. All three mutants were also tested for methyltransferase activity with SA (4), indole-3-acetic acid (5) and gibberellic acid (6), but did not show any activity.

2.4. The structural basis of changed substrate specificity of the N361S mutant of PtJMT1

Based on a previous study, the relative reduction of specific activity of the PtJMT1 S153Y mutant with JA (1) is probably because this change occludes much of the active site near the SAM/SAH binding site, which makes the binding of JA (1) in the mutant less sterically favorable than the wild type enzymes (Zubieta et al., 2003). To further understand the structural basis underlying the specific activity changes involving N361S, homology-based structural models of the complexes of PtJMT1 and its N361S mutant with substrate JA (1) (Fig. 4A and B) and BA (3) (Fig. 4C and D) were created. The alignment between the carboxylate of BA (3) and SAH did not change significantly in going from the wild-type (Fig. 4C) to the mutated enzyme (Fig. 4D). By contrast, a significant change was observed in the alignment between the carboxylate of JA (1) and SAH in going from wild-type (Fig. 4A) to the mutant (Fig. 4B). The homology modeling and docking calculations suggest that the reduced specific activity of N361S mutant with JA (1) (Fig. 3) was partly due to the relatively poor alignment of JA (1) in the active site.

2.5. Expression of PtJMT1 in different tissues

To determine in what plant tissues *PtJMT1* is expressed, total RNA was isolated from young leaves, old leaves, stems and roots of one-year-old poplar trees and used for gene expression analysis by semi-quantitative RT-PCR. *PtJMT1* expression was observed in all four tissues examined with the highest level of expression detected in stems (Fig. 5).

2.6. Expression of PtJMT1 in poplar leaves under stress conditions

To determine the expression patterns of *PtJMT1* under stress conditions, black cottonwood seedlings were treated with three biotic stress factors: SA (4), MeJA (2) and a fungal elicitor alamethi-

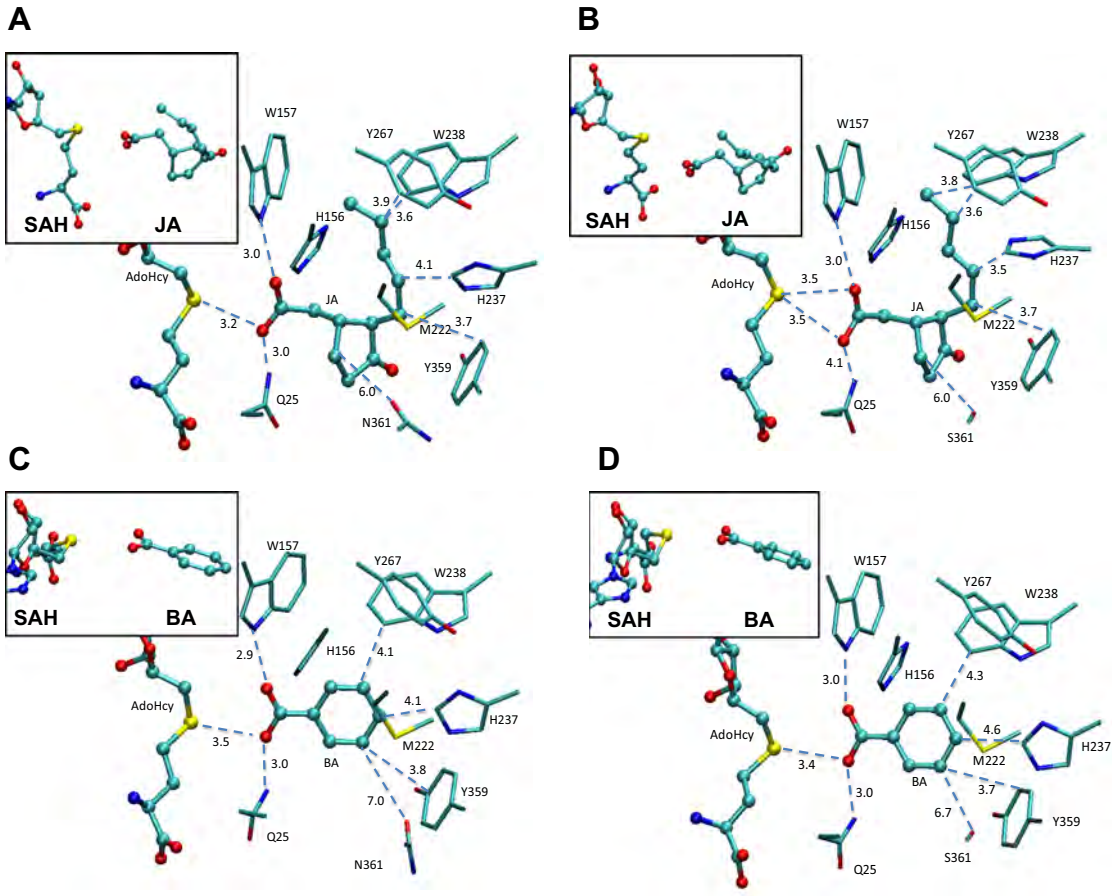

Fig. 4. Homology models of the active site of wild-type PtJMT1 and the mutant of PtJMT1 (N361S) complexed with *S*-adenosyl-L-homocysteine (AdoHcy or SAH) and jasmonic acid (1) (JA) or benzoic acid (2) (BA). PtJMT1 and its mutant (N361S) are shown in sticks. SAH, JA and BA (3) are shown in balls and stick models. Some distances are given. The relative orientation of SAH and the carboxylate group in each structure is shown on the top (left). (A) The wild-type PtJMT1 complexed with SAH and JA (1). (B) The PtJMT1 mutant (N361S) complexed with SAH and JA (1). (C) The wild-type PtJMT1 complexed with SAH and BA (3). (D) The PtJMT1 mutant (N361S) complexed with SAH and BA (3).

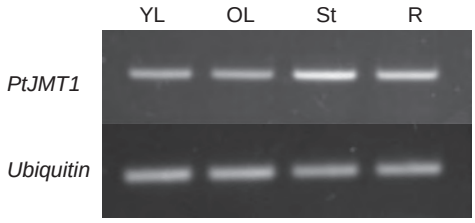

Fig. 5. Semi-quantitative RT-PCR analysis of *PtJMT1* expression. Young leaves (YL), old leaves (OL), stems (St) and roots (R) were collected from 1-year-old poplar trees grown in a greenhouse. Total RNA was extracted and used for RT-PCR analysis. PCR with primers for a ubiquitin gene (Ubiquitin) was used to judge equality of concentration of cDNA templates in different samples.

cin. Leaf tissues were collected for gene expression analysis (Fig. 6). SA treatments at both 2 h and 24 h caused significant induction of *PtJMT1* expression. At 24 h, the expression increased one fold. For MeJA (2), while the shorter time treatment (40 min) did not change the expression of *PtJMT1*, 4 h treatment led to more than one fold induction. The treatment by alamethicin for 2 h also led to significant induction in *PtJMT1* expression (Fig. 6).

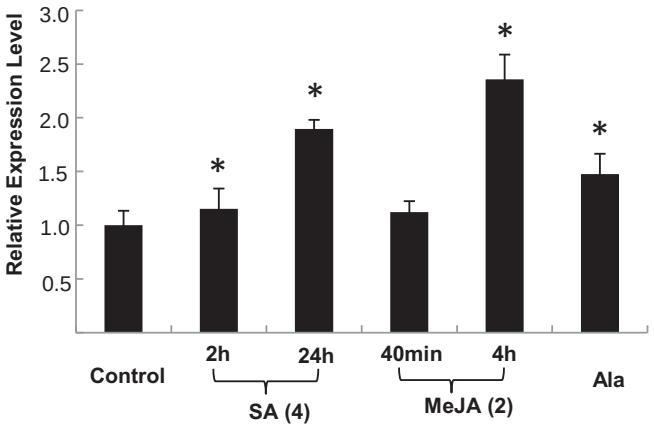

Fig. 6. Expression of *PtJMT1* under stress conditions. Seedlings of black cottonwood were treated with salicylic acid (3) (SA) for 2 h and 24 h, methyl jasmonate (2) (MeJA) for 40 min and 4 h, alamethicin (Ala) for 2 h. Leaves were collected and used for quantitative RT-PCR analysis. The expression level of *PtJMT1* in untreated control was arbitrarily set at 1.0. Three biological replicates and three technical replicates were performed for gene expression analysis. The expression levels were presented as mean + SE. Bars marked with asterisk (*) indicate that *PtJMT1* showed a significant difference in expression levels in treated vs. control plants ($p < 0.05$).

2.7. Phylogenetic analysis of PtJMT1 with other known SABATH proteins

To understand the evolutionary relatedness between PtJMT1 with other known SABATH proteins, a phylogenetic tree was reconstructed using a number of biochemically characterized MTs (Fig. 7). The SABATH proteins analyzed include PtJMT1, PtSABATH2, PtSABATH4, AtJMT, tomato JMT (SlJMT), CbSAMT, IAMTs from Arabidopsis, poplar and rice, farnesoic acid methyltransferase from Arabidopsis (AtFAMT) and two gibberellic acid methyltrans-

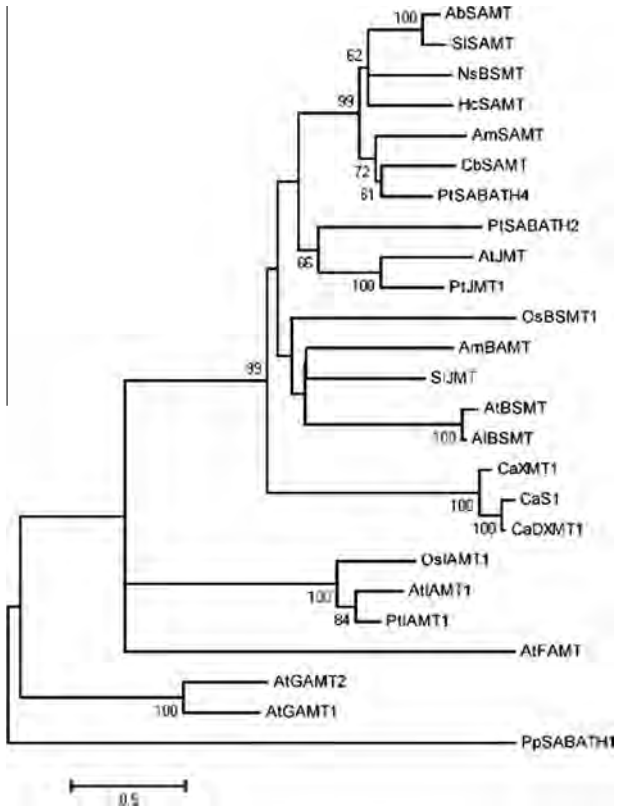

Fig. 7. Phylogenetic analysis of PtJMT1 with other known SABATH proteins. CbSAMT, *C. breweri* SAMT (Accession # AF133053); AmSAMT, *Antirrhinum majus* SAMT (Accession # AF515284); HcSAMT, *Hoya carnosa* SAMT (Accession # AJ863118); AmBAMT: *A. majus* BAMT (Accession # AF198492); NsBSMT, *Nicotiana suaveolens* BSMT (Accession # AJ628349); AtBSMT, *Arabidopsis thaliana* BSMT (Accession # BT022049); AlBSMT, *Arabidopsis lyrata* BSMT (Accession # AY224596); AbSAMT, *Atropa belladonna* SAMT (Accession # AB049752); OsBSMT1, *Oryza sativa* BSMT1 (Accession # XM467504); SlSAMT, *Solanum lycopersicum* SAMT (Accession # NM001247880); AtJMT, *Arabidopsis thaliana* JMT (Accession # AY008434); SlJMT, *Solanum lycopersicum* JMT (EST cLEI13O14); AtFAMT, *Arabidopsis thaliana* FAMT (Accession # AY150400); AtIAMT1, *Arabidopsis thaliana* IAMT1 (Accession # AK175586); PtIAMT1, *Populus trichocarpa* IAMT1 (fgenesh4_pg.C_LG_I002808); OsIAMT1, *Oryza sativa* IAMT1 (Accession # EU375746); AtGAMT1, *Arabidopsis thaliana* GAMT1 (At4g26420); AtGAMT2, *Arabidopsis thaliana* GAMT2 (At5g56300); Cas1, *Coffea arabica* caffeine synthase 1 (Accession # AB086414); CaXMT1, *Coffea arabica* xanthosine methyltransferase 1 (Accession # AB048793); CaDXMT1, *Coffea arabica* 3,7-dimethylxanthine methyltransferase 1 (Accession # AB084125); PtSABATH2, *Populus trichocarpa* SABATH2 (Accession # KC894592); PtSABATH4, *Populus trichocarpa* SABATH4 (Accession # KC894591). PpSABATH1, *P. patens* SABATH1 (gw1.47.108.1). PpSABATH1 was used as outgroup. Branches are drawn to scale with the bar indicating 0.1 substitutions per site.

ferases from Arabidopsis, selected characterized SAMTs from a variety of plant species and three caffeine synthases (CaXMT1, CaDXMT1, CaS1). A SABATH protein from the moss *Physcomitrella patens* (PpSABATH1, Zhao et al., 2012) was used as an outgroup (Fig. 7). Phylogenetic analysis showed that PtJMT1 was most closely related to AtJMT, while the tomato JMT showed closer relatedness to BAMT and BSMTs than to PtJMT1 and AtJMT (Fig. 7).

3. Discussion

In this article, the isolation, biochemical characterization, structure–function analysis and expression analysis of the *PtJMT1* gene from black cottonwood is reported encoding jasmonic acid methyltransferse. *PtJMT1* is the third plant *JMT* gene, after *AtJMT* from Arabidopsis (Seo et al., 2001) and the tomato *JMT* (Tieman et al., 2010), to be isolated and biochemically characterized. Phylogenetic analysis suggests that *PtJMT1* and *AtJMT* are apparent orthologs, implying the existence of a *JMT* gene in the last common ancestor of the poplar and Arabidopsis lineages. In contrast, the tomato *JMT* gene shows a closer relatedness to *BSMTs*/*SAMTs* than to *PtJMT1* and *AtJMT* (Fig. 7). Because the tomato genome has recently been fully sequenced (Tomato Genome Consortium, 2012), the tomato genome sequence (http://solgenomics.net/tools/blast/index.pl) was searched for all putative *SABATH* genes. This analysis did not identify an apparent ortholog of *PtJMT1* and *AtJMT* in tomato, which implies independent evolution of *JMT* in poplar/Arabidopsis and tomato lineages.

Despite their close evolutionary relatedness, PtJMT1 and AtJMT displayed striking differences in substrate specificity. While both enzymes showed the highest level of catalytic activity with JA (1), PtJMT1 and AtJMT showed different degrees of promiscuity. PtJMT1 can also use BA (3) as a substrate, while AtJMT does not (Seo et al., 2001). The tomato JMT also has the highest level of relative activity with JA (1). It can also use BA (3) as a substrate, resembling PtJMT1. This raises an intriguing question about substrate specificity evolution of JMT and JMT-related enzymes. From previous studies, *IAMT* was found to be conserved in rice, Arabidopsis, poplar and white spruce, indicating that *IAMT* is conserved in seed plants (Zhao et al., 2008). It was hypothesized that *JMT* and *SAMT*/*BAMT* may have evolved from *IAMTs*. However, the relationship between *JMT* and *SAMT*/*BAMT* is not clear. A previous study suggested that the ancestor of *SAMT* and *BSMT* may have *BAMT* activity (Chen et al., 2003a). Because both PtJMT1 and the tomato JMT exhibit activity with BA (3), it is tempting to speculate that JMT evolved from BAMT. If this hypothesis is proven, it would suggest that the specific activity of AtJMT for JA (1) but not BA (2) represents a selection for specificity. The evolution of specificity can be achieved by changes of a few amino acids. As shown in our site-directed mutagenesis studies, the relative specificity of PtJMT1 using JA (1) and BA (2) as substrates can be readily reversed by the change of one of the two amino acids tested (Fig. 3). In the case of SAMT/BSMT, a positive selection of a single amino acid appears to play a critical role in the relative specificity with SA (4) and BA (2) (Barkman et al., 2007). It will be interesting to determine whether positive selection is also a mechanism responsible for the evolution of JMT and BAMT. Continued isolation and functional characterization of *JMT*, *SAMT*, and *IAMT* genes from plants of key phylogenetic positions will open new avenues for the understanding of the trajectory and mechanisms underlying substrate specificity evolution of the SABATH family.

In a previous study (Yao et al. 2011), it was demonstrated that a good alignment between the transferable methyl group of SAM and the lone pair of electrons on the oxygen of the substrate carboxylic acids is of importance for the efficient methyl transfer. For CbSAMT, a good alignment could be achieved in the reactant complex of this enzyme containing SA (4) and SAM, but not in the complex containing 4-hydroxybenzoate (4HA) and SAM. These results are consistent with experimental observation that for CbSAMT the activity on 4HA is only 0–0.8% of that on SA (4) (Zubieta et al., 2003). In this study, the result of the homology-based structural modeling of the PtJMT1 mutant N361S reinforces this notion: the N361S mutant showed reduced catalytic efficiency (*Kcat*/*Km*) with JA (1) than the wild type enzyme, which could be partly explained by the changed alignment of JA (1) in the active site (Fig. 4). While the structural basis for the increased activity of the N361S mutant with BA (2) is unclear, this result does suggest that the residue at the 361 position of PtJMT1 plays an important role in determining its relative specific activities towards JA (1) and BA (2).

In addition to being a model tree species, poplar is also considered an important bioenergy crop (Davis, 2008). Improving poplar trees for increased resistance to biotic stresses is integral to the ge-

netic improvement of poplar trees for higher biomass yield. Understanding natural defense mechanisms of poplar trees will be of profound importance for such endeavors. The expression of *PtJMT1* was induced by a number of stress factors tested (Fig. 6), suggesting that this gene has an important role in regulating the defense response of poplar trees against biotic stresses. It will be useful to elucidate the molecular mechanisms underlying *PtJMT1*-mediated plant resistance. At the same time, *PtJMT1* may be used as an important gene for rationally designed genetic engineering of poplar trees for improved resistance against biotic stresses.

4. Concluding remarks

PtJMT1 is the third S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase gene, after the Arabidopsis gene *AtJMT* and tomato *JMT*, to be isolated and biochemically characterized. Among all SABATH proteins in poplar and Arabidopsis, PtJMT1 is most similar to AtJMT, implying that the genes encoding them are orthologs. JA (1) is the most preferred substrate for PtJMT1, which also displayed activity with BA (3). It is not yet clear which of the two enzymes, PtJMT1 or AtJMT, represents the ancestral state of substrate specificity of JMT. Site-directed mutagenesis studies identified two amino acids in the active site of PtJMT1 that are important for determining the relative specificity between JA (1) and BA (3). The expression of *PtJMT1* was induced by a number of stress factors, suggesting that this gene has a role in the defense of poplar trees against biotic stresses.

5. Experimental

5.1. Sequence retrieval and analysis

The protein sequence of AtJMT (Accession: AY008434) was used initially as a query to search against the poplar genome database (www.phytozome.net) using the BlastP algorithm (Altschul et al., 1990). The poplar gene encoding a protein that showed the highest level of sequence similarity with AtJMT was chosen for further analysis. Multiple protein sequence alignments were constructed using the ClustalX software (Thompson et al., 1997), and displayed using GeneDoc (http://www.psc.edu/biomed/genedoc/). The phylogenetic trees were produced using Paup3.0 and viewed using the TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

5.2. Plant material and chemicals

The female black cottonwood (*P. trichocarpa*) clone 'Nisqually-1', which was used for whole genome sequencing, was used for gene cloning and expression analysis of *PtJMT1*. Young leaves (newly-emerged leaves), old leaves (5 cm in length), stems and roots were collected from one-year-old poplar trees and used for examining tissue specificity of *PtJMT1* expression. To examine *PtJMT1* expression under stress conditions, leaf tissues were collected from poplar plants at the eight-leaf stage that were vegetatively propagated grown on MS medium. These plants were treated with either alamethicin for 2 h, SA (4) for 2 h and 24 h, or MeJA (2) for 40 min and 4 h, as previously described (Zhao et al., 2009). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise noted.

5.3. Cloning full-length cDNA of PtSABATH genes

Total RNAs from different poplar tissues were extracted using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). An on-column DNase (Qiagen, Valencia, CA, USA) treatment was performed to remove genomic DNA contamination. 1.5 μg of total RNA for each sample was reverse- transcribed into first strand cDNA in a 15 μl reaction volume using the First-strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA), as previously described (Chen et al., 2003b). Full-length cDNA of *PtSABATH* genes were amplified from mixed cDNAs. Primers used for gene cloning are listed in Supplemental Table 2. The PCR products were cloned into pEXP5/CT TOPO vector using the protocol recommended by the vendor (Invitrogen, Carlsband, CA). The cloned cDNAs in pEXP5/CT TOPO vector were sequenced using T7 primers.

5.4. Purification of recombinant PtJMT1

The full-length cDNA of *PtJMT1* in pEXP5/CT TOPO was subcloned into a protein expression vector pET32a. To express recombinant PtJMT1 in *E. coli*, the protein expression construct was transformed into *E. coli* strain BL21 Codon Plus (Stratagene, La Jolla, CA). Protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) for 18 h at room temperature, and the cells were lysed by sonication. His-tagged PtJMT1 protein was purified from the *E. coli* cell lysate using Ni–NTA agarose following the manufacturer instructions (Invitrogen, Carlsband, CA). Protein purity was verified by SDS–PAGE, and protein concentrations were determined by the Bradford assay (Bradford, 1976).

5.5. Radiochemical activity assay

Radiochemical assay was performed with a 50 μl volume containing 50 mM Tris–HCl, pH 7.5, 1 mM JA (1) and 0.4 μl ^{14}C-adenosyl-L-methionine (SAM) (Perkin Elmer, Boston, MA). The assay was started by the addition of SAM and kept at room temperature for 30 min. The reaction was stopped by the addition of EtOAc 150 μl, vortexed, and phase-separated by a 1 min centrifugation at 14,000*g*. The upper organic phase was counted using a liquid scintillation counter (Beckman Coulter, Fullerton, CA), as previously described (D'Auria et al., 2002). The amount of radioactivity in the organic phase indicated the amount of synthesized MeJA (2). All assays were conducted with three replicates.

5.6. Determination of kinetic parameters of PtJMT1

In all kinetic analyses, the appropriate enzyme concentrations and incubation times were chosen so that the reaction velocity was linear during the reaction time period. For the PtJMT1 and PtJMT1 mutants, to determine the *Km* for JA (1) and BA (3), then concentrations were independently varied from 10 μM to 200 μM, while SAM was held constant at 200 μM. Assays were conducted at 25 °C for 30 min. The kinetic parameters *Km* and *Vmax* were calculated with GraphPad Prism 5 software for Windows (GraphPad Software Inc.), using standard settings for non-linear regression curve fitting in the Michaelis–Menten equation.

5.7. pH optimum for PtJMT1 activity

PtJMT1 activities were determined in 50 mM Bis–tris buffer with pH ranging from 6.0 to 7.5, and 50 mM Tris–HCl buffer with pH ranging from 7.5 to 10.0 using the standard radiochemical activity assay. The data presented are an average of three independent assays.

5.8. Effectors

A standard radiochemical activity assay was carried out in the presence of one of the following cations present at a final concentration of 5 mM: K^+, Ca^{2+}, NH_4^+, Na^+, Mg^{2+}, Mn^{2+}, Cu^{2+},

Fe^{2+}, Fe^{3+}, Zn^{2+}. Results presented are an average of three independent assays.

5.9. Semi-quantitative RT-PCR analysis of PtJMT1 expression

Total RNA extraction from young leaves, old leaves, stems and roots of one-year-old poplar trees, and subsequent first-strand cDNA synthesis were performed as described in Section 5.3. For PCR analysis of *PtJMT1* expression in different organs, primers were designed to amplify a *PtJMT1* fragment of 566 bp as follows: forward primer 5′-TATGCCAAATGCAGAGAGTTGG-3′ and reverse primer 5′-GACCTTTTCTTTCTCGACGAGC-3′. Two primers used for PCR amplification of an Ubiquitin gene were designed as previously described (Kohler et al., 2004): forward primer 5′-CAGGGAAACAGTGAGGAAGG-3′ and reverse primer 5′-TGGAC TCACGAGGACAG-3′. PCR analysis was performed as previously described (Zhao et al., 2007). All PCRs were replicated twice using first-strand cDNA made from two independent RNA preparations.

5.10. Quantitative RT-PCR

Quantitative PCR was performed on an ABI7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR green fluorescence dye (Bio-Rad Laboratories), as previously described (Yang et al., 2006). The gene-specific primers were designed as followed: forward primer 5′-CCCACCACGGACGA-GAGT-3′ and the reverse primer 5′-CCCCTCAGAAACCATGCTCAT-3. The two primers used for the PCR amplification of a Ubiquitin gene were designed as the internal control: forward primer 5′-CAG-GGAAACAGTGAG GAAGG-3′ and reverse primer 5′-TGGACT CACGAGGACAG-3′.

5.11. Site-mutagenesis of PtJMT1

The cDNAs for encoding PtJMT1 mutants, S153Y, N361S and double mutant S153Y/N361S were produced using a PCR method. For the mutant S153Y, two totally complementary primers, which contain the mutation site, were designed as follows: the forward mutant primer 5′-GTGTGCACTCTTCTTATAGTCTTCACTGGCTC-3′ and the reverse mutant primer 5′-GAGCCAGTGAAGACTATAAGAA GAGTGCACAC-3′. The plasmid containing the full-length *PtJMT1* was used as template for PCR amplification. Two fragments were amplified: one using the forward primer of *PtJMT1* and the mutant reverse primer; the other using the reverse primer of *PtJMT1* and the mutant forward primer. These two fragments were put together as templates for the second round PCR amplification, for which the primers for the full-length cDNA of *PtJMT1* were used. The PCR product was cloned into pEXP5/CT TOPO vector and fully sequenced. For the mutant N361S, because the mutation site is located near the 3′ end of the gene, only one reverse primer containing the mutated nucleotide was designed: 5′-TTAATTTCTAACC ATTGAAATGACCAAGCTGGTGTACTTG-3′. The full length of mutant was amplified using this reverse primer and the *PtJMT1* forward primer. The double mutant S153Y/N361S was constructed on the basis of these two mutants using the same method.

5.12. Structural modeling

Based on the X-ray structure of CbSAMT (PDB ID: 1M6E), a homology model of wild type PtJMT1 was built by using the homology modeling program in the molecular operation environment (MOE). SAH in the model of the PtJMT1 complex was built based on the superposition of the model with the X-ray structure of the CbSAMT complex that contains SAH. JA (1) and BA (3) were then docked, respectively, into the PtJMT1 active site by using the MOE docking program to generate PtJMT1 complexes with JA and BA. The N361S mutant of PtJMT1 complexed with JA/BA (1/3) and SAH were built based on manually mutating Asn-361 into Ser-361 in the corresponding wild-type complex. Finally, the energy minimization was carried on for each of four complexes to generate the final models used in this paper. The attempts to generate the models containing SAM and substrate (JA or BA) were unsuccessful.

5.13. Statistical analysis

Statistical analysis of *PtJMT1* expression in control and treated plants was conducted using SAS (Version 9.2) (SAS Institute, NC) based on three biological replicates and three technical replicates. Levels of significance were calculated using Student's *t*-test at $p < 0.05$.

Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov) with the accession numbers KC894590 (PtJMT1), KC894591 (PtSABATH4) and KC894592 (PtSABATH2).

The homology model of PtJMT1 was deposited in the PMBD protein model database (http://www.caspur.it/PMDB/) with the accession number of PM0079066.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.06.014.

References

Altschul, S.F., Stephen, F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Barkman, T.J., Martins, T.R., Sutton, E., Stout, J.T., 2007. Positive selection for single amino acid change promotes substrate discrimination of a plant volatile-producing enzyme. Mol. Biol. Evol. 24, 1320–1329.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Bradshaw, H.D., Ceulemans, R., Davis, J., Stettler, R., 2000. Emerging model systems in plant biology: poplar (*Populus*) as a model forest tree. J. Plant Growth Regul. 19, 306–313.
Chen, F., D'Auria, J.C., Tholl, D., Ross, J.R., Gershenzon, J., Noel, J.P., Pichersky, E., 2003a. An *Arabidopsis* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. Plant J. 36, 577–588.
Chen, F., Tholl, D., D'Auria, J.C., Farooq, A., Pichersky, E., Gershenzon, J., 2003b. Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. Plant Cell 15, 481–494.
Cheong, J.J., Choi, Y.D., 2003. Methyl jasmonate as a vital substance in plants. Trends Genet. 19, 409–413.
Creelman, R.A., Tierney, M.L., Mullet, J.E., 1992. Jasmonic acid methyl Jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc. Natl. Acad. Sci. USA 89, 4938–4941.
Creelman, R.A., Mullet, J.E., 1995. Jasmonic acid distribution and action in plants – regulation during development and response to biotic and abiotic stress. Proc. Natl. Acad. Sci. USA 92, 4114–4119.
D'Auria, J.C., Chen, F., Pichersky, E., 2003. The SABATH family of MTs in *Arabidopsis thaliana* and other plant species. In: Romeo, J.T. (Ed.), Recent Advances in Phytochemistry, vol. 37. Elsevier Science Ltd., Oxford, pp. 253–283.
D'Auria, J.C., Chen, F., Pichersky, E., 2002. Characterization of an acyltransferase capable of synthesizing benzylbenzoate and other volatile esters in flowers and damaged leaves of *Clarkia breweri*. Plant Physiol. 130, 466–476.

Davis, J.M., 2008. Genetic improvement of poplar (*Populus* spp.) as a bioenergy crop. In: Vermerris, W. (Ed.), Genetic Improvement of Bioenergy Crops. Springer, New York, pp. 397–419.

Howe, G.A., 2005. Jasmonates as signals in the wound response. J. Plant Growth Regul. 23, 223–237.

Kessler, A., Baldwin, I.T., 2001. Defensive function of herbivore induced plant volatile emissions in nature. Science 291, 2141–2144.

Knudsen, J.T., Tollsten, L., Bergstrom, L.G., 1993. Floral scents – a checklist of volatile compounds isolated by headspace techniques. Phytochemistry 33, 253–280.

Kohler, A., Blaudez, D., Chalot, M., Martin, F., 2004. Cloning and expression of multiple metallothioneins from hybrid poplar. New Phytol. 164, 83–93.

Koo, A.J., Gao, X., Jones, A.D., Howe, G.A., 2009. A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. Plant J. 59, 974–986.

Murfitt, L.M., Kolosova, N., Mann, C.J., Dudareva, N., 2000. Purification and characterization of *S*-adenosyl-L-methionine: benzoic acid carboxyl methyltransferase, the enzyme responsible for biosynthesis of the volatile ester methyl benzoate in flowers of *Antirrhinum majus*. Arch. Biochem. Biophys. 382, 145–151.

Nojavan-Asghari, M., Ishizawa, K., 1998. Inhibitory effects of methyl jasmonate on the germination and ethylene production in cocklebur seeds. J. Plant Growth Regul. 17, 13–18.

Qin, G., Gu, H., Zhao, Y., Ma, Z., Shi, G., Yang, Y., Pichersky, E., Chen, F., Liu, M., Chen, Z., Qu, L.J., 2005. Regulation of auxin homeostasis and plant development by an indole-3-acetic acid carboxyl methyltransferase in Arabidopsis. Plant Cell 17, 2693–2704.

Reymond, P., Farmer, E.E., 1998. Jasmonate and salicylate as global signals for defense gene expression. Curr. Opin. Plant Biol. 1, 404–411.

Ross, J.R., Nam, K.H., D'Auria, J.C., Pichersky, E., 1999. *S*-Adenosyl-L-methionine: salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. Arch. Biochem. Biophys. 367, 9–16.

Seo, H.S., Song, J.T., Cheong, J.J., Lee, Y.H., Lee, Y.W., Hwang, I., Lee, J.S., Choi, Y.D., 2001. Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc. Natl. Acad. Sci. USA 98, 4788–4793.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The Clustalx windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Tieman, D., Zeigler, M., Schmelz, E., Taylor, M.G., Rushing, S., Jones, J.B., Klee, H.J., 2010. Functional analysis of a tomato salicylic acid methyl transferase and its role in synthesis of the flavor volatile methyl salicylate. Plant J. 62, 113–123.

Tomato Genome Consortium, 2012. The tomato genome sequence provides insights into fleshy fruit evolution. Nature 485, 635–641.

Tuskan, G.A., DiFazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U., Putnam, N., Ralph, S., Rombauts, S., Salamov, A., Schein, J., Sterck, L., Aerts, A., Bhalerao, R.R., Bhalerao, R.P., Blaudez, D., Boerjan, W., Brun, A., Brunner, A., Busov, V., Campbell, M., Carlson, J., Chalot, M., Chapman, J., Chen, G.L., Cooper, D., Coutinho, P.M., Couturier, J., Covert, S., Cronk, Q., Cunningham, R., Davis, J., Degroeve, S., Dejardin, A., Depamphilis, C., Detter, J., Dirks, B., Dubchak, I., Duplessis, S., Ehlting, J., Ellis, B., Gendler, K., Goodstein, D., Gribskov, M., Grimwood, J., Groover, A., Gunter, L., Hamberger, B., Heinze, B., Helariutta, Y., Henrissat, B., Holligan, D., Holt, R., Huang, W., Islam-Faridi, N., Jones, S., Jones-Rhoades, M., Jorgensen, R., Joshi, C., Kangasjarvi, J., Karlsson, J., Kelleher, C., Kirkpatrick, R., Kirst, M., Kohler, A., Kalluri, U., Larimer, F., Leebens-Mack, J., Leple, J.C., Locascio, P., Lou, Y., Lucas, S., Martin, F., Montanini, B., Napoli, C., Nelson, D.R., Nelson, C., Nieminen, K., Nilsson, O., Pereda, V., Peter, G., Philippe, R., Pilate, G., Poliakov, A., Razumovskaya, J., Richardson, P., Rinaldi, C., Ritland, K., Rouze, P., Ryaboy, D., Schmutz, J., Schrader, J., Segerman, B., Shin, H., Siddiqui, A., Sterky, F., Terry, A., Tsai, C.J., Uberbacher, E., Unneberg, P., et al., 2006. The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313, 1596–1604.

Varbanova, M., Yamaguchi, S., Yang, Y., McKelvey, K., Hanada, A., Borochov, R., Yu, F., Jikumaru, Y., Ross, J., Cortes, D., Ma, C., Noel, J.P., Mander, L., Shulaev, V., Kamiya, Y., Rodermel, S., Weiss, D., Pichersky, E., 2007. Methylation of gibberellins by Arabidopsis GAMT1 and GAMT2. Plant Cell 19, 32–45.

Yang, Y., Yuan, J.S., Ross, J., Noel, J.P., Pichersky, E., Chen, F., 2006. An *Arabidopsis thaliana* methyltransferase capable of methylating farnesoic acid. Arch. Biochem. Biophys. 448, 123–132.

Yao, J., Xu, Q., Chen, F., Guo, H., 2011. QM/MM free energy simulations of salicylic acid methyltransferase: the effects of transition state stabilization on substrate specificity. J. Phys. Chem. B 115, 389–396.

Zhao, N., Ferrer, J.L., Moon, H.S., Kapteyn, J., Zhuang, X., Hasebe, M., Stewart Jr., N.C., Gang, D.R., Chen, F., 2012. A SABATH methyltransferase from the moss *Physcomitrella patens* catalyzes S-methylation of thiols and has a role in detoxification. Phytochemistry 81, 31–41.

Zhao, N., Ferrer, J.L., Ross, J., Guan, J., Yang, Y., Pichersky, E., Noel, J.P., Chen, F., 2008. Structural, biochemical, and phylogenetic analyses suggest that indole-3-acetic acid methyltransferase is an evolutionarily ancient member of the SABATH family. Plant Physiol. 146, 455–467.

Zhao, N., Guan, J., Ferrer, J.L., Engle, N., Chern, M., Ronald, P., Tschaplinski, T.J., Chen, F., 2010. Emission, biosynthesis and regulation of insect-induced methyl salicylate and methyl benzoate from rice. Plant Physiol. Biochem. 48, 279–287.

Zhao, N., Guan, J., Forouhar, F., Tchaplinski, T., Cheng, Z.M., Tong, L., Chen, F., 2009. Two poplar methyl salicylate esterases display comparable biochemical properties but divergent expression patterns. Phytochemistry 70, 32–39.

Zhao, N., Guan, J., Lin, H., Chen, F., 2007. Molecular cloning and biochemical characterization of indole-3-acetic acid methyltransferase from poplar. Phytochemistry 68, 1537–1544.

Zubieta, C., Koscheski, P., Ross, J.R., Yang, Y., Pichersky, E., Noel, J.P., 2003. Structural basis for substrate recognition in the salicylic acid carboxyl methyltransferase family. Plant Cell 15, 1704–1716.