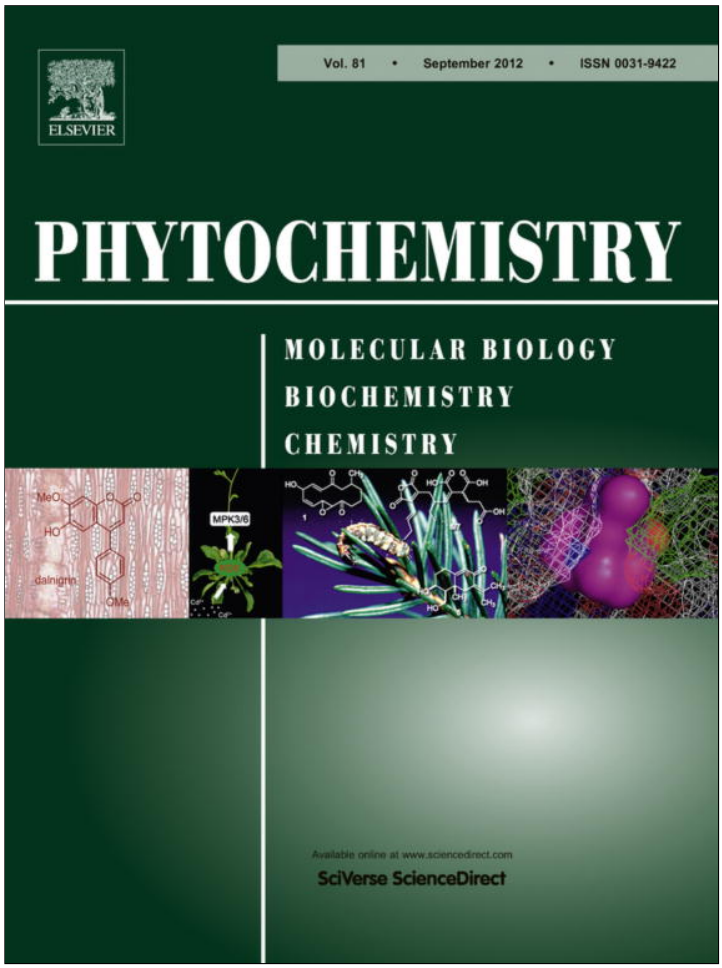

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A SABATH Methyltransferase from the moss *Physcomitrella patens* catalyzes *S*-methylation of thiols and has a role in detoxification

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ABSTRACT

Known SABATH methyltransferases, all of which were identified from seed plants, catalyze methylation of either the carboxyl group of a variety of low molecular weight metabolites or the nitrogen moiety of precursors of caffeine. In this study, the SABATH family from the bryophyte *Physcomitrella patens* was identified and characterized. Four *SABATH*-like sequences (*PpSABATH1*, *PpSABATH2*, *PpSABATH3*, and *PpSABATH4*) were identified from the *P. patens* genome. Only *PpSABATH1* and *PpSABATH2* showed expression in the leafy gametophyte of *P. patens*. Full-length cDNAs of *PpSABATH1* and *PpSABATH2* were cloned and expressed in soluble form in *Escherichia coli*. Recombinant PpSABATH1 and PpSABATH2 were tested for methyltransferase activity with a total of 75 compounds. While showing no activity with carboxylic acids or nitrogen-containing compounds, PpSABATH1 displayed methyltransferase activity with a number of thiols. PpSABATH2 did not show activity with any of the compounds tested. Among the thiols analyzed, PpSABATH1 showed the highest level of activity with thiobenzoic acid with an apparent Km value of 95.5 μM, which is comparable to those of known SABATHs. Using thiobenzoic acid as substrate, GC–MS analysis indicated that the methylation catalyzed by PpSABATH1 is on the sulfur atom. The mechanism for *S*-methylation of thiols catalyzed by PpSABATH1 was partially revealed by homology-based structural modeling. The expression of *PpSABATH1* was induced by the treatment of thiobenzoic acid. Further transgenic studies showed that tobacco plants overexpressing *PpSABATH1* exhibited enhanced tolerance to thiobenzoic acid, suggesting that *PpSABATH1* have a role in the detoxification of xenobiotic thiols.

1. Introduction

S-adenosyl-L-methionine (SAM)-dependent methylation is one of the most important reactions involved in the biosynthesis of plant specialized metabolites. Members of many classes of plant specialized metabolites, including phenylpropanoids, flavonoids, alkaloids, sterols, purines and thiols, are known to be methylated (D'Auria et al., 2003). The atoms that can be methylated include oxygen, carbon, nitrogen, sulfur and halogens. In plants, SAM-dependent methylation of metabolites is catalyzed by several classes of methyltransferases (MTs). While the members of the same protein family of MTs often use substrates of similar structure, the members of different gene families do not share sequence similarity, implying independent evolutionary origins. How plant MTs have evolved, especially with respect to diversification of substrate specificity, has been an important question in the study of plant MTs (Noel et al., 2003).

The SABATH family of MTs is one class of small molecule MTs found in plants. "SABATH" was coined from the first three enzymes to be characterized in the family: salicylic acid MT, benzoic acid MT and theobromine synthase (D'Auria et al., 2003). The majority of characterized SABATH MTs catalyze the methylation of carboxylic acids. In addition to salicylic acid MT (SAMT) (Ross et al., 1999; Chen et al., 2003; Zhao et al., 2010) and benzoic acid MT (BAMT) (Murfitt et al., 2000), other known carboxylic acid SABATH MTs include indole-3-acetic acid MT (IAMT) (Qin et al., 2005; Zhao et al., 2007; Zhao et al., 2008), gibberellic acid MT (GAMT) (Varbanova

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et al., 2007), jasmonic acid MT (JMT) (Seo et al., 2001), cinnamate/*p*-coumarate carboxyl MT (CCMT) (Kapteyn et al., 2007), and loganic acid MT (LAMT) (Murata et al., 2008). Some SABATH proteins catalyze nitrogen methylation. All known nitrogen MTs of the SABATH family are involved in caffeine biosynthesis (Kato et al., 2000; Ogawa et al., 2001; Mizuno et al., 2003; Yoneyama et al., 2006).

The characterized *SABATH* genes have important biological functions. Arabidopsis *IAMT* (*AtIAMT1*) has a role in leaf development (Qin et al., 2005). Arabidopsis *GAMTs* were shown to regulate seed germination (Varbanova et al., 2007). Arabidopsis *JMT* has a function in plant defense against fungi (Seo et al., 2001). *SAMT* functions in multiple biological processes. Methyl salicylate, the product of SAMT, has been demonstrated to be a mobile signal for systemic acquired resistance (Park et al., 2007), a component of floral scent (Knudsen et al., 1993), and a constituent of insect-induced plant volatiles (Zhao et al., 2010). The nitrogen-containing compounds produced by SABATHs are toxic and therefore have a role in plant defense (Uefuji et al., 2005).

Most known SABATHs exhibit strict substrate specificity. Nitrogen MTs of the SABATH family cannot use carboxylic acids as substrate and carboxylic acid MTs do not catalyze nitrogen methylation. Of carboxylic acid MTs, most do not exhibit substrate promiscuity. For example, GAMT cannot methylate indole-3-acetic acid, jasmonic acid or salicylic acid (Varbanova et al., 2007). JMT does not methylate indole-3-acetic acid and salicylic acid (Seo et al., 2001). Nevertheless, some promiscuous activity has been observed with SAMT and BAMT. Some SABATH MTs possess dual activity capable of methylating both salicylic acid and benzoic acid. Such enzymes have been called BSMTs (Chen et al., 2003; Pott et al., 2004; Zhao et al., 2010). The promiscuous activity of BSMTs from some plants, such as Arabidopsis (Chen et al., 2003) and rice (Zhao et al., 2010), appears to be biologically relevant, responsible for the *in planta* production of both methyl salicylate and methyl benzoate. In *Nicotiana*, functional divergence of duplicated genes appears to have played an important role in the evolution of *SABATH* methyltransferase genes encoding SAMT, BAMT and BSMT (Hippauf et al., 2010). Structural studies have been an important tool for understanding the mechanisms governing substrate specificity of SABATH MTs. To date, the three-dimensional structures of four proteins, including SAMT from *Clarkia breweri* (CbSAMT) (Zubieta et al., 2003), two nitrogen SABATH MTs (McCarthy and McCarthy, 2007) and AtIAMT1 (Zhao et al., 2008), have been reported. In addition to describing the mechanism of methylation, these structural studies revealed some amino acid residues critical for governing substrate specificity of these enzymes (Zubieta et al., 2003).

In several plant species whose genomes have been fully sequenced, *SABATH* genes appear to exist as multi-gene families. The Arabidopsis and rice genomes contain 24 and 41 *SABATH* genes, respectively (D'Auria et al., 2003; Zhao et al., 2008). The presence of multiple members of the SABATH family having distinct substrates in a single plant species poses an intriguing question about the substrate specificity evolution of the SABATH family. Because of the significance of the substrates of SABATHs (e.g., hormones and signaling molecules) to plant physiology, maintenance of strict substrate specificity for many SABATH MTs is probably important for their specific biological functions. Our recent study combining structural biology, biochemical characterization and comparative genomics suggests that IAMT is an evolutionarily ancient member of the SABATH family in seed plants, and that SAMT and JMT may have evolved from IAMT (Zhao et al., 2008; Zhao et al., 2009). To further understand the evolution of the SABATH family, especially the evolutionary trajectory of the substrate specificity of its members, it is important to continue to isolate and characterize *SABATH* genes from a wide range of plant species of diverse taxa, especially non-seed plants, which were noted to contain SABATH-like sequences (Kapteyn et al., 2007).

In this study, *SABATH* genes were characterized in a non-seed, non-vascular plant: the moss *Physcomitrella patens*. *P. patens* is a representative of bryophytes that arose about 450 million years ago (Rensing et al., 2008). Residing at a critical position in plant phylogeny and with the availability of whole genome sequence (Rensing et al., 2008), *P. patens* is a useful model for studying *SABATH* genes and gaining a more complete understanding about the evolution of substrate specificity in the SABATH family. The *P. patens* genome was found to contain a small family of *SABATH* genes comprised of only four members. One member of the *P. patens* SABATH family, PpSABATH1, was found to catalyze methylation of the sulfur atom of specific thiols. The *S*-methylating activity of PpSABATH1 was demonstrated to be biologically relevant, as the protein appears to have a role in the detoxification of xenobiotic thiols.

2. Results

2.1. Identification of SABATH genes in the P. patens genome and sequence analysis

Using CbSAMT, the founding member of the SABATH family, as a query, four SABATH-like sequences were identified in the *P. patens* genome, compared to the more than twenty members typically found in higher plants (Chen et al., 2003; Zhao et al., 2008). These four genes, gw1.47.108.1, e_gw1.327.25.1, e_gw1.204.84.1 and Scaffold_72 were renamed *PpSABATH1* through *PpSABATH4*, respectively. Through manual annotation, *PpSABATH1* and *PpSABATH2* were determined to encode a protein of 384 and 381 amino acids, respectively. *PpSABATH3* encodes a shorter protein with 293 amino acids. *PpSABATH4* encodes the longest protein among the four genes, which consists of 405 amino acids. Among the four proteins, PpSABATH1 and PpSABATH2 are most similar to each other with a 46% similarity (Supplementary Fig. 1). The sequence similarity of PpSABATHs to two known SABATH proteins, CbSAMT and AtIAMT1, is approximately 35%. There are significant variations in the structure of these four genes. *PpSABATH1*, *PpSABATH2* and *PpSABATH4* each contain two introns. The positions of the two introns are conserved among *PpSABATHs* and with *SABATHs* from other plants (Supplemental Fig. 2). In contrast, *PpSABATH3* does not contain any intron.

To understand the evolutionary relationships of PpSABATHs to other SABATHs, a phylogenetic tree containing the entire set of SABATH proteins from *P. patens* and selected known SABATHs from other plants was constructed using the maximum likelihood method (Fig. 1). The four *P. patens* SABATH proteins clustered together and they are most closely related to GAMTs and IAMTs (Fig. 1).

2.2. Initial screening of substrates for PpSABATH1 and PpSABATH2

To determine whether the four *PpSABATH* genes were expressed, quantitative RT-PCR was performed using total RNA extracted from the leafy gametophyte of *P. patens* grown on agar plates. While no expression was detected for *PpSABATH3* and *PpSABATH4*, *PpSABATH1* and *PpSABATH2* showed expression with *PpSABATH1* exhibiting a higher level of expression (Fig. 2).

Full-length cDNAs for *PpSABATH1* and *PpSABATH2* were cloned into a protein expression vector and their corresponding recombinant proteins were produced in *E. coli*. Crude enzymes of PpSABATH1 and PpSABATH2 were screened against a total of 68 compounds (Supplemental Table 1) using radiochemical-based assays as previously described (Zhao et al., 2009). In this method, the amount of transmethylation activity is indicated by the amount of

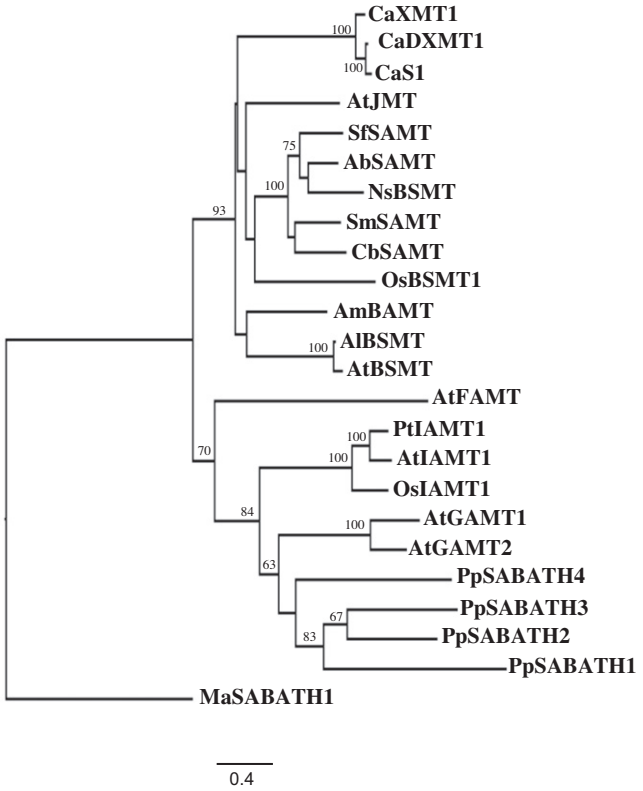

Fig. 1. Phylogenetic tree constructed by the maximum likelihood method using the protein sequences of *P. patens* SABATHs and selected SABATHs from other plant species. CbSAMT, *C. breweri* SAMT (accession # AF133053); AmSAMT, *Antirrhinum majus* SAMT (accession # AF515284); SfSAMT, *Stephanotis floribunda* SAMT (accession # AF308570); 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); AtJMT, *Arabidopsis thaliana* JMT (accession # AY008434); 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* XMT1 (accession # AB048793); CaDXMT1, *Coffea arabica* DXMT1 (accession # AB084125); MaSABATH1, a SABATH-like sequence from a bacterium *Mycobacterium avium* (accession # YP881389). MaSABATH1 was used as outgroup. Branches are drawn to scale with the bar indicating 0.1 substitutions per site.

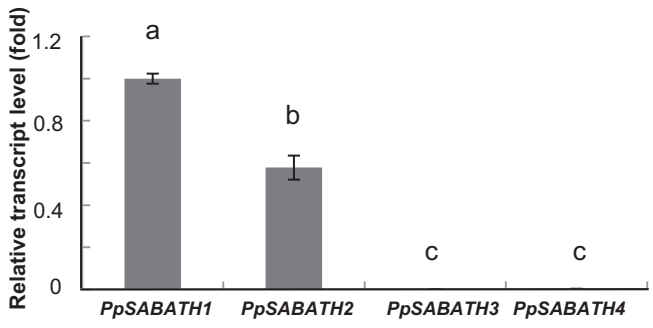

Fig. 2. Expression analysis of *PpSABATH1*, *PpSABATH2*, *PpSABATH3*, and *PpSABATH4*. Expression values were normalized to the levels of 18S rRNA in respective samples. The level of *PpSABATH1* expression in leaves is arbitrarily set at 1.0. The values are the average of three independent measurements. Bars marked with different letters indicate that the expression levels showed significant difference ($p < 0.05$).

radioactivity conferred by methylesters, in which the transferred methyl group is derived from ^{14}C-SAM. A very low level of background count is always present in the detection of ^{14}C using a liquid scintillation counter. Using this method, PpSABATH1 and PpSABATH2 did not show significant activity with any of these compounds. However, the presumed background levels of transmethylation activity for PpSABATH1 were significantly higher than those for PpSABATH2 and known SABATH enzymes. This suggested that PpSABATH1 had a methyltransferase activity with a component in the reaction mixture. When β-mercaptoethanol was omitted from the reaction buffer, the background activity for PpSABATH1 was reduced to a normal level. This suggested that PpSABATH1 had an activity with β-mercaptoethanol. To test whether PpSABATH1 had activity with other reducing agents, dithiothreitol (DTT) was tested, and the assays again demonstrated significantly higher background when DTT was used as a reducing agent in the assay and a normal level of background when DTT was omitted. Both β-mercaptoethanol and DTT contain two types of functional groups: hydroxyl and sulfhydryl. To gain insight into the atom to which the methyl group was transferred to, threitol was tested as a potential substrate. PpSABATH1 did not catalyze the methylation of threitol, suggesting that the methylation catalyzed by PpSABATH1 occurred on the sulfur atom. PpSABATH2 did not display the ability to methylate these sulfur-containing compounds.

2.3. Substrate specificity of PpSABATH1

Our initial screening of substrates suggested that PpSABATH1 had methyltransferase activity with thiols. To better characterize this apparent thiol-specific methyltransferase activity, *E. coli*-expressed PpSABATH1 was purified without a reducing agent (Supplemental Fig. 3) and tested with a number of thiol containing compounds for activity. These compounds were chosen based on their structural similarity to known substrates of other SABATHs. In addition, a number of alcohols and acids that are structurally very similar to the thiols were also tested to verify the specific S-methylating activity of PpSABATH1 (Fig. 3). Among the compounds tested, PpSABATH1 showed the highest level of specific activity with thiobenzoic acid (**1**) (1156 ± 79 pkat/mg protein), which was about three times higher than that for DTT (**7**). Thiophenol (**3**) and furfuryl thiol (**5**) served equally well as substrates for PpSABATH1. Their activity was about 66% of that for thiobenzoic acid (**1**). In contrast, PpSABATH1 did not show activity with any of the alcohols or acids tested, including benzoic acid (**2**), phenol (**4**), furfuryl alcohol (**6**) and threitol (**8**) (Fig. 3). These results strengthened the conclusion that the methylation of thiols catalyzed by PpSABATH1 occurs on the sulfur atom. To provide further evidence, the product produced from PpSABATH1 in *in vitro* enzyme assay using thiobenzoic acid (**1**) as a substrate was extracted and analyzed using GC–MS. The assay product exhibited a retention time and mass spectrum identical to those of methylthiobenzoate (Fig. 4), confirming the conclusion that the methylation of thiobenzoic acid (**1**) catalyzed by PpSABATH1 occurs on the sulfur atom.

2.4. Biochemical properties of PpSABATH1 using thiobenzoic acid as substrate

The biochemical properties of PpSABATH1 using thiobenzoic acid (**1**) as substrate were determined in detail. PpSABATH1 was found to be relatively unstable above 4 °C, where it lost 20%, 57% and 64% of its activity when incubated at 10, 20 and 30 for 30 min. When kept at 50 °C for 30 min, PpSABATH1 lost all of its activity (Fig. 5A). The optimum pH of PpSABATH1 was determined to be 7.5 (Fig. 5B). At pH 7.0, pH 8.0 and pH 9.0, the enzyme had about 70%, 54% and 33% of its maximal activity, respectively. PpSABATH1 activity can be affected by metal ions (Fig. 5C). K^+ was found to stimulate PpSABATH1 activity by 70%. Ca^{2+} had a mild inhibitory effect (<25%). Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+} and

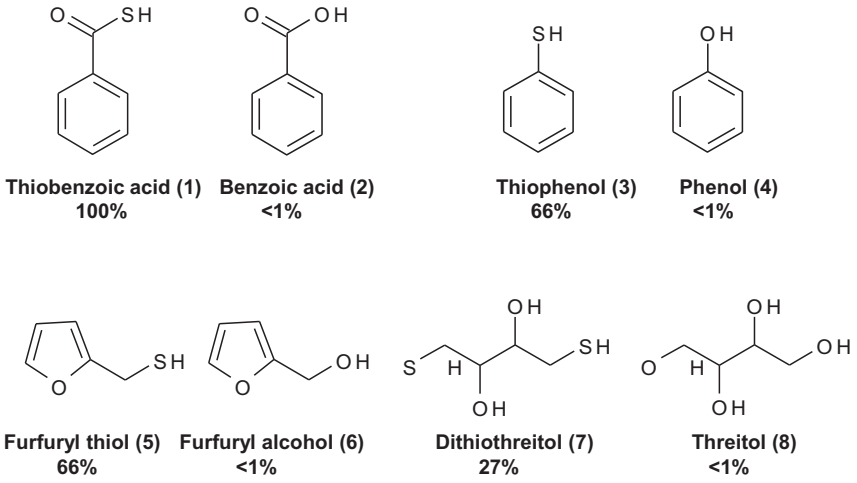

Fig. 3. Activity of PpSABATH1 with aromatic and aliphatic thiols and structurally related alcohols. The values were normalized to the activity with thiobenzoic acid (**1**), with 100% equal to a specific activity of 1156 ± 79 pkat/mg.

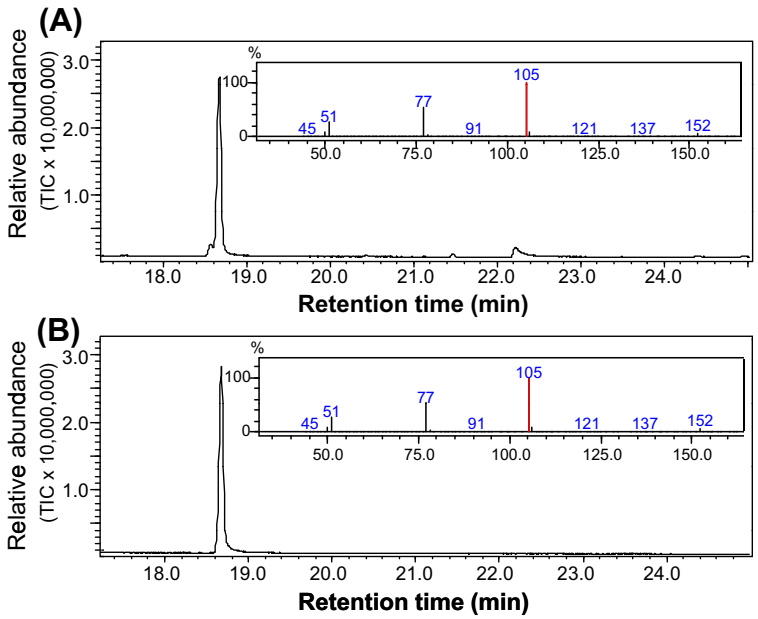

Fig. 4. Identification of the catalytic product of PpSABATH1. (A) GC chromatogram of the product formed by the action of PpSABATH1 proteins using thiobenzoic acid (**1**) as substrate. Inset shows mass spectrum of the assay product. (B) GC chromatogram of authentic thiobenzoic acid methylthioester. Inset shows mass spectrum of thiobenzoic acid methylthioester.

Zn^{2+} all had a strong inhibitory effect on PpSABATH1 activity (50–100%). PpSABATH1 displayed pseudo Michaelis–Menton kinetics, with apparent K_M values of 83.2 ± 5.2 μM for thiobenzoic acid (**1**) (Fig. 5D) and 94.2 ± 8.3 μM for SAM. The Kcat of PpSABATH1 with thiobenzoic acid (**1**) as substrate was determined to be 0.046 s^{-1}.

2.5. Structural modeling of PpSABATH1

To understand the structural basis of thiol methylation, a homology model of PpSABATH1 was constructed using the experimentally determined structure of AtIAMT1 (Zhao et al., 2008; PDB code IB5I) as template. *In silico* docking of the four thiol compounds that showed significant activity with PpSABATH1 (Fig. 4) was performed. Because of the limited reliability of the homology model, the search for a good docking site was not possible for all of the compounds. However, among the thiols that showed activity with PpSABATH1, furfuryl thiol (**5**) exhibited an interesting configuration with the protein, with its thiol at 4.6 Å from the sulfur in SAM (Fig. 6), which is about the same distance observed in the CbSAMT structure between the carboxyl oxygen atom of salicylic acid and the sulfur of *S*-adenosyl-L-homocysteine (4.3 Å) (Zubieta et al., 2003).

In CbSAMT, Trp151 and Gln25 (in the *N*-terminal loop) form hydrogen bonds with the carboxyl moiety of the substrate to maintain its close proximity to the methyl group of SAM to be transferred. Trp151 in CbSAMT is replaced by Tyr163 in PpSABATH1. Trp151 of CbSAMT makes a hydrogen bond with one of the oxygen atoms of the reactive carboxyl moiety of the substrate (Zubieta et al., 2003). Correspondingly, Tyr163 in PpSABATH1 makes a hydrogen bond with the ring oxygen of furfuryl thiol, contributing to its stabilization in the proper orientation. At a position equivalent to Gln25 in CbSAMT is Ser33, which however is too far away for interaction with the thiol sulfur of PpSABATH1 substrate. Instead, Gln17, at a position equivalent to Lys10 in

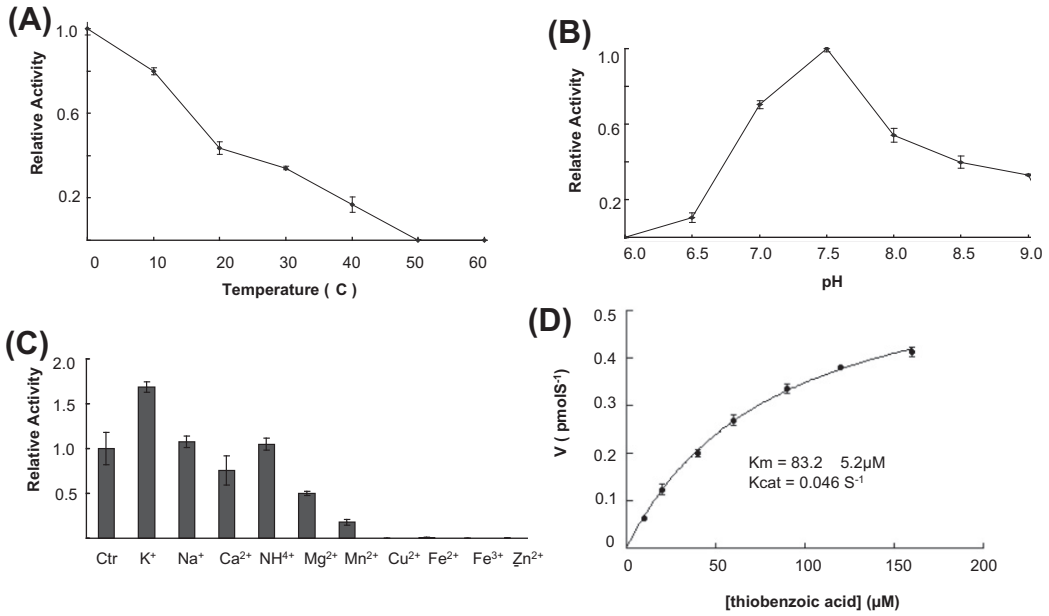

Fig. 5. Biochemical properties of PpSABATH1 using thiobenzoic acid (**1**) as substrate. (A) Thermostability of PpSABATH1. The activity of PpSABATH1 with incubation at 0 °C for 30 in was arbitrarily set at 1.0. (B) pH effect on PpSABATH1 activity. The level of PpSABATH1 activity in the buffer at pH 7.5 was arbitrarily set at 1.0. (C) Effects of metal ions on activity of PpSABATH1. The level of PpSABATH1 activity without any metal ion added as control (Ctr) was arbitrarily set at 1.0. (D) Steady-state kinetic measurements of PpSABATH1. Each value is an average of three independent measurements.

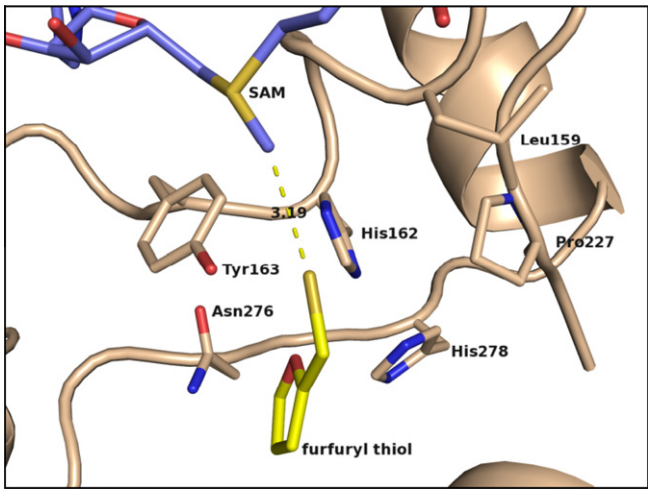

Fig. 6. A homology model of PpSABATH1 (in wheat color), which was calculated with Modeller (Sali and Blundell, 1993) based on the experimentally determined AtIAMT1 and CbSAMT structures (in salmon and orange respectively). Secondary structures are represented as ribbons and most of the active site residues involved in substrate recognition are represented as sticks with color-coded atoms. SAM is represented as blue sticks, and furfuryl thiol as yellow sticks. This figure was produced with PyMOL (http://www.pymol.org) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

CbSAMT, appears to play a role similar to that by Gln25 in CbSAMT; one difference is that it sits on the opposite end of the substrate. The ring of furfuryl thiol lies in a position similar to the ring of salicylic acid in CbSAMT. This is likely a consequence of the binding of the thiol sulfur at the proper position to make possible the methyl transfer. Therefore, despite the significant differences in their sequences, PpSABATH1 and CbSAMT bind similarly to the reactive moiety of the substrate.

Other variations were observed among the residues comprising the active sites of CbSAMT, AtIAMT1 and PpSABATH1. Tyr147 and Phe158 (respectively, in CbSAMT and AtIAMT1) are replaced by Leu159 in PpSABATH1; Trp226 and Phe243 are replaced by Ala247; Leu210 and Leu 226 are replaced by Pro227; Val311 and Val326 are replaced by Gly334 (Fig. 7). These changes led to an enlarged binding pocket in PpSABATH1 in comparison to those in CbSAMT and AtIAMT1. Consequently, there are very few significant interactions between PpSABATH1 and its substrate, apart from the hydrogen bonds with the sulfur.

2.6. Characterization of PpSABATH1 gene expression under thiobenzoic acid treatment

Because aromatic thiols have been shown to be toxic to plants (Tillekeratne et al., 1987), it could be hypothesized that the methylation of thiols by PpSABATH1 may have a role in detoxification. To test this hypothesis, we analyzed the expression of *PpSABATH1* in *P. patens* plants was analyzed in response to the treatment with thiobenzoic acid (**1**), the most preferred substrate among all the compounds tested (Fig. 3). First, the toxicity of thiobenzoic acid to *P. patens* plants was determined. *P. patens* plants were grown on agar plates supplemented with various concentrations of thiobenzoic acid. While 1 mM thiobenzoic acid appeared to be lethal, *P. patens* plants survived on other concentrations of it (**1**). For gene expression analysis, two concentrations of thiobenzoic acid (**1**), 250 μM and 500 μM, were chosen for plant treatments. Plant tissues were collected at two time points, 2 h and 24 h, after the treatments. The expression of *PpSABATH1* in thiobenzoic acid (**1**)-treated plants was reduced at 2 h after the treatment in comparison to that in control plants. At 24 h after treatment, expression of *PpSABATH1* was up-regulated by eight-fold in plants treated with 250 μM thiobenzoic acid (**1**) and 16-fold in plants treated with 500 μM thiobenzoic acid (**1**) (Fig. 8).

2.7. Production and analysis of transgenic tobacco plants overexpressing PpSABATH1

To further test the hypothesis that *PpSABATH1* has a role in detoxification of thiols, transgenic tobacco plants were generated that constitutively express the *PpSABATH1* gene under the control

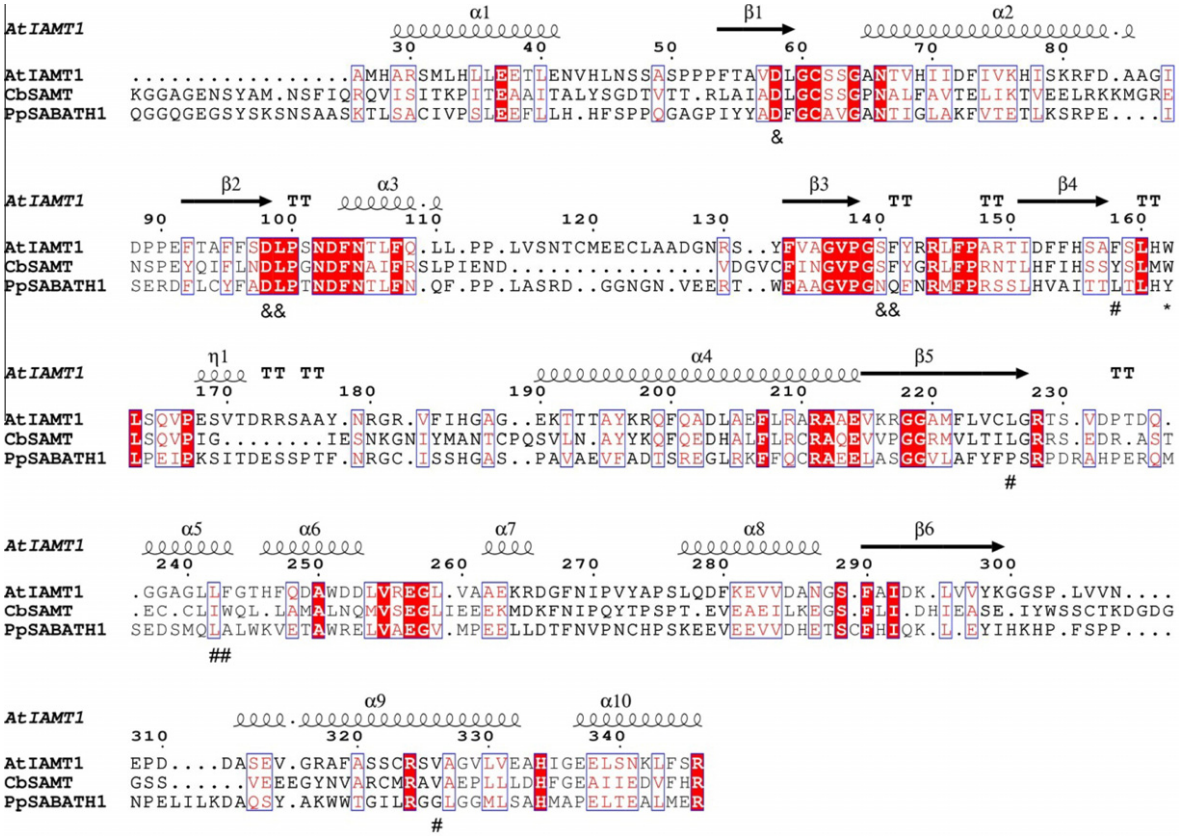

Fig. 7. Structure-based sequence alignment of PpSABATH1, AtIAMT1 and CbSAMT. The sequence alignment, which was restricted to the part used to build the PpSABATH1 homology model, was modified by hand to include the structural information available for AtIAMT1 (Zhao et al., 2008) and CbSAMT (Zubieta et al., 2003). The blue frames indicate conserved residues, white characters in red boxes indicate strict identity and red characters in white boxes indicate similarity. The secondary structure elements indicated above the alignment are those of AtIAMT1. Residues marked with "&" below the alignment indicate the residues for SAM/SAH binding. Residues marked with "∗" are residues that interact with the carboxyl moiety of indole-3-acetate. Residues indicated with "#" interact with the aromatic moiety of the substrate, and are important for substrate selectivity. This figure was prepared with ESPript (Gouet et al., 1999) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

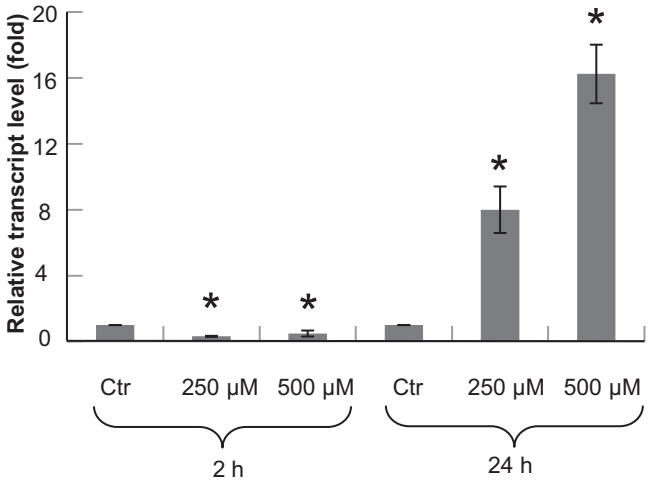

Fig. 8. Expression of *PpSABATH1* in *P. patens* plants with 0 (Ctr), 250 and 500 μM thiobenzoic acid (**1**) for 2 and 24 h. Expression values were normalized to the levels of 18S rRNA gene expression in respective samples. The level of *PpSABATH1* expression in the control plant is arbitrarily set at 1.0. Values are the average of three independent measurements. Bars marked with an asterisk (∗) indicate that *PpSABATH1* showed a significant difference in expression levels in treated vs. control plants using Student's *t*-test ($p < 0.05$).

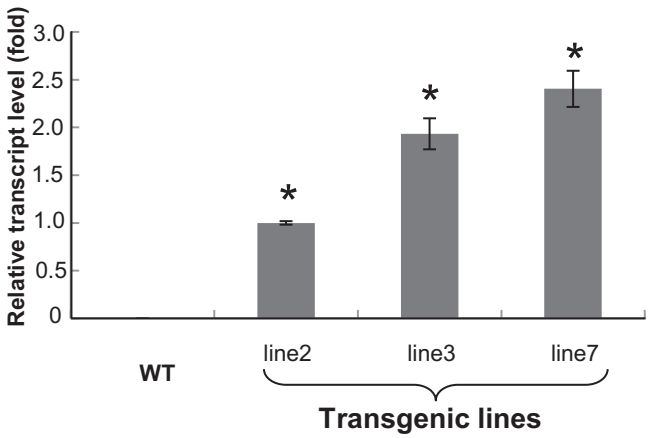

Fig. 9. Expression analysis of *PpSABATH1* in transgenic lines (line2, line3 and line7) and nontransgenic (WT) tobacco plants using quantitative RT-PCR analysis. Expression values were normalized to the levels of tobacco actin gene expression in respective samples. The level of *PpSABATH1* expression in transgenic line2 is arbitrarily set at 1.0. The values are the average of three independent measurements. Levels of significance were calculated using student's *t*-test at $p < 0.05$. Bars marked with an asterisk (∗) indicate that *PpSABATH1* showed a significant difference in expression levels in treated vs. control plants using Student's *t*-test ($p < 0.05$).

of the CaMV 35S promoter. The expression of *PpSABATH1* in three transgenic lines (lines 2, 3 and 7) was examined by quantitative RT-PCR. All three lines showed expression of *PpSABATH1*. The expression levels of *PpSABATH1* in line3 and line7 were comparable, which was approximately two-fold higher than that observed in line2 (Fig. 9). The three transgenic plants overexpressing

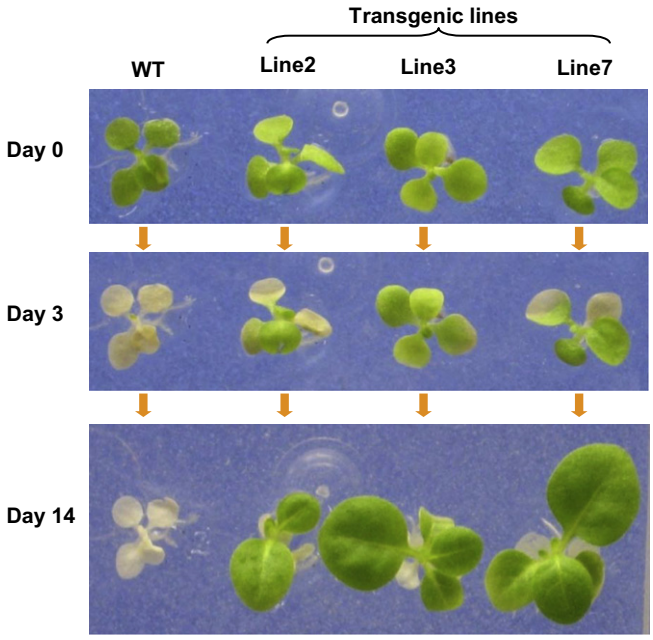

Fig. 10. Transgenic tobacco plants overexpressing *PpSABATH1* showed higher resistance to thiobenzoic acid than nontransgenic tobacco plants (WT). Three independent events (Line2, Line3 and Line7) were analyzed. Seedlings of transgenic and nontransgenic plants at a same developmental stage were transferred from the thiobenzoic acid (**1**)-free medium to the thiobenzoic acid-containing medium at the same time. Ten plants per line were analyzed in each experiment, which was repeated three times. The plants of a same line showed same phenotype. The picture showed the phenotypes for one representative nontransgenic plant and one representative plant from each of the three transgenic lines immediately after the transfer (Day 0), at 3 days after the transfer (Day 3) and at 14 days after the transfer (Day 14).

PpSABATH1 did not show obvious phenotypic differences in comparison to nontransgenic tobacco plants when grown under standard conditions.

Transgenic tobacco plants overexpressing *PpSABATH1* were then analyzed for their resistance to thiobenzoic acid (**1**). Seeds of nontransgenic plants and the three transgenic lines were sterilized and germinated on agar plates. Plants with their first true leaves fully unfolded were used for toxicity assays. The lethality of thiobenzoic acid (**1**) to wild-type tobacco seedlings was determined as described above. After 3 days, plants growing on plates containing 350 μM or higher concentrations of thiobenzoic acid (**1**) exhibited bleached leaves, and eventually died. The plants grown on lower concentrations of thiobenzoic acid (**1**) survived. Therefore, 350 μM thiobenzoic acid (**1**) was selected as the minimum lethal concentration to test the resistance of transgenic tobacco plants to this chemical. Three days after the treatment, the leaves of transgenic plants overexpressing *PpSABATH1* retained normal color and had normal growth (Fig. 10). At 14 days after initial exposure, transgenic plants continued to grow normally (Fig. 10). Among the three transgenic lines, the plants of line3 and line7 exhibited notably less bleaching than the plants of line2 at day 3, corresponding positively with transcript levels. At day 14, the recovered line2 plants were smaller in size than those of line3 and line7 (Fig. 10).

3. Discussion

3.1. PpSABATH1 encodes a thiol MT

In this study, it was demonstrated that PpSABATH1 functions as a thiol MT. As the first characterized *SABATH* gene isolated from a non-seed plant, PpSABATH1 is also the only member of the SABATH family that has been shown to catalyze *S*-methylation of thiols. Different from other characterized SABATH proteins, which often show strict substrate specificity, PpSABATH1 exhibits activity with substrates of distinct structures, with the ring-containing thiols being the preferred substrates among the compounds tested (Fig. 4). The kinetic properties of PpSABATH1 are comparable to those of other characterized SABATH proteins. The K_M of PpSABATH1 for thiobenzoic acid (**1**), which was the most preferred substrate among the thiols tested, was 83.2 μM. In comparison, the K_M of rice indole-3-acetic methyltransferase (OsIAMT) for indole-3-acetic acid was 17.9 μM (Zhao et al., 2008). The catalytic efficiencies of PpSABATH1 and OsIAMT are also comparable, which were 0.046 s^{-1} and 0.025 s^{-1}, respectively.

The mechanism of methylation catalyzed by PpSABATH1 is partly understood by structural modeling (Fig. 6). The homology model, together with the *in silico* docking experiment, provides clues to the thiol methylation preference of PpSABATH1. Based on pKa, the ligand thiol sulfur is expected to be protonated at cellular pH values. This is different from other known substrates of the SABATH family, the reactive groups of which are fully or predominantly deprotonated in the cellular environment (Zubieta et al., 2003). Therefore, the catalytic mechanism of PpSABATH1 may involve a general base to deprotonate its thiol substrate prior to or during the methyl transfer. One candidate is His162. The weak hydrogen bond distance between the NE2 nitrogen and the thiol group is coherent with this hypothesis. It also positions the thiol sulfur 3.2 Å from the methyl group of SAM. Apart from the enzymatic mechanism, the binding pocket itself may account for the thiol methylation preference. The distances between the residues involved in the thiol binding in PpSABATH1 are increased, compared to the corresponding distances in CbSAMT. This model is in agreement with the differences expected in the interaction distances between a sulfur and an oxygen.

Prior to this study, two types of thiol MT have been reported in plants. The first type of thiol MT was identified in cabbage (*Brassica oleracea*), which was involved in the production of volatiles upon glucosinolate degradation (Attieh et al., 2000a,b, 2002). One cabbage thiol MT (cTMT1) was shown to methylate thiocyanate, one degradation product of glucosinolates. In addition, cTMT1 was shown to have activity with benzene thiol, with the latter being the much preferred substrate (Attieh et al., 2002). The second type of plant thiol MT was isolated from *Catharanthus roseus*. The only representative is CrSMT1, which belongs to the type I *O*-methyltransferase family (Coiner et al., 2006). This enzyme has a broad substrate range, with many sulfhydryl-containing compounds (Coiner et al., 2006), some of which, such as DTT, thiobenzoic acid (**1**) and furfuryl thiol (**5**), are also the substrates of PpSABATH1 (Fig. 3). Despite that PpSABATH1, cTMT1 and CrSMT1 catalyze the *S*-methylation of the same thiols, such as thiobenzoic acid (**1**), PpSABATH1, cTMT1 and CrSMT1 do not share significant sequence similarity at the protein level. The existence of MTs with non-homologous protein sequences that nonetheless posses similar biochemical activities invokes convergent evolution, a mechanism playing an important role in the evolution of plant specialized metabolism (Pichersky and Lewinsohn, 2011).

3.2. Evolution of the SABATH family

The significant sequence similarity and conserved gene structure between *PpSABATHs* with *SABATH* genes from seed plants (Fig. 1) suggest that plant SABATH genes have a common evolutionary origin. The size of the *SABATH* family in the *P. patens* genome, which contains four members, is much smaller than has been found in flowering plants. For example, the Arabidopsis and rice genomes contain 24 and 41 *SABATH* genes, respectively (Chen et al., 2003; Zhao et al., 2008). The small size of the SABATH family

in *P. patens* is not due to the size of its genome. The *P. patens* genome is 480 Mb, which is larger than both Arabidopsis and rice, which are 125 Mb and 389 Mb, respectively. This indicates that the SABATH family has expanded in the seed plants, or has been reduced in *P. patens*. Phylogenetic analysis showed that the *P. patens SABATH* genes form a monophyletic clade (Fig. 2), suggesting that the divergence of these genes occurred after the split of the *P. patens* lineage and the lineage leading to higher plants. The four *SABATH* genes in *P. patens* are localized to different scaffolds, suggesting that they are the consequence of segmental or whole genome duplication. The lack of introns in *PpSABAPT3* suggests that this gene resulted from a processed pseudogene. *PpSABATH3* contains a stop codon at position 882, which causes the premature termination of protein translation compared to the other SABATHs, suggesting that *PpSABATH3* encodes a pseudogene. This was supported by the lack of expression for this gene under all conditions, although it is possible that under other conditions this gene may be expressed.

The presence of a mid-size family of *SABATH* genes in flowering plants is consistent with the diversity of substrates synthesized by these plants and the biological functions conferred by the methyl esters. The known substrates of *SABATH* genes that are ubiquitously found in flowering plants include gibberellic acid, indole-3-acetic acid, jasmonic acid and salicylic acid. The methyl esters of these metabolites catalyzed by corresponding *SABATH* genes are involved in regulating plant developmental processes (Qin et al., 2005; Varbanova et al., 2007) and plant interactions with the environment (Chen et al., 2003). However, some of these metabolites, such as gibberellic acid, are not synthesized in *P. patens* (Hirano et al., 2007). This suggests that the expansion of the *SABATH* family and the evolution of new activities in flowering plants may be associated with the unique biology of flowering plants and commensurate with their expanding ecological niches and diversity.

The strict substrate specificity of individual *SABATH* genes poses an intriguing question regarding the evolutionary trajectory of the *SABATH* family in plants. An analysis of *SABATH* genes in flowering plants using a combination of phylogeny, structural biology and biochemical analysis suggests that IAMT is an evolutionary ancient member of the SABATH family in seed plants (Zhao et al., 2008; Zhao et al., 2009). The phylogenetic analysis conducted in this study showed that PpSABATHs cluster with IAMTs and GAMTs (Fig. 1), whereas SAMTs, JMTs and NMTs grouped in a different clade (Fig. 1). Two hypotheses can be invoked in regards to the evolution of thiol MT in *P. patens*. One is that thiol MT activity may have been a more ancient activity of the SABATH family, preceding the evolution of IAMTs. The other possibility is that thiol MT evolved through divergent evolution within the SABATH family, in a lineage-specific manner that has occurred only in non-vascular plants. It will be informative to determine whether some members of the SABATH family in seed plants, the majority of which remain functionally unknown, have thiol MT activity.

3.3. The biological function of PpSABATH1

In contrast to many other classes of specialized metabolites, the number of thioesters known to be produced by plants is very limited. Methyl thiocyanate is a metabolite produced by *Brassica*, which is the product of cTMT1 (Attieh et al., 2000a,b, 2002). Because of its toxicity, methyl thiocyanate has been proposed to have a role in plant defense against biotic stress (Attieh et al., 2002). The biological function of CrSMT1, the thiol MT identified from *C. roseus* (Coiner et al., 2006), remains to be determined. While the biological functions of *S*-methylated thiols in plants are generally poorly understood, the functions of thiol MTs in animals and microorganisms have been relatively well characterized. In the latter, thiol MTs are involved in the detoxification of both endogenous and xenobiotic thiols (Drotar and Fall, 1986; Drotar et al., 1987). Xenobiotic thiols can also be toxic to plants. For example, aromatic thiols can degrade plant pigments such as carotene and oxidized carotenoids of the xanthophyll type leading to a bleaching phenotype (Tillekeratne et al., 1987). The induced expression of *PpSABATH1* in *P. patens* plants by the treatment of thiobenzoic acid (**1**) suggested that this gene may have a role in tolerance to toxic thiols (Fig. 8). The assays with transgenic tobacco plants clearly showed that plants overexpressing *PpSABATH1* had enhanced tolerance to thiobenzoic acid (**1**), relative to the nontransgenic plants (Fig. 10). The level of chemical resistance appeared to be positively associated with the levels of gene expression, as line3 and line7 possessed two-fold higher transgene expression relative to that found in line2 (Fig. 9); these two lines had higher tolerance to thiobenzoic acid than line2 (Fig. 10). Direct genetic manipulation of *PpSABATH1* in *P. patens* and characterization of the occurrence of thiols as xenobiotics of either natural or anthropogenic sources will provide stronger evidence with regards to the biological role of this gene.

4. Conclusions

P. patens is the first non-vascular plant in which the *SABATH* family of methyltransferases is experimentally studied. In comparison to higher plants such as Arabidopsis and rice, *P. patens* contains a small SABATH family with only four members. One of these four proteins, PpSABATH1, was demonstrated to catalyze a novel reaction that has not been previously identified with the SABATH family: it catalyzes the *S*-methylation of thiols displaying the highest level of activity with thiobenzoic acid among the thiols tested. As a xenobiotic, thiobenzoic acid induced the expression of *PpSABATH1*. Overexpression of *PpSABATH1* in tobacco led to enhanced tolerance of tobacco plants to thiobenzoic acid. The biochemical and transgenic evidence suggests that *PpSABATH1* may have a role in the detoxification of xenobiotic thiols.

5. Experimental

5.1. The culture of P. patens, plant treatment and chemicals

The leafy gametophyte of *P. patens* (Gransden) was grown on agar plates containing BCD medium (Knight et al., 2002) at 25 °C under 16 h light/8 h dark photoperiod. One-month-old culture was used for chemical treatment and RNA isolation. For thiobenzoic acid (**1**) treatment, one-month-old culture was transferred to the BCD medium supplemented with certain concentrations of thiobenzoic acid (**1**), including 0, 10, 50, 100, 250, 500 and 1 mM. The plants treated with 0, 250 and 500 μM were collected for expression analysis of *PpSABATH1*. All chemicals were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise noted.

5.2. Database search and sequence analysis

To identify putative *P. patens SABATH* genes, the protein sequence of CbSAMT was used to search the *P. patens* genome database (http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html) using BlastP and TBlastn algorithm (Altschul et al., 1990). In phylogeny reconstruction, multiple protein sequence alignments of SABATHs were performed using the ClustalX2 program. PhyML v2.4.4 (Guindon and Gascuel, 2003) was used to build maximum likelihood (ML) trees using the JTT model. 100 replicates of bootstrap analyses were conducted.

5.3. Full-length cDNA cloning for PpSABATH1 and PpSABATH2

Extraction of total RNA from *P. patens* plants and RT-PCR was conducted as previously described (Chen et al., 2003). Primers used for gene cloning are listed in Supplemental Table 2. PCR amplicons for *PpSABATH1* and *PpSABATH2* were purified from the gel using QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and cloned into pEXP5-CT/TOPO vector (Invitrogen, Carlsband, CA, USA). The cloned cDNAs were fully sequenced.

5.4. Radiochemical MT enzyme assays

A high-throughput biochemical procedure (Zhao et al., 2009) was used in initial screening of potential substrates (Supplemental Table 1) for PpSABATH1 and PpSABATH2. Radiochemical MT assays were performed in a 50 μl volume containing 50 mM Tris–HCl, pH 7.5, 1 mM substrates, 10 mM β-mercaptoethanol, 0.4 μg purified enzyme, and 3 μM ^{14}C-*S*-adenosyl-L-methionine (SAM) with a specific activity of 51.4 mCi/mmol. Radiochemical assays for sulfhydryl groups were performed the same as the high-throughput biochemical assays, except that β-mercaptoethanol was not included.

The reactions were incubated at 25 °C for 30 min and then extracted with 180 μL ethyl acetate. The upper organic phase was counted using a liquid scintillation counter (Beckman Coulter, Fullerton, CA). The amount of radioactivity extracted into the organic phase is assumed to be equivalent to the amount of methyl ester formed by the recombinant enzyme using ^{14}C-SAM as a cofactor.

5.5. Purification from E. coli-expressed recombinant proteins

PpSABATH1 full-length cDNA was cloned into the pET100/D-TOPO vector (Invitrogen, Carlsband, CA). Expression of PpSABATH1 in *E. coli* and purification of His-tagged PpSABATH1 protein was conducted as previously reported (Zhao et al., 2007).

5.6. Biochemical characterization of PpSABATH1

In all of the kinetic analyses, the appropriate enzyme concentrations and incubation times were chosen so that the reaction velocity was linear during the incubation time period. To determine an apparent K_M value for SAM, concentrations of SAM were varied from 3 to 120 μM, while thiobenzoic acid (**1**) was held constant at 1 mM. To determine the K_M value of thiobenzoic acid (**1**), the concentration of SAM was fixed at the saturated level and the concentration of thiobenzoic acid (**1**) was varied from 10 to 500 μM. 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 Michaelis–Menten equation.

To determine pH optimum of PpSABATH1, PpSABATH1 enzyme assay was carried out in two buffer systems. Reactions were carried out 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 9.0. To determine the effect of metal ions on PpSABATH1 activity, enzyme assays were performed with the addition of one of the metal ions in the form of chloride salts in assay buffer at the final concentration of 5 mM: K^+, Ca^+, NH_4^+, Na^+, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}. To determine the temperature stability of PpSABATH1, PpSABATH1 protein was incubated at temperatures ranging from 0 to 60 °C for 30 min, chilled on ice, and then used in standard assays.

5.7. Product identification using GC/MS

A reaction containing 150 μg purified PpSABATH1, 1 mM thiobenzoic acid and 600 μM unlabelled SAM was incubated in a 1 mL reaction containing 50 mM Tris-HCl, pH 7.5 at 25 °C for 4 h. The reaction product was extracted with hexanes (1.5 mL), concentrated under N_2 gas and analyzed by Shimadzu GC (GC-17A)/MS (QP 5050A) system (Columbia, MD). A DB-5 column (30 m × 0.25 mm id × 0.25 μm thickness) was used with helium as carrier gas at a flow rate of 1 mL min^{-1}. As a control, a similar reaction was performed, except that PpSABATH1 protein was denatured by boiling at 100 °C for 10 min before being added to the assay. Thiobenzoic acid methylthioester authentic standard was dissolved in EtOH at the concentration of 0.5 μg/μL and 1 μg thiobenzoic acid methylthioester was injected into the GC in split (1/30) mode. The GC temperature program was as follows: 2 min at 80 °C followed by a ramp of 8 °C min^{-1} to 320 °C. Identity of the product was confirmed by comparison of GC retention times and mass spectra with that of the authentic standard.

5.8. Structural modeling of PpSABATH1

Homology models were built with Modeller (Sali and Blundell, 1993) by generating a first model (3D alignment on template) and running 200 cycles of molecular dynamics on the basis of simulated annealing. An *in silico* docking experiment was performed with the Glide program (Friesner et al., 2004), which is part of the Schrödinger^{TM} suite (Schrödinger, LLC, New York, NY, 2005). A highest structural homology with AtIAMT1 was assumed based on the highest sequence identity (32%, for 349 aligned residues), compared to that with CbSAMT (30%, for 292 aligned residues). However, as residues 1–25 of AtIAMT1 are not visible in its crystal structure, the corresponding N-terminal fragment of PpSABATH1 was modeled based on the CbSAMT structure (PDB code 1M6E), where clear density was visible for the N-terminal loop followed by helix alpha1, in a position suggesting that this loop closes the active site to sequester the substrate. Assuming that this flexible N-terminal fragment may adapt to the substrate actually bound to the active site, it was not included in the model used as a target for the *in silico* calculation presented below. Instead, it was introduced in the *a posteriori* analysis of the binding mode of the substrate indicated by the calculation. In the same way, the truncated model used for *in silico* experiments was completed with SAH, to avoid a steric clash between the methyl group of SAM and the substrate, whereas SAM is presented for the complete model used for analysis of methyl transfer mechanism.

5.9. Tobacco transformation

The cDNA of *PpSABATH1* was first cloned into a Gateway entry vector pENTR/D-TOPO (Invitrogen, Carlsband, CA). It was then mobilized into a Gateway binary vector pCHF3-GW (Varbanova et al., 2007) under the control of the 35S promoter. The binary construct containing PpSABATH1 was transformed into the *Agrobacterium tumefaciens* strain EHA105. *Agrobacterium*-mediated tobacco transformation method and tissue culture system were based on a previously published protocol (Horsch et al., 1985).

Briefly, tobacco (*Nicotiana tabacum* L. 'Xanthi') seeds were sterilized with 10% commercial chlorine bleach and EtOH-H_2O (7:3, v/v). Sterilized seeds were grown on MS medium (Murashige and Skoog, 1962) containing B5 vitamin. Tobacco leaves were cut into 1–1.5 cm^2 disks and co-incubated with *Agrobacterium* for 30 min. Infected leaf disk explants were placed on antibiotic-free DBI medium containing indole-3-acetic acid (1 mg/L) for tobacco shoot organogenesis and co-cultivated for 48 hr. Then, the explants were transferred to selective DBI medium containing kanamycin (200 mg/L) and timentin (400 mg/L). Regenerated shoots were removed from each callus and moved to MS media for root development. All cultures were maintained at 24 °C under a 16/8 h light/dark photoperiod. Rooted shoots were transplanted to soil and

acclimated for 2 weeks. T_1 seeds were produced by self-fertilization of each independent transgenic event. Transgenic T_1 seeds were selected on MS medium containing kanamycin (200 mg/l) and then ascertained by PCR using genomic DNA isolated using the CTAB (*N*-acetyl-*N*, *N*,*N*-trimethylammonium bromide) method (Murray and Thompson, 1980). Plants from T_1 lines showing ~3:1 kanamycin resistant to sensitive ratios were then transplanted to the greenhouse, self-fertilized and resulted in the production of T_2 seeds. Homozygous T_2 lines were selected by kanamycin resistance and used for thiobenzoic acid (**1**) treatment experiments.

5.10. Quantitative RT-PCR analysis of gene expression

Quantitative RT-PCR experiments were performed to determine the expression of the four members of the *PpSABATH* family, the expression of *PpSABATH1* in *P. patens* plants treated by thiobenzoic acid (**1**) and the expression of *PpSABATH1* in transgenic tobacco plants. The primers used in these experiments are listed in Supplemental Table 3. Quantitative RT-PCR was conducted as previously described (Yang et al., 2006). Statistical analysis of quantitative RT-PCR expression 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 $p < 0.05$.

5.11. Thiobenzoic acid treatment of transgenic tobacco

To test the lethal concentration of thiobenzoic acid (**1**) to tobacco plants, the seeds of wild-type plants were sterilized and germinated on the MS medium. When the first true leaves were fully unfolded, plants were transferred to MS medium supplemented with varying concentrations of thiobenzoic acid (**1**), including 10, 50, 100, 250, 350, 500, and 1 mM. After the lethal concentration was determined, the experiment was repeated with only 350 μM thiobenzoic acid (**1**). Photographs were taken after 3 days and 14 days of treatment.

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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.2012.06.011.

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