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Research article

Biosynthesis and emission of insect-induced methyl salicylate and methyl benzoate from rice

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ABSTRACT

Two benzenoid esters, methyl salicylate (MeSA) and methyl benzoate (MeBA), were detected from insect-damaged rice plants. By correlating metabolite production with gene expression analysis, five candidate genes encoding putative carboxyl methyltransferases were identified. Enzymatic assays with *Escherichia coli*-expressed recombinant proteins demonstrated that only one of the five candidates, OsBSMT1, has salicylic acid (SA) methyltransferase (SAMT) and benzoic acid (BA) methyltransferase (BAMT) activities for producing MeSA and MeBA, respectively. Whereas OsBSMT1 is phylogenetically relatively distant from dicot SAMTs, the three-dimensional structure of OsBSMT1, which was determined using homology-based structural modeling, is highly similar to those of characterized SAMTs. Analyses of *OsBSMT1* expression in wild-type rice plants under various stress conditions indicate that the jasmonic acid (JA) signaling pathway plays a critical role in regulating the production and emission of MeSA in rice. Further analysis using transgenic rice plants overexpressing *NH1*, a key component of the SA signaling pathway in rice, suggests that the SA signaling pathway also plays an important role in governing *OsBSMT1* expression and emission of its products, probably through a crosstalk with the JA signaling pathway. The role of the volatile products of OsBSMT1, MeSA and MeBA, in rice defense against insect herbivory is discussed.

1. Introduction

Upon insect herbivory, many plants emit elevated levels of volatile organic compounds [26]. These volatiles can defend plants indirectly by attracting carnivorous enemies of the herbivores (predators and parasitoids) [38]. Methyl salicylate (MeSA) is a frequent constituent of insect-induced plant volatiles. It has been detected in the headspace of many insect-infested plants, such as lima bean [2], Arabidopsis [5], tomato [1], and soybean [47]. When tested in its pure, synthetic form, MeSA can attract several types of carnivores [8,10,47], suggesting that MeSA, singly or in combination with other volatiles, is an active signal for indirect defense of tested plants. In addition, MeSA released from cabbage plants infested by cabbage moth was shown to inhibit the oviposition of conspecific mated female moths [40], indicating that MeSA can also be detected by herbivores. Methyl benzoate is structurally highly similar to MeSA. It is occasionally detected from insect-damaged plants [5].

In plants, MeSA and MeBA are synthesized by the action of S-adenosyl-L-methionine (SAM):salicylic acid (SA) carboxyl methyltransferase (SAMT) and SAM:benzoic acid (BA) carboxyl methyltransferase (BAMT), respectively. SAMT and BAMT belong to a protein family called the SABATH [7]. In addition to SA and BA, other known carboxylic acid substrates of the SABATH family include jasmonic acid (JA) [34], indole-3-acetic acid [31,44–46], gibberellic acids [41], farnesoic acid [42], cinnamic acid and *p*-coumaric acid [16] and loganic acid [21]. Other members of the SABATH family are involved in caffeine biosynthesis by catalyzing nitrogen methylation [25]. Some SAMTs possess BAMT activity and vice versa. Such an enzyme has been named BSMT. Genes encoding SAMT, BAMT and BSMT have been isolated and biochemically characterized from a number of plant species that include *Clarkia breweri* [32], snapdragon (*Antirrhinum majus*) [24], *Stephanotis floribunda* [29], *Nicotiana suaveolens* [29], and Arabidopsis [5]. The majority of

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these genes are involved in producing MeSA and/or MeBA as a floral scent compound. The Arabidopsis gene, *AtBSMT1*, is the only gene involved in herbivore-induced MeSA and MeBA production [5]. Interestingly, AtBSMT1 is phylogenetically more closely related to JA methyltransferase (JMT) than to other SAMTs, although it does not use JA as substrate [5]. Whether the production of insect-induced MeSA in other plant species is catalyzed by *SABATH* genes, and if so, how those genes are related, remains unknown.

Recently, we established rice as a model for studying the molecular and genomic basis of volatile-mediated indirect defense [43]. Rice plants when damaged by fall armyworm (*Spodoptera frugiperda*) larvae (FAW) emit about 30 volatiles including MeSA and MeBA. FAW-induced volatiles are highly attractive to female parasitic wasps (*Cotesia marginiventris*), carnivorous enemies of FAW. In the same study, about 20 genes potentially involved in the production of insect-induced rice volatiles were identified based on the correlation between volatile production and gene expression profiling using microarray analysis. The functions of three terpene synthase genes were biochemically verified [43]. Our long term goal of this project is to identify all key genes for synthesizing all insect-induced volatiles in rice and subsequently to study the roles of individual volatiles in indirect defense. Here we report the isolation and characterization of rice genes for the production of insect-induced MeSA and MeBA. Our previous study reported that the rice genome contains 41 *SABATH* genes [45]. One of them OsSABATH1 encodes indole-3-acetic acid methyltransferase (IAMT). Another one Os02g48770 was shown to have SAMT and BAMT activities [17]. This gene was used to study the function of *BSMT* in plant defense against pathogens in transgenic Arabidopsis plants overexpressing Os02g48770 [17]. However, the role of Os02g48770 in the rice biology has not been investigated. For example, it is not clear whether Os02g48770 is involved in the production of insect-induced MeSA and MeBA. In this report, candidate genes that regulate the production of insect-induced benzenoid esters were systematically identified, based on the correlation of MeSA and MeBA production with expression of all rice *SABATH* genes.

In addition to the identification of methyltransferase genes for synthesizing herbivore-induced benzenoid esters, it is also our interest to understand how the production of insect-induced MeSA is regulated. Previous studies have shown that the JA signaling pathway is involved in regulating MeSA emission. When applied with exogenous JA, lima bean plants release elevated levels of MeSA similar to those induced by herbivores [9]. When tomato mutant plants deficient in the accumulation of JA were infested with spider mites, emission of MeSA was significantly reduced compared to wild-type plants [1]. These data support that the JA pathway plays an important regulatory role in MeSA production and emission. The SA pathway is another central defense pathway in plants. In addition to mediating systemic acquired resistance (SAR), the SA signaling pathway interacts with the JA pathway through a crosstalk to regulate plant defense responses [18]. Little is known on whether the SA pathway has a role in the regulation of MeSA production. Presumably the SA pathway can modulate MeSA production at two levels: via substrate availability and via signal transduction. Measurement of SA contents in tissues of insect-treated rice plants would help clarify the role of substrate regulation. Analysis of MeSA emission from insect-treated mutant rice plants with altered SA signaling would help elucidate the role of the SA signal transduction pathway on MeSA production. Previous studies indicated that NH1 is a key component of the SA signal transduction pathway in rice. Overexpressing *NH1* in rice plants (NH1ox) leads to enhanced resistance to pathogens [6]. In this study, NH1ox plants were studied for understanding the regulatory role of the SA signaling pathway in production of MeSA induced by herbivores.

2. Results

2.1. Insect-damaged rice plants emit elevated levels of MeSA

Two-week old rice seedlings grown under normal conditions in a growth chamber emitted a low level of MeSA with an emission rate of 2.7 ± 0.4 ng g^{-1} h^{-1}. When the rice seedlings were damaged by FAW larvae overnight, the emission rate of MeSA increased approximately 23 fold to 64.8 ± 1.9 ng g^{-1} h^{-1} (Fig. 1A). MeBA, a benzenoid ester structurally related to MeSA, was detected from FAW-damaged rice plants with an emission rate of 22.4 ± 2.1 ng g^{-1} h^{-1}. This compound was, however, not detectable from control rice plants (Fig. 1B).

2.2. Identification of a rice SABATH gene encoding SAMT

The rice genome contains 41 *SABATH* genes, which were named *OsSABATH1* to *OsSABATH41* [45]. Our previous microarray analysis examining gene expression changes in control vs. FAW-treated rice plants showed that two *SABATH* genes, *OsSABATH3* (Os02g48770) and *OsSABATH9* (Os05g01140), were significantly up-regulated by FAW feeding [43]. However, the coverage of the *SABATH* family on the microarray slides that we used previously was very low, with only three *SABATH* genes present. To identify additional candidate genes for MeSA and MeBA biosynthesis, the RT-PCR approach was used to compare expression of all rice *SABATH* genes in control vs. FAW-treated rice plants.

RT-PCR analysis demonstrated that three *SABATH* genes, *OsSABATH13* (Os06g13460), *OsSABATH14* (Os06g13470) and *OsSABATH34* (Os11g15060), were up-regulated by FAW feeding (Fig. 2). Combined with the two *SABATH* genes identified from microarray analysis, five candidates were chosen for further study. Full-length cDNAs of the five genes were cloned using RT-PCR and fully sequenced. The presence of an intron-like sequence in multiple independent clones containing cDNA of *OsSABATH14* suggests that it encodes a pseudogene. The remaining four genes were expressed in *Escherichia coli* and recombinant proteins tested for methyltransferase activity using SA and BA as substrates. Results demonstrated that only OsSABATH3 (Os02g48770) exhibited activity with

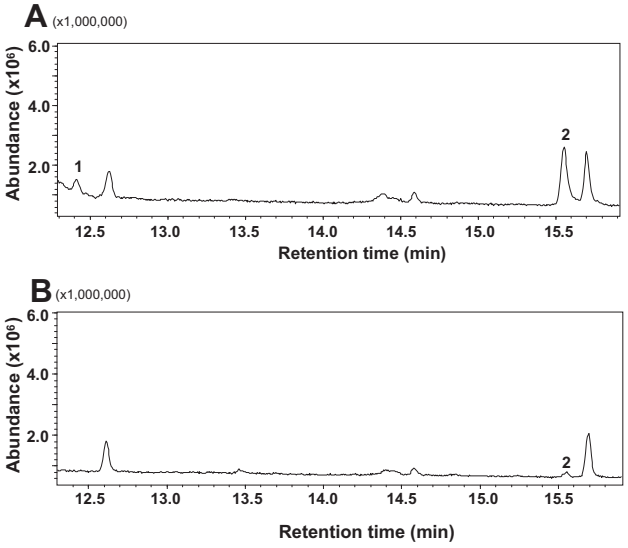

Fig. 1. Emission of benzenoid esters from rice plants. Numbers "1" and "2" depict MeBA and MeSA respectively. A, insect-treated rice plants emitted both MeSA and MeBA. B, untreated control plants emitted only MeSA. MeBA was not detectable from control plants.

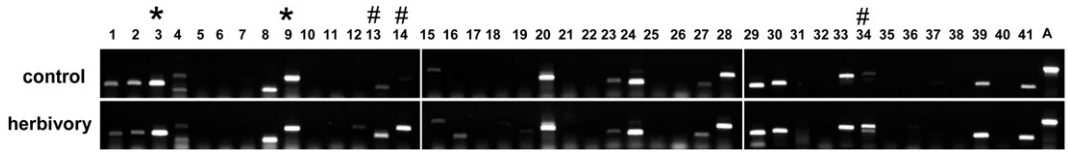

Fig. 2. RT-PCR expression analysis of rice *SABATH* genes. Total RNA was extracted from aerial parts of intact and FAW-damaged rice plants of two-week old seedlings and used for RT-PCR. The numbers 1–41 on the top of the figure denote *OsSABATH1* to *OsSABATH41*. The expression of an actin gene "A" was used as an internal control. "*" Denotes the genes identified from a previous microarray study (Yuan et al., 2008). "#" Denotes the genes identified in this study.

both SA and BA. The other three proteins did not display methyltransferase activity with either SA or BA, or other chemicals listed in Table 2. As we cannot rule out the possibility that other rice SABATH proteins having SAMT and/or BAMT activities, we adopted the name OsBSMT1, the name designated for this gene previously [17], for OsSABATH3.

2.3. Biochemical properties of OsBSMT1

Recombinant OsBSMT1 was purified in its native form using two steps of ion exchange chromatography (Fig. 3). Purified OsBSMT1 was used for detailed biochemical characterization. In methyltransferase enzyme assays with a group of potential substrates, OsBSMT1 had the highest level of specific activity with SA. The specific activity of OsBSMT1 with BA is about 42% of the activity with SA. OsBSMT1 also displayed activity with nicotinic acid and anthranilic acid, the activities of which are approximately 16% and 14.5% of the activity with SA, respectively. In addition, myristic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid can also serve as substrates for OsBSMT1 with lower activities (Table 2).

Under steady-state conditions, OsBSMT1 exhibited Km values of 37.4 ± 0.9 μM and 43.2 ± 3.7 μM for SA and BA, respectively. The kcat values of OsBSMT1 using SA and BA as substrate were 0.003 s^{-1} and 0.004 s^{-1} respectively.

2.4. Phylogenetic analysis OsBSMT1 and related proteins

To understand the phylogenetic relationship between OsBSMT1 with other known SABATH proteins, a phylogenetic tree containing OsBSMT1 and representative SABATHs, including all known SAMTs were constructed (Fig. 4). Five clades were identified. Clade I contains IAMTs from Arabidopsis (*Populus trichocarpa*), poplar and rice, farnesoic acid methyltransferase from Arabidopsis (AtFAMT) and two gibberellic acid methyltransferases from Arabidopsis. Clade II consists of the members of the SABATH family catalyzing nitrogen methylation for caffeine biosynthesis. Clade III contains BSMT from Arabidopsis and its relative *Arabidopsis lyrata* and one BAMT from snapdragon. All other characterized SAMTs from a variety of plant species form clade IV, which also includes jasmonic acid methyltransferase. The newly-identified OsBMST1, along with its homolog identified from sorghum (*Sorghum bicolor*), forms clade V.

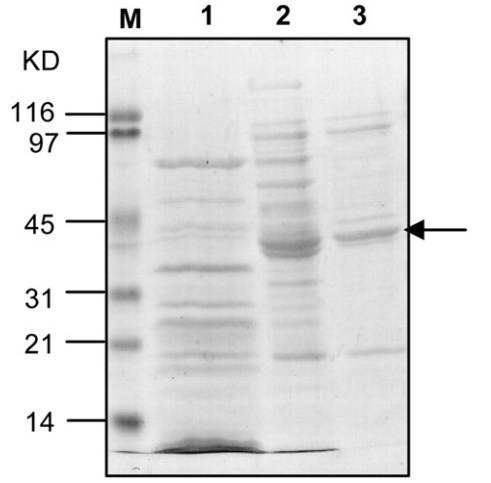

Fig. 3. SDS-PAGE of purified recombinant OsBSMT1. Protein expressed in *E. coli* was purified as described in Materials and methods. Lane M contained protein molecular weight markers. Lane 1 contained OsBSMT1crude extract, and lane 2 contained 1 μg of DE53 fraction. Lane 3 contained 1 μg of mono-Q fraction. The gel was stained with Coomassie Blue. Arrow indicates the target band for OsBSMT1.

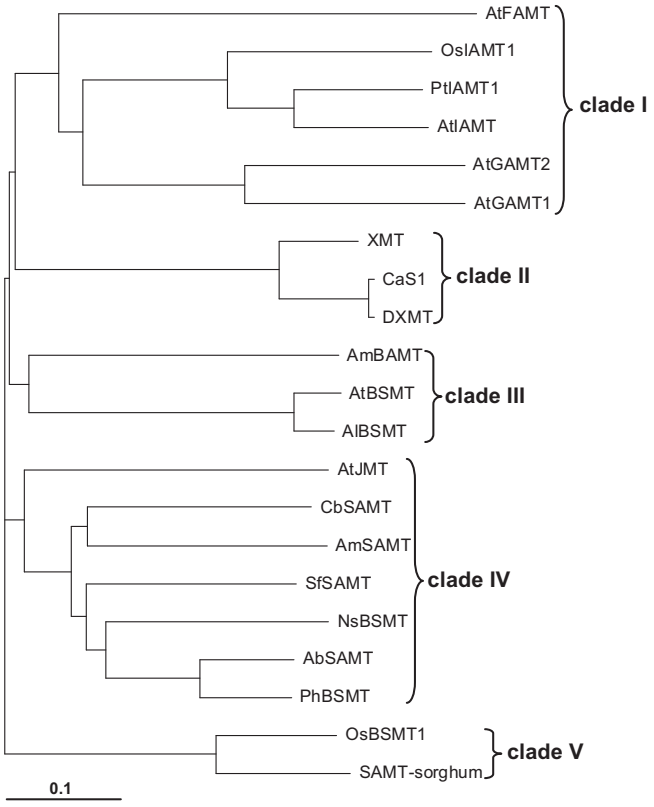

Fig. 4. A neighbor joining phylogenetic tree based on protein sequence alignment of functionally characterized SAMTs and other selected SABATHs from plants using the clustalX program. CbSAMT, *C. breweri* SAMT (AF133053); AmSAMT, *A. majus* (snapdragon) SAMT (AF515284); SfSAMT, *S. floribunda* SAMT (AJ308570); AmBAMT, *Antirrhinum majus* BAMT (AF198492); NsBSMT, *N. suaveolens* BSMT (AJ628349); AtBSMT, *Arabidopsis thaliana* BSMT (BT022049); AlBSMT, *A. lyrata* BSMT (AY224596); AbSAMT, *Atropa belladonna* SAMT (AB049752); PhBSMT, *Petunia hybrida* BSMT (AY233465); AtJMT, *A. thaliana* JMT (AY008434); AtFAMT, *A. thaliana* FAMT (AY150400); OsIAMT1, *Oryza sativa* IAMT1 (EU375746); PtIAMT1, *Populus trichocarpa* IAMT1 (XP_002298843); AtIAMT, *A. thaliana* IAMT (AK175586); AtGAMT1, *A. thaliana* GAMT1 (At4g26420); AtGAMT2, *A. thaliana* GAMT2 (At5g56300); Cas1, *Coffea arabica* caffeine synthase 1 (AB086414); CaXMT1, *C. arabica* XMT1 (AB048793); CaDXMT1, *C. arabica* DXMT1 (AB084125). SAMT-sorghum represents a putative sorghum SAMT gene (gene ID Sb07g028690). Five clades were identified. Branches were drawn to scale with the bar indicating 0.1 substitutions per site.

2.5. Structural modeling of OsBSMT1

A homology model of OsBSMT1 was built by using the experimentally-determined structure of *C. breweri* SAMT (CbSAMT) [48] as the template. This model was then used as a target for an *in silico* docking experiment to rank the affinities for the different potent substrates and to analyze their binding mode. SA and BA were observed to be placed at exactly the same position and at the right distance from *S*-adenosyl-L-homocysteine (SAH), with the right orientation of their carboxyl group for methylation (Fig. 5). The aromatic ring of SA in OsBSMT1 is rotated 180° with respect to that observed in the CbSAMT structure, presenting its hydroxyl group to nothing and therefore generating no significant interactions. As a consequence, SA and BA dock in the same manner with very close scores (Gscore values of −5.98 and −5.65 respectively). The corresponding theoretical K_d values of OsBSMT1 were calculated to be 35 μM and 62 μM with SA and BA respectively.

2.6. Expression of OsBSMT1 and emission of MeSA under various stress conditions

To understand the defense role of *OsBSMT1*, expression of *OsBSMT1* under a number of stress conditions was analyzed using quantitative real-time PCR. Plants were treated with physical wounding, a fungal elicitor alamethicin, and JA. Physical wounding lowered the expression of *OsBSMT1*. In contrast, alamethicin treatment led to a slight induction of *OsBMST1* expression. JA treatment led to significant up-regulation of *OsBSMT1* expression. The induced levels were similar to those induced by insects (Fig. 6A).

To determine whether the emission of MeSA and MeBA correlated with the expression of *OsBSMT1*, the emission of MeSA and MeBA from rice plants treated with the same stress factors used for gene expression studies were analyzed using headspace analysis. Physical wounding reduced the emission of MeSA. In contrast, alamethicin treatment led to slight induction in emission of MeSA. JA treatment led to significant increases in emission of MeSA, which was, however, lower than that from insect-treated plants (Fig. 6B). MeBA emission was detected from insect-damaged rice plants but not from rice plants treated with other stresses (data not shown).

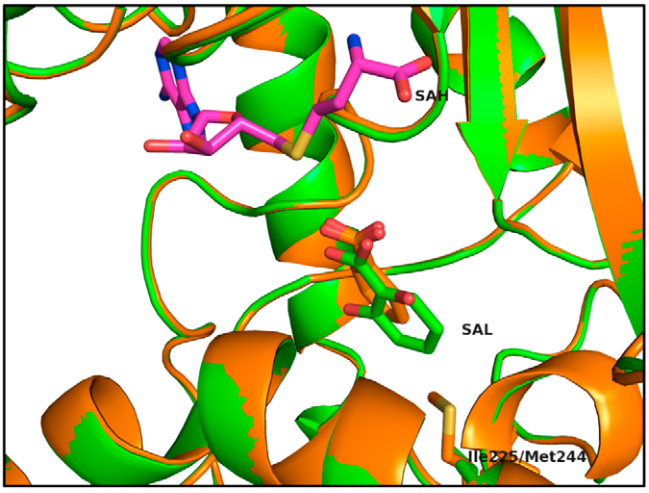

Fig. 5. Homology model of OsBSMT1 active site (orange), calculated with Modeller (Sali and Blundell, 1993), based on CbSAMT structure (green). Secondary structure is represented as ribbons. In this figure, the N-terminal loop–helices (residues 1–30) are omitted to make a better view of the substrate binding pocket. OsBSMT1 Met244 active site residues, as well as its structural equivalent Ile225 in CbSAMT, are represented as bonds. Structurally observed salicylic acid in CbSAMT crystal structure is represented as green bonds. Salicylic acid and benzoic acid, both positioned in the OsBSMT1 active site by *in silico* docking using Glide program (Friesner et al., 2004), are represented as orange bonds. This figure was produced with PyMOL (http://www.pymol.org). SAL stands for salicylic acid and benzoic acid. SAH stands for *S*-adenosyl-L-homocysteine.

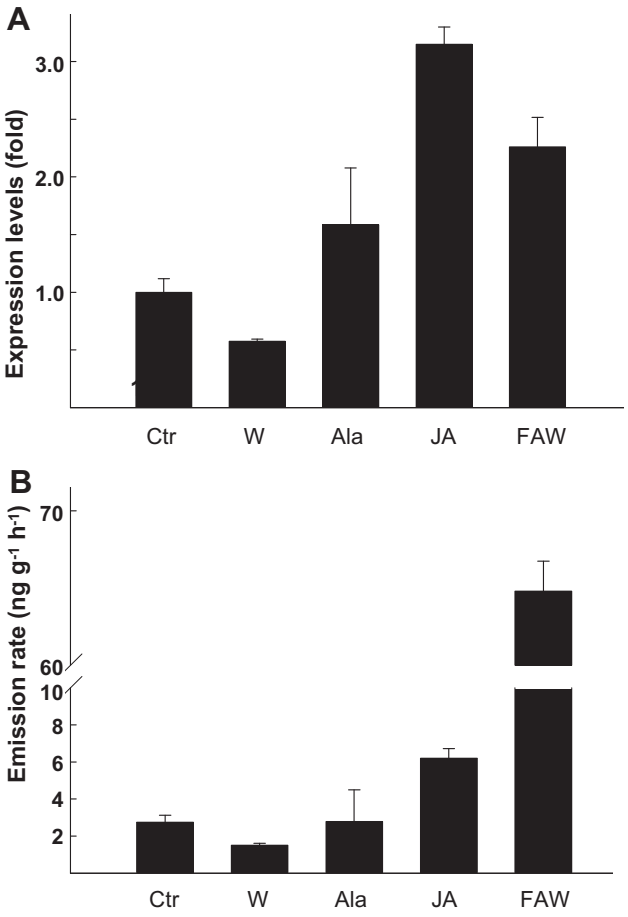

Fig. 6. Effects of stress factors on expression of *OsBSMT1* (A) and emission of MeSA (B). Wild-type Nipponbare plants were treated with physical wounding (W), alamethicin (Ala) and jasmonic acid (JA). Aerial parts of the plants were collected and used for quantitative real-time PCR analysis. Additional plants treated with the same conditions were also used for headspace collection. Volatiles were analyzed using GC–MS. The values are the average of three independent measurements.

2.7. Role of the SA signaling pathway in regulating OsBSMT1 expression, product emission and SA contents

To understand the potential regulation of the SA pathway on emission of MeSA, transgenic rice plants with altered SA pathway were analyzed for emission of MeSA upon FAW-feeding. NH1, which is the ortholog of Arabidopsis NPR1, is an important component of the SA signaling pathway [6]. Previous studies reported that SA-dependent plant defenses in transgenic rice plants overexpressing *NH1* gene (NH1ox) were altered [6]. Quantitative real-time PCR was performed to examine expression of *OsBSMT1* in NH1ox and LiaoGeng (LG), the wild-type background of NH1ox. FAW-feeding induced the expression by about 2.8 fold in LG, which is similar to that in Nipponbare plants (Fig. 6A). Expression of *OsBSMT1* was induced 5.6 fold by insect feeding in NH1ox plants (Fig. 7A).

Emissions of MeSA and MeBA from LG and NH1ox plants with or without insect treatment were analyzed. Both untreated LG and

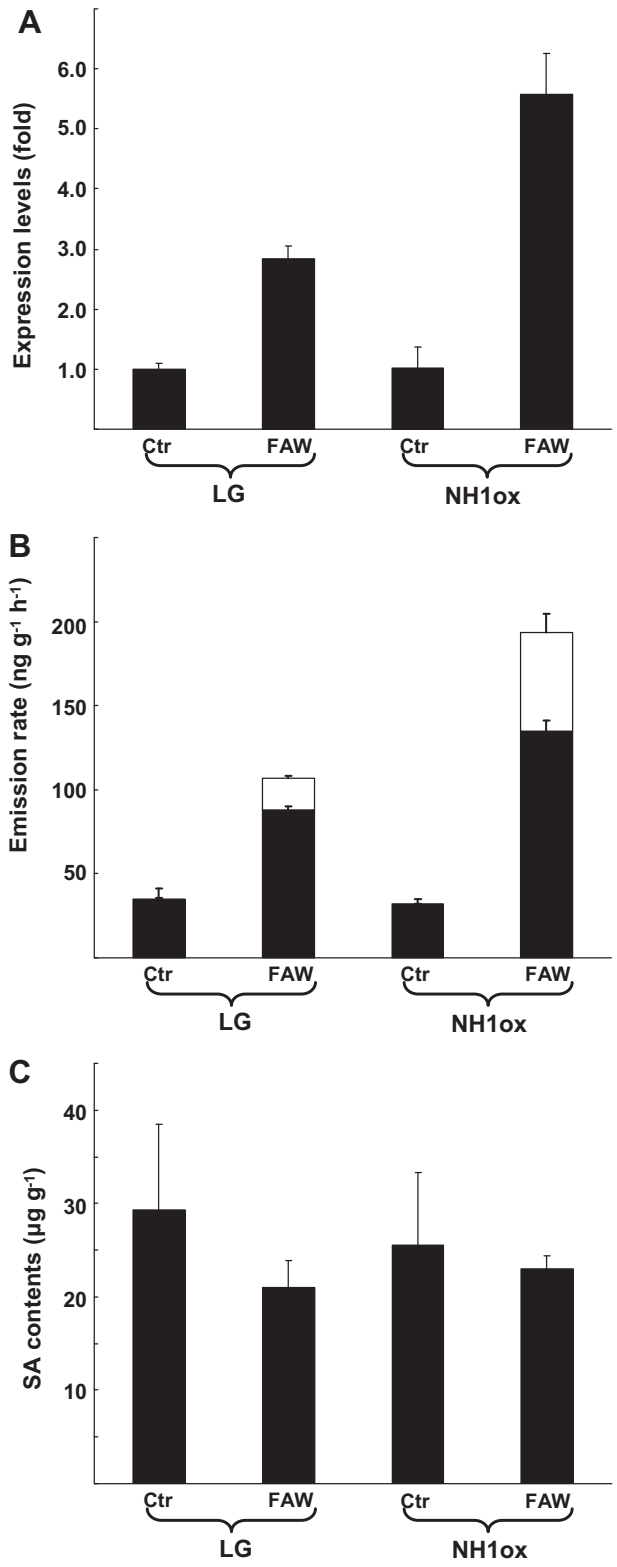

Fig. 7. Comparison of *OsBSMT1* expression, emission of MeSA and MeBA, and contents of SA. A. Expression of *OsBSMT1* in aerial parts of the untreated (Ctr) and FAW-treated (FAW) LG and NH1ox plants analyzed using quantitative real-time PCR. B. Emission of MeSA (black bars) and MeBA (open bars) from untreated (Ctr) and FAW-treated (FAW) LG and NH1ox plants based on headspace analysis. C. Contents of SA in aerials parts of the untreated (Ctr) and FAW-treated (FAW) LG and NH1ox plants. The values are the average of three independent measurements.

NH1ox plants emitted MeSA. The emission rates were 34.8 ± 6.6 ng g^{-1} h^{-1} and 31.9 ± 3.1 ng g^{-1} h^{-1} respectively. The emission from both LG and NH1ox plants was induced by FAW feeding. FAW-damaged NH1ox plants displayed a higher emission rate of MeSA, which was approximately 135 ± 6.3 ng g^{-1} h^{-1}. The emission rate of MeSA from FAW-damaged LG plants was approximately 87.8 ± 2.4 ng g^{-1} h^{-1} (Fig. 7B). MeBA was not detected from either untreated LG or NH1ox plants. However, insect-feeding induced the emission of MeBA from LG and NH1ox plants at the rates of 18.8 ± 1.8 ng g^{-1} h^{-1} and 58.4 ± 11.2 ng g^{-1} h^{-1} respectively (Fig. 7B).

The contents of free SA in control and insect-treated LG and NH1ox plants were also determined. The contents of SA in control NH1ox plants were similar to those in wild-type LG plants. For both LG and NH1ox plants, insect-feeding did not cause significant changes in the contents of SA (Fig. 7C).

3. Discussion

In this paper, we report the isolation and functional characterization of rice *OsBSMT1*. Recombinant OsBSMT1 displayed specific methyltransferase activities with SA and BA. The bifunctionality of OsBSMT1 is biologically relevant as both MeSA and MeBA, the product of SAMT and BAMT activities respectively, were detected from insect-damaged rice plants. Structural analysis based on homology modeling revealed that the active site of OsBSMT1 can accommodate SA and BA equally well, providing another line of evidence that both SA and BA are *in planta* substrates for OsBSMT1. JA was shown to regulate the production and emission of MeSA. Analysis of MeSA and MeBA levels in transgenic rice plants overexpressing a key component of the SA signaling pathway, *NH1*, indicates that the SA signaling pathway also plays an important role in regulating MeSA production and emission.

3.1. Identification of rice OsBSMT1 and SAMT evolution

Despite the fact that a number of *SAMT* genes have been isolated from a variety of plant species prior to this study [3,5,11,12,14,22–24,29,30,32], the identification of the rice gene for MeSA production was not straightforward. The rice genome contains 41 *SABATH* genes [45]. On the phylogenetic tree, there is not a single rice *SABATH* gene belonging to the same clade as known SAMTs or BSMTs (Fig. 4). By correlating MeSA emission with expression of all rice *SABATH* genes, the number of candidate genes was narrowed down from 41 to 5. Biochemical analyses indicated that only one of them has SAMT/BAMT activity. Whether this is the only gene responsible for biosynthesis of MeSA and MeBA in rice still needs to be determined. The SABATH family is best studied in Arabidopsis, which contains 24 members. Only one of the 24 genes has SAMT/BAMT activity. This suggests that it is possible that the rice genome contains only one BSMT gene.

OsBSMT1 is the first SAMT/BAMT to be isolated from a monocot. Phylogenetic analysis showed that OsBSMT1 is not closely related to dicot SAMTs (Fig. 4). The presence of SAMTs in three clades suggests that either SAMTs have evolved multiple times from related SABATH proteins or SAMTs in certain plant species underwent a positive selection. In addition, the evolution of bifunctionality of OsBSMT1 is intriguing. The study with Arabidopsis BSMT suggested that the ancestor of AtBSMT1 may have higher activity with BA, i.e., the SAMT activity of AtBSMT1 was evolved from BAMT activity [5]. In addition to rice, other monocots, such as maize [15] and sorghum (Zhuang & Chen, unpublished) also emit MeSA after insect herbivory. Insights into the evolutionary trajectory and trend of OsBSMT1 activities will be provided with the continued isolation

and characterization of bona fide SAMTs from maize, sorghum and other plant species of diverse taxon groups.

3.2. Biochemical properties of OsBSMT1 and its structural features

The biochemical properties of OsBSMT1 had been previously reported [17]. The Km values of OsBSMT1 with SA and BA in that report were 78 μM and 80 μM, respectively, which is about twice the Km values determined in this analysis. The discrepancy is probably due to the different recombinant enzymes examined. In the previous study, OsBSMT1 was purified with an N-terminal tag of 20 kDa [17]. It has been reported that the presence of a tag at the N-terminal of CbSAMT affect the kinetics of the enzyme [32]. In this study, OsBSMT1 was purified in its native form through two steps of ion-exchange chromatography. Therefore, the kinetic parameters reported in this study are probably more accurate.

The bifunctionality of OsBAMT1 is supported by two additional lines of evidence. When *OsBSMT1* was overexpressed in Arabidopsis, transgenic plants displayed higher levels of production of both MeSA and MeBA [17]. In addition, molecular modeling provides a structural explanation of how OsBSMT1 uses SA and BA as substrates with almost equal efficiency. Experiments of *in silico* docking performed on the homology model show that both SA and BA bind at the same position. However, it revealed a preferred orientation for the SA aromatic ring different with respect to CbSAMT, with a 180° flip so that it presents its hydroxyl group in a pocket closed by the loop–helices N-terminal fragment. The reduced contribution of this hydroxyl group in the binding interactions may explain the similarity in the binding mode of SA and BA, and as a consequence the close values for calculated affinity. The calculated K_d values of OsBSMT1 are compatible with the measured affinity, providing a validation of the *in silico* docking calculation.

3.3. Biological roles of OsBSMT1

OsBSMT1 is expressed in multiple tissues of rice plants grown under normal conditions [45], suggesting that this gene has a role in the normal biology of healthy plants. MeSA and MeBA may be toxic to certain microbial pathogens. The continued production of these compounds may provide a defense role in various tissues, such panicles, the reproductive structure of the rice plant [45]. Recently, MeSA was shown to be a mobile signal for SAR in tobacco (*Nicotiana tabacum*) [27]. It will be interesting to determine whether MeSA plays the same role in rice functioning as an internal signal.

The specific induction of *OsBSMT1* by herbivory and enhanced emission of MeSA support that MeSA has a specific role in plant responses to insect herbivory. While FAW-induced rice volatiles as a whole are attractive to *C. marginiventris* [43], it will be interesting to determine whether MeSA acts as an active signal. Recent studies have reported that insect-induced plant volatiles can also function in plant–plant communications, thereby volatiles emitted from insect-damaged plants can prime or activate defense responses in nearby healthy plants [39]. MeSA as an air-borne signal has been shown to mediate such inter-plant interactions in the context of plant–viral interactions [36]. Incubation with the transgenic Arabidopsis plants overexpressing *OsBSMT1*, which has higher levels of MeSA emission, was sufficient to trigger PR-1 induction in neighboring wild-type plants [17], supporting the role of MeSA as an air-borne signal in plant–plant communications. MeSA may play a similar role for plant defense against insect pests.

The biological significance of the bifunctionality of OsBSMT1 using SA and BA as substrates is not fully understood. Although OsBSMT1 indicate similar catalytic efficiencies with SA and BA, rice plants emit MeSA and MeBA at different rates, suggesting that the concentrations of their corresponding substrates, SA and BA, may play a critical role in determining their levels of emission. Whether MeBA has similar roles as MeSA under various conditions, such as defense against insects, needs to be further investigated.

3.4. Regulation of production of herbivore-induced air-borne MeSA

Application of exogenous JA led to induction of MeSA emission from rice plants (Fig. 6B), suggesting that the JA signaling pathway plays a central role in regulation of production of insect-induced MeSA, as in other plant species. While this phenomenon has been observed previously [19], our study suggests that at least part of this regulation is through the modulation of *OsBSMT1* expression, which was induced by the JA treatment (Fig. 6A). In both wild-type and NH1ox plants, herbivory did not cause significant changes in SA content (Fig. 7), indicating that the levels of SA are not limiting, or are a minor factor, in regulating the production of MeSA. Whether such regulation is species-specific awaits determination. Rice appears to have an SA metabolism different from that in Arabidopsis or tobacco. Rice is one of the plant species that contain high levels of SA in tissues of plants grown under normal conditions [37]. The high levels of SA may explain why substrate availability is not a critical factor for regulating MeSA production.

Our data support that *NH1*, a key component of the SA signaling pathway leading to plant defense responses in rice, plays an important role in MeSA production. *NH1* is the ortholog of Arabidopsis *NPR1*, is a key regulator of the SA-mediated systemic acquired resistance (SAR) [4,35]. *NPR1* encodes a novel protein with a bipartite nuclear localization sequence. Under unchallenged conditions, NPR1 protein exists as an oligomer and is excluded from the nucleus. When SAR is induced, monomeric NPR1 is formed, which accumulates in the nucleus and activates the expression of PR genes [20]. In addition to its role in SAR, NPR1 functions in crosstalk between SA- and JA-dependent defense signaling pathways (review see [28]). NH1 appears to have a similar role as NPR1. Overexpression of *NH1* in rice led to enhanced resistance to *Xanthomonas oryzae* [6]. Our data indicate that *NH1* is also involved in regulating rice–insect interactions. In particular, *NH1* has a role in the regulation of the production of insect-induced air-borne MeSA, mainly through the regulation of *OsBSMT1* expression. Because of the central role of the JA signaling pathway in regulating insect-induced MeSA emission, we hypothesize that the regulatory role of *NH1* on MeSA production is through the interaction of the SA signaling pathway with the JA pathway. It will be interesting to determine whether the same components mediating the SA–JA crosstalk involved in plant–pathogen interactions, such as *WRKY70* [18], are also involved in regulating the SA–JA crosstalk in plant–insect interactions.

4. Materials and methods

4.1. Rice lines, plant growth and treatments

Nipponbare and LiaoGeng (LG), both of the japonica subspecies, were used as wild-type plants. NH1 lines were produced in the LG background [6]. Rice seeds were dehulled and germinated at 30 °C for two days. Then seedlings were planted (eight plants per 200 ml glass jar) and grown in the growth chamber at 26 °C with 16 h of light for two weeks. Insect treatment using fall armyworm (*S. frugiperda*) was performed as previously described [43]. For physical wounding, leaves were cut with a sterile razor blade to produce one lateral incision on each side of the midvein. For alamethicin treatment, leaves were cut detached from the base of

the petiole and then stood up in a small glass beaker containing 10 ml of 5 μg/ml alamethicin (dissolved 1000-fold in water from a 5 mg/ml stock solution in 100% methanol). The glass beaker was then sealed with Saran wrap and placed in a growth chamber. Leaves were collected 15 h after the treatment. For JA treatment, JA was dissolved in ethanol to make 50 mM stock solution, which was diluted five times to make a working solution. The JA working solution was applied evenly with a cotton swab to incisions produced by wounding treatment. Plants were placed in a 5-L glass jar for 15 h.

4.2. Volatile collection and identification

Volatile collection and identification were performed as previously described [43].

4.3. RNA isolation

Total RNA was isolated with Plant RNA Isolation Reagent (Invitrogen Inc.) according to the manufacture's protocol. The total RNA were then cleaned up with the RNeasy Plant Mini Kit (Qiagen, Inc.) according to the RNA cleanup protocol provided by the manufacture, and DNA contamination was removed with an on-column DNase (Qiagen) treatment.

4.4. RT-PCR

Total RNA extracted from treated or untreated rice plants was used for expression analysis of rice *SABATH* genes. RT-PCR expression analysis was performed as previously described [45]. Primer sequences and sizes of PCR products of rice *SABATH* genes and the effectiveness of these primers confirmed with genomics DNA were described in Zhao et al. [45]. The PCR reaction was performed under the following conditions: an initial denature at 95 °C for 2 min followed by 30 cycles of 95 °C for 45 s, 54 °C for 45 s and 72 °C for 60 s, and then followed by an extension step of 72 °C for 10 min.

4.5. Full-length cDNA cloning

1.5 μg of total RNA was reverse transcribed into first strand cDNA in a 15-μL reaction volume using the First-strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ), as previously described [5]. Primers used for PCR amplification are shown in Table 1. PCR reaction was set as follows: 94 °C for 2 min followed by 30 cycles at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. The PCR product was separated on 1.0% agarose gel. The target band was sliced from the gel and purified using QIAquick Gel Extraction kit (Qiagen, Valencia, CA). The PCR product was cloned into pEXP5/CT TOPO vector using the protocol recommended by the vendor (Invitrogen, Carlsbad, CA). The cloned cDNA in pEXP5/CT TOPO vector was sequenced using T7 primers.

Table 2
Relative activity of OsBSMT1 with salicylic acid, benzoic acid and related substrates.

Substrates	Relative activity (%)
Salicylic acid	100
Jasmonic acid	<1
Benzoic acid	42
Indole-3-acetic acid	<1
Gibberellic acid (GA3)	<1
Farnesoic acid	<1
2,4-Dichlorophenoxyacetic acid	<1
3-Hydroxybenzoic acid	1.3
4-Hydroxybenzoic acid	2.7
7-Methylxanthine	<1
Anthranilic acid	14.5
Abscisic acid	<1
Acetic acid	<1
Caffeic acid	<1
Cinnamic acid	<1
p-Coumaric acid	<1
Geranic acid	<1
Indolebutyric acid	<1
Lactic acid	<1
Lauric acid	<1
Linolenic acid	<1
Myristic acid	3.8
Nicotinic acid	16
Octanoic acid	<1
Vanillic acid	<1

4.6. Protein expression and purification

To express OsBSMT1, the corresponding protein expression constructs were transformed into *E. coli* strain BL21 (DE3) CodonPlus (Stratagene, La Jolla, CA, USA). Protein expression was induced by IPTG for 18 h at 22 °C and the cells were lysed by sonication. The protein expressed in pEXP5/CT TOPO vector was purified via two steps of ion exchange chromatography using an FPLC system, as previously described [5]. Cell lysate was first loaded onto a DE53 cellulose column (Whatman, Maidstone, England) preequilibrated with buffer A (containing 50 mM Tris–HCL, pH 7.0, 10% glycerol, 10 mM β-mercaptoethanol). The loaded column was washed with buffer A then eluted with buffer A containing a linear gradient of KCl from 0 to 400 mM of KCl. Fractions were collected and assayed for SAMT activity. The fractions with positive SAMT activity were pooled, diluted threefold with buffer A, and then purified using a HiTrap Q FF column (GE Health Life Sciences, San Francisco, CA), following the same procedure as described for the DE53 column. Protein purity was verified by SDS-PAGE and protein concentration was determined by the Bradford assay.

4.7. Radiochemical methyltransferase activity assay

Radiochemical methyltransferase activity assays were performed with a total volume of 50 μL containing 50 mM Tris–HCl pH 7.5 buffer, 5 μL protein, 0.5 μL [^{14}C]-SAM (specific activity 52.7 mCi/mmol, Perkin Elmer Instruments, Shelton, CT), and 1 μL substrate of 50 mM. The reaction was incubated at room temperature for

Table 1
Primers for cloning of full-length cDNAs of *OsSABATH* genes.

Gene name	Forward primer sequences (5′–3′)	Reverse primer sequences (5′–3′)
OsSABATH3	ATGAAGGTAGAGCAGGACCTCCACATG	GGCACGCTACTTATAAAATGCAACATC
OsSABATH9	ATGGTGGTAAACTGGCATGTACACATG	CTGACACGTGGTATATATTGTAGGTA
OsSABATH13	ATGGTCAATATCGAAGGCGATTTACAC	CTATATTTTCTTCAAGGACATGACGATGAC
OsSABATH14	ATGATTTCTATATCTATATATGCTACTTGTG	TTAGGAAACCCTCACCAATCCGACTCC
OsSABATH33	ATGAAGATCGAGCGAGATTTCCACA	GCATACCATGAGCGCACTTTACTCTAT

30 min, after which the products of the assay were extracted with 150 μL ethylacetate and counted in a scintillation counter.

4.8. Determination of kinetic parameters of OsBSMT1

In all kinetic analyses, the appropriate enzyme concentrations and incubation time were chosen so that the reaction velocity was linear during the reaction time period. To determine a Km value for SAM, concentrations of SAM were independently varied from 3 to 120 μM, while SA was held constant at 1 mM. To determine the Km values for SA and BA, concentrations of SA and BA were independently varied from 10 to 200 μM, while SAM was held constant at 200 μM. Assays were conducted at 25 °C for 30 min. Lineweaver–Burk plots were used to calculate apparent Km values. Final values represent the average of three independent measurements.

4.9. Homology-based structural modeling and in silico docking experiments

The structural homology model of OsBSMT1 was built with Modeller [33]. A sequence alignment of OsBSMT1 with CbSAMT was performed with Blast (http://www.ncbi.nih.gov/BLAST/) using Blosum62 matrix, followed by the generation of a first model (3D alignment on template) and 200 cycles of molecular dynamics-based simulated annealing. *In silico* docking of potential substrates was then performed with the Glide program [13], which is part of the Schrödinger™ suite (Schrödinger, LLC, New York, NY, 2005).

4.10. Salicylic acid measurement

Approximately 50–120 mg leaf tissue that were ground in liquid nitrogen were quantitatively transferred to scintillation vials containing 2.5 ml 80% ethanol (aqueous) and 100 μL of sorbitol (0.100 g/100 ml) added to the vial as an internal standard to correct for sample loss during extraction and heating and differences in derivatization efficiency. The samples were extracted for 24 h, and the supernatant was transferred to another vial and the leaf residue was re-extracted with 2.5 ml 80% ethanol for another 24 h. The supernatants were combined and a 0.5-mL aliquot was dried down in a helium stream. The dried samples were dissolved in 500 μL of silylation–grade acetonitrile, followed by the addition of 500 μL N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce Chemical Co., Rockford, IL, USA), and then heated for 1 h at 70 °C to generate trimethylsilyl (TMS) derivatives. After 2 days, 1-μL aliquots were injected into a ThermoFisher DSQII gas chromatograph–mass spectrometer (GC–MS), fitted with an Rtx-5MS (crosslinked 5% PH ME Siloxane) 30 m × 0.25 mm × 0.25 μm film thickness capillary column (Restek, Bellefonte, PA, USA). The standard quadrupole GC–MS was operated in electron impact (70 eV) ionization mode, with 6 full-spectrum (70–650 Da) scans per second. Carrier gas (helium) flow was set at 1.1 ml per minute with the injection port configured in the splitless mode. The injection port and detector temperatures were set to 220 °C and 300 °C, respectively. The initial oven temperature was held at 50 °C for 2 min and was programmed to increase at 20 °C per min to 325 °C and held for another 11.25 min, before cycling back to the initial conditions. The SA peak was quantified by extracting 267 *m*/*z* to minimize integration of co-eluting metabolites. Peaks were quantified by area integration and the concentrations were derived from an external calibration curve of amount of SA injected versus peak area integration of the extracted *m*/*z*. Final values are an average of three independent measurements.

4.11. Quantitative real-time PCR

Quantitative real-time PCR was conducted on an ABI7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR green fluorescence dye (Bio-Rad Laboratories). The gene-specific primers were designed as followed: forward primer 5′-TCAAGAGAAGGCCATCCTGAA-3′ and the reverse primer 5′-AGA GCGACGCGTGAGCTT. The two primers used for the PCR amplification of *actin* were designed as the internal control: forward primer 5′-GACTCTGGTGATGGTGTCAGCGAC-3′ and reverse primer 5′-CTGC TGGAATGTGCTGAGAGATGC-3′. Data analysis was performed as previously described [42].

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