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Dedication

I dedicate my dissertation work to my parents, Qiang Zhao and Hongshu Zhang for their unconditional support and encouragement through the years. They always emphasized the importance of education and sacrificed a lot to support my studies. Without their continued support and counsel I could not have completed this process. The dissertation is also dedicated to my wife, Jingyu Lin, who accompanied me through all the hardships and shared the many uncertainties, challenges and frustrations for completing this dissertation.

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Abstract

Known members of the plant SABATH family of methyltransferases (MTs) have important biological functions by methylating hormones, signaling molecules and other metabolites. This dissertation aims to systematically investigate the biochemical and biological functions and evolution of *SABATH* genes in plants. The genomes of rice and poplar have been fully sequenced, which provides unprecedented opportunities for cross-species comparison of the *SABATH* family. Using a comparative genomic approach, 41 and 33 *SABATH* genes were identified in rice and poplar, respectively. The expression of these genes in different tissue was analyzed using RT-PCR approach and some genes highly expressed in multiple tissues were cloned. The cloned cDNAs were expressed in *E. coli* to produce enzymes. Recombinant proteins were tested with a large number of compounds for MT activities. In poplar, two proteins were determined to have MT activities with indole-3-acetic acid (IAA) and jasmonic acid (JA), respectively. Biochemical and gene expression evidence suggests that poplar *IAMT* (*PtIAMT1*) has an important role in poplar development through IAA methylation. Poplar *JMT* (*PtJBMT1*) shows high levels of expression at multiple tissues under normal conditions, and the expression of *PtJBMT1* was slightly induced by wounding and methyl jasmonate treatment, suggesting it has a role in poplar development and defense. Two *SABATH* genes, *IAMT* (*OsIAMT1*) and *BSMT* (*OsBSMT1*), were identified in rice. The high level of *OsIAMT1* transcripts in rice roots and panicles implies that *OsIAMT1* is involved in root and panicle development. *OsBSMT1* has MT activities with both salicylic acid

and benzoic acid. The specific induction of *OsBSMT1* by herbivory and enhanced emission of methylsalicylate support that it has a specific role in plant response to insects. The evolution of the *SABATH* gene family was also investigated based on the identified *SABATH* genes in plants using comparative genomics analysis. Phylogenetic analysis revealed that IAMTs in Arabidopsis, rice and poplar are highly conserved, suggesting IAMTs are evolutionarily ancient. However, SAMTs in these plant species are divergent, indicating it is possible that SAMTs were evolved after the split of these plant lineages.

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**Chapter I. Literature Review: The Plant SABATH
Family of Methyltransferases: Biochemical and
Biological Functions, Evolution, and Gene Discovery**

I. Plant secondary metabolism: a brief introduction

Plant secondary metabolites are a broad spectrum of low molecular weight compounds involved in a myriad of complex processes in plants. Some secondary metabolites serve as attractants for pollinators and seed dispersing animals; some protect plants from attacks by insects and pathogens; others are involved in the survival of the plants under abiotic stresses (Zhao et al., 2005). More than 100,000 structures of secondary metabolites have been described (Wink, 1988), and this is likely to be the tip of iceberg of total secondary metabolites made by the plant kingdom (Osbourn et al., 2003). Secondary metabolites often appear to be species specific, i.e., a given plant species produces a subset of unique secondary metabolites that are related to the unique physiological and ecological processes of the species.

A large portion of the plant genomes is involved in secondary metabolism. For example, approximately 20% of the genes in the Arabidopsis and rice genomes are annotated to be involved in the biosynthesis of secondary metabolites (The Arabidopsis Genome Initiative, 2000; Goff et al., 2002). To date, only a small portion of these genes has been functionally characterized. The biochemical and biological/ecological functions of the majority of the genes involved in secondary metabolism are yet to be unveiled. Experimental characterization of these genes may provide important insights into the evolution and function of secondary metabolism in different plant species. Moreover, a deeper understanding of plant secondary metabolism may provide important knowledge and tools for the development of

sustainable agricultural systems for effective pest management and abiotic stress resistance.

Despite the enormous structural diversity, the biosynthesis of most secondary metabolites involves a limited number of enzymatic reactions, such as hydroxylation, glycosylation, methylation and acylation (Noel et al., 2005). Methylation is catalyzed by a group of methyltransferases that transfer of the methyl group from a methyl donor molecule to the oxygen, nitrogen, or carbon atoms of various acceptor molecules. S-adenosyl-L-methionine (SAM) is the most widely used methyl donor. Almost all types of plant metabolites of both primary and secondary metabolism, such as DNA, protein, lipid and small molecular metabolites, are known to be methylated.

II Small molecule metabolites methyltransferases

Based on the chemical nature of substrates, plant small molecule methyltransferases (MTs) can be divided into different types: carbon MT (CMT), nitrogen MT (NMT), sulfur MT (SMT) and oxygen MT (OMT). To date, a large variety of enzymes that mediate the methylation of small molecule metabolites have been identified in plants and most of them are OMTs. Based on their structures and functions, OMTs can be classified into three types: the Type I, II and III OMTs (Figure 1.1; Noel et al., 2003).

Type I OMTs

Type I OMTs consist of a large group of proteins with a molecular mass of approximately 40 kDa, whose known substrates include phenylpropanoids, flavonoids, isoflavonoids, alkaloids, eugenol, chavicol, coumarins, orcinols, and stilbenes. For example, isoflavone OMT (IOMT) catalyzes the formation of 4'-hydroxy-7-methoxyisoflavone, which is an essential intermediate in the biosynthesis of phytoalexins in legumes (Wu et al., 1997). This group of OMTs does not require divalent cations for activity compared with type II OMTs. The crystal structure of several Type I OMTs has been solved, such as the chalcone O-methyltransferase (ChOMT) and the isoflavone O-methyltransferase (IOMT) from alfalfa (Zubieta et al., 2001).

Type II OMT

Type II OMTs contain a family of proteins with a molecular mass of 23-28 kDa. All of these proteins use CoA-esters of phenylpropanoids as substrates, and their main role is related in lignin biosynthesis. Type II OMTs typically require a divalent cation to mediate the methyl group transfer. Caffeoyl Coenzyme A 3-O-methyltransferase (CCoAOMT) is the representative of these enzymes. CCoAOMT is a bifunctional enzyme, which catalyzes the conversion of caffeoyl CoA to feruloyl-CoA and 5-Hydroxyferuloyl-CoA to sinapoyl-CoA in lignin biosynthesis. The crystal structure of alfalfa CCoAOMT has been solved (Ferrer et al., 2005); it shows high structural similarity to the animal catechol OMT.

Type III OMTs (SABATHs)

The members of Type III OMTs share sequence similarity to each other but have no significant sequence similarity to other groups of OMTs. Characterized members of this group catalyze the transfer of methyl group from SAM to the carboxyl group or the nitrogen group of the substrate, forming the small molecule methyl esters such as methylsalicylate (MeSA) or N-methylated compounds such as caffeine. Based on the first three members functionally characterized (SAMT, BAMT, Theobromine synthase), this family was named the SABATH family (D'Auria, 2003). All SABATH proteins have a molecular mass of appropriately 40 kDa. The crystal structure has been resolved with *clarkia breweri* Salicylic acid methyltransferase (CbSAMT) (Zubieta et al., 2003).

III. Known biochemical and biological functions of the SABATH family of methyltransferases

Known plant SABATH MTs methylate small molecules of diverse structures (D'Auria, 2003). Some of these substrates are important plant hormones and signaling molecules, such as indole-3-acetic acid (IAA; Qin et al., 2005), jasmonic acid (JA; Seo et al., 2001), salicylic acid (SA; Ross et al., 1999; Chen et al., 2003), and farnesoic acid (FA; Table 1.1; Yang et al., 2006). Methylating these compounds may have important impacts on plant growth and development (Qin et al., 2005).

IAA and indole-3-acetate (MeIAA): biosynthesis, metabolism and biological functions

IAA is the most abundant naturally occurring auxin in higher plants. It is not only involved in a diverse array of plant growth and development processes, but also in plant interactions with the environment (Cohen et al., 2003). Evidence shows that there are multiple pathways of IAA biosynthesis in plants, making it possible for plants to keep the necessary level of IAA in vivo and inflect the essential role of IAA in plant growth and development. To date, the physiological effects of IAA in plants have been well studied (Taiz and Zeiger, 2006). IAA is involved in diverse biological processes including cell elongation, cell division, gravitropism, apical dominance, lateral root formation and vascular differentiation (Taiz and Zeiger, 2006). In addition to the effects of IAA in plant growth and development, growing evidence suggests IAA might have a critical role in plant response to pathogens (Donnell et al., 2003; Navarro et al., 2006).

Plants utilize a variety of mechanisms to regulate IAA concentration, including biosynthesis, conjugation/deconjugation, degradation, and down-stream signaling transduction (Ljung et al., 2002; Woodward and Bartel, 2005). Actually, only a small part of IAA exists as free IAA, and most IAA molecules exist as IAA-ester or IAA amide conjugates in plants. Two main groups of conjugates have been identified in a variety of plant species: ester-type conjugates, in which the carboxyl group of IAA is linked to sugars, such as glucose; and amide-type conjugates, in which the carboxyl group forms an amide bond with amino acids or polypeptides (Ljung et al., 2002).

Some IAA conjugates such as IAA-Ala and IAA-Glucose showed auxin activities when applied exogenously to plants, and data showed they could be hydroxylated back into IAA. While some other conjugates such as IAA-Asp and IAA-Glu were not capable of inducing auxin responses when applied exogenously and therefore are considered as intermediates for IAA degradation (Ljung et al., 2002; Woodward and Bartel, 2005).

Recently, a new type of conjugation of IAA was reported with the identification of an IAA-methyltransferase (*IAMT1*) from *Arabidopsis thaliana* (*A. thaliana*), which can methylate IAA to form the methyl ester of IAA: MeIAA (Figure 1.2; Qin et al., 2005). Data showed *IAMT1* is involved in *Arabidopsis* leaf development. Down-regulating *IAMT1* expression leads to dramatic epinastic leaf phenotypes, which is consistent with IAA overproduction mutants, and transgenic plants with overexpression of *IAMT1* display leaf phenotypes opposite to those of *IAMT1* RNAi lines. These suggest *IAMT1* has a role in regulating IAA homeostasis (Qin et al., 2005). Furthermore, exogenous MeIAA displayed similar but stronger hypocotyl inhibition than IAA in the dark, indicating that MeIAA, like other IAA conjugates, may be hydrolyzed into IAA by a putative esterase (Qin et al. 2005). This hypothesis has been recently proved by the identification of several methyl esterases in *Arabidopsis* (Yang et al., 2008).

SA and MeSA: biosynthesis, metabolism and biological function

SA is an important plant signaling molecule, involved in a diverse array of physiological processes, such as flower induction, stomatal closure and heat production. In addition, SA plays a central role in plant resistance against pathogens (Klessig et al., 1994).

The biological function of SA has been studied for several decades. In the early 1980s, White and co-workers found that tobacco leaves treated with SA or acetyl salicylic acid (aspirin) exhibited enhanced resistance to TMV infection and increased pathogenesis-related (PR) protein accumulation (White, 1979; Antoniwand et al., 1980). Later, reports demonstrated that exogenous SA treatment enhances resistance to many bacterial, fungal, and viral pathogens and induces PR gene expression in a variety of plant species (Malamy et al., 1992; Raskin et al., 1992; Klessig et al., 1994). The importance of SA in plant defense has been demonstrated in a large number of mutants and transgenic experiments. Several *Arabidopsis* mutants which constitutively express systemic acquired resistance (SAR) accumulated high level of SA (Bowling et al., 1994; Bowling et al., 1997). And another evidence for the function of SA in plant defense is the transgenic *Arabidopsis* and tobacco overexpressing the *Pseudomonas putida* nahG gene (Seskar et al., 1998). The nahG gene encodes salicylate hydroxylase, which converts SA to the biologically inactive catechol. When these transgenic plants were infected by pathogens, SA can not accumulate, resulting in failure of inducing PR gene expression and SAR (Seskar et

al., 1998). In addition to inducing plant defense in the damaged site, SA is also considered as the endogenous long distance signal molecule which accumulates in non-infected region to induce plant defense response, which is supported by the detection of SA in the phloem of pathogen-infected tobacco and cucumber (Mettraux et al., 1990; Rasmussen et al., 1991). However, its role as the long distance signal has been challenged. The grafting experiments suggested that SA may not be a primary mobile signal in SAR but it is necessary for the induction of SAR (Vernooij et al., 1994). Besides, several studies have suggested SA may play a role in activating cell death as the signal. And some reports also showed SA were capable of restricting the invading pathogen to the area immediately surrounding the site of infection (Dempsey et al., 1999).

Several recent researches showed the application of exogenous SA could provide protection against several types of abiotic stresses such as high or low temperature and heavy metals (Eszter et al., 2007; Dorothea et al., 2005). Recently, researchers generated SA-deficient transgenic rice by overexpressing the nahG gene, and results showed transgenic lines are more susceptible to oxidative damage caused by avirulent isolates (Yang et al., 2004).

In higher plants, the modification of SA also plays critical roles in plant defense processes. The glycosylation, methylation and amino acid conjugation are three main kinds of modification of SA. The major conjugate of SA is non-toxic

2-O-b-D-glucoside SA synthesized under the catalyzation of UDP-glucosyltransferase (UGT). Evidence showed that glucosyl SA is immobile and biologically inactive, which may function as the storage form of SA (Hennig et al., 1993; Lee et al., 1995).

By contrast, data indicated that the methylation form of SA (MeSA) has an important role in multiple trophic interactions, acting as attractants of insect pollinators and seed dispersers, and inducing defense against microbial pathogens. Several reports have shown that MeSA is the constituent of floral flavor, which can attract the insect pollinators (Knudsen et al., 1993). The level of MeSA increased dramatically in TMV-inoculated tobacco plants, suggesting it may act as an air-borne signal to induce the SAR against pathogen in the healthy tissues of the infected plant and neighboring uninfected plants (Seskar et al., 1998; Shulaev et al., 1997). Recently, MeSA has been proved as the endogenous signal inducing SAR (Park et al., 2007).

MeSA is also reported involved in the tritrophic interactions, the concentration of MeSA was detected to be highly increased in the insect damaged plants (Ozawa et al., 2000; De Boer and Dicke, 2004; Zhu and Park, 2005). In the meantime, researchers studied the role of MeSA as part of the volatile blend in the foraging behavior of the predatory mite by using a Y-tube olfactometer. Results showed the predatory mites prefer the MeSA-containing volatile blends and MeSA attracted predatory mites in an amount dependent way (De Boer and Dicke, 2004). These suggested MeSA may be important in plant indirect defense.

The salicylic acid MT (SAMT), which uses the SA as substrate, catalyzing the formation of MeSA (Figure 1.3), has been isolated from a variety of plant species (Table 1.1). The first several *SAMT* genes are identified from flowers of some flowering plants, such as *clarkia breweri*, snapdragon (*Antirrhinum majus*), and *Stephanotis floribunda* (Ross et al., 1999; Negre et al., 2002; Pott et al., 2002). However, there is no induced expression of SAMT during various treatments reported in these papers. Later, a new *SAMT* identified from Arabidopsis, was named *AtBSMT1*, which has both BAMT and SAMT activities. Although identifying another enzyme having BAMT/SAMT activity is not novel, *AtBSMT* was the first one of *SAMT* genes to be shown to have a defense role (Chen et al., 2003). In *A. thaliana* leaves, the expression of *AtBSMT1* is induced by the treatment with alamethicin, *plutella xylostella* herbivory, uprooting, physical wounding, and methyl jasmonate (Chen et al., 2003).

JA and Methyljasmonate (MeJA): biosynthesis, metabolism and biological function

JA-signaling pathway plays critical roles in plant developmental programs as well as plant interactions with the environmental responses. JA is important for root growth, flower development, and seed germination (Creelman and Mullet, 1997). JA is also known as a wounding signal. When plants are injured by the chewing insects or larger herbivores, a large variety of defense compounds are produced to cope with the wounding, such as proteinase inhibitors (PIs) that affect the activity of digestive

enzymes in the insect gut, toxic compounds that exert toxic and antifeedant effects on herbivores, or volatiles which attract the predators and parasites of the herbivores. Evidences showed all of these wounding induced responses are regulated by complex signal transduction events, and JA and its derivatives are regarded as the central signals in the wounding responses (Howe et al., 2005; Leon et al., 2001).

Genetic analysis provides powerful evidences for the identification of the function of JAs in the wounding defense. A JA-deficient *acx1* tomato mutant was found to be defective in acyl-CoA oxidase (ACX1), an important enzyme in JA biosynthesis. The mutant shows increased susceptibility to tobacco hornworm attack (Li et al., 2005). Another evidence comes from the transgenic tomatoes that are silenced in the expression of allene oxide cyclase (AOC), which are shown to lack wound induced PIs expression (Stenzel et al., 2003). Arabidopsis Coronatine Insensitive1 (*COI1*) gene encodes an F-box protein that is the key regulator of JA biosynthesis (Devoto et al., 2002). The *coi1* mutants exhibit more susceptible to *B. impatiens* and *M. perzicae* (Stintzi et al., 2001; Stotz et al., 2002).

In addition to the local defense, plants are found to exhibit systemic wounding defense. Several experiments supported JAs serve as the long distance wounding signal. A radioactivity experiment demonstrates, after applied to one leaf of tobacco, the ¹⁴C-JA is found in roots, where the nicotine is synthesized to resist the wounding (Zhang et al., 1997). Grafting experiments with wounding signaling mutants further

proved that the systemic signaling molecule might be JAs. Grafting experiment with wild type tomato and *jar1* mutant (a JA resistant mutant) shows that *jar1* mutant plants can produce the signal to induce PIs expression in the wild type scion leaves, and wild type plants can not result to the PIs expression in *jar1* mutant scion leaves, suggesting that the signal is related with JA responsive gene. On the contrary, grafting experiment with wild type tomato and *spr2* mutant (a JA synthesis deficient mutant) shows *spr2* mutant can not induce the PIs expression in the wild type scion leaves and wild type plants can produce the signal to induce the PI expression in *spr2* mutant scion leaves, suggesting JA synthesis is necessary for the signal production (Li et al., 2002).

It has been shown in recent years that many related compounds, including JA precursors and conjugates, have biological activity (Stintzi et al., 2001). For example, MeJA has been proven to be ubiquitously distributed in plant kingdom (Hamberg and Gardner, 1992).

MeJA is reported as an important regulator of various developmental processes, such as seed germination, flowering, fruit development, and leaf senescence (Creelman and Mullet, 1995; Seo et al., 2001). MeJA has been reported as a constituent of floral scent of many species, possibly serving as an attractant of pollinators. In addition, MeJA has a role in plant defense response against pathogens (Reymond and Farmer, 1998) and mechanical wounding (Creelman et al., 1992).

Evidence supports that MeJA, as an air-borne signal molecule, induces the defense response in uninfected region of the infected plant and uninfected neighboring plants (Reymond and Farmer, 1998; Kessler and Baldwin, 2001). In particular, recent study isolated an esterase from tomato that has MeJA esterase activity (Stuhlfelder et al., 2004). Therefore, it is possible that MeJA acts as a mobile signal of the JA signaling pathway.

The MeJA biosynthesis is catalyzed by jasmonic acid methyltransferase (JMT) using JA as the substrate (Figure 1.4). To date, there is only one JMT reported, which was characterized from *Arabidopsis* (AtJMT; Seo et al., 2001). The transgenic plants over-expressing *AtJMT* exhibited enhanced resistance against the virulent fungus *Botrytis cinerea* (Seo et al., 2001).

Interaction of SA and JA pathway

Among many mechanisms that plants utilize to defense against biotic stresses, JA and SA signaling pathways are two critical ones. SA mediated pathway mainly functions as plant defense against pathogens, and JA mediated pathway plays an important defense role against herbivores. The interaction between SA-dependent signaling pathway and JA-dependent pathway has been well documented in plants. The SA and JA signaling pathways are mutually antagonistic, which provides the plant with a regulatory ability to fine-tune the defense reaction in response to the different attacker encountered (Felton et al., 1999; Kunkel et al., 2002). A wealth of

reports show SA has the inhibitory effect on JA signaling pathway. Early in 1988, Doherty et al. found aspirin and related hydroxybenzoic acids can inhibit the wounding response in tomato plants. Later, several experiments indicated the SA can prevent wounding induced gene expression and PIs by interfering with the JA biosynthesis pathway (Pena-Cortes et al., 1993; Doares et al., 1995; Engelberth et al., 2001).

Genetic analysis also provides evidence for this antagonistic function. Several *Arabidopsis* mutants have been found to interfere with SA signaling, such as *eds4* and *pad4* mutants that impair the SA biosynthesis, *npr1* mutant which affects the signaling downstream of SA. Researches demonstrated these mutants exhibit enhanced induction of JA-mediated gene expression (Penninckx et al., 1996; Clarke et al., 1998; Gupta et al., 2000). The transgenic tobacco that is silenced in the expression of PAL provides further evidence. When induced by wounding, the transgenic plants produce higher level of JA, leading to enhanced insect resistance (Felton et al., 1999).

In recent study using the *Arabidopsis* mutant *npr1*, researches found a regulatory protein NPR1, which was shown to play a critical role in regulating SA-mediated inhibition on JA signaling pathway (Spoel et al., 2003). More important, a transcriptional factor WRKY70 is identified recently. Result showed WRKY70 acts as the activator of SA-mediated gene expression and the repressor of JA induced gene expression (Li et al., 2004). In conclusion, there is a negative cross-talk between

SA-dependent signaling pathway and JA-dependent pathway.

Farnesoic acid (FA) and Methyl farnesoate (MeFA)

FA is reported as an important immediate in insect juvenile hormone biosynthetic pathway. It can be methylated to form MeFA, which is further converted to Juvenile hormone III (JH III) by an enzyme-catalyzed 10, 11-epoxidation reaction (Shinoda and Itoyama, 2003). The enzyme responsible for the conversion of FA to MeFA (FAMeT) has been identified from several animal species (Holford et al., 2004; Burtenshaw et al., 2008; Ho Lam Hui et al., 2008). Interestingly, the FAMT was recently characterized from *Arabidopsis*, and the expression level of *FAMT* increased when treated with several defense-related compounds (Yang et al., 2006). Therefore, the existence of FAMT in *Arabidopsis* is speculated to have a role in plant defense against insects by interfering with insect development.

N-methyltransferases in SABATH family

Besides the above mentioned compounds, this new family of MTs also includes several N -methyltransferases involved in the biosynthesis of caffeine (Kato et al., 2000; Ogawa et al., 2001). The caffeine is synthesized from xanthosine through a step of ribose removal and three steps of N-methylation. The three N-methylation processes are catalyzed by different enzymes, including 7-methylxanthosine synthase, theobromone synthase and caffeine synthase. Genes encoding the enzymes have been isolated from tea (Kato et al., 2000) and coffee (Mizuno, 2001; Mizuno et al., 2003a;

Mizuno et al., 2003b). Sequence analysis showed that the N-MTs belong to the SABATH family.

IV. Many SABATH sequences remain functionally unknown

Continued genomics projects with various plant species reveal the existence of a large number of *SABATH* genes in plants (D'Auria, 2003). Taking *Arabidopsis* as an example, it contains 24 *SABATH* genes (Chen et al., 2003; D'Auria, 2003). These genes are scattered on all five chromosomes with some of them localized in clusters (D'Auria, 2003). Six of the 24 *AtSABATH* genes have been biochemically characterized. They have five biochemical activities, including JMT, BSMT, IAMT, FAMT and GAMT. *AtJMT* is the first *AtSABATH* gene to be characterized (Seo et al., 2001). Its identification was based on the hypothesis that some members of the SABATH family may methylate JA because MeJA is ubiquitous in plants and JA bears structural similarity to SA (Seo et al., 2001). BSMT represents BAMT and SAMT as the enzyme has both activities (Chen et al., 2003). *AtBSMT* was identified using a biochemical genomics approach that correlated emission patterns of methyl salicylate and methyl benzoate from plants and expression patterns of *AtSABATH* genes under various conditions (Chen et al., 2003). Identification of *AtIAMT* was based on structural modeling using the three-dimensional structure of *CbSAMT* as a template (Zubieta et al., 2003). *AtFAMT* was initially identified using a high-throughput biochemical assays (Yang et al., 2006). In this assay system, individual SABATH proteins were tested with a pool of chemicals including known

substrates of SABATH proteins and the compounds that are structurally similar to known substrates (Yang et al., 2006).

Although some *SABATH* genes may have redundant functions in plants, the preliminary biochemical analysis did not identify other AtSABATH proteins having JMT, BSMT, IAMT or FAMT activity except the six enzymes just described. This suggests that the remaining 18 AtSABATH proteins likely possess novel biochemical functions not identified. Expression analysis showed that the majority of the *AtSABATH* genes have specific expression patterns, supporting that *AtSABATHs* have diverse biological roles (Chen et al., 2003; D'Auria, 2003). In addition to unknown *AtSABATH* genes, a growing set of *SABATH* genes that have been/are being identified from various plant species through genomics need to be characterized biochemically. To determine biochemical function of unknown *SABATH* genes, a combination of molecular genetics, biochemistry, and functional genomics is required.

Overall, although significant progress has been gained in the understanding of biochemical and biological functions and structures of the SABATH family, a number of interesting questions still await to be addressed. First, how ancient is the SABATH family? All the characterized *SABATH* genes were isolated from angiosperm species. Are they also present in gymnosperm and lower plants? If so, do they have conserved functions among different plant lineages? Second, how diverse are biochemical reactions catalyzed by SABATH MTs? As discussed in previous section, the

Arabidopsis genome contains 24 *SABATH* genes. So far, only 6 of them have been biochemically characterized. Determination of function of unknown *SABATH* genes in Arabidopsis and other plants species remains a challenging task. Research in this area will help answer the question whether certain *SABATH* genes have species-specific biochemical functions that contribute to the unique biology and ecology of the species. Third, what are the determinants that govern substrate specificity of individual *SABATH* proteins? Some information has been obtained with structural elucidation of *CbSAMT*, *XMT* and *DXMT* (Ross et al., 1999; McCarthy, 2007). Nonetheless, to more accurately identify the structural determinants responsible for substrate specificity among *SABATH* proteins, the empirically determined three-dimensional structure for other members of the *SABATH* family is necessary. Fourth, what is the evolutionary trajectory that leads to functional diversification observed in the contemporary *SABATH* family? To answer all these questions, *SABATH* genes need to be isolated and characterized from various plant species. Such analysis, especially identification of orthologous *SABATH* genes from diverse plant species, will help understand evolution of *SABATH* genes within and among plant species.

V. *SABATH* gene discovery with comparative functional genomic approach

Functional genomics and comparative genomics are effective approaches for the gene discovery. Functional genomics is to study functions of genes at a large scale, for example, microarray analysis can help to identify the candidate genes. Comparative

functional genomics is to study functions of genes at a large scale based on genomic comparison of a variety of plant species. The genomes of three plant species, including Arabidopsis, rice and poplar, have been fully sequenced (Arabidopsis Genome, 2000; Goff et al., 2002; Yu et al., 2002; Tuskan et al., 2006). Genome sequencing for many other plant species is underway. This provides unprecedented opportunities for cross-species comparison of gene families.

Comparative analysis can be carried out by searching for conservation among genome sequences, as functional regions are believed to be under stabilizing selection and should be preferentially conserved during evolutionary process. Taking the flowering pathways as an example, a large group of orthologous genes that regulate floral induction and flowering time were found in rice and Arabidopsis by the BLAST analysis of Arabidopsis proteins with the rice genome. Comparison of orthologous genes in rice and Arabidopsis suggests the flowering pathways are evolutionarily conserved between short-day plants and long-day plants (Izawa et al., 2003). As such, comparative gene analyses will provide valuable insights about the evolutionary aspects of gene functions in higher plants.

In this thesis, the protein sequence of CbSAMT (accession: AF133053) will be used to blast search the rice and poplar genome databases to identify the *SABATH* family genes from rice and poplar. Then the phylogenetic analysis among Arabidopsis, rice and poplar *SABATH* genes will be performed to identify the orthologous genes.

Such genes will be isolated and biochemically characterized. Once biochemical function is confirmed, the biological functions of selected genes in the three plant species will be analyzed.

In this thesis, rice and poplar are used as the plant models. Rice has several advantages as a model plant. First, rice has a small genome compared with the other crops (about 430 Mbp; Goff et al., 2002). Second, the whole genome of rice has been fully sequenced (Goff et al., 2002; Yu et al., 2002) and a large amount of functional genomics tools have been established, such as the whole genome long-oligo arrays, the proteomics database and the insertional mutant database. Third, production of transgenic rice plants is relatively easy compared to that of other major cereals through efficient use of *Agrobacterium*-mediated transformation. As a model species for the monocotyledonous plants and the cereal crops, the results obtained from rice can also be used as an important reference for the other cereal crops, such as maize and wheat.

Poplar has several advantages as the model of perennial woody species. It has a small genome size (about 480 Mbp). Recent completion of genome sequencing for poplar enables the establishment of various functional genomics tools (Tuskan et al., 2006). Poplar also has the characteristics of rapid growth and prolific sexual reproduction. Furthermore, the ease for the gene cloning and poplar transformation facilitates the study of physiology and ecology of forest trees (Bradshaw et al., 2000).

Taking advantage of fully sequenced genomes of rice and poplar, **I propose to use a comparative functional genomics approach to identify the SABATH families in rice and poplar and then investigate the biochemical and biological functions and evolution of selected members of the SABATH family. The goal of this PhD study is to improve the understanding on function and evolution of the SABATH family.**

This dissertation contains five specific objectives. In objective 1, identification, expression analysis and phylogenetic analysis will be carried out to identify the SABATH members from rice and poplar. In the objective 2, poplar IAMT will be cloned and its biochemical and biological functions will be studied. In the objective 3, the rice IAMT will be cloned. Phylogenetic and structural analysis will be performed to understand the evolution of the SABATH family. A large amount of evidences showed the concentration of MeSA highly increased in the insect damaged plants, suggesting it may be important in plant indirect defense. Therefore, the objective 4 is proposed to study the rice SAMT and its relationship with indirect defense. Jasmonic acid is an important plant defense hormone, objective 5 is aimed to identify and characterize JMT from poplar and study the biological function of this gene.

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Appendix

Table 1.1 SABATH genes in plants

Gene	Host plant	Reference
C-methyltransferase		
Salicylic acid methyltransferase (CbSAMT)	<i>Clarkia breweri</i>	(Ross et al., 1999)
Salicylic acid methyltransferase (AmSAMT)	Snapdragon (<i>Antirrhinum majus</i>)	(Negre et al., 2002)
Salicylic acid methyltransferase (SfSAMT)	<i>Stephanotis floribunda</i> (Asclepiaceae)	(Pott et al., 2002)
Salicylic acid methyltransferase (AbSAMT)	<i>Atropa belladonna</i>	(Fukami et al., 2002)
Benzoic acid methyltransferase (AmBAMT)	Snapdragon (<i>Antirrhinum majus</i>)	(Murfitt et al., 2000; Dudareva et al., 2000)
Benzoic acid:salicylic acid methyltransferase (AtBSMT)	<i>Arabidopsis (thaliana)</i>	(Chen et al., 2003)
Benzoic acid:salicylic acid methyltransferase (AIBSMT)	<i>Arabidopsis (lyrata)</i>	(Chen et al., 2003)
Benzoic acid:salicylic acid methyltransferase (PhBSMT)	<i>Petunia hybrida</i>	(Negre et al., 2003)
Benzoic acid:salicylic acid methyltransferase (NsBSMT)	<i>Nicotiana suaveolens</i>	(Pott et al., 2004)
Jasmonic acid methyltransferase (AtJMT)	<i>Arabidopsis</i>	(Seo et al., 2001)
Indole-3-acetic acid methyltransferase (AtIAMT)	<i>Arabidopsis</i>	(Qin et al., 2005)
Farnesoic acid methyltransferase (AtFAMT)	<i>Arabidopsis</i>	(Yang et al., 2006)
N-methyltransferase		
Caffeine synthase (TCS1)	tea (<i>Camellia sinensis</i>)	(Kato et al., 1999; Kato et al., 2000)
7-Methylxanthine Methyltransferase (MXMT)	Coffee (<i>C. arabica</i>)	(Ogawa et al., 2001)
7-Methylxanthine Methyltransferase (XRS1)	Coffee (<i>C. arabica</i>)	(Mizuno et al., 2003a)
Caffeine synthase (CCS1)	Coffee (<i>C. arabica</i>)	(Mizuno et al., 2003b)
Theobromine synthase (CTS1, CTS2)	Coffee (<i>C. arabica</i>)	(Mizuno et al., 2003b)
Xanthosine methyltransferase (XMT1)	Coffee (<i>C. arabica</i>)	(Uefuji et al., 2003)
Theobromine synthase (MXMT2)	Coffee (<i>C. arabica</i>)	(Uefuji et al., 2003)
Caffeine synthase (DXMT1)	Coffee (<i>C. arabica</i>)	(Uefuji et al., 2003)

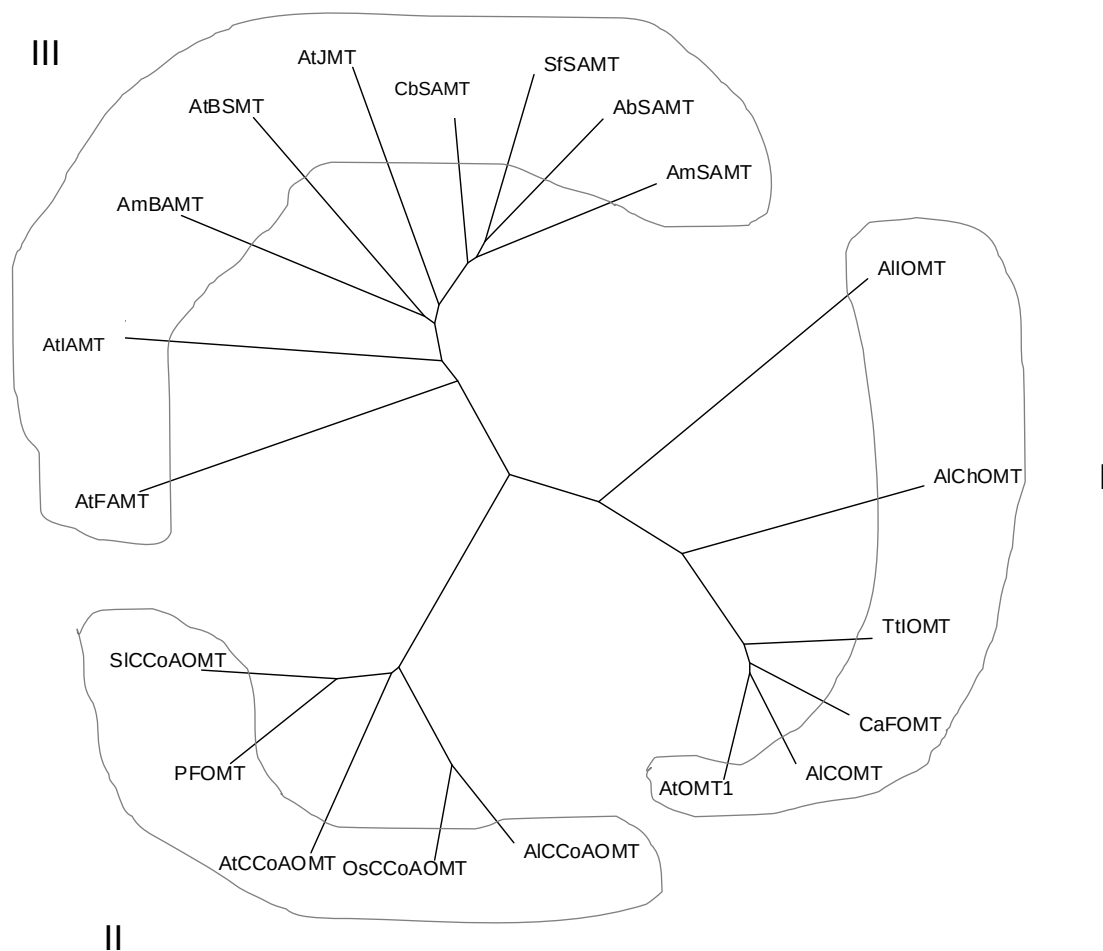


Figure 1.1. A neighbor-joining tree based on the degree of sequence similarity between the Type I, II and III OMTs from plants using the clustalX program. Type I OMTs: AICOMT, alfalfa COMT (AAB46623); AtOMT1, *Arabidopsis thaliana* OMT (U70424); AIChOMT, alfalfa chalcone O-methyltransferase (AAB48059); AIIOMT, alfalfa isoflavone O-methyltransferase (AAC49927); TtIOMT, *Thalictrum tuberosum* isoquinoline OMT (AAD29844); CaFOMT, *Capsicum annuum* flavonoid OMT (AAC17455); Type II OMTs: AICCoAOMT, alfalfa CCoAOMT (AAC28973.1); PFOMT, *Mesembryanthemum crystallinum* PFOMT (AY145521); SICCoAOMT,

Figure 1.1. Continued

Stellaria longipes CCoAOMT (L22203); AtCCoAOMT, *Arabidopsis thaliana* CCoAOMT (AAM64800); OsCCoAOMT, *Oryza sativa* CCoAOMT (BAA78733);
Type III OMTs: CbSAMT, *Clarkia breweri* SAMT (AF133053); AmSAMT, *Antirrhinum majus* (snapdragon) SAMT (AF515284); SfSAMT, *Stephanotis floribunda* SAMT (AJ308570); AmBAMT, *Antirrhinum majus* BAMT (AF198492);
AtBSMT, *Arabidopsis thaliana* BSMT (BT022049); AbSAMT, *Atropa belladonna* SAMT (AB049752); AtJMT, *Arabidopsis thaliana* JMT (AY008434); AtFAMT, *Arabidopsis thaliana* FAMT (AY150400); AtIAMT, *Arabidopsis thaliana* IAMT (AK175586).

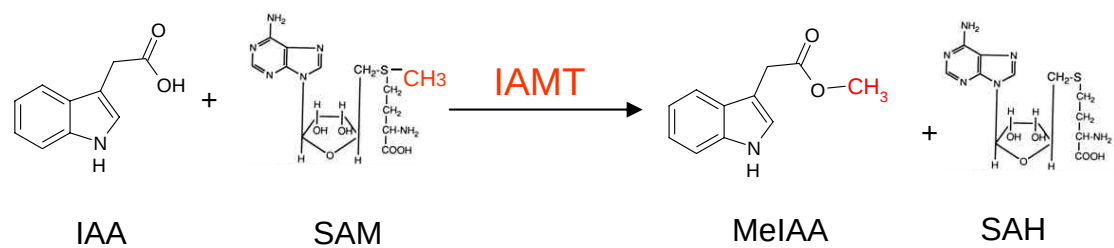


Figure 1.2. MeIAA biosynthesis

MeIAA: methyl indole-3-acetate

IAMT: Indole-3-acetic acid Methyltransferase

IAA: Indole-3-acetic acid

SAM: s-adenosyl-l-methionine

SAH: S-adenosyl-L-homocysteine

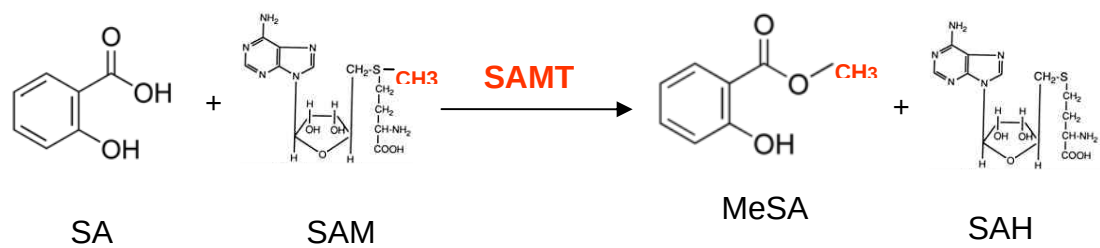


Figure 1.3. MeSA biosynthesis

MeSA: Methyl Salicylate

SMT: salicylic acid methyltransferase

SA: salicylic acid

SAM: s-adenosyl-l-methionine

SAH: S-adenosyl-L-homocysteine



Figure 1.4. MeJA biosynthesis

MeJA: Methyl Jasmonate

JAMT: Jasmonic acid methyltransferase

JA: Jasmonic acid

SAM: s-adenosyl-l-methionine

SAH: S-adenosyl-L-homocysteine

Chapter II. Molecular Cloning and Biochemical Characterization of Indole-3-acetic Acid Methyltransferase from Poplar

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Phytochemistry 68: 1537-1544

Abstract

Indole-3-acetic acid (IAA) is the most active endogenous auxin and is involved in various physiological processes in higher plants. Concentrations of IAA in plant tissues are regulated at multiple levels including *de novo* biosynthesis, conjugation/deconjugation, and degradation. In this chapter, I report molecular isolation and biochemical characterization of a novel gene *PtIAMT1* from poplar encoding IAA methyltransferase (IAMT), which plays a role in regulating IAA homeostasis. *PtIAMT1* was identified from the poplar genome based on sequence similarity to *Arabidopsis* IAMT. A full-length cDNA of *PtIAMT1* was cloned from poplar roots via RT-PCR. Recombinant PtIAMT1 expressed in *E. coli* was purified to electrophoretic homogeneity. Enzyme assays combined with GC-MS verified that PtIAMT1 catalyzes formation of methyl indole-3-acetate using S-adenosyl-L-methionine (SAM) as a methyl donor and IAA as a methyl acceptor. PtIAMT1 had a temperature optimum at 25 °C and a pH optimum at pH 7.5. Its activity was promoted by K⁺ but inhibited by Fe²⁺, Cu²⁺ and Zn²⁺. Under steady-state conditions, PtIAMT1 exhibited apparent *K_m* values of 23.1 μM and 30.4 μM for IAA and SAM, respectively. Gene expression analysis showed that *PtIAMT1* had the highest level of expression in stems, a moderate level of expression in young leaves, and a low level of expression in roots. Presence of *PtIAMT1* transcripts in several organs suggests that *PtIAMT1* is involved in development of multiple organs in poplar.

Keywords: Black cottonwood; Poplar; *Populus trichocarpa*; Salicaceae;
Methyltransferase; SABATH; Indole-3-acetic acid; IAMT

Introduction

Indole-3-acetic acid (IAA) is the most abundant and most active endogenous auxin in higher plants. It modulates diverse aspects of plant growth and development ranging from embryogenesis, tropic responses to light and gravity, lateral and adventitious root formation, to induction of vascular differentiation (Taiz and Zeiger, 2006). Concentrations of IAA in plant tissues are maintained through regulation of its *de novo* biosynthesis, IAA degradation, and both conjugation and deconjugation of IAA with amino acids, peptides, and sugars (Ljung et al., 2002). A novel enzyme, IAA methyltransferase (IAMT), was recently identified from *Arabidopsis*. *Arabidopsis* IAMT (AtIAMT) catalyzes formation of methyl indole-3-acetate (MeIAA) using S-adenosyl-L-methionine (SAM) as a methyl donor and IAA as a methyl acceptor (Zubieta et al., 2003). Exogenous MeIAA is much more potent than exogenous IAA in inhibiting hypocotyl elongation of dark-grown *Arabidopsis* seedlings (Qin et al., 2005), indicating IAA activities can be effectively regulated by methylation.

The gene encoding AtIAMT, *At5g55250* or *IAMT1*, is involved in *Arabidopsis* leaf development (Qin et al., 2005). Expression of *IAMT1* is developmentally regulated in *Arabidopsis* leaves. Before the eighth true leaf emerges, *IAMT1* is expressed ubiquitously in rosette leaves. After emergence of the eighth true leaf, *IAMT1* expression gradually fades away from the leaf center to the leaf edges (Qin et al., 2005). Down-regulating *IAMT1* expression leads to dramatic epinastic leaf phenotypes (Qin et al., 2005), which is consistent with IAA overproduction mutants.

IAMT1 over-expressors display leaf phenotypes opposite to those of *IAMT1* RNAi lines. Roles for *IAMT1* in developmental processes other than leaf development in *Arabidopsis* are not yet clear. Regardless, over-expression of *IAMT1* in *Arabidopsis* affects auxin responses, for example, by disrupting plant response to gravity (Qin et al., 2005).

AtIAMT was originally identified based on structural modeling using the three-dimensional structure of *Clarkia breweri* salicylic acid methyltransferase (CbSAMT) as a template (Ross et al., 1999; Zubieta et al., 2003). Both AtIAMT and CbSAMT belong to a protein family called SABATH (D'Auria et al., 2003). Other known members of the family include benzoic acid/salicylic acid methyltransferase (BSMT) (Chen et al., 2003a; Pott et al., 2004), benzoic acid methyltransferase (BAMT) (Murfitt et al., 2000), jasmonic acid methyltransferase (JMT) (Seo et al., 2001), farnesoic acid methyltransferase (Yang et al., 2006) and nitrogen methyltransferases involved in caffeine biosynthesis (Kato et al., 2000; Yoneyama et al., 2006). Continued characterization of SABATH proteins with novel biochemical activities raises an intriguing question about the evolutionary trajectory of these enzymes. Because IAA is universal plant hormone and methylation of IAA catalyzed by IAMT has important consequences on plant growth and development, I am interested in understanding whether IAMTs in different plant species are evolutionarily conserved. In this chapter, I report the molecular isolation and biochemical characterization of an *IAMT* gene from poplar (*Populus trichocarpa*, Torr.

& Gray), a perennial woody species. A high degree of sequence similarity between *PtIAMT1* and *AtIAMT*, as well as comparable kinetic properties between the two, imply that IAMT is a conserved enzyme. Gene expression analysis suggests *PtIAMT1* is involved in development of multiple organs in poplar.

Material and Methods

Plant material and chemicals

The female black cottonwood (*Populus trichocarpa*) clone ‘Nisqually-1’, previously employed for whole genome sequencing (Tuskan et al., 2006), was used for gene cloning and expression analysis of *PtIAMT1*. Tissues used for gene expression analysis, including young leaves, old leaves, stems and roots, were collected from one-year-old poplar trees grown in a greenhouse.

Farnesoic acid was purchased from Echelon (Salk Lake City, UT). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Database search and sequence analysis

To identify putative poplar *IAMT* genes, the protein sequence of *AtIAMT* was used to search the genome sequence database of poplar (<http://genome.jgi-psf.org/Poptr1/Poptr1.home.html>) using BlastP algorithm (Altschul et al., 1990). A poplar gene encoding a protein with the highest level of sequence similarity (86%) to *AtIAMT* was chosen for further analysis.

Multiple protein sequence alignments were made using ClustalX software (Thompson et al. 1994), and displayed using GeneDoc (<http://www.psc.edu/biomed/genedoc/>). Phylogenetic trees were constructed using ClustalX program and viewed using TreeView software (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

Cloning full-length cDNA of PtIAMT1

Root tissues of plants grown on tissue medium were collected from poplar seedlings at the six-leaf stage. Total RNA was extracted using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA) with DNA contamination removed using an on-column DNase treatment (Qiagen, Valencia, CA). After purification, total RNA (1.5 µg) 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 (Chen et al., 2003b). *PtIAMT1* full-length cDNA was amplified using forward primer 5'-TGGCTCCTAAAGGTGACAATGTTGTTG-3' and reverse primer 5'-CCCTTGTTCTCAAGCAAAAGAAAGAGA-3' corresponding with the beginning and end of *PtIAMT1* coding region, respectively. The PCR was conducted using the following program: 94 °C for 2 min followed by 30 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. Products of PCR were separated on 1.0% agarose gel. The target band was sliced from the gel, purified using QIAquick Gel Extraction kit (Qiagen, Valencia, CA), and

cloned into a pCRT7/CT-TOPO vector using the protocol recommended by the vendor (Invitrogen, Carlsband, CA). Cloned cDNA in pCRT7/CT-TOPO vector was sequenced using T7 and V5 primers.

Purification of PtIAMT1 expressed in E. coli

Two primers, the forward primer 5'-CACCATGGCTCCTAAAGGTGACAATGTTG-3' and the reverse primer 5'-CCCTTGTTCTCAAGCAAAAGAAAGAGA-3', were used to subclone *PtIAMT1* cDNA in pCRT7/CT-TOPO into the vector of pET100/D-TOPO (Invitrogen, Carlsband, CA). To express the PtIAMT1 protein, the protein expression construct was transformed into the *E. coli* strain BL21 (DE3) CodonPlus (Stratagene, La Jolla, CA). Protein expression was induced by isopropyl β -D-1-thiogalactopyranoside (IPTG) at a concentration of 500 μ M for 18 h at 25 °C, with cells lysed by sonication. His-tagged PtIAMT1 protein was purified from the *E. coli* cell lysate using Ni-NTA agarose following the manufacturer instructions (Invitrogen, Carlsband, CA). Protein purity was verified by SDS-PAGE and protein concentrations were determined by the Bradford assay (Bradford, 1976).

Radiochemical IAMT activity assay (standard IAMT assay)

Radiochemical IAMT assays were performed with a 50 μ L volume containing 50 mM Tris-HCl, pH 7.5, 1 mM IAA, and 3 μ M ^{14} C-SAM with a specific activity of 51.4 mCi/mmol (Perkin Elmer, Boston, MA). The assay was initiated by addition of

SAM, maintained at 25 °C for 30 min, and stopped by addition of EtOAc (150 µL). After phase separation by one min centrifugation at 14,000g, the upper organic phase was counted using a liquid scintillation counter (Beckman Coulter, Fullerton, CA) as previously described (D'Auria et al., 2002). Radioactivity counts in the organic phase indicated the amount of synthesized MeIAA.

Assays for PtIAMT1 with indole-3-butyric acid, salicylic acid, benzoic acid, jasmonic acid and farnesoic acid were conducted as described for IAMT assay. Three independent assays were performed for each compound.

Determination of kinetic parameters of PtIAMT1

Increases in reaction rate by increasing concentrations of SAM and IAA were evaluated through the radiochemical assay described above and were found to obey Michaelis–Menten kinetics. Appropriate enzyme concentrations and incubation times were determined in time-course assays, such that the reaction velocity was linear during the assay period. To determine a K_m value for SAM, concentrations of SAM were independently varied from 3 to 120 µM, while IAA was held constant at 1 mM. To determine the K_m for IAA, concentrations of IAA were independently varied from 2 to 150 µM, while SAM was held constant at 200 µM. Assays were conducted at 25 °C for 30 min, as described in section 3.5. Lineweaver-Burk plots yielded apparent K_m values as previously described (Chen et al., 2003a). Final values represent the average of three independent measurements.

Optimal temperature for PtIAMT1 activity

A standard IAMT assay was carried out at one of the following temperatures: 4, 10, 20, 25, 30, 40 and 50 °C. Data presented are the average of three independent assays.

pH optimum for PtIAMT1 activity

PtIAMT1 activity was determined in 50 mM Bis-Tris propane buffer for the pH range across 6.5 to 10.0 using the standard IAMT assay. Data presented are the average of three independent assays.

Effectors

To examine effects of metal ions on PtIAMT1 activity, standard IAMT assays were performed in the independent presence of each of the following salts at 5 mM final concentration: KCl, CaCl₂, NH₄Cl, NaCl, MgCl₂, MnCl₂, CuCl₂, FeCl₂ and ZnCl₂. Results presented are the average of three independent assays.

Product identification

A reaction containing 150 µg purified PtIAMT1, 1 mM IAA 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 hexane (1.5 mL), concentrated under N₂ gas and analyzed by Shimadzu GC (GC-17A)-MS (QP 5050A)

system (Columbia, MD). A DB-5 column (30 m × 0.25id x 0.25µm) was used with He as carrier gas at a flow rate of 1 mL min⁻¹. As a control, a similar reaction was performed, except that PtIAMT1 protein was denatured by boiling at 100 °C for 10 min before added to the assay. MeIAA authentic standard was dissolved in EtOH at the concentration of 0.5 µg mL⁻¹ and 1µg MeIAA was injected into the GC in a 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.

Semi-quantitative RT-PCR analysis of PtIAMT1 expression

Total RNA extraction from young leaves, old leaves, stems and roots of one year-old poplar trees and subsequent first-strand cDNA synthesis were performed as described in section 3.3. For PCR analysis in different organs, primers were designed to amplify a *PtIAMT1* fragment of 566 bp as follows: forward primer 5'-AGAAACCCTAGATAGGGTGCAC-3' and reverse primer 5'-CCAAGCAGACAAGAAACATGGA-3'. Two primers used for PCR amplification of *Ubiquitin* were designed as previously described (Kohler et al., 2004): forward primer 5'-CAGGGAAACAGTGAG GAAGG-3' and reverse primer 5'-TGGACTCACGAGGACAG-3'. Initially, PCR analysis was performed with *Ubiquitin*-specific primers using 0.1 µL, 0.2 µL, 0.5 µL and 1.0 µL cDNA. The program used to amplify *Ubiquitin* was as follows: 94°C for 2 min followed by 30

cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 10 min. Amplified products were separated on 1.0% agarose gel. Gels were stained with ethidium bromide, visualized under UV-light, and quantified using the Bio-Rad Quantity One software (Bio-Rad, Hercules, CA). Analysis showed amounts of amplified products with the *Ubiquitin*-specific primers increased linearly with increasing amounts of template cDNA. Therefore, 0.2 µL cDNA was chosen as the optimal template concentration for PCR analysis with *PtIAMT1*-specific primers. The program used to amplify the *PtIAMT1* fragment was as follows: 94 °C for 2 min followed by 35 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. All PCRs were replicated twice using first strand cDNA made from two independent RNA preparations.

Results and Discussion

Identification and sequence analysis of poplar IAMT

To identify the putative poplar *IAMTs*, the protein sequence of AtIAMT, which is the only reported IAMT in plants (Zubieta et al., 2003), was used to blast search the sequenced poplar genome (Tuskan et al., 2006). Gene fgenes1_pg.C_LG_I002776 was identified to be the most similar to AtIAMT. The protein encoded by fgenes1_pg.C_LG_I002776 displayed IAMT activity. Because biochemical activity for all poplar SABATH proteins were not analyzed, it was not possible to rule out the possibility that other poplar SABATH proteins might have IAMT activity. Thus, fgenes1_pg.C_LG_I002776 was named *PtIAMT1*.

PtIAMT1 is localized on poplar chromosome I and encodes a protein of 385 amino acids. *PtIAMT1* and *AtIAMT* are 76% identical at the amino acid sequence level. Both genes contain three introns and four exons, with positions of introns conserved in the two genes. The SAM binding site in *CbSAMT* has been determined (Zubieta et al., 2003). Sequence comparison revealed that amino acids comprising the SAM binding site in *PtIAMT1* are identical to those of *CbSAMT* (Figure 2.2A). In *CbSAMT*, Trp226 is one of the amino acids that form the salicylic acid binding site. In *AtIAMT*, Trp226 is replaced by a Gly residue. The loss of Trp226 at the active site location in *AtIAMT* creates a large and spacious pocket for recognizing and binding to the indole-ring of IAA (Zubieta et al., 2003). In *PtIAMT1*, Trp226 is also replaced with a Gly (Figure 2.2A), providing further evidence to support an important role of Trp226 for binding with IAA.

PtIAMT1 is the second *IAMT* gene to be identified in plants. To understand the evolutionary relationship between *PtIAMT1* and other known SABATH proteins including *AtIAMT*, a phylogenetic tree was constructed consisting *PtIAMT1*, *AtIAMT*, *CbSAMT*, *Arabidopsis* jasmonic acid methyltransferase (*AtJMT*), *Arabidopsis* benzoic acid/salicylic acid methyltransferase (*AtBSMT1*), *Arabidopsis* farnesoic acid methyltransferase (*AtFAMT*), snapdragon benzoic acid methyltransferase (*AmBAMT*), and coffee caffeine synthase (*CCS1*). *PtIAMT1* is most related to *AtIAMT* (Figure 2.2B), implying the presence of *IAMT* predated the divergence of

poplar and *Arabidopsis* lineages.

Purification of recombinant PtIAMT1 and determination of its substrate specificity

A *PtIAMT1* full-length cDNA was amplified from poplar root tissues by RT-PCR, cloned into the vector of pCRT7/CT-TOPO, and fully sequenced. *PtIAMT1* was expressed in *E. coli* in its native form. The crude protein extract of *PtIAMT1* showed IAMT activity (data not shown).

To purify *PtIAMT1* expressed in *E. coli*, *PtIAMT1* full-length cDNA in pCRT7/CT-TOPO was subcloned into the vector of pET100/D-TOPO. In the latter vector, *PtIAMT1* coding sequence is fused to an N-terminal sequence containing codons for six histidine residues. His-tagged *PtIAMT1* was expressed in *E. coli* then purified using Ni-NTA agarose to electrophoretic homogeneity (Figure 2.3A).

Purified *PtIAMT1* recombinant protein was assayed with IAA, indole-3-butyric acid (IBA), salicylic acid (SA), benzoic acid (BA), jasmonic acid (JA) and farnesoic acid (FA). *PtIAMT1* had the highest level of catalytic activity with IAA, exhibiting a specific activity of 60 pkat/mg protein. It also exhibited activity with indole-3-butyric acid, another naturally occurring auxin. Specific activity of *PtIAMT1* with indole-3-butyric acid was about 11% (6.6 pkat/mg protein) of IAMT activity. *PtIAMT1* had no activity with other compounds tested that are known substrates of other SABATH proteins.

To determine the chemical structure of the PtIAMT1 product, the compound produced from the PtIAMT1 enzyme assay using IAA as a substrate was extracted with hexane and analyzed using GC-MS. As shown in Figure 2.3B, the product had an identical retention time and mass spectrum to that of the authentic MeIAA standard, confirming that PtIAMT1 catalyzes formation of MeIAA using SAM as a methyl donor and IAA as a methyl acceptor.

Biochemical properties of PtIAMT1

Under steady-state conditions, PtIAMT1 exhibited apparent K_m values of 23.1 μM and 30.4 μM for IAA (Figure 2.3C) and SAM (Figure 2.3D), respectively. Calculated catalytic efficiency (K_{cat}/K_m) of PtIAMT1 is $4782 \text{ s}^{-1} \text{ M}^{-1}$. Kinetic properties of PtIAMT1 are comparable to those of AtIAMT, which displayed a K_m value of 13 μM for IAA and a catalytic efficiency of $2200 \text{ s}^{-1} \text{ M}^{-1}$ (Zubieta et al., 2003). Taken together, the phylogenetic, structural and biochemical evidence suggests that IAMT is a conserved enzyme.

PtIAMT1 had an optimal temperature at 25 °C (Figure 2.4A). Activity at 4 °C and 40 °C was about 40% of the maximal activity. The optimum pH for PtIAMT1 was determined to be pH 7.5. At pH 6.5, the enzyme had ~34% of its maximal activity, whereas at pH 9.0, the enzyme displayed ~40% of the maximal activity (Figure 2.4B). PtIAMT1 activity can also be affected by metal ions. K^+ stimulated PtIAMT1 activity

by more than one fold, suggesting that the presence of K^+ in reaction solution may induce protein conformational changes and/or activation of PtIAMT1 active site leading a stimulatory effect on PtIAMT1 activity. Ca^{2+} , NH_4^+ and Na^+ had a mild stimulation on PtIAMT1 activity. By contrast, Mg^{2+} had a mild inhibitory effect and Mn^{2+} decreased the specific activity of PtIAMT1 by ~46%. Strong inhibitory effects on PtIAMT1 activity were observed with Cu^{2+} , Fe^{2+} , and Zn^{2+} (Figure 2.4C). The partial or complete inhibition of PtIAMT1 activity by Mn^{2+} , Cu^{2+} , Fe^{2+} , and Zn^{2+} suggests that these metal ions may induce protein conformational changes and/or inhibition of PtIAMT1 active site limiting PtIAMT1 catalytic ability.

Gene expression analysis of PtIAMT1

To identify plant tissues where *PtIAMT1* was expressed, total RNA was isolated from newly emerged leaves (young leaves), old leaves (5 cm in length), stems and roots of one-year-old poplar trees and used for semi-quantitative RT-PCR analysis. The highest level of *PtIAMT1* transcripts was observed in stems. Young leaves also showed an abundant expression of *PtIAMT1*, whereas no expression of *PtIAMT1* was detected in old leaves. A low level of expression of *PtIAMT1* was also detected in roots (Figure 2.5).

Biological roles of PtIAMT1

Presence of *PtIAMT1* transcripts in leaves, roots and stems (Figure 2.5) suggests involvement of this gene in development of these organs in poplar. In leaves,

PtIAMT1 showed an expression pattern similar to *AtIAMT1*. Both genes displayed high expression levels in young leaves than old leaves (Qin et al., Figure 2.5), implying that *PtIAMT1* has a role similar to *AtIAMT1* in leaf development. *PtIAMT1* may also be involved in root development. IAA is an important signal regulating root architecture. Although the mode of action is not well understood, the concentrations of IAA appear important (Wang et al., 2003). *PtIAMT1* may have a role in poplar root development by regulating IAA concentrations. As a perennial woody species, poplar differs from annuals such as *Arabidopsis* by displaying a number of distinctive developmental and anatomical characteristics. Of these, wood formation is arguably the most important. Wood formation in poplar is regulated by IAA (Moyle et al., 2002). A high level expression of *PtIAMT1* in poplar stems indicates that this gene may play a role in wood formation by regulating IAA activities.

Concluding remarks

Pathways that regulate IAA activity in plants are complicated and imperfectly understood (Leyser 2002). Identification of IAMT that appears to be evolutionarily conserved in different plant species adds a new layer of complexity to this network. Although physiological functions of MeIAA generally have been inconclusive, biochemical and gene expression evidence suggests an important role of *PtIAMT1* in poplar development likely through IAA methylation. Transgenic poplar trees including both *PtIAMT1* over-expressors and RNAi lines are being generated in my lab, which will help reveal biological roles of this gene.

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Appendix

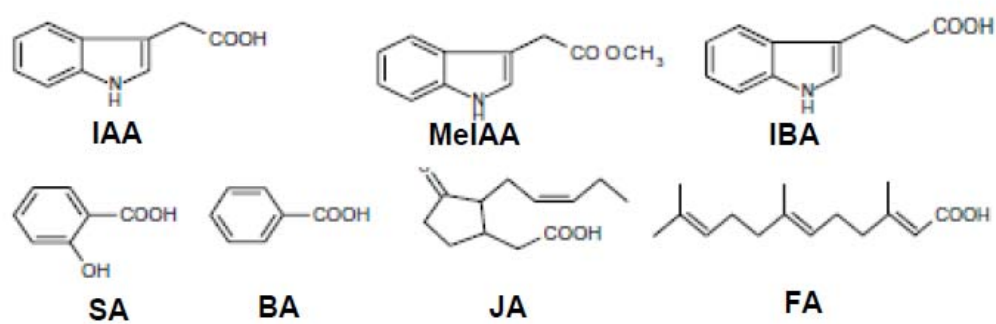


Figure 2.1. Structures of compounds.

a

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PtIAMT1 : -----MAPKGDNVVSSMKTEKIQMGEGENSYANNISOALHARSMLHETEDRHHNSP : 60
AtIAMT : -----MGSKGDNVAVCMKTERLLSMRGEGCOTSYANNISOALHARSMLHETEDRHHNS : 60
AtJMT : -----MEIMRVLRHNNNGESTSYANNSTACSNIIISLGRVLDHAKKVMLSN : 47
CbSMT : -----MDVROVLRHNNNGEGENSYANNSTACSNIIISLGRVLDHAKKVMLSN : 47
AtBSMT1 : MDPRTINTTIPSLRYDDDKCDEYAFVKALQMSGGEGANSYSANRLCKVLMAKPVIVRNTTEEMNL : 69
AtFAMT : -----NSTSFTNIGGEGPNVSRERHVKYQALVIAAKEKINHAIS-TRDID : 45

PtIAMT1 : EFP--FCVADLCSSGNNTIHIIDVILKHKIKRPFESSGLE-PPEFSAFFADLESNDFNLLFQILP : 127
AtIAMT : ASPPPPTAVDLGCSSGANTVHIIDFIVVGLISKRFDAAGID-PPEFTAFFSDFLESNDFNLLFQILP : 129
AtJMT : SEISSIGTADLCSSGANSLLSISNIVDTIHNLCPLDLRP-VPBLRVSLNDLESNDFNLLFQILP : 116
CbSMT : TVITRLAIADLCSSGEMALFVTLIKTVLEELRKKMGRENSPVQIFLNDLESNDFNLLFQILP : 112
AtBSMT1 : DFPTTYIKVADLCSSGONSFLAIFETIINTINVLCQHVKNK-SPEIDCCCLDLESNDFNLLFQILP : 138
AtFAMT : FTSNIVNIADLCSSGENTFTAVQILDAVENKYKESNIEGIEPQVFFDSSNDFNLLFQILP : 113

PtIAMT1 : GGSMEECIAAS--GHRNVFAAGVPGSPHRLFEARSHDVPSAFSLAWLSQVEECVLDK----- : 190
AtIAMT : -TCMEECIAAD--GNRSYFAAGVPGSPHRLFEARTDFFPSAFSLAWLSQVEESVTDK----- : 191
AtJMT : VNNKKEGLGFRGGGSGSPVSAVPGSPGRLEPFRSLHFVHSSSLAWLSQVEECVLDK----- : 186
CbSMT : IENDVDGV-----GPIGVPGPSFGRLEPFRSLHFVHSSSLAWLSQVEECVLDK----- : 162
AtBSMT1 : LMITNKSS-----GPIGVPGPSFGRLEPFRSLHFVHSSSLAWLSQVEECVLDK----- : 188
AtFAMT : -----LVFASGVGPSFGRVLEKNSLHVGVSYSLEFVSKVEKPKDRD-----SLV : 160

PtIAMT1 : KGVFPIHNAS-EHTTNAYKQPCDLAGFLSARSGEKSGSSPFIACVCGRTSADPTDQGGAGLLPETH : 259
AtIAMT : KGVFPIHAG-EKTTTAYKQPCDLAGFLSARSGEKSGSSPFIACVCGRTSADPTDQGGAGLLPETH : 260
AtJMT : MGVFIYSKTSPKSAHKAVALQPCDFWVFLSSSEELVPGGFWLSPLGRSL--DPTTEESCQWELLA : 254
CbSMT : KGVFIYANTCPQSVLNAYKQPCDLAGFLSARSGEKSGSSPFIACVCGRTSADPTDQGGAGLLPETH : 230
AtBSMT1 : KGVFIYNTSSPQSAKAYLNQPCDFWVFLSSSEELVPGGFWLSPLGRSL--DPTTEESCQWELLA : 257
AtFAMT : WNKDIHCSSSSKEVVKILGQVKKHVGSLFARAGELVSGGILHLLGSGRTG-VQMFETVCGMIDF : 229

PtIAMT1 : DAWDDIVCGELTSEKRFNPNIPVYASLQDFREYVANGSPFIKKEVFKGGSPLVNVHPDNE----- : 323
AtIAMT : DAWDDIVRGLVAERKPCPNIPVYASLQDFREYVANGSPFIKKEVFKGGSPLVNVHPDNE----- : 324
AtJMT : QALMSVAREGIDEEKKIDAFVYASSEELVPMIEKGGSPFIKKEVFKGGSPLVNVHPDNE----- : 324
CbSMT : MALNIVVSEGLDEEKIDAFVYASSEELVPMIEKGGSPFIKKEVFKGGSPLVNVHPDNE----- : 298
AtBSMT1 : NSLRDVPFGLVSESKIDAFVYASSEELVPMIEKGGSPFIKKEVFKGGSPLVNVHPDNE----- : 319
AtFAMT : SSINRIVANGLIDQKIDAFVYASSEELVPMIEKGGSPFIKKEVFKGGSPLVNVHPDNE----- : 292

PtIAMT1 : ---AEVSHAMANSQSSAGVIVDAHICDQSEEPFLVEHRAKSHAKELLEKLOPHIVASISFA-- : 385
AtIAMT : ---SEVGRAPASSQSSAGVIVDAHICDQSEEPFLVEHRAKSHAKELLEKLOPHIVASISFA-- : 386
AtJMT : KPEALASGRHVSNTIRAVVERPLEPTGGENVDELPERYAKIVGEY--VSSPRYAVILSLVITG- : 389
CbSMT : ---EEGVNARCMAAERHLLDHEGEAIEDVPHRYKLLIIRMS--KEKTKINIVISIRKSD : 359
AtBSMT1 : ---FEAGNEANGIRASEHLLAHGGEIIDTLPQYAYHVTQHAN--CRNKTTVSIVVSTTK-- : 379
AtFAMT : -----LTSAPKVTVGGSVASLGGDCMEKTYELVKEKQEMLPQIAKAKPGMQLIVIRN-- : 348

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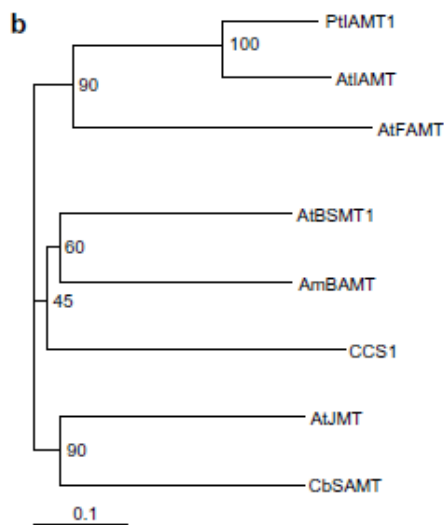


Figure 2.2. (A) Alignment of deduced amino acid sequences of PtIAMT1 with AtIAMT and other representative carboxyl SABATH enzymes including CbSMT (AF133053), *Arabidopsis* jasmonic acid methyltransferase (AtJMT, At1g19640), *Arabidopsis* benzoic acid/salicylic acid methyltransferase (AtBSMT1, At3g11480)

Figure 2.2. Continued

and *Arabidopsis* farnesoic acid methyltransferase (AtFAMT, At3g44860), using the ClustalX program. Conserved or semi-conserved amino acids in four or more sequences are shown in white letters on black or grey backgrounds, respectively. Amino acids indicated with asterisks are SAM binding residues. Amino acids (positions Gly 255 of PtIAMT1 and Trp 226 of CbSAMT) critical for determining substrate specificity of SABATH enzymes are marked with a frame. (B) A neighbor joining phylogenetic tree based on protein sequence alignment of PtIAMT1, AtIAMT, AtJMT, AtBSMT1, AtFAMT, AmBAMT (Q9FYZ9) and CCS1 (BAC43760) using the ClustalX program. Branches were drawn to scale with the bar indicating 0.1 substitutions per site.

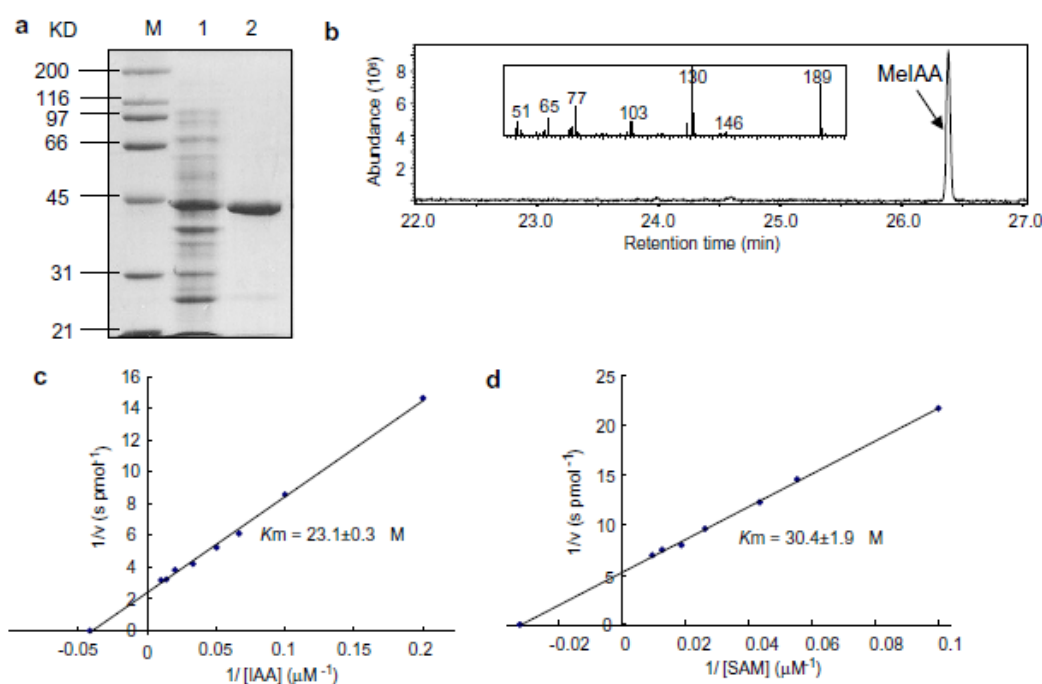


Figure 2.3. Biochemical analysis of PtIAMT1. (A) SDS-PAGE of purified recombinant PtIAMT1 protein. His-tagged PtIAMT1 expressed in *E. coli* was purified as described in Experimental. Lane M contained protein molecular weight markers. Lane 1 contained crude extract, and lane 2 contained 1 μg of purified PtIAMT1 protein. The gel was stained with Coomassie Blue. (B) Identification by GC-MS of the product of the enzyme assay catalyzed by recombinant PtIAMT1 protein using IAA as substrate. The product had a retention time of 26.4 min. Inset shows mass spectrum of the assay product. Both retention time and mass spectrum match those of authentic MeIAA. (C) Steady-state kinetic measurements of PtIAMT1 using IAA as the substrate. One example of a Lineweaver-Burk plot is shown. (D) Steady-state kinetic measurements of PtIAMT1 using SAM as the substrate. One example of the Lineweaver-Burk plot is shown. In both (C) and (D), K_m values shown were not absolute. However, they were apparent under the limited reaction conditions.

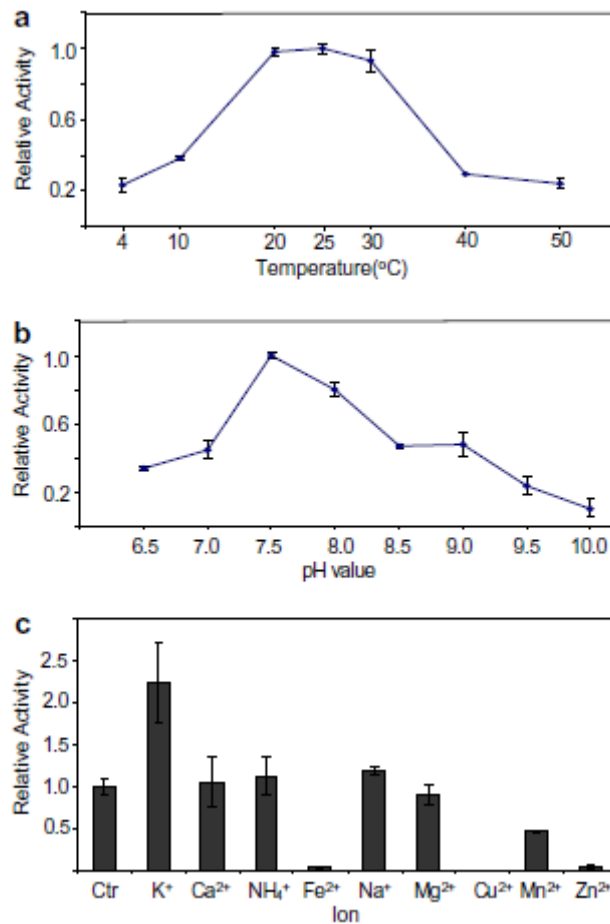


Figure 2.4. Biochemical properties of PtIAMT1. (A) Optimal temperature of PtIAMT1. Level of PtIAMT1 activity at 25 °C was arbitrarily set at 1.0. (B) Effects of buffer pH on activity of PtIAMT1. Level of PtIAMT1 activity in the buffer of pH 7.5 was arbitrarily set at 1.0. (C) Effects of metal ions on activity of PtIAMT1. Metal ions were added to reactions in the form of chloride salts at 5 mM final concentrations. Level of PtIAMT1 activity without any metal ion added as control (Ctr) was arbitrarily set at 1.0. In all three panels, actual specific activity for 1.0 is 60 pkat/mg protein.

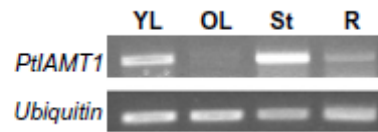


Figure 2.5. Semi-quantitative RT-PCR analysis of *PtIAMT1* expression. Young leaves (YL), old leaves (OL), stems (St) and roots (R) were collected from one year-old poplar trees grown in a greenhouse. Total RNA was extracted and used for RT-PCR analysis. PCR with primers for *Ubiquitin* was used to judge equality of concentration of cDNA templates in different samples.

Chapter III. Structural, Biochemical and Phylogenetic Analyses Suggest that Indole-3-acetic Acid Methyltransferase Is An Evolutionarily Ancient Member of the SABATH Family

Adapted from:

Nan Zhao, Jean-Luc Ferrer, Jeannine Ross, Ju Guan, Yue Yang, Eran Pichersky, Joseph P. Noel, and Feng Chen (2008) Structural, Biochemical and Phylogenetic Analyses Suggest that Indole-3-acetic Acid Methyltransferase Is An Evolutionarily Ancient Member of the SABATH Family. *Plant Physiol* 146: 455 - 467.

Abstract

The plant SABATH protein family encompasses a group of related small molecule methyltransferases (MTs) that catalyze the S-adenosyl-L-methionine-dependent methylation of natural chemicals of widely divergent structures. Indole-3-acetic acid (IAA) methyltransferase (IAMT) is a member of the SABATH family that modulates IAA homeostasis in plant tissues through the methylation of IAA's free carboxyl group. The crystal structure of *Arabidopsis thaliana* IAMT (AtIAMT1) was determined and refined to 2.75 Å resolution. The overall tertiary and quaternary structures closely resemble the two-domain bi-lobed monomer and the dimeric arrangement, respectively, previously observed for the related salicylic acid (SA) carboxyl methyltransferase from *Clarkia breweri* (CbSAMT). To further the understanding of the biological function and evolution of SABATHs, especially of IAMT, I analyzed the SABATH gene family in the rice (*Oryza sativa*) genome. Forty one OsSABATH genes were identified. Expression analysis showed that more than half of the OsSABATH genes were expressed in one or multiple organs. The OsSABATH gene most similar to AtIAMT1 is OsSABATH4. *E. coli*-expressed OsSABATH4 protein displayed the highest level of catalytic activity towards IAA, and was therefore named OsIAMT1. OsIAMT1 exhibited kinetic properties similar to AtIAMT1 and poplar IAMT (PtIAMT1). Structural modeling of OsIAMT1 and PtIAMT1 using the experimentally determined structure of AtIAMT1 reported here as a template revealed conserved structural features of IAMTs within the active site cavity that are divergent from functionally

distinct members of the SABATH family such as CbSAMT. Phylogenetic analysis revealed that IAMTs from Arabidopsis, rice and poplar form a monophyletic group. Thus, structural, biochemical and phylogenetic evidence supports the hypothesis that IAMT is an evolutionarily ancient member of the SABATH family likely to play a critical role in IAA homeostasis in a wide range of plants.

Keywords: Methyltransferase; SABATH family; Indole-3-acetic acid; IAMT; Rice

Introduction

A group of structurally and phylogenetically related methyltransferases (MTs) called the SABATH family was recently identified in plants (D'Auria et al., 2003). Biochemically characterized members of the SABATH family methylate the nitrogen containing or carboxyl groups of a variety of plant small molecules. Salicylic acid (SA) MT (SAMT), the first characterized member of the family, catalyzes the formation of methyl salicylate (MeSA) from SA and S-adenosyl-L-methionine (SAM) (Ross et al., 1999; Negre et al., 2002). Benzoic acid (BA) MT (BAMT) uses BA to produce methyl benzoate (MeBA) (Murfitt et al., 2000). Some SABATH proteins possess dual functionality exhibiting both SAMT and BAMT activities and are therefore called BSMTs (Chen et al., 2003; Pott et al., 2004). Both MeSA and MeBA are components of the floral scents of some plants, and are also produced by vegetative parts of plants in response to environmental challenge (Chen et al., 2003).

Jasmonic acid MT (JMT) accepts jasmonic acid as a substrate to produce methyl jasmonate (MeJA). In *Arabidopsis*, the *JMT* gene is involved in plant defense (Seo et al., 2001). Farnesoic acid MT (FAMT) converts farnesoic acid to methyl farnesoate. *Arabidopsis* FAMT (AtFAMT) was identified using high-throughput biochemical assays (Yang et al., 2006). Gene expression analysis suggests that the *AtFAMT* gene, like other members of the SABATH gene family, has an as yet unidentified role in plant defense (Yang et al., 2006). Indole-3-acetic acid (IAA) MT (IAMT) catalyzes the methylation of the free carboxyl end of the plant hormone IAA.

IAMT has been characterized in *Arabidopsis* (Zubeita et al., 2003) and poplar (Zhao et al., 2007). Genetic analysis suggests the *Arabidopsis IAMT (AtIAMT1)* gene plays a role in leaf development (Qin et al., 2005). More recently, gibberellic acid methyltransferases (GAMTs) were shown to specifically methylate several types of gibberellins (Varbanova et al., 2007).

In addition to carboxyl MTs, the SABATH family includes a number of nitrogen-directed MTs involved in caffeine biosynthesis (Ogawa et al., 2001). Caffeine (1,3,7-trimethylxanthine) is an alkaloid secondary metabolite produced by certain plant species, with coffee (*Coffea arabica*) and tea (*Camellia sinica*) the best known. Caffeine is synthesized from xanthosine after three nitrogen-directed methylation steps, catalyzed by xanthosine MT (XMT), 7-methylxanthine MT (MXMT or theobromine synthase) and 3,7-methylxanthine MT (DXMT or caffeine synthase), respectively (Ashihara et al., 1996). A number of genes encoding these enzymes have been isolated from coffee (Ogawa et al., 2001; Mizuno et al., 2003) and tea plants (Kato et al., 2000; Yoneyama et al., 2006). Sequence analysis showed that all these genes belong to the SABATH family (Yoneyama et al., 2006).

The continued identification and biochemical characterization of SABATH proteins will greatly benefit from the identification of the structural features responsible for substrate recognition that must have undergone evolutionary variation to refine their selectivity towards specific small molecules critical to plant

development and survival in a variety of ecological niches. To date, the three-dimensional crystal structure of three SABATH proteins have been determined, including *C. breweri* SAMT (Zubieta et al., 2003) and coffee XMT and DXMT (McCarthy and McCarthy, 2007). In addition, the three-dimensional structure of the active site of CbSAMT served as a useful template for constructing homology models of the active sites of other SAMTs (Pott et al., 2004) as well as the active sites of functionally distinct members of the SABATH family including IAMT (Zubieta et al., 2003; Qin et al., 2005) and FAMT (Yang et al., 2006). These analyses provided useful insights into the structural basis for natural variations of substrate specificity among SABATH proteins. Nonetheless, to more accurately identify the structural determinants responsible for substrate specificity among SABATH proteins, an experimentally determined three-dimensional structure for each member of the SABATH family is necessary, particularly when subtle structural rearrangements or insertions and deletions occur within the core primary sequence of the growing family of SABATH enzymes. This task is particularly daunting as a large number of SABATH sequences have been found in many plant genomes through genomic and EST sequencing projects (D'Auria et al. 2003; F. Chen, unpublished). Comparative genomic analysis of SABATH genes in these plant species, in particular the identification of orthologous genes and the determination of the substrate specificity of the enzymes they encode, will also aid in providing useful insight into the evolution of SABATH proteins within and among plant species and the physiological relevance of small molecule methylation reactions in plant growth and development.

Here, the determination of the three-dimensional structure of the Arabidopsis IAMT, the biochemical analysis of IAMT from rice, as well as the phylogenetic analysis of the entire SABATH family from rice and Arabidopsis were reported. The three-dimensional structure of Arabidopsis IAMT was further used to model the active site of rice IAMT as well as of poplar IAMT, whose biochemical activity has recently been demonstrated (Zhao et al., 2007).

Material and Methods

Plant Materials and Chemicals

Rice (*Oryza sativa* ssp. Japonica cv. Nipponbare) seeds were obtained from Dale Bumpers National Rice Research Center at Arkansas. Dehulled seeds were germinated on filter paper. After four days, the seedlings were transferred to soil and placed in a growth chamber. The plants were grown under 14 h light/10 h dark photoperiods. The temperature was fluctuated between 26°C (day) and 22°C (night). The relative humidity was 80%, and light intensity was 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Four-week-old seedlings, which were approximately 15 cm tall, were used to collect leaves, stems and roots for RNA extraction. Panicles were collected from 4-month old flowering plants. For the seed sample, rice seeds were dehulled and then placed on wet filter paper in growth chambers (26°C). After 18 h, seeds were collected for RNA extraction. All chemicals were purchased from Sigma-Aldrich (St. Louis, MR) unless otherwise noted.

Protein X-Ray Crystallography

A full-length cDNA of AtIAMT1 was cloned into the pHIS8 expression vector (Jez et al., 2000). N-terminal His8-tagged protein was expressed in *E. coli* BL21 (DE3) cells. Tagged AtIAMT1 was purified from sonicates using a Nickel column (Ni-NTA, Qiagen). The histidine tag was removed using thrombin protease digestion, and the cleaved protein was purified to greater than 99 % homogeneity by gel filtration chromatography on a Superdex-S200 (Amersham Biosciences, Inc.) FPLC column, equilibrated in 500 mM KCl, 25 mM Hepes- Na^+ , pH 7.5, 2 mM DTT. AtIAMT1-containing fractions were combined and concentrated to 33 mg/mL. An in-house crystallization screen was used to find the initial crystallization conditions [7 % (w/v) PEG 8000, 0.25 M KSCN, 100 mM sodium succinate (pH 5.5) or 100 mM PIPES- Na^+ (pH 6.5), and 14 % (w/v) PEG 8000, 0.25 M KSCN, 100 mM PIPES- Na^+ (pH 6.5) or 100 mM MOPSO- Na^+ (pH 7.0) in the presence of 1 mM SAH], yielding 0.5 mm by 0.1 mm by 0.05 mm crystals. This first crystallization screen was followed by crystal size and growth optimization varying the concentrations and constituents of the crystallization matrix. Larger more ordered crystals (0.5 mm by 0.1 mm by 0.1 mm) were obtained at 4°C using a protein concentration of 33 mg/mL, with the following crystallization conditions: 7 % (w/v) PEG 20,000, 1 M urea, 0.3 M KNO_3 , 100 mM MOPSO- Na^+ (pH 7.0) and 3 mM SAH. The cryoprotectant used prior to freezing crystals in liquid nitrogen consisted of a final concentration of 17 % (v/v) ethylene glycol added to the mother liquor noted above for crystal growth. A 2.75 Å resolution

dataset was collected at the National Synchrotron Light Source (NSLS). Data reduction was performed with the XDS program (Kabsch, 1993). The space group was P21 ($a=67.3 \text{ \AA}$, $b=129 \text{ \AA}$, $c=68.3 \text{ \AA}$, $\beta=112.3^\circ$) with two molecules per asymmetric unit (solvent content of 63%). The AtIAMT1 structure was determined by molecular replacement using Molrep (Vagin and Teplyakov, 1997) and *Clarkia breweri* CbSAMT (PDB code: 1M6E, 34 % sequence identity) as a template. A first structure solution, including 345 residues out of the 374 residues of AtIAMT1, was built manually into the experimental electron density maps displayed with Coot (Emsley and Cowtan, 2004) and refined with CNS (Brunger et al., 1998) and Refmac5 (Murshudov et al., 1997). Clear electron density was observed in the active site and interpreted as a SAH molecule. Despite several attempts at soaking IAA into existing crystals or growing crystals in the presence of IAA, no IAA was observed in the active site.

In silico Docking Experiments

In silico docking experiments were carried out with the software package Schrödinger (www.schrodinger.com), and more specifically with the sub-programs LigPrep (version 1.6, Schrödinger, LLC, New York, NY, 2005) for energy minimization, Glide (version 3.5, Schrödinger, LLC, New York, NY, 2005) for docking and Maestro for visualization. All programs were run on a bi-OpteronTM 64b Linux computer. Each docking experiment was carried out over an extended area, exceeding the volume of the active site.

Sequence Retrieval and Analysis

The protein sequence of CbSAMT (accession number AF133053) was used initially as a query sequence to search against the translated rice genome database (<http://www.tigr.org/tdb/e2k1/osa1>) using the BlastP algorithm (Altschul et al., 1990). Newly identified SABATH-like sequences were used reiteratively to search the same sequence database. The cutoff e-value was set to e^{-6} . The chromosome locations of the rice *SABATH* genes were generated by Map Viewer (<http://www.ncbi.nlm.nih.gov/mapview/static/MVstart.html>). Phylogenetic trees were produced using PAUP4.0 based on multiple sequence alignments made with ClustalX (Thompson et al., 1997) and viewed using the TreeView software (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

Gene Expression Analysis via RT-PCR

Semi-quantitative RT-PCR expression analysis of rice *SABATH* genes were performed as previously described (Chen et al., 2003). Primer sequences are shown in the Table 3.3. Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA). 1.0 µg total RNA was synthesized into first strand cDNA in a 20 µL reaction volume using the iScript cDNA synthesis kit (Bio-rad Laboratories). 1.0 µL of the resulting cDNA mixture was used in each PCR using the following conditions: initial denaturation at 95°C for 2 min followed by 30 cycles at 95°C for 45 sec, 54 °C for 45 sec and 72 °C for 60 sec, and then followed by a final extension step at 72 °C for 10 min.

Purification of Recombinant OsIAMT1

OsIAMT1 full-length cDNA was amplified from rice root tissues by RT-PCR using the forward primer 5'- CACCATGGCTCCTAAAGGTGACAATGTTG-3' and the reverse primer 5'-CTACTACTATGCGGATGCTGCTATAC -3'. The resulting PCR product was cloned into the pET100/D-TOPO vector (Invitrogen, Carlsband, CA). The construct was transformed into *E. coli* strain BL21 Codon Plus (Invitrogen, Carlsband, CA). Protein expression was induced by IPTG for 18 h at room temperature. His-tagged *OsIAMT1* protein was purified from *E. coli* cell lysate using Ni-NTA agarose following manufacturer's instructions (Invitrogen, Carlsband, CA). Protein purity was verified by SDS-PAGE and protein concentrations were determined using the Bradford assay.

Radiochemical MT Activity Assay

Radiochemical MT assays were performed in a 50 μ l volume containing 50 mM Tris-HCl, pH 7.5, 1 mM substrate (IAA, 2, 4-D, salicylic acid, jasmonic acid, farnesoic acid or gibberellic acid) and 0.4 μ l ^{14} C-SAM (Perkin Elmer, Boston, MA). The assay was initiated with the addition of SAM and maintained at 25 °C for 30 min. The reaction was stopped by the addition of 150 μ l ethyl acetate, vortexed and phase-separated using a 1 min centrifugation at 14,000g. The upper organic phase was counted using a liquid scintillation counter (Beckman Coulter, Fullerton, CA). The amount of radioactivity extracted into the organic phase correlated to the amount of methyl ester formed by the recombinant enzyme and ^{14} C-SAM. Three independent

assays were performed for each compound.

Determination of Kinetic Parameters of OsIAMT1

In all kinetic analyses, the appropriate enzyme concentrations of OsIAMT1 and incubation times were chosen so that the reaction velocity was linear during the reaction time period examined. To determine the K_m values for IAA and SAM, one substrate concentration was fixed at a saturating level and the concentration of the other substrate was varied. K_m values and maximum velocity values were obtained as previously described (Chen et al., 2003). Final values were an average of three independent measurements after non-linear regression analyses using the Michaelis-Menten equation.

pH Optimum for OsIAMT1 Activity

OsIAMT1 activity was determined in a 50 mM Bis-Tris propane buffer for the pH range 6.5 to 10 using the standard IAMT assay described above. The resultant kinetic constants used for determination of the pH optimum were an average of three independent assays.

Effectors

To examine effects of metal ions on OsIAMT1 activity, standard IAMT assays were performed in the independent presence of each of the following salts at 5 mM final concentration: KCl, CaCl_2 , NH_4Cl , NaCl, MgCl_2 , MnCl_2 , CuCl_2 , FeCl_2 and

ZnCl₂. Results presented were an average of three independent assays.

Product Identification

A reaction containing 150 µg of purified OsIAMT1, 1 mM IAA and 600 µM SAM was incubated in a 1 ml reaction volume containing 50 mM Tris-HCl, pH 7.5, for 4 h at 25 °C. The product was extracted with 1.5 ml hexane, the hexane layer concentrated under N₂ gas, and the resultant organic concentrate analyzed on a Shimadzu GC (GC-17A)-MS (QP 5050A) system. A DB-5 column (30 m by 0.25id by 0.25 µm) was used with helium as carrier gas at a flow rate of 1 ml/min. As a control, a similar reaction was performed, except that OsIAMT1 protein was denatured by boiling at 100 °C for 10 min before addition to the assay. A MeIAA authentic standard was dissolved in ethanol, and a volume containing 1µg MeIAA was injected into the GC-MS in a split (1/30) mode. The GC program was as follows: 2 min at 80 °C, ramp to 300 °C at 8 °C per min followed by a 5 min hold at 300 °C. The compound was identified by comparison of GC retention times and mass spectra with those of the authentic standard.

Homology-based Structure Modeling

Based on the structure of AtIAMT1, homology models of OsIAMT1 and PtIAMT1 were calculated. First, a sequence alignment with AtIAMT1 was performed with Blast (<http://www.ncbi.nih.gov/BLAST/>) using the Blosum62 matrix. Then the homology model was calculated with the program Modeller (Sali and Blundell, 1993)

by generating a first model (3D alignment on template) and running 200 cycles of molecular dynamics based simulated annealing. The OsIAMT1-IAA complex and PtIAMT1-IAA complex were then built by hand, based on the position of IAA in AtIAMT1, as calculated by *in silico* docking.

Results

Three-Dimensional Architecture of AtIAMT1

The overall structure of AtIAMT1 (Figure 3.1) is similar to CbSAMT (Zubieta et al., 2003; Figure 3.2), with a root mean square distance (rmsd) for aligned alpha carbons of 2.0 Å (rmsd calculated after structural alignment using the DALI server, www.ebi.ac.uk/dali/). The monomer fold comprises two clearly delineated domains, a C-terminal catalytic domain involved in SAM/S-adenosyl-L-homocysteine (SAH) and substrate binding, comprising residues 149 to 374 (α helices $\alpha 4$ - $\alpha 11$; 3_{10} helix $\eta 1$; β strands $\beta 4$ - $\beta 7$; Figure 3.3), and an N-terminal capping domain spanning residues 1 to 148 (α helices $\alpha 1$ - $\alpha 3$; β strands $\beta 1$ - $\beta 3$; Figures 3.3 and 3.4) involved in dimer formation and a portion of which extends over the carboxyl-bearing substrate (Zubieta et al., 2003) to orient the methyl acceptor near SAM. The SAM binding C-terminal domain possesses the commonly observed α/β fold of a large superfamily of SAM-dependent MTs that diverge in the number and size of accessory domains (Figure 3.1).

The quaternary structure of AtIAMT1 (one homodimer observed per asymmetric unit, Figure 3.1) again is shared with CbSAMT (Zubieta et al., 2003). The surface area buried in the dimerization interface is about 1025 Å², which represents only 6.3% of the total surface area of each monomer. As in CbSAMT, and unlike most of the plant small molecule O-methyltransferases structurally characterized to date (Zubieta et al., 2001; Zubieta et al., 2002), the dyad-related monomer of IAMT does not contribute to the active site of its partner molecule (Figure 3.1).

The first 49 residues of AtIAMT1 in this particular set of crystal structures form a mobile loop. In fact, recognizable electron density attributable to residues 1-24 is noticeably absent from the refined AtIAMT1 crystal structure which also lacks a bound IAA substrate. The mobile active site capping loop in CbSAMT equivalent to residues 1-24 in AtIAMT1 closes the active site, forming a series of inter-atomic interactions with the carboxyl group of the bound salicylate substrate (Zubieta et al., 2003).

Active Site Topology of AtIAMT1

As expected from sequence alignments, the residues of AtIAMT1 likely to interact with the carboxyl moiety of the IAA substrate are strictly conserved with respect to CbSAMT. These residues include Lys10, Gln25 (not observed in the electron density and located on the mobile N-terminal active site capping loop) and Trp162 (Trp151 in CbSAMT). The majority of the carboxyl-bearing substrate binding

pocket in AtIAMT1 is noticeably hydrophobic and rich in aromatic residues as previously seen in CbSAMT (Figure 3.4). Residues include Phe158 (Tyr147 in CbSAMT), Leu226 (Leu210 in CbSAMT), Leu242 (Ile225 in CbSAMT), Phe243 (Trp226 in CbSAMT), Val326 (Val311 in CbSAMT) and Phe364 (Phe347 in CbSAMT).

Identification of the SABATH Gene Family in Rice

To identify the complete *SABATH* gene family from the fully sequenced rice genome (International Rice Genome Sequencing Project, 2005), the protein sequence of CbSAMT was initially used to search the genome sequence database of rice using the BlastP algorithm (Altschul et al., 1990). The new *SABATH*-like sequences detected were in turn used reiteratively to search the same sequence database. Through this iterative sequence search, forty-one sequences encoding proteins bearing significant similarity to known *SABATH* proteins were identified in the rice genome (Table 3.2). It should be noted that among the 41 *OsSABATH* sequences, fifteen of them appear to encode proteins shorter than 300 amino acid residues. Some of the shorter proteins may be due to inaccurate annotation (Rouze et al., 1999), and some of them may represent pseudogenes. Some of the genes annotated to encode proteins over 350 amino acid residues in length may also be pseudogenes, as shown in Figure 3.10. Additional efforts to characterize these genes will clarify whether individual *OsSABATH* sequences code for intact genes or pseudogenes.

Mapping the physical locations of the 41 *OsSABATH* genes revealed that these genes are scattered on seven chromosomes that include chromosomes 1, 2, 4, 5, 6, 10 and 11 (Figure 3.5). More than half of the *OsSABATH* genes (22) are localized on chromosome 6. In contrast, chromosomes 3, 5 and 10 each contain only one *SABATH* gene. Twenty two *OsSABATH* genes are localized in six clusters in which *OsSABATH* genes are adjacent or separated by one unrelated gene. Cluster C6 contains eight *OsSABATH* genes (Figure 3.5).

Expression analysis of OsSABATH Genes

To obtain information on the biological processes in which *OsSABATH*s may be involved, comprehensive gene expression analyses using semi-quantitative RT-PCR were performed for all *OsSABATH* genes. Gene expression analyses were performed with leaves, roots and stems from one-month old seedlings, panicles from four month-old flowering plants and germinating seeds.

In these experiments, results for different genes in the same organ are directly comparable, because an identical aliquot of cDNA from the original RT reaction was used in each PCR. To determine whether equal amounts of cDNA were used in the reactions involving different organs, I also performed RT-PCR with primers designed to detect actin mRNA. After RT-PCR, amplified fragments from mRNAs of 23 of the 41 *OsSABATH* genes were obtained from at least one organ (Figure 3.6). The expression of 20 genes was detected in roots, 13 genes in stems, 16 genes in leaves,

16 genes in panicles and 10 genes in germinating seeds. Nine genes showed expression in all tissues examined. In contrast, sixteen genes exhibited no expression in any of the tissues examined.

Identification of OsIAMT1 and Its Biochemical Properties

The rice *SABATH* gene that is most similar to *AtIAMT1* is *OsSABATH4*. *OsSABATH4* encodes a protein spanning 404 amino acid residues with a calculated molecular mass of 43.8 kD. At the amino acid sequence level, *OsSABATH4* is 61% identical to *AtIAMT1*. To determine whether this gene encodes rice IAMT, full length cDNA of *OsSABATH4* was cloned and protein expressed in *E. coli*. The protein was purified and tested with a group of potential substrates including IAA, salicylic acid, jasmonic acid, farnesoic acid and gibberellic acid. 2,4-D, a synthetic compound and is structurally highly similar to IAA, was also tested as a substrate. *OsSABATH4* displayed the highest level of catalytic activity with IAA. The enzyme also displayed activity with 2,4-D (5% of the activity measured with IAA), but exhibited no activity with salicylic acid, jasmonic acid, farnesoic acid and gibberellic acid used as substrates. *OsSABATH4* therefore encodes IAMT. As I have not yet analyzed all rice *SABATH* proteins and can not rule out the possibility that other *OsSABATH*s also possess IAMT activity, *OsSABATH4* was named *OsIAMT1*.

To determine the chemical structure of the product of *OsIAMT1*, the compound produced from the *OsIAMT1* enzyme assay with IAA as a substrate was extracted

with hexane and analyzed using a GC-MS. As shown in Figure 3.7, the product showed the same retention time and mass fragmentation spectrum as the authentic methyl indole-3-acetate (MeIAA) standard, confirming that OslAMT1 catalyzes the formation of MeIAA using SAM as a methyl donor and IAA as a methyl acceptor.

To determine the pH optimum of the enzymatic assays, OslAMT1 was assayed with IAA at buffers with differing pH values between pH 6.5 to pH 10.0. The optimum pH was determined to be pH 7.5. At pH 6.5, the enzyme showed 20% of its maximal activity. At pH 9.0, the activity was 30% of the maximum. As observed for other SABATH proteins that have been biochemically characterized, OslAMT1 activity can be affected by metal ions. K^+ , NH_4^+ , and Na^+ all stimulated OslAMT1 activity by more than 1.5 fold. Ca^{2+} and Mg^{2+} had a mild inhibitory effect on OslAMT1 activity. In contrast, Mn^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} and Zn^{2+} all had a strong inhibitory effect on OslAMT1 activity. Kinetic parameters for OslAMT1 were also determined. Under steady-state conditions, OslAMT1 exhibited K_m values of $17.9 \pm \mu M$ and $7.3 \pm \mu M$ for IAA and SAM, respectively, and a k_{cat} of $0.025 \pm s^{-1}$.

Molecular Modeling of Rice and Poplar IAMTs

Homology models of OslAMT1 (Figure 3.8B) and PtlAMT1 (not shown) were built with Modeller (Sali and Blundell, 1993) based on the experimental AtIAMT1 structure reported here. These models show a high degree of similarity of the overall structure among AtIAMT1, OslAMT1 and PtlAMT1 (Figure 3.8), which is a

predictable consequence of the protein sequence similarity. Furthermore, these models also exhibit the hydrophobic residues that form the substrate binding site previously observed in AtIAMT1 (Phe158, Leu226, Leu242, Phe243, Val326 and Phe364 in AtIAMT1 sequence) (Figure 3.4).

Phylogenetic Analysis of SABATHs

Arabidopsis was the first plant species in which the complete *SABATH* gene family was identified (Chen et al., 2003; D'Auria et al., 2003). To understand the evolutionary relationships among *SABATH* proteins, a phylogenetic tree containing the entire set of rice and Arabidopsis *SABATH* proteins and selected *SABATH* proteins from other plants was constructed (Figure 3.9). When only *SABATH*s from rice are considered, 41 Os*SABATH*s group into three clades (I, II and III). Clade I contains 23 Os*SABATH*s, subdivided into two subclades. One subclade contains the majority of the Os*SABATH* genes found on chromosome 6. The other subclade contains all Os*SABATH* genes localized on chromosome 11. When Os*SABATH* proteins and At*SABATH* proteins are analyzed together, five clades emerge. Clade I appears to be rice-specific. In contrast, clades III and V contain only Arabidopsis *SABATH*s. Considering known Arabidopsis *SABATH* proteins, AtFAMT resides in clade III and AtBSMT resides in clade V. Clades II and IV contain *SABATH*s from both Arabidopsis and rice. Two At*SABATH* proteins, including AtJMT, and ten Os*SABATH*s cluster in clade II. Three At*SABATH*s (AtIAMT1, AtGAMT1 and AtGAMT2) and eight Os*SABATH*s localize in clade IV.

Previously identified SABATH proteins from other plants group in clades II, IV and V (Figure 3.9). CbSAMT and SAMTs isolated from snapdragon, *Stephanotis floribunda* and *Nicotiana suaveolens* sit in clade II. Snapdragon BAMT and coffee caffeine synthase (CCS1) reside in clade V. Notably, AtIAMT1, OsIAMT1 and PtIAMT1 form a monophyletic group, which is closely related to putative SABATHs identified from gymnosperm species (Figure 3.9).

Discussion

Structural Basis for Substrate Specialization of SABATH Proteins

Comparison of the previously determined CbSAMT structure obtained with SAH (demethylated SAM) and salicylate bound in the active site with the AtIAMT1 structure obtained with SAH bound but no visible IAA strongly suggests that the N-terminal active site capping loop absent in the current structure of AtIAMT1 but well ordered in the previously published CbSAMT structure acts as a dynamic lid to lock down substrates and desolvate the carboxyl group undergoing methylation. This hypothesis is supported not only by the apparent mobility of the capping loop which allows substrate entry but also by the absolute conservation of the capping loop residues interacting with the substrate's carboxyl moiety including Lys10 and Gln25. Through hydrogen bonding interactions, these residues ensure that water molecules solvating the carboxyl group and reducing its reactivity are eliminated. This desolvation mechanism is a prerequisite for enhancing the intrinsic reactivity of the

negatively charged carboxyl oxygens now abutting the electrophilic methyl group of bound SAM. Moreover, 42% of the residues located on the mobile loop (1-24) are strictly conserved among the four MTs shown in Figure 3.4.

Interestingly, standard sequence alignments and homology-based models calculated from the previously published CbSAMT structure predict that Trp226 of CbSAMT is replaced by Gly244 in AtIAMT1, leading to an intuitively simple explanation for the IAA specificity of IAMT1 (Zubieta et al., 2003). However, as a warning against over-reliance on homology models even for proteins possessing a high degree of sequence identity/similarity, it was observed in the experimentally determined AtIAMT1 crystallographic structure that loop $\alpha 5$ is shifted by one residue compared to the sequence alignments, thus superimposing Phe243 of AtIAMT1 with Trp226 of CbSAMT. This significant readjustment of the actual structure relative to the previously published homology model (Zubieta et al., 2003) leaves little room in this portion of the active site compared to CbSAMT. The more restricted AtIAMT1 active site relative to the original homology model based on CbSAMT suggests that in AtIAMT1 the IAA substrate will assume a quite different conformation to avoid a clash of the indole ring of IAA with Phe243.

In order to build a model of IAA bound to AtIAMT1 that takes into account the differences between the IAMT and SAMT active sites noted here, *in silico* docking techniques were used. The first attempts to computationally calculate a binding

orientation for IAA without spatial restraints failed. This lack of initial success is likely due to the absence of the critical N-terminal active site capping loop responsible for highly specific interactions with the carboxyl moiety of bound substrates. A second computational attempt was undertaken, this time using hard constraints to ensure one oxygen of the carboxyl group is located within the appropriate distance for methyl transfer from SAM, and within a sphere of 1 Å diameter centered on the equivalent position in CbSAMT complexed with salicylate. The resulting docking model with favorable docking scores show that the indole ring of IAA points away from Phe243 and forms energetically favorable van der Waals and aromatic-aromatic interactions with Phe158 and Phe364 (Figures 3.3A and 3.8).

Based on these computational results, Phe158, Pro303 and Ser322 appear to be primarily responsible for the IAA specificity of AtIAMT1 with Ser322 possibly involved in forming a specific hydrogen bond with the indole ring nitrogen of IAA (upon rotation with respect to C α -C β bond, O γ atom of Ser322 resides as close as 2.9 Å from the indole nitrogen atom). Indeed, all three residues contribute to the IAA binding pocket, and, based on the structure-based sequence alignments, are conserved residues in the three IAMTs listed and are consistently different in SAMTs (Figures 3.3 and 3.4).

Biological Roles of IAMTs

IAA is the most abundant and most active endogenous auxin in higher plants

(Leyser, 2002). It modulates diverse aspects of plant growth and development including embryogenesis, lateral and adventitious root formation and induction of vascular differentiation (Teale et al., 2006). Methylation of IAA is likely to have multiple biological consequences. *OsIAMT1*, *AtIAMT1* and *PtIAMT1* all exhibited expression in multiple tissues (Figure 3.6; Qin et al., 2005; Zhao et al., 2007), suggesting that IAMT is involved in a variety of biological processes. *AtIAMT1* was shown to play a role in leaf development (Qin et al., 2005). *AtIAMT1* exhibited ubiquitous expression in young leaves and edge-specific expression in old leaves. Down-regulating *AtIAMT1* expression leads to dramatic epinastic leaf phenotypes (Qin et al., 2005). Poplar *IAMT1* also showed higher expression in young leaves than old leaves (Zhao et al., 2007). Additionally, *PtIAMT1* also displayed high expression levels in stems. Because IAA is involved in secondary wood development (Moyle et al., 2002), it was speculated that *PtIAMT1* may serve a critical role in xylem formation in perennial woody species (Zhao et al., 2007).

OsIAMT1 also exhibited expression in multiple tissues. *OsIAMT1* showed high levels of expression in roots and panicles (Figure 3.6). In rice, IAA plays critical roles in root development, including the elongation of the primary roots, the initiation and elongation of lateral roots, the formation of adventitious roots and root gravitropism (Chhun et al., 2003). Although the mode of action in these cases is not well understood, the concentration of IAA appears to be important in regulating IAA activities in rice roots (Wang et al., 2003). The presence of *OsIAMT1* transcripts in

rice roots suggest that *OsIAMT1* is involved in root development by regulating the homeostasis of IAA in the tissue. IAA also appears to play a role in rice grain development. During grain filling, a dramatic reduction in the levels of IAA in panicles has been observed (Yang et al., 2000). The high level of *OsIAMT1* transcripts in rice panicles (Figure 3.6) implies that *OsIAMT1* is involved in the reduced level of IAA found there. Expression of *IAMTs* in leaves of different plant species suggests that their function in leaf development may be evolutionarily conserved. Their divergent expression patterns in certain tissues, for instance, high expression levels in poplar stems and very low expression levels in rice stems, imply that *IAMTs* may also have acquired lineage-specific roles in different plant species.

Evolution of the SABATH Gene Family

Rice is the second plant species, after Arabidopsis, in which the entire *SABATH* gene family has been identified. The majority of *OsSABATH* genes exhibited expression in one or multiple tissues under normal growing conditions (Figure 3.6), suggesting that the gene products possess roles in the normal physiology of rice plants. Elucidating the biochemical functions of *OsSABATH* proteins will be critical for understanding their biological roles. Identification of the complete family of *SABATH* genes in rice provides unprecedented opportunities for cross-species analysis of the *SABATH* gene family. Phylogenetic analyses of all rice and Arabidopsis *SABATHs* suggests that the expansion of the *SABATH* families in rice and Arabidopsis occurred after the split of the two lineages. It will be interesting to test whether some rice and

Arabidopsis SABATHs catalyze divergent biochemical reactions that contribute to the unique biology and ecology of rice and Arabidopsis plants, respectively.

Among all *OsSABATHs* and *AtSABATHs*, *OsIAMT1* is most related to *AtIAMT1*, implying that they are likely orthologous genes. In contrast, whether the rice genome encodes *AtSABATH* proteins with the same catalytic activity as *AtJMT*, *AtBSMT* and *AtFAMT*, respectively, is difficult to predict from this phylogenetic analysis because each of them is more related to other *AtSABATHs* than to any *OsSABATHs* (Figure 3.9). Rather, the phylogenetic analysis suggests that *AtJMT*, *AtBSMT* and *AtFAMT* evolved after the divergence of Arabidopsis and rice lineages. If the rice genome also contains genes encoding such activities, these genes likely evolved independently. A recent study showed that *OsSABATH3* has BSMT activity in vitro (Koo et al., 2007). The phylogenetic placements of *OsSABATH3* in clade I that is a rice-specific, *AtBSMT* in clade V, and other known SAMTs in clade II (Figure 3.9) suggest that SAMTs emerged several times during the course of SABATH gene evolution. It is interesting to note that caffeine biosynthetic pathways seem to have evolved independently several times in plants as well. This conclusion was drawn based on the observation that the sequence identity between theobromine synthase and caffeine synthase within the genus of *Camellia* is very high, while the sequence identity among the functionally orthologous N-methyltransferases between *Camellia* and *Theobroma* is lower (Yoneyama et al., 2006).

As previously demonstrated, the emergence of a novel SABATH MT activity can occur rapidly, and small changes in primary protein sequences can lead, as for other enzymes of specialized metabolism, to the functional emergence of SABATH proteins with altered substrate preferences (Zubieta et al., 2003; Pichersky et al, 2006). This observation poses difficulties for the functional assessment of SABATH proteins based only on overall sequence similarity to a biochemically characterized protein. Identification of conserved IAMTs from rice, Arabidopsis and poplar, however, implies that the presence of IAMT predated the divergence of the monocotyledous and dicotyledous lineages. Therefore, IAMT is likely an ancient member of the SABATH protein family, from which other SABATH MT activities, including JMT, SAMT and FAMT, may have emerged. This hypothesis will be tested with the continued identification of *JMT*, *SAMT* and *FAMT* in rice and poplar, their biochemical characterization and structurally-guided approaches for delineating residues that dictate substrate specificity.

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Appendix

Table 3.1. Crystallographic data and refinement statistics of AtIAMT1

Dataset	apo form
X-ray Source	NSLS
Space group	p21
Cell parameters (Å and °)	a=67.3, b=129, c=68.3, β=112.3
Wavelength (Å)	1.009321
Resolution (last shell) (Å)	50-2.75 (2.82-2.75)
Total reflections (last shell)	60638 (4761)
Unique reflections (last shell)	20426 (1626)
Redundancy (last shell)	2.97 (2.93)
Completeness (last shell) (%)	72.4 (79.5)
R_{sym}^a (last shell) (%)	9.4 (46.9)
I/σ (last shell)	12.1 (2.72)
R_{cryst}^b (last shell) (%)	25.6 (30.2)
R_{free}^c (last shell) (%)	28.2 (35.7)
R.m.s.d bonds (Å)	0.012
R.m.s.d angles (°)	1.45
Non-hydrogen protein atoms	5278
Non-hydrogen ligand atoms	56
Water molecules	150
Average B factor (Å ²)	50.8
Ramachandran plot	87.4/11.4/1.2/0

Values in parenthesis are for the outer resolution shell.

^a $R_{sym} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the intensity of a reflection and $\langle I \rangle$ is the average intensity of that reflection.

^b $R_{cryst} = \sum ||F_{obs}| - |F_{calc}|| / \sum |F_{obs}|$.

^c 5% of the data were set aside for R_{free} calculation.

^d Percentage of residues in most-favored/additionally allowed/generously allowed/disallowed regions of the Ramachandran plot.

Table 3.2. Rice SABATH methyl transferase genes

Gene	TIGR ID	Chr ^a	Strand	Chromosome Location	Protein size
<i>OsSABATH1</i>	LOC_Os01g50480	1	-	29312863 - 29311390	379
<i>OsSABATH2</i>	LOC_Os01g50610	1	-	29380292 - 29377624	380
<i>OsSABATH3</i>	LOC_Os02g48770	2	+	29844503 - 29846574	381
<i>OsSABATH4</i>	LOC_Os04g56950	4	-	33733503 - 33731186	405
<i>OsSABATH5</i>	LOC_Os04g57050	4	+	33782242 - 33786150	380
<i>OsSABATH6</i>	LOC_Os04g57070	4	+	33795025 - 33796687	295
<i>OsSABATH7</i>	LOC_Os04g57080	4	+	33798360 - 33800490	379
<i>OsSABATH8</i>	LOC_Os04g57090	4	+	33803567 - 33805014	384
<i>OsSABATH9</i>	LOC_Os05g01140	5	-	96310 - 94475	374
<i>OsSABATH10</i>	LOC_Os06g13310	6	-	7322202 - 7317600	448
<i>OsSABATH11</i>	LOC_Os06g13350	6	-	7343868 - 7341145	376
<i>OsSABATH12</i>	LOC_Os06g13390	6	-	7369766 - 7368205	352
<i>OsSABATH13</i>	LOC_Os06g13460	6	-	7407610 - 7405038	169
<i>OsSABATH14</i>	LOC_Os06g13470	6	-	7421148 - 7416745	401
<i>OsSABATH15</i>	LOC_Os06g13490	6	-	7432792 - 7428731	287
<i>OsSABATH16</i>	LOC_Os06g13510	6	+	7454886 - 7455585	109
<i>OsSABATH17</i>	LOC_Os06g13520	6	+	7458468 - 7460721	268
<i>OsSABATH18</i>	LOC_Os06g13530	6	-	7468414 - 7467751	147
<i>OsSABATH19</i>	LOC_Os06g13550	6	-	7474599 - 7473909	186
<i>OsSABATH20</i>	LOC_Os06g13560	6	-	7481195 - 7479074	375
<i>OsSABATH21</i>	LOC_Os06g20630	6	-	11889148 - 11886861	346
<i>OsSABATH22</i>	LOC_Os06g20770	6	-	11975246 - 11972576	366
<i>OsSABATH23</i>	LOC_Os06g20790	6	+	11991873 - 11983849	371
<i>OsSABATH24</i>	LOC_Os06g20920	6	-	12087594 - 12081450	339
<i>OsSABATH25</i>	LOC_Os06g20960	6	-	12117524 - 12114576	360
<i>OsSABATH26</i>	LOC_Os06g21020	6	-	12144412 - 12140325	400
<i>OsSABATH27</i>	LOC_Os06g21760	6	+	12571596 - 12573253	240
<i>OsSABATH28</i>	LOC_Os06g21820	6	+	12603825 - 12605643	350
<i>OsSABATH29</i>	LOC_Os06g21830	6	+	12607804 - 12608319	172
<i>OsSABATH30</i>	LOC_Os06g22440	6	+	13040901 - 13043159	367
<i>OsSABATH31</i>	LOC_Os10g09360	10	-	4821809 - 4821396	138
<i>OsSABATH32</i>	LOC_Os11g15030	11	+	8451461 - 8454568	388
<i>OsSABATH33</i>	LOC_Os11g15040	11	+	8461158 - 8463673	204
<i>OsSABATH34</i>	LOC_Os11g15060	11	+	8472066 - 8474370	376
<i>OsSABATH35</i>	LOC_Os11g15130	11	+	8523516 - 8525669	288
<i>OsSABATH36</i>	LOC_Os11g15180	11	+	8556112 - 8558343	366
<i>OsSABATH37</i>	LOC_Os11g15300	11	+	8643774 - 8645126	350
<i>OsSABATH38</i>	LOC_Os11g15310	11	+	8647797 - 8650728	312
<i>OsSABATH39</i>	LOC_Os11g15340	11	+	8671640 - 8673396	203
<i>OsSABATH40</i>	LOC_Os11g15410	11	+	8726937 - 8728351	266
<i>OsSABATH41</i>	LOC_Os06g21720	6	+	12554481 - 12554816	112

Table 3.3. Primers for gene expression analysis of *OsSABATHs* via RT-PCR

Gene Name	Forward Primer Sequences (5'-3')	Reverse Primer Sequences (5'-3')
<i>OsSABATH1</i>	ACCAACGACTTCAACACCATCTTCT	ACCTGAGGAACAGGCTGAAGTCTCTC
<i>OsSABATH2</i>	AGGGAAAGATGTACATATCAAGCACGAG	GTACGTCTGCACGTAGTCCATCCTGAAC
<i>OsSABATH3</i>	GAAGGCTCTGCCTCCCTACTACATT	AAGAAGCCCATACATGTAGCTCACT
<i>OsSABATH4</i>	ACGTGTTACCTCCACCTTCTCTTT	GGATGTTGAAGCTGTCCCTCTTCTC
<i>OsSABATH5</i>	TATGAAGTGATTGCAGCATGTTGAG	TTACACCAAGTGAAAGCGAGCATAGAAT
<i>OsSABATH6</i>	ACGGATCAAGGCTACATCTATGAAGTG	GAAAGTGAGCATAGAATATGGGGGAAG
<i>OsSABATH7</i>	CAGCTTCTATACGGCGCCATGTTT	CAGCGAAAGGGAACACACAATATG
<i>OsSABATH8</i>	CGTACAACAAGGAGAAGGTGTTGCTC	GACGTTGAAGCTGTCCATCTCTCTC
<i>OsSABATH9</i>	GATAGCCTGGACTACGTGGTGTGCTG	CTAGATCTGCTTGGGATTGGCATGAG
<i>OsSABATH10</i>	ACTAGAGGCTAGTGGAATCTCTCTTG	GACTCCAGGACTGACCTAATGAGATTAC
<i>OsSABATH11</i>	CACAAAGGAGATATACTACCTGCATACC	CATTTACATCTCCACTATATGGGTCCTC
<i>OsSABATH12</i>	AAGGTAACCCAAGAAGTATACACTGGTC	GTCCTTGTAGAACTGCTTGGAAATAGT
<i>OsSABATH13</i>	AGGATGAGGATGTATACAATGGAAGTC	CTATCAAAGACTCCAGCACAGATCTAA
<i>OsSABATH14</i>	AAGACTACCCCTAAATCGGTGATAAAG	GAAGAGCACGCTAGTATAGATTCTCCA
<i>OsSABATH15</i>	ATCTAGTGGTCAGATGCTACTCACTTTC	ATAGCACTCCATCTCCATATGTTCTTG
<i>OsSABATH16</i>	GACCGAATACACTAGTCTTCATCTCTGA	ATGTAGGGAATACGATGAATGGAAGAG
<i>OsSABATH17</i>	GTGACTACACCTGAAGAAGTGATAAAGC	ATGAGTAGCAAAACAACCTCTCCATAAC
<i>OsSABATH18</i>	AGTTGTACCAGGATCAGTTCCAGAAG	ATGACAAGACTATGACCGAGTACTTCAAC
<i>OsSABATH19</i>	AAACTATGCCAACAACCTCTAGGCTTC	AGCCCAGAGATATAGAATGGAGGTAAC
<i>OsSABATH20</i>	AGTAGCACTGCCTCCATTCTATATCTCT	ATACAGTGGTAGGTTGAATGACTCCAG
<i>OsSABATH21</i>	AGGACTTTTCATTGTTCTCTCACACT	GTACATAGCCCTTCTTTCTCAAGATG
<i>OsSABATH22</i>	CTACCTGGCAATGACTTTAACTACGTCT	GAACAGTGAGAAGTCTCTTTGAACTT
<i>OsSABATH23</i>	AGAAAAGCTTGACTCCTTCTACATTC	TCACATCGCTCTAGTAAGAGACAAACA
<i>OsSABATH24</i>	CTCCAGATGATCTTAAGGAGGGTAAAA	GTGCGTAGAGTGGTATGTAGAAGGAAT
<i>OsSABATH25</i>	GTCATTCTATGAGAGGCTTTTCACTTC	GCTACAGATACCACTTTCCATACTTGAG
<i>OsSABATH26</i>	CATGGTACCTGGGTCATTTTATGATAG	GTGCATAGATAGGTATGTGGAATGTGTC
<i>OsSABATH27</i>	GGTGGGAGTATGGACAGCTTCAAC	CTCTCCATCCGGACAAATAGCTTG
<i>OsSABATH28</i>	GGAGTCCAGTTACCTCAAAAACAGTAA	AAGGTGGAGGTGAACACGTCGATAGAT
<i>OsSABATH29</i>	GGGACGATGGACAGCTTCAACAT	GTGGAAGTGGACCCCATCTCGT
<i>OsSABATH30</i>	AGCTCTGCGTACTCCTCAACGATCT	AGTTCTACAGCCCTCATCTTCAGAAAC
<i>OsSABATH31</i>	AGATTTGGCGTGGCTGACTTGGACT	AGGCTGAAAGAAGAGCAGACGAGAT
<i>OsSABATH32</i>	GATCCAATTCTTTCTCAATGACCTACC	CTGAATACATCTGTGCTATTCTCCCTA
<i>OsSABATH33</i>	AAGGAGAAGCTGGACTCTTTCAACATT	GCATACCATGAGCGCACTTTACTCTAT
<i>OsSABATH34</i>	AGGACCATAACAACACTATCAAGGAGAG	ATCTCATTGGGGAAGTGTGAGAAGT
<i>OsSABATH35</i>	GGTGACAAAGCTCTACCAAAATCAGTT	TCATCGAGTATAGAATCCCCGAAAT
<i>OsSABATH36</i>	GGGTTACAACCTCCTCCGTACTATGTC	CTTGTGAAAGCAACTCGAAAATAGTGG
<i>OsSABATH37</i>	GGCAACATTTATATAGGGGTGACTACAC	GCTTTGTTGTACTAGCTGCTTCAGTTC
<i>OsSABATH38</i>	GAGGTCCAGATATTTCTCAATGACCTAC	CTCAAACTGGTCCAAGTAGAGTTTTCTC
<i>OsSABATH39</i>	AAGAGAGCCAGTTGTTGACATCATAG	AGTGACACAGAAATGGCCATAATGC
<i>OsSABATH40</i>	CCGAGGTATAGCTTACAAGGAGGTATTT	CACTGCAAAGAGGTATCGATTATT
<i>OsSABATH41</i>	ATGGACAGCTTCAACATCCCGTCGTA	CTCTCCATCCGAACAAACAGCTCAT

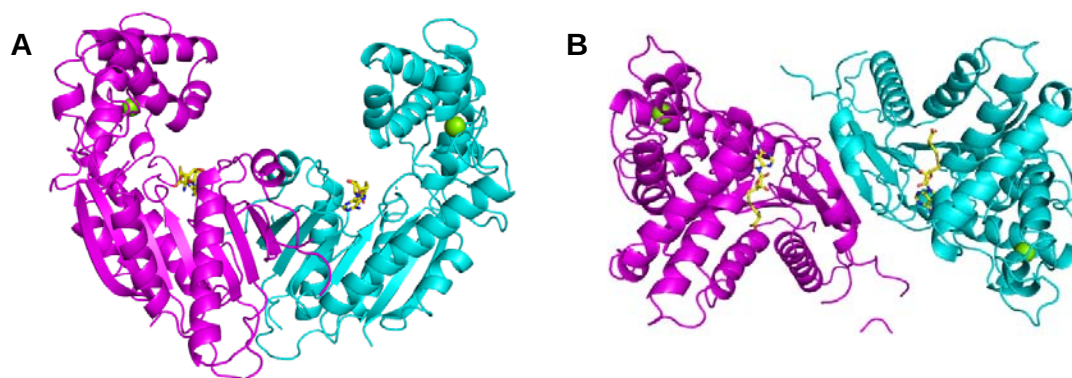


Figure 3.1. Ribbon diagram of the AtIAMT1 dimer. A, front view. B, top view. The two monomers are represented in magenta and cyan respectively. SAH is represented as yellow sticks. This figure was produced with PyMOL (www.pymol.org).

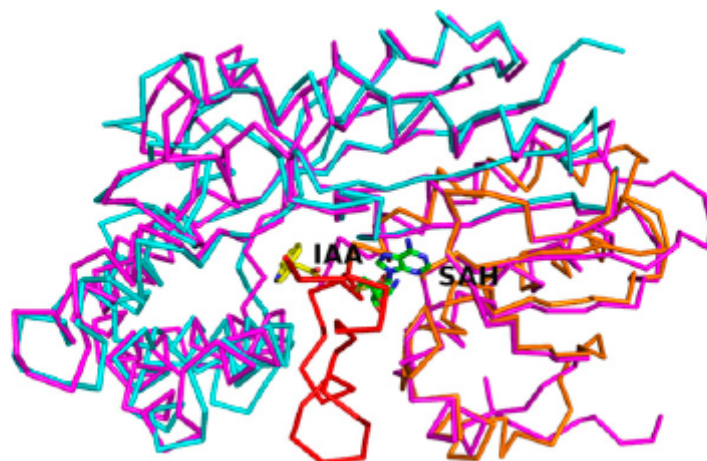


Figure 3.2. Superposition of CbSAMT and AtIAMT1 (magenta). SAH and IAA (from docking calculations) are represented as green and yellow sticks, respectively. The mobile N-terminal active site capping loop, the N-terminal cap-like domain and the C-terminal SAM/substrate binding domain of CbSAMT are colored red, orange and cyan, respectively. This figure was produced with PyMOL (www.pymol.org).

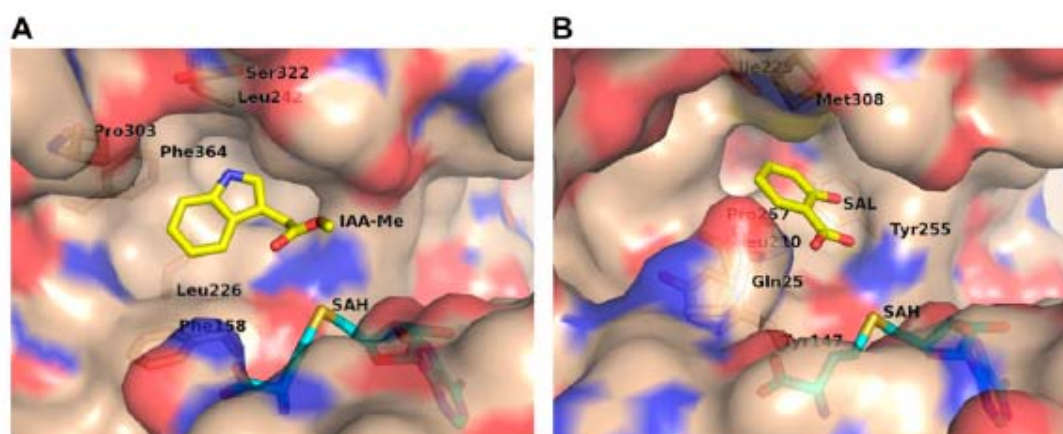


Figure 3.3. Molecular surface view of the active site of AtIAMT1 (A) and CbSAMT (B). In (B), residues 1-24, 307 and 311 are omitted. This figure was produced with PyMOL (www.pymol.org).

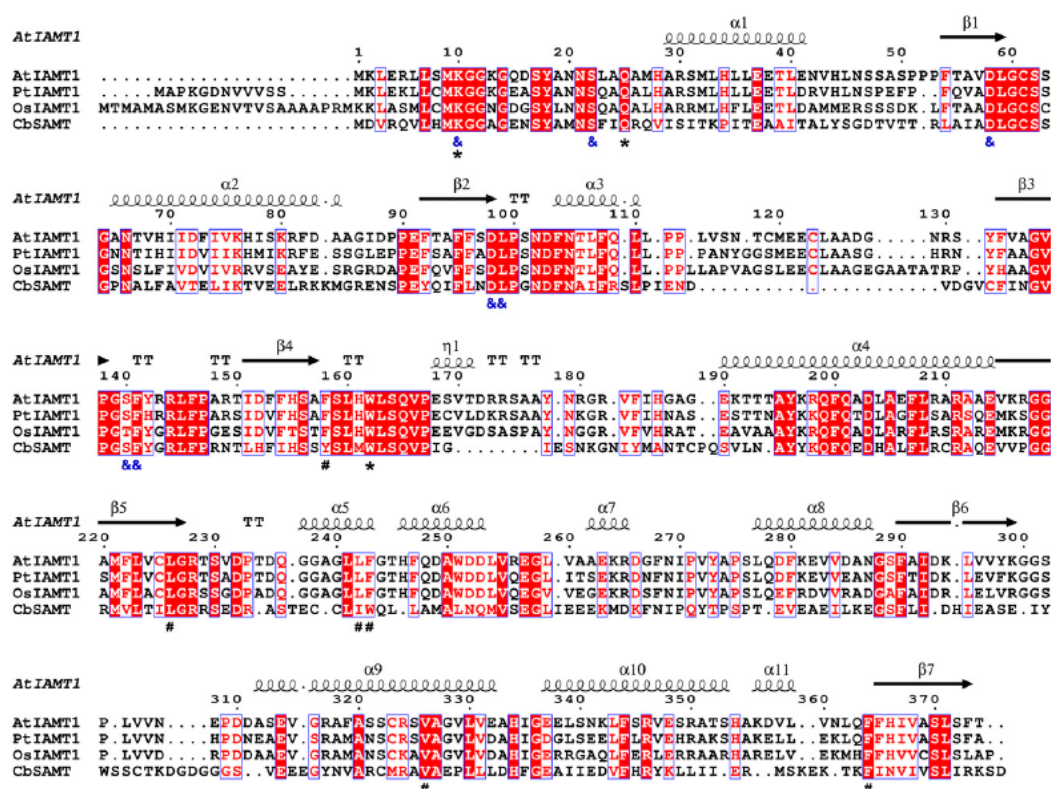


Figure 3.4. Structure-based sequence alignment of IAMTs and CbSAMT. The blue frames indicate conserved residues, white characters in red boxes indicate strict identity and red characters in white boxes indicate similarity. The secondary structure elements indicated above the alignment are those of AtIAMT1, whose structure has been experimentally determined and described here. Residues indicated with “&” below the alignment are SAM/SAH binding residues. Residues indicated 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 the substrate selectivity. CbSAMT is *Clarkia breweri* SAMT (Ross et al., 1999). PtIAMT1 is poplar IAMT (Zhao et al., 2007). OsIAMT1 is rice IAMT (this study). This figure was prepared with ESPript (Gouet et al., 1999).

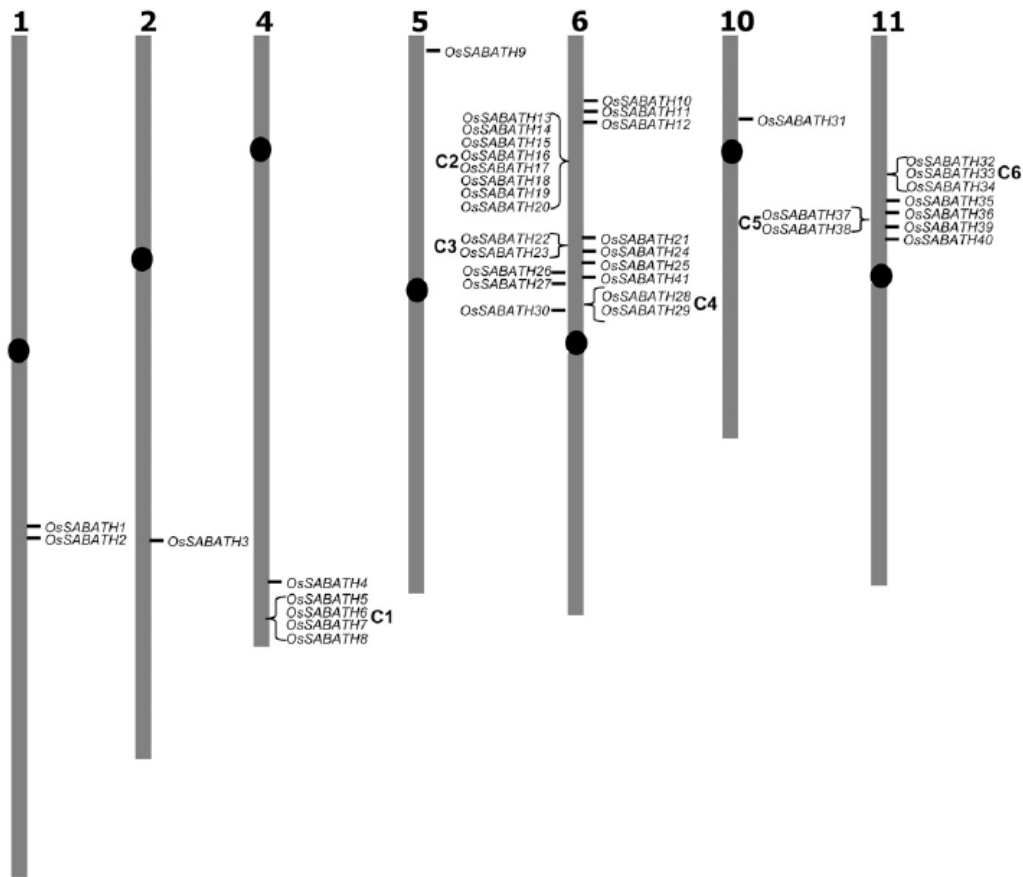


Figure 3.5. Chromosomal location of *OsSABATH* genes. Forty one *OsSABATH* genes are localized on seven chromosomes (1, 2, 4, 5, 6, 10 and 11). Twenty two genes are situated in six clusters (C1 to C6). In each cluster, neighboring genes are found either as tandem repeats or separated by one gene that is not a *SABATH* family member.

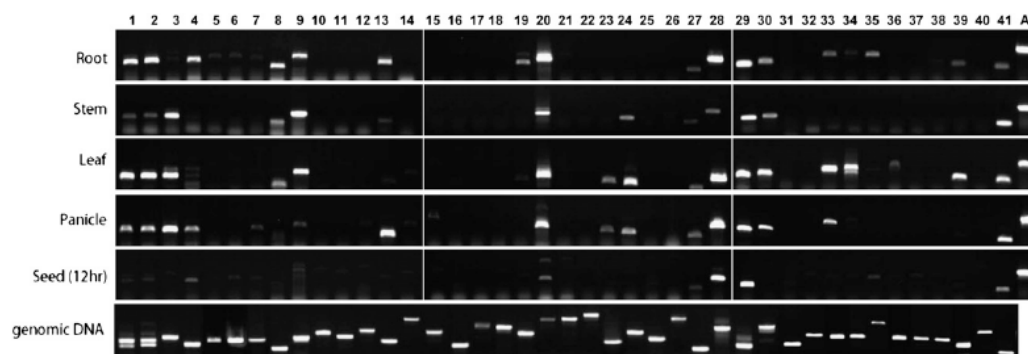


Figure 3.6. Expression analysis of rice *SABATH* genes. Total RNA was extracted from leaves, stems, roots of two-week old seedling, panicles from four-month old plants and germinating seeds, and used for RT-PCR. The numbers 1 to 41 on the top of the figure denote *OsSABATH1* to *OsSABATH41*. The expression of an actin gene (A) was used as an internal control. PCR using rice genomic DNA as template was performed to confirm the effectiveness of the primers used in the RT-PCRs.

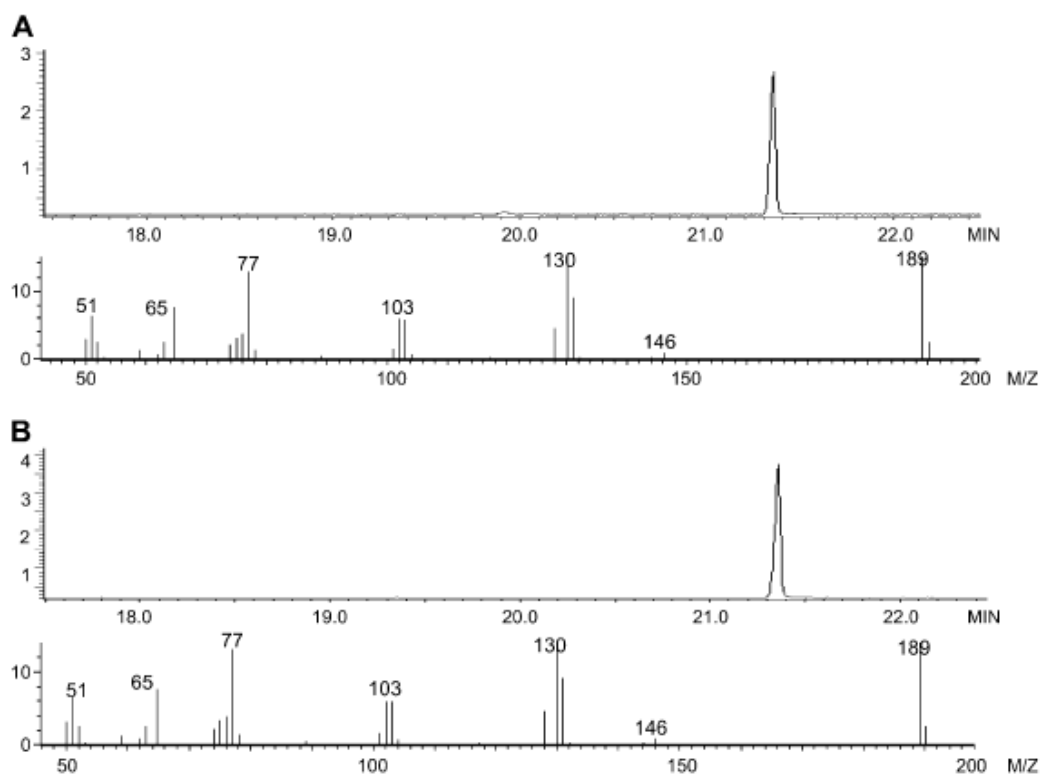


Figure 3.7. GC-MS analysis of the product of the enzyme assay catalyzed by recombinant OstIAMT1 protein with IAA as the substrate. A, GC chromatogram of the hexane extract of an OstIAMT1 enzyme assay with IAA and the mass fragmentation spectrum of the methylated product. B, GC chromatogram of a MeIAA authentic standard and its mass fragmentation spectrum.

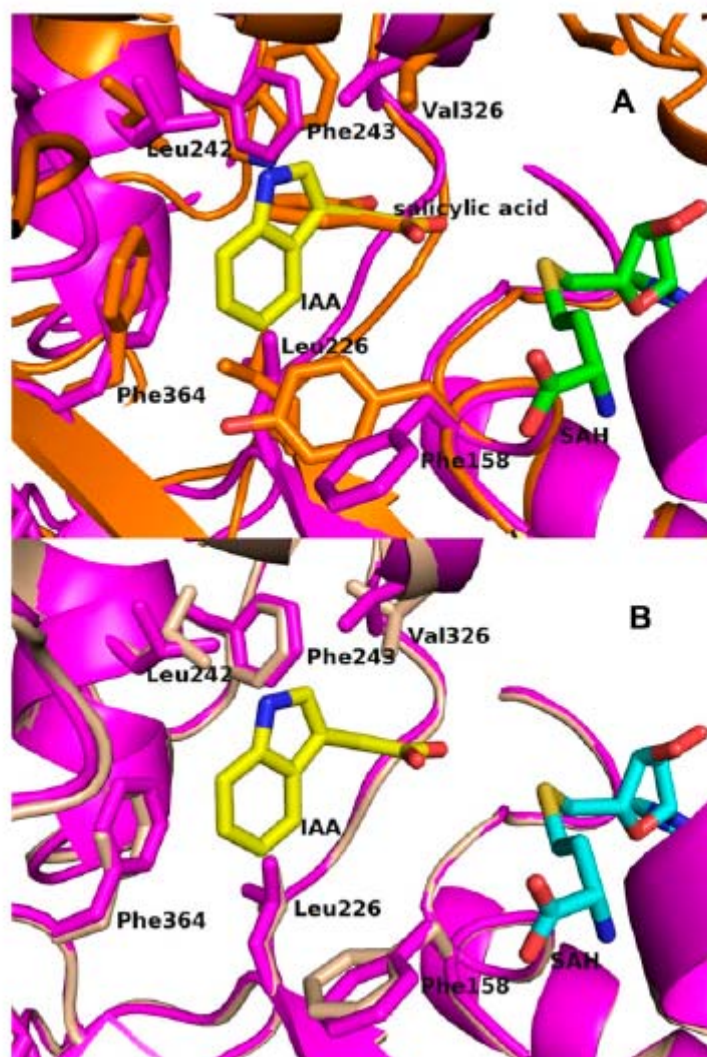


Figure 3.8. A, Comparison of AtIAMT (magenta) and CbSAMT (orange) active sites. B, Comparison of the active sites of AtIAMT1 (magenta) and a homology model of OsIAMT1 (wheat). The latter was calculated with Modeller (Sali and Blundell, 1993) based on the experimentally determined AtIAMT1 structure (magenta). Secondary structures are represented as ribbons and most of the active site residues involved in substrate recognition are represented as filled and color-coded bonds. SAH is represented as green sticks, IAA as yellow sticks and SAH as orange sticks. This figure was produced with PyMOL (<http://www.pymol.org>).

Figure 3.9. Continued

synthase 1 (accession # AB086414); pine 1 and spruce 1 represent SABATH-like proteins identified from pine and white spruce, respectively. Branches were drawn to scale with the bar indicating 0.1 substitutions per site.

```

      M K I E R D F H M M K G D S E F S Y A K
1  ATGAAGATCG AGCGAGATTT CCACATGATG AAAGGGGACA GTGAGTTCAG TTATGCCAAG
      N S R I Q K R V V L A A K P I V E K A V
61 AATTCAAGGA TCCAAAAAAG AGTTGTTCTT GCTGCCAAAC CAATAGTTGA GAAAGCTGTA
      R E V C I D L H P Q S M V I A D L G C S
121 CGGGAAGTGT GCATAGATCT TCATCCTCAA TCAATGGTCA TTGCTGACCT TGGCTGCTCC
      F G A N T L L F I S E V I T T I Y E D Y
181 TTCGGTGCAA ACACACTCCT CTTCATCTCT GAGGTGATCA CCACAATATA TGAGGACTAT
      N N T I K E S P M E V Q F F L N D L P S
241 AACAAACACCA TCAAGGAGAG CCCCATGGAG GTCCAGTTCT TCCTCAATGA CCTACCAAGC
      N D F N H I F Q S L E Q F E Q L I T Q Y
301 AATGACTTTA ACCACATCTT CCAATCACTA GAGCAGTTCG AGCAGTTGAT AACGCAATAT
      C A C K G L Q P P P H F V A G L P G S F
361 TGCCTTGCA AAGGGTTACA ACCTCCCCCG CACTTTGTCG CAGGCCTGCC AGGTTCC TTC
      Y T R L F P C N S V H L F H S S M S V M
421 TACACTAGAC TCTTCCCTTG TAATAGCGTC CATCTCTTCC ATTCCTCCAT GAGCGTCATG
      *
481 TGACTCTCTC AGGTTCTGTA GCAGCTTGAT GGCAGCATGA ATGAGGGGAA CATTACATA
541 GGAGCGACTA CACCACCATC CGTGGCAAAG CTCTACCAAA ATCAGTTTGA GAAAGACTTC
601 TCACGGTTCC TCCAGATGAG ATGCATGGAG ATTGTGCCCC GAGGCCGGAT GGTGCTGACG
661 GTTGCTGGGA GGAAGAGCAA AGATGTGTTC AATGCAGGAG GGACGACCAC GATATTTGAT
721 TTGCTTTTCA AAGGGCTACG TATTCTTGTT GCTGAGGGTC GTGTTGCCAA GGAGAAGCTG
781 GACTCTTTCA ACATTCCAGT GTACTGCCCT TCAGCTGATG AGCTGACGCA GCTGGTGCA
841 CAGTGCGAGC TGCTTGACAT AAGTGACATT CAACTCTTCG AGATGGATGA GAACCGCATG
901 CATGACTCGG AGCAAGCAGA GGGCACCACC GCCGCTCACA CAGCGGGACA GAGCATGTCT
961 GCAACTCTAA GGGTGGCGAC AGAGTCCCTG GTAGCAAGCC ATTTTGGGGA GGACATACTC
1021 GAGGAGATCT TCACGGTGTT TGCACGTAAT TTCACTAGTT ATATTGAGAG TGAGGTTGAG
1081 AAAAGTGGCA TCACCATCAT CACACTGTAC TTGCAGGCAA AACACTAG

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Figure 3.10. *OsSABATH33* is a pseudogene. It encodes a truncated protein due to the presence of a premature stop codon at the positions of 481 to 483 of the coding sequence.

Chapter IV. Emission, Biosynthesis and Regulation of Insect-induced Benzenoid Esters from Rice Plants

Adapted from:

Nan Zhao, Ju Guan, Jean-Luc Ferrer, Nancy Engle, Mawsheng Chern, Pamela Ronald, Timothy J. Tschaplinski, and Feng Chen. Emission, Biosynthesis and Regulation of Insect-induced Benzenoid Esters from Rice Plants. Submitted

Abstract

Two benzenoid esters, methyl salicylate (MeSA) and methyl benzoate (MeBA), are among the volatiles emitted 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 *E. coli*-expressed recombinant proteins showed that only one of the five candidates, OsBSMT1, has salicylic acid (SA) methyltransferase (SAMT) and benzoic acid (BA) methyltransferase (BAMT) activities for making MeSA and MeBA, respectively. Recombinant OsBSMT1 in its native form was purified from *E. coli* crude extract via two steps of ion exchange chromatography. Kinetic studies showed that OsBSMT1 has K_m values of 37.4 μ M and 43.2 μ M with SA and BA, respectively. While 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. 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. Such regulation appears to modulate the production and emission of not only MeSA and MeBA but also other

insect-induced volatiles such as terpenoids.

Keywords: Salicylic acid; Benzoic acid; Methyl salicylate; Methyl benzoate; Methyltransferase; SABATH; OsBSMT1; NH1; Rice

Introduction

Upon insect herbivory, many plants emit elevated levels of volatile organic compounds (Pare and Tumlinson, 1997). These volatiles can defend plants indirectly by attracting carnivorous enemies of the herbivores (predators and parasitoids) (Takabayashi and Dicke, 1996). Methyl salicylate (MeSA) is a frequent constituent of insect-induced plant volatiles. It has been detected in the headspace of insect-infested plants of lima bean (Arimura et al., 2002), *Arabidopsis* (Chen et al., 2003), tomato (Ament et al., 2004), and soybean (Zhu and Park, 2005). When tested in its pure, synthetic form, MeSA can attract several types of carnivores (Dicke et al., 1990, De Boer and Dicke, 2004; Zhu and Park, 2005), 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 (Ulland et al., 2008), indicating that MeSA can also be explored by herbivores.

In plants, MeSA is synthesized by the action of S-adenosyl-L-methionine (SAM):salicylic acid (SA) carboxyl methyltransferase (SAMT) using SAM as the methyl donor and SA as the methyl acceptor (Ross et al., 1999). SAMT belongs to a protein family called the SABATH (D'Auria et al., 2003). In addition to SA, other known carboxylic acid substrates of the SABATH family include benzoic acid, a benzenoid structurally highly similar to SA, jasmonic acid (JA) (Seo et al., 2001), indole-3-acetic acid (Qin et al., 2005; Zhao et al., 2008), and gibberellic acids

(Varbanova et al., 2007). Some members of the SABATH family are involved in caffeine biosynthesis by catalyzing nitrogen methylation (Ogawa et al., 2001). *SAMT* genes have been isolated and biochemically characterized from a number of plant species that include *Clarkia breweri* (Ross et al., 1999), snapdragon (Negre et al., 2002), *Stephanotis floribunda* (Pott et al., 2004), *Nicotiana suaveolens* (Pott et al., 2004), and Arabidopsis (Chen et al., 2003). The majority of these genes are involved in making MeSA as a floral scent compound. The Arabidopsis gene, *AtBSMT1*, is the only gene involved in making herbivore-induced MeSA (Chen et al., 2003). Interestingly, *AtBSMT1* is phylogenetically more related to JA methyltransferase (JMT) than to other SAMTs (Chen et al., 2003). 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, rice was established as a model for studying the molecular and genomic basis of volatile-mediated indirect defense in my lab (Yuan et al., 2008). Rice plants when damaged by fall armyworm (*Spodoptera frugiperda*) larvae (FAW) emit about 30 volatiles including MeSA. 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 making insect-induced rice volatiles were identified based on the correlation between volatile production and gene expression profiling using microarray. Three terpene synthase genes were biochemically verified (Yuan et al., 2008). The 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, including MeSA, in indirect defense. Here I report the isolation and characterization of rice genes for production of insect-induced MeSA. Previous study showed that the rice genome contains 41 *SABATH* genes (Zhao et al., 2008). One of them encodes indole-3-acetic acid MT (IAMT). Another one was shown to have SAMT and BAMT activities (Koo et al., 2007). In this report, candidate genes for making insect-induced MeSA would be systematically identified based on correlation of MeSA production with expression of all rice *SABATH* genes.

In addition to identification of *SAMT* genes for making herbivore-induced MeSA, it is also my interest to understand how the production of insect-induced MeSA is regulated. Previous studies showed that the JA signaling pathway is involved in regulating MeSA emission. When applied with exogenous JA, both lima bean (Dicke et al., 1999) and rice (Lou et al., 2005) plants release elevated levels of MeSA similar to those induced by herbivores. When tomato mutant plants deficient in accumulation of JA were treated with spider mites, emission of MeSA was significantly reduced compared to wild type plants (Ament et al., 2004). 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 crosstalk to regulate plant defense responses (Li et al., 2004). Little

is known on whether the SA pathway has a role in the regulation of MeSA production. Presumably the SA pathway can regulate MeSA production at two levels: the substrate availability level and the signal transduction level. Measurement of SA levels in tissues of insect-treated rice plants will help us understand the role of substrate regulation. Analysis of MeSA emission from insect-treated mutant rice plants with altered SA signaling pathway will help understand the role of signal transduction pathway on MeSA production. Previous studies showed 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 (Chern et al., 2005). In this study, NH1ox plants were studied for understanding the regulatory role of the SA signaling pathway in production of MeSA induced by herbivores.

Material and Methods

Rice Lines, Plant Growth and Treatments

Nipponbare and LiaoGeng (LG), both of japonica subspecies, were used as wild types plants. NH1 lines were produced in the LG background (Chern et al., 2005). Rice seeds were dehulled and germinated at 30 °C for five days. The seedlings were planted and grown in the growth chamber at 26 °C with 16 hours of light for two weeks. The rice plants were then subjected to the treatment of rice fall armyworm (*Spodoptera frugiperda*) for 15 hours with about four armyworms per plant. 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 off

from the base of the petiole and detached leaves stood up in a small glass beaker containing 10 ml of 5 $\mu\text{g/ml}$ alamehithicin (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 15h after the treatment. For JA treatment, Jasmonic acid (Sigma-Aldrich, St. Louis, MO) were dissolved in 100% ethanol to make 50mM stock solution. The jasmonic acid working solution is made by one to five dilution of stock solution in water. The jasmonic acid working solution was evenly applied to incisions produced by wounding treatment with cotton swab. Plants were placed in a 1 liter glass jar for 15 hours. After the 15 hours' treatments, the rice plants were separated to two groups, one group was used for volatile collection, and the other group was used for RNA extraction and SA measurement. Plants after treatments were subject to either headspace volatile collection. For RNA isolation or measurement of SA levels, above-ground parts of the plants were collected and grounded in liquid nitrogen. The powder was used for either RNA extraction or SA measurement.

Volatile Collection and Identification

Volatile collection was performed as previously described (Yuan et al., 2008). Eight treated or untreated rice plants grown in a single glass jar were wrapped in aluminum foil for the root part and put in a 1-L bell jar, respectively. Emitted volatiles were collected for four hours on activated charcoal traps that had been fitted into a steel column under continuous air circulation. Volatiles were eluted with 50 μL of

CH₂Cl₂. Samples from volatile collections were eluted into methylchloride and analyzed with Shimadzu QP5050A GC-MS instrument. The experiments were carried out at the following conditions, flow rate of 5mL/min of carrier gas Helium, a splitless injection of 3 µL, and a temperature gradient of 5°C/min from 40°C (3-min hold) to 240°C.

RNA Isolation

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

Semi-quantitative RT-PCR

Total RNA extraction from control and insect damaged rice plants was used for expression analysis of rice *SABATH* genes. Semi-quantitative RT-PCR expression analysis was performed as previously described (Chen et al., 2003). Primer sequences and sizes of PCR products of rice *SABATH* genes are shown in Table 4.1. All primers were tested with genomic DNA to confirm their effectiveness. 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 sec, 54 °C for 45 sec and 72 °C for 60 sec, and then followed by an extension step of 72 °C for 10 min.

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 (Chen et al., 2003). Primers used for PCR amplification were shown in table 4.2. 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, Carlsband, CA). The cloned cDNA in pEXP5/CT TOPO vector was sequenced using T7 primers.

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). 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 with FPLC system as previously described (Ross et al., 1999). The cell lysis was loaded onto the DE53 cellulose column (Whatman, Maidstone, England), which was installed in a Pharmacia FPLC apparatus and preequilibrated with buffer A (containing 50 mM Tris-HCL, pH 7.0, 10% glycerol, 10mM β-mercaptoethanol). Flow rate was 1 ml/min.

After the enzyme was loaded, the column was washed with 20 ml buffer A containing a linear gradient of KCL from 0 to 200mM, and eluted with a linear gradient from 200 to 400 mM KCL in buffer A. Fractions were collected and assayed for enzyme activity. The fractions with enzyme activity were pooled together and diluted threefold with buffer A, and then were loaded onto the HiTrap Q FF column (GE Health Life Sciences, San Francisco, CA), which was installed in a Pharmacia FPLC apparatus and preequilibrated with buffer A. Flow rate was 0.5 ml/min. After that, the column was washed with a linear gradient from 0 to 100 mM KCL and eluted with a linear gradient from 100 to 400 mM KCL in buffer A. Fractions were collected and enzyme activity was determined. Protein purity was verified by SDS-PAGE and protein concentration was determined by the Bradford assay.

Screen for SAMT Specificity

For the substrate screening, radiochemical assays were performed with a total volume of 50 μ L containing 50 mM Tris-HCl pH 7.5 buffer, 5 μ L protein, 0.5 μ L [^{14} C]-SAM (specific activity 52.7 mCi/mmol, Perkin Elmer Instruments, Shelton, CT), and 1 μ L of 50 mM SA as substrate. The reaction was incubated at room temperature for 30 min, after which the products of the assay were extracted with 150 μ L ethylacetate and counted in scintillation counter.

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 K_m 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 K_m 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 yielded apparent K_m values as previously described (Chen et al., 2003). Final values represent the average of three independent measurements.

Homology-based Structural Modeling and In Silico Docking Experiments

The structural homology model of OsSBMT1 was built with Modeller (Sali and Blundell, 1993): first, a sequence alignment against 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 potent substrates was then performed with the Glide program (Friesner et al., 2004), which is part of the SchrödingerTM suite (Schrödinger, LLC, New York, NY, 2005).

Salicylic Acid Measurement

Approximately 50mg grounded powers were quantitatively transferred to scintillation vials and dried down in a helium stream. The dried samples were dissolved in 500 μL of silylation–grade acetonitrile followed by the addition of 500

1- μ L N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce Chemical Co., Rockford, IL), and then heated for 1 h at 70 °C to generate trimethylsilyl (TMS) derivatives. After 2 days, 1- μ L aliquots were injected into a ThermoFisher DSQII GC-MS, fitted with an Rtx-5MS (crosslinked 5% PH ME Siloxane) 30 m x 0.25 mm x 0.25 μ m film thickness capillary column (Restek, Bellefonte, PA). The standard quadrupole GC-MS was operated in electron impact (70 eV) ionization mode, with 6 full-spectrum (70-650 Da) scans per second. 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 (1) 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 (1) injected versus peak area integration of the extracted m/z. Final values are an average of three independent measurements.

Quantitative Real-time PCR

To determine the expression of OsBSMT1 after treatments, Nipponbare was treated by physical wounding, JA, alamethicin (Ala) and fall armyworm. And LG and NH1 lines were treated by fall armyworm. After treatments, above ground parts

of the plants were used for quantitative real-time RT-PCR analysis. Quantitative real-time quantitative PCR (RT-PCR) was conducted on an ABI7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR green fluorescence dye (Bio-Rad Laboratories) as previously described (Yang et al., 2006). The gene-specific primers were designed as followed: forward primer 5'-TCAAGAGAAGGCCATCCTGAA -3' and the reverse primer 5'-AGAGCGACGCGTGAGCTT. The two primers used for the PCR amplification of *actin* were designed as the internal control: forward primer 5'-GACTCTGGTGATGGTGTGTCAGCGAC -3' and reverse primer 5'-CTGCTGGAATGTGCTGAGAGATGC-3'.

Results

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 ± 0.37 ng/g FW/h. When the rice seedlings were damaged by FAW larvae for overnight, the emission of MeSA increased approximately 23 fold to an emission rate of 64.8 ± 1.9 ng/g FW/h (Figure 4.1A). 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 FW/h. This compound was, however, not detectable from control rice plants (Figure 4.1B).

Identification of A Rice SABATH Gene Encoding SAMT

The rice genome contains 41 *SABATH* genes, which were named *OsSABATH1* to *OsSABATH41* (Zhao et al., 2008). Previous microarray analysis examining gene expression changes in control vs. FAW-treated rice plants showed that two genes, *OsSABATH3* (Os02g48770) and *OsSABATH9* (Os05g01140), were significantly up-regulated by FAW feeding (Yuan et al., 2008). However, the coverage of the *SABATH* family on the microarray slides in the previous analysis was very low, with only three *SABATH* genes present. To identify additional candidate genes for MeSA and MeBA biosynthesis, RT-PCR approach was used to compare expression of all rice *SABATH* genes in control vs. FAW-treated rice plants.

Semi-quantitative RT-PCR showed that three *SABATH* genes, *OsSABATH13* (Os06g13460), *OsSABATH14* (Os06g13470) and *OsSABATH34* (Os11g15060), were up-regulated by FAW feeding (Figure 4.2). 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 *E. coli* and recombinant proteins tested for methyltransferase activity using SA and BA as substrates. Results showed only *OsSABATH3* (Os02g48770) exhibited activity with both SA and BA. The other three proteins did not show methyltransferase activity with either SA or BA. As there is still the

possibility that other rice SABATH proteins having SAMT and/or BAMT activities, I adopted the name *OsBSMT1*, the name designated for this gene previously (Koo et al., 2007), for *OsSABATH3*.

Biochemical Properties of OsBSMT1

Recombinant *OsBSMT1* was purified in its native form using two steps of ion exchange chromatography (Figure 4.3). Purified *OsBSMT1* was used for detailed biochemical characterization. Purified *OsBSMT1* was assayed with a group of potential substrates for SABATH proteins. The most efficient activity was observed in the reactions with SA. *OsBSMT1* also showed activity with BA, the relative activity of BA is about 42% of SA activity. It also showed high activity with Nicotinic acid and Anthranilic acid, the relative activity is about 16% and 14.5%, respectively. *OsBSMT1* showed some activity with myristic acid, 3-hydroxyl benzoic acid and 4-hydroxyl benzoic acid. No activity was tested with other substrates (Table 4.3). Under steady-state conditions, *OsBSMT1* exhibited K_m values of $37.4 \pm 0.9 \mu\text{M}$ and $43.2 \pm 3.7 \mu\text{M}$ for SA and BA, respectively. The k_{cat} values of *OsBSMT1* using SA and BA as substrate were 0.003 s^{-1} and 0.004 s^{-1} respectively.

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 (Figure 4.4).

While the majority of characterized SAMTs cluster together, rice OsBSMT1 and Arabidopsis AtBSMT1 were situated in different clades.

Structural Modeling of OsBSMT1

A homology model of OsBSMT1 was built by using the experimentally determined structure of *Clarkia breweri* SAMT (CbSAMT) (Zubieta et al., 2003) with SA as the template. This model was then used as a target for *in silico* docking experiment, in order to rank the affinities for the different potent substrates, and to analyze their binding mode. Interestingly, both SA and BA come at exactly the same place, at the right distance from the SAH, with the right orientation of their carboxyl group for methylation (Figure 4.5). The aromatic ring of SA is rotated 180° with respect to the one observed in CbSAMT structure, presenting its OH to nothing, making no significant interaction with it. As a consequence, SA and BA dock in the same way with very close score (Gscore values of -5.98 and -5.65 respectively). The corresponding theoretical K_d values of OsBSMT1 was calculated to be 35 μ M and 62 μ M with SA and BA respectively.

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 known as a potent volatile-inducer, and JA. Physical wounding appeared to lower the expression of

OsBSMT1. In contrast, alamethicin treatment led to a slight induction of *OsBSMT1* expression. JA treatment led to significant up-regulation of *OsBSMT1* expression. The induced levels were similar to those induced by herbivory (Figure 4.6A).

To determine whether the emission of MeSA and MeSA correlated with the expression of *OsBSMT1*, the emission of MeSA and MeSA from rice plants treated with the same stress factors used for gene expression analysis were analyzed using headspace analysis. Physical wounding reduced the emission of MeSA. In contrast, alamethicin treatment led to slight induction in emission of the compounds. JA treatment led to significant increases in emission of MeSA. The emission was however much lower than that from insect-treated plants (Figure 4.6B).

Role of the SA Signaling Pathway in Regulating OsBSMT1 Expression, Product Emission and SA Contents

To further understand the potential regulation of the SA pathway on emission of MeSA, transgenic rice plants with altered SA pathway was 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 (Chern et al., 2005). Previous studies showed SA-dependent plant defenses in transgenic rice plants overexpressing NH1 gene (NH1ox) were altered (Chern et al., 2005). Quantitative real-time PCR was performed to examine expression of *OsBSMT1* in NH1ox and 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 (Figure 4.6A). Expression of *OsBSMT1* was induced 5.6 fold in NH1ox plants (Figure 4.7A).

Emissions of MeSA and MeBA from LG and NH1ox plants with or without insect treatment were analyzed. Both untreated LG and NH1ox plants emitted MeSA. The emission rates were 34.8 ± 6.6 ng/g FW/h and 31.9 ± 3.1 ng/g FW/h respectively. The emission in both LG and NH1ox was induced by FAW feeding. FAW-damaged NH1ox plants displayed a higher emission rate of MeSA, about 135.0 ± 6.3 ng/g FW/h compared to FAW-damaged LG plants, the emission rate of which is 87.8 ± 2.4 ng/g FW/h (Figure 4.7B).

The contents of free SA in control and insect-treated LG and NH1ox plants were also determined. Similar to previous studies, the contents of SA in control NH1ox plants were lower than those in wild type LG plants. For both LG and NH1ox plants, insect-feeding led to a slight increase in the levels of SA (Figure 4.7C).

Emission of other FAW-induced Volatiles from LG and NH1ox Plants

FAW-feeding induced the emission of a large number of volatile in addition to MeSA and MeBA (Yuan et al., 2008). Emission rates of these volatiles from LG and NH1ox plants treated by FAW larvae were compared. Two compounds derived from fatty acid degradation, nonanal and decanal, showed lower levels of emission from NH1ox plants than those from LG plants. In contrast, the emissions of terpenoids, for

example linalool, (E)- β -caryophyllene and zingiberene, from FAW-damaged NH1ox plants were significantly higher than those from FAW-treated LG plants (Figure 4.8).

Discussion

In this chapter, I report the isolation and functional characterization of rice *OsBSMT1*. Recombinant *OsBSMT1* displayed specific methyltransferase activities with SA and BA. The dual activity 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 of *OsBSMT1*. The JA signaling pathway was shown to regulate the production and emission of MeSA. Analysis using 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.

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 (Ross et al., 1999; Negre et al., 2002; Pott et al., 2002; Fukami et al., 2002; Murfitt et al., 2000; Dudareva et al., 2000; Chen et al., 2003; Negre et al., 2003; Pott et al., 2004; Effmert et al., 2005; Barkman et al., 2007),

the identification of the rice gene for making MeSA was not straightforward. The rice genome contains 41 *SABATH* genes (Zhao et al., 2008). On the phylogenetic tree, there is no a single rice *SABATH* gene that is clustered in the same clade as known SAMTs or BSMTs (Figure 4.4). By correlating MeSA emission with expression of all rice *SABATH* genes, the number of candidate genes was narrowed down from 41 to 5. Biochemical study showed 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 need 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 to be isolated from a monocot. Phylogenetic analysis showed that OsBSMT1 is not closely related to dicot SAMTs (Figure 4.4). 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 negative selection. In addition to rice, other monocots, such as maize (Hammack, 2001) and sorghum (Zhuang and Chen, unpublished) also emit MeSA. In the preliminary sequence analysis of the sorghum genome, one sorghum gene highly related to OsBSMT1 was identified. The isolation and characterization of this sorghum gene is underway. If this gene is verified to be a SAMT, it would strongly suggest that monocot SAMTs were evolved after the split of monocot and dicot lineages. In addition, the evolution of dual activities of OsBSMT1 is intriguing. The

study with *Arabidopsis* BSMT showed that the ancestor of AtBSMT1 may have higher activity with BA, i.e., the SAMT activity of AtBSMT1 was evolved from BAMT activity (Chen et al., 2003). Insights into the evolutionary trajectory and trend of OsBSMT1 activities will be provided with the continued isolation and characterization of bona fide SAMTs from a wide range of plant species covering diverse taxons.

Biochemical Properties of OsBSMT1 and Its Structural Features

The biochemical properties of OsBSMT1 had been previously reported (Koo et al., 2007). The K_m values of OsBSMT1 with SA and BA in that report were 80 and 80, respectively, which is about one fold higher than K_m values determined in this analysis. The discrepancy is probably due to the recombinant enzyme. In the previous study, OsBSMT1 was purified with an N-terminal tag of 20 KD (Koo et al., 2007). It has been reported that the presence of a tag at the N-terminal of CbSAMT affects the kinetics of the enzyme (Ross et al., 1999). 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 much closer to the *in vivo* kinetics of OsBSMT1.

The *in vitro* dual activity of OsBAMT1 is supported by structural studies. Indeed, *in silico* docking experiments performed on the homology model show that both SA and BA bind at the same place that SA in SAMT, presenting their carboxyl at

the right distance and with the right orientation to SAM. However, it revealed a preferred orientation for the SA aromatic cycle different with respect to SAMT, with a 180° flip so that it presents its OH in the pocket closed by the loop-helices N-terminal fragment. The reduced contribution of this hydroxyl 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. It can be noticed that calculated K_d are compatible with the measured affinity, providing a validation of the *in silico* docking calculation.

Biological Roles of OsBSMT1

OsBSMT1 is expressed in multiple tissues of rice plants grown under normal growing conditions (Zhao et al., 2008), suggesting that this gene has a role in the normal biology of healthy plants. In Arabidopsis, *AtBSMT1* also showed expression in multiple tissues of healthy plants. In leaf, *AtBSMT1* is specifically expressed in trichomes (Chen et al., 2003). In Arabidopsis, *AtBSMT1* was suggested to produce MeSA and MeBA as toxic compounds. *OsBSMT1* may have a similar role. Interestingly, *OsBSMT1* showed expression in panicle (Zhao et al., 2008), where the gene may also have a defense role. Recently, the product of SAMT, MeSA, was shown to be a mobile signal for SAR (Park et al., 2007). It will be interesting to determine whether MeSA plays a same role in rice. In that case, the MeSA will function as an internal signal.

The specific induction of *OsBSMT1* by herbivory and enhanced emission

support that MeSA has a specific role in plant response to insects. In the context of indirect plant defense, MeSA has been shown to be an active signal in attracting a number of carnivorous species (Dicke et al., 1990, De Boer and Dicke, 2004; Zhu and Park, 2005). The induced emission of MeSA in rice may play a same role. The role of MeSA in direct defense should also be considered. Recent study showed that insect-induced plant volatiles can function in plant-plant communications, thereby volatiles can prime or activate defense responses in nearby healthy plants (Ton et al., 2007). MeSA as an air-borne signal has been shown to mediate such inter-plant interactions in the context of plant-viral interactions (Shulaev et al., 1997). MeSA may play a same role for plant defense against insect pests.

The biological significance of dual activity of OsBSMT1 with SA and BA is not clear. The higher level production of MeSA suggests that the SAMT activity is more biologically important. Our data showed the in vitro activity of OsBSMT1 with BA is biologically relevant. Because OsBSMT1 showed highly similar activities with SA and BA, the production of MeSA or MeBA may be determined by the availability of their precursors: SA and BA. The role of MeBA is less investigated. The highly homology of the structures of MeBA and MeSA suggest that MeBA may play a similar role as MeSA.

Regulation of Production of Herbivore-induced Airborne MeSA

Application of exogenous JA led to induction of MeSA emission from rice plants

(Figure 4.6B), suggesting that the JA signaling pathway plays a central role in regulation of production of insect-induced MeSA. At least part of this regulation is through the regulation of transcription of OsBSMT1, which expression was induced by JA treatment (Figure 4.6A). In both wild type and NH1ox plants, herbivory did not cause significant changes in SA content (Figure 4.8), suggesting that the levels of SA is not important, or a minor factor, in regulating production of MeSA. Whether such regulation is species-specific awaits to be determined. Rice appears to have a SA metabolism different from that in Arabidopsis or tobacco. Rice is one of the plant species that contains higher levels of SA at tissues of plants grown under normal conditions (Silverman et al., 1995). The high levels of SA may explain why substrate availability is not a critical factor of regulation of 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. NPR1 is a key regulator of the SA-mediated system acquired resistance (SAR) (Cao et al., 1994; Shah et al., 1997). NPR1 encodes a novel protein with a bipartite nuclear localization sequence. Under unchallenged conditions, NPR1 protein exists as oligomer and is excluded from the nucleus. When SAR is induced, monomeric NPR1 is formed, which accumulate in the nucleus and activate the expression of PR genes (Mou et al., 2003). In addition to its role in SAR, NPR1 functions in cross-talk between SA- and JA-dependent defense signaling pathways (review see Pieterse and Van Loon, 2004). NH1 has a similar role

as that of NPR1. Overexpression of NH1 in rice led to enhanced resistance to *Xanthomonas oryzae* (Chern et al., 2005). Our data indicates 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 airborne MeSA, mainly through the regulation of *OsBSMT1* expression. Based on the fact that the JA plays an essential role in insect-induced MeSA emission, our data further suggest that NH1 regulates MeSA production by interacting with the JA signaling pathway. It will be interesting to determine whether the same components mediating SA-JA crosstalk involved in plant-pathogen interactions are also involved in regulating SA-JA crosstalk in plant-insect interactions.

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Appendix

Table 4.1. Primers for gene expression analysis of *OsSABATHs* via RT-PCR

Gene Name	Forward Primer Sequences (5'-3')	Reverse Primer Sequences (5'-3')
<i>OsSABATH1</i>	ACCAACGACTTCAACACCATCTTCT	ACCTGAGGAACAGGCTGAAGTCTCTC
<i>OsSABATH2</i>	AGGGAAAGATGTACATATCAAGCACGAG	GTACGTCTGCACGTAGTCCATCCTGAAC
<i>OsSABATH3</i>	GAAGGCTCTGCCTCCCTACTACATT	AAGAAGCCCATACATGTAGCTCACT
<i>OsSABATH4</i>	ACGTGTTACCTCCACCTTCTCTTT	GGATGTTGAAGCTGTCCCTCTTCTC
<i>OsSABATH5</i>	TATGAAGTGTATTGCAGCATGTTGAG	TTACACCAAGTGAAAGCGAGCATAGAAT
<i>OsSABATH6</i>	ACGGATCAAGGCTACATCTATGAAGTG	GAAAGTGAGCATAGAATATGGGGGAAG
<i>OsSABATH7</i>	CAGCTTCTATACGGCGCCATGTTT	CAGCGAAAGGGAACACACAATATG
<i>OsSABATH8</i>	CGTACAACAAGGAGAAGGTGTTGCTC	GACGTTGAAGCTGTCCATCCTCTC
<i>OsSABATH9</i>	GATAGCCTGGACTACGTGGTGTGCT	CTAGATCTGCTTGGGATTGGCATGAG
<i>OsSABATH10</i>	ACTAGAGGCTAGTGGAAAATCTCTCTTG	GACTCCAGGACTGACCTAATGAGATTAC
<i>OsSABATH11</i>	CACAAAGGAGATATACTACCTGCATACC	CATTTACATCTCCACTATATGGGTCCTC
<i>OsSABATH12</i>	AAGGTAACCCAAGAAGTATACTGAGTC	GTCCTTGTAAGTGTCTTGGAAATAGT
<i>OsSABATH13</i>	AGGATGAGGATGTATACAATGGAAGTC	CTATCAAAGACTCCAGCACAGATCTAA
<i>OsSABATH14</i>	AAGACTACCCCTAAATCGGTGATAAAG	GAAGAGCACGTCTAGTATAGATTCTCCA
<i>OsSABATH15</i>	ATCTAGTGGTCAGATGCTACTCACTTTC	ATAGCACTCCATCTCCATATGTTCTTG
<i>OsSABATH16</i>	GACCGAATACACTAGTCTTCATCTCTGA	ATGTAGGGAATACGATGAATGGAAGAG
<i>OsSABATH17</i>	GTGACTACACCTGAAGAAGTGATAAAGC	ATGAGTAGCAAAACACCTCTCCATAAC
<i>OsSABATH18</i>	AGTTGTACCAGGATCAGTTCAGAAG	ATGACAAGACTATGACCGAGTACTTCAAC
<i>OsSABATH19</i>	AACTATGCCAACAACCTAGGCTTC	AGCCCAGAGATATAGAATGGAGGTAAC
<i>OsSABATH20</i>	AGTAGCACTGCCTCCATTCTATATCTCT	ATACAGTGGTAGGTTGAATGACTCCAG
<i>OsSABATH21</i>	AGGACTTTTCATTGTTCTCTCACACT	GTACATAGCCCTTCTTTCTCAAGATG
<i>OsSABATH22</i>	CTACCTGGCAATGACTTTAATACGTCT	GAACAGTGAGAAGTCTCTTTGAAGTT
<i>OsSABATH23</i>	AGAAAAGCTTGACTCCTTCTACATTCC	TCACATCGCTCTAGTAAGAGACAAACA
<i>OsSABATH24</i>	CTCCAGATGATCTTAAGGAGGGTAAAA	GTGCGTAGAGTGGTATGTAGAAGGAAT
<i>OsSABATH25</i>	GTCATTCTATGAGAGGCTTTTCACTTC	GCTACAGATACCACTTTCCATACTTGAG
<i>OsSABATH26</i>	CATGGTACCTGGGTCAATTTATGATAG	GTGCATAGATAGGTATGTGGAATGTGTC
<i>OsSABATH27</i>	GGTGGGAGTATGGACAGCTTCAAC	CTCTCCATCCGGACAAATAGCTTG
<i>OsSABATH28</i>	GGAGTCCAGTTACCTCAAAAACAGTAA	AAGGTGGAGGTGAACACGTCGATAGAT
<i>OsSABATH29</i>	GGGACGATGGACAGCTTCAACAT	GTGGAAGTGGACCCCATCTCGT
<i>OsSABATH30</i>	AGCTCTGCGTACTCCTCAACGATCT	AGTTCTACAGCCCTCATCTTCAGAAAC
<i>OsSABATH31</i>	AGATTTGGCGTGGCTGACTTGGACT	AGGCTGAAAGAAGAGCAGACGAGAT
<i>OsSABATH32</i>	GATCCAATTCTTTCTCAATGACCTACC	CTGAATACATCTGTGCTATTCTCCCTA
<i>OsSABATH33</i>	AAGGAGAAGCTGGACTCTTTCAACATT	GCATACCATGAGCGCACTTTACTCTAT
<i>OsSABATH34</i>	AGGACCATAACAACATATCAAGGAGAG	ATCTCATTTGGAGGAACTGTGAGAAGT
<i>OsSABATH35</i>	GGTGACAAAGCTCTACAAAATCAGTT	TCATCGAGTATAGAATCCCCGAAAT
<i>OsSABATH36</i>	GGGTTACAACCTCCTCCGTACTATGTC	CTTGTGAAAGCAACTCGAAAATAGTGG
<i>OsSABATH37</i>	GGCAACATTTATATAGGGGTGACTACAC	GCTTTGTTGTACTAGCTGCTTCAGTTC
<i>OsSABATH38</i>	GAGGTCCAGATATTTCTCAATGACCTAC	CTCAAAGTGGTCCAAGTAGAGTTTTCTC
<i>OsSABATH39</i>	AAGAGAGCCAGTTGTTGACATCATAG	AGTGACACAGAAATGGCCATAATGC
<i>OsSABATH40</i>	CCGAGGTATAGCTTACAAGGAGGTATTT	CACTGCAAAGAGGTGATCGATTATT
<i>OsSABATH41</i>	ATGGACAGCTTCAACATCCGTCGTA	CTCTCCATCCGAACAACAGCTCAT

Table 4.2. Primers for cloning of full-length cDNAs of *OsSABATH* genes

Gene Name	Forward Primer Sequences (5'-3')	Reverse Primer Sequences (5'-3')
<i>OsSABATH3</i>	ATGAAGGTAGAGCAGGACCTCCACATG	GGCACGCTACTTATAAAATGCAACATC
<i>OsSABATH9</i>	ATGGTGGTAAACTGGCATGTACACATG	CTGACACGTGGTATATATTGTAGGTA
<i>OsSABATH13</i>	ATGGTCAATATCGAAGGCGATTTACAC	CTATATTTTCTTCAAGGACATGACGATGAC
<i>OsSABATH14</i>	ATGATTTCTATATCTATATATGCTACTTGTG	TTAGGAAACCCTCACCAATCCGACTCC
<i>OsSABATH33</i>	ATGAAGATCGAGCGAGATTTCACA	GCATACCATGAGCGCACTTTACTCTAT

Table 4.3. Enzymatic activities with other substrates

Substrates	Relative activity (%)
SA	100
JA	<1
BA	42
IAA	<1
GA3	<1
FA	<1
2,4 D	<1
3Hydroxyl benzoic acid	1.3
4Hydroxyl benzoic acid	2.7
7methxyl xanthanine	<1
Anthranilic acid	14.5
ABA	<1
acetic acid	<1
caffeic acid	<1
cinnamic acid	<1
coumaric acid	<1
Geranic acid	<1
IBA	<1
Cysteine	<1
Glycine	<1
Histidine	<1
lactic acid	<1
lauric acid	<1
linolenic acid	<1
myristic acid	3.8
Nicotinic acid	16
octanoic acid	<1
vanillic acid	<1
Phenol	<1
thiophenol	<1

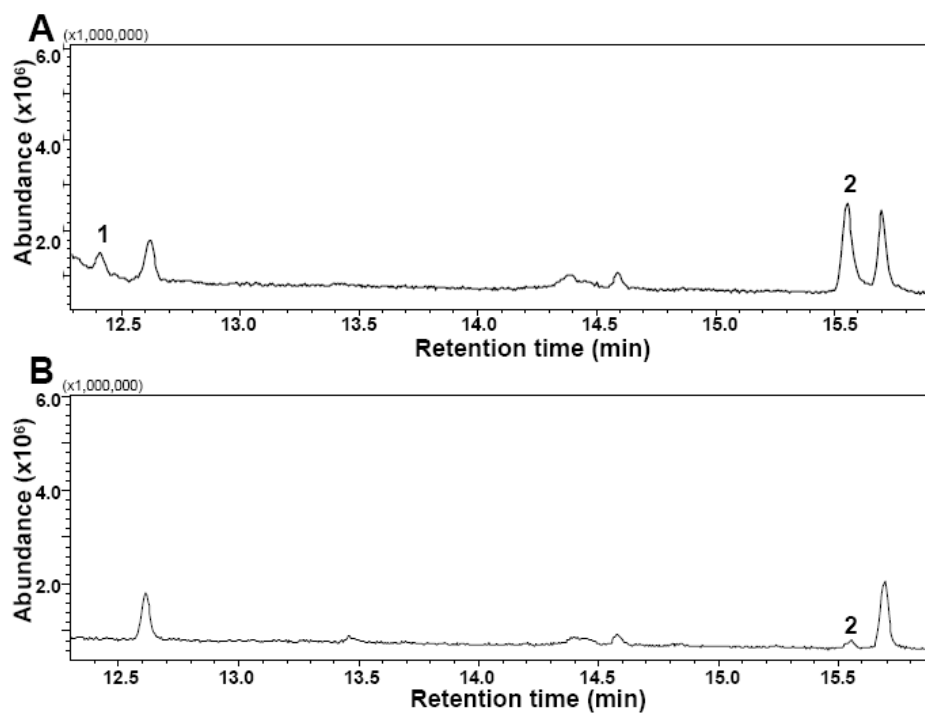


Figure 4.1. Emission of benzenoid esters from rice plants. Numbers “1” and “2” depict MeBA and MeSA respectively. A, insect-treated rice plants emitted both MeSA and MeBA. B, untreated control plants emitted only MeSA. MeBA was not detectable from control plants.

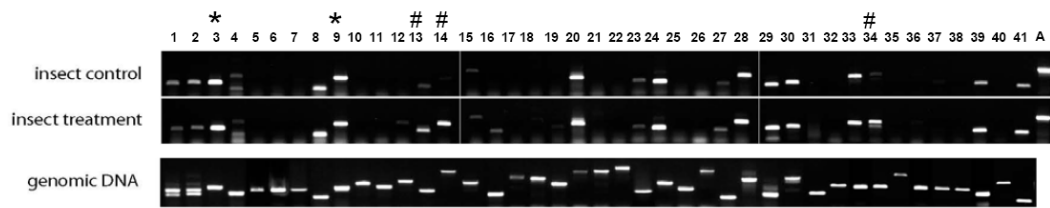


Figure 4.2. RT-PCR expression analysis of rice SABATHs. Total RNA was extracted from above-ground part of intact and FAW-damaged rice plants of two-week old seedlings and used for RT-PCR. The numbers 1 to 41 on the top of the figure denote *OsSABATH1* to *OsSABATH41*. The expression of an actin gene “A” was used as an internal control. PCR using rice genomic DNA as template was also performed to confirm the effectiveness of primers.

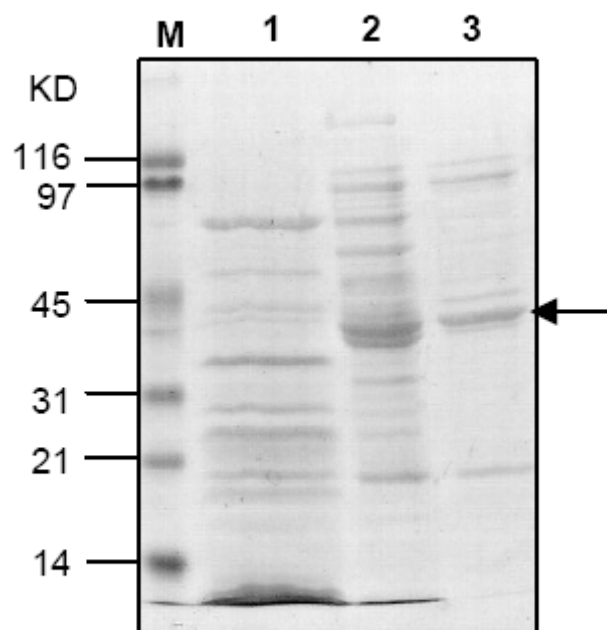


Figure 4.3. SDS-PAGE of purified recombinant OsBSMT1. Protein expressed in *E. coli* was purified as described in Materials and methods. Lane M contained protein molecular weight markers. Lane 1 contained OsBSMT1 crude extract, and lane 2 contained 1 μ g of DE53 fraction. Lane 3 contained 1 μ g of mono-Q fraction. The gel was stained with Coomassie Blue. Arrow indicates the target band for OsBSMT1.

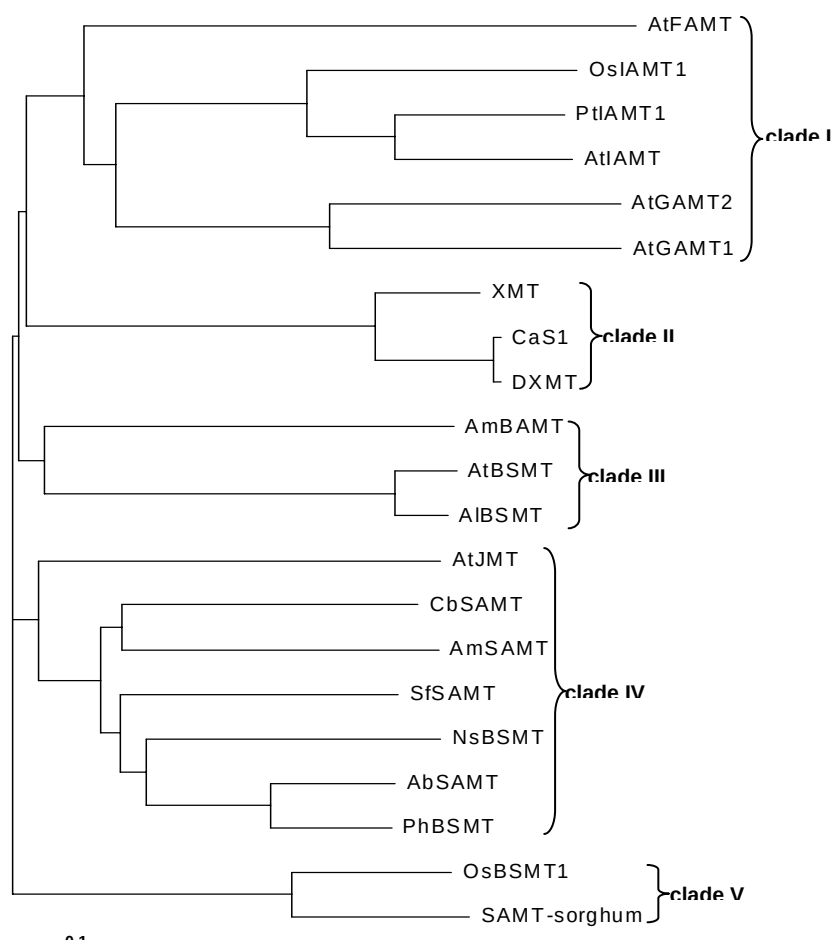


Figure 4.4. A neighbor joining phylogenetic tree based on protein sequence alignment of functionally characterized SAMTs and other selected SABATHs from plants using the clustalX program. CbSAMT, *Clarkia breweri* SAMT (AF133053); AmSAMT, *Antirrhinum majus* (snapdragon) SAMT (AF515284); SfSAMT, *Stephanotis floribunda* SAMT (AJ308570); AmBAMT, *Antirrhinum majus* BAMT (AF198492); NsBSMT, *Nicotiana suaveolens* BSMT (AJ628349); AtBSMT, *Arabidopsis thaliana* BSMT (BT022049); AIBSMT, *Arabidopsis lyrata* BSMT (AY224596); AbSAMT, *Atropa belladonna* SAMT (AB049752); PhBSMT, *Petunia hybrida* BSMT (AY233465); AtJMT, *Arabidopsis thaliana* JMT (AY008434); AtFAMT, *Arabidopsis*

Figure 4.4. Continued

thaliana FAMT (AY150400); OsIAMT1, *Oryza sativa* IAMT1 (EU375746); PtIAMT1, *Populus trichocarpa* IAMT1 (fgenesh4_pg.C_LG_I002808); AtIAMT, *Arabidopsis thaliana* IAMT (AK175586); AtGAMT1, *Arabidopsis thaliana* GAMT1 (At4g26420); AtGAMT2, *Arabidopsis thaliana* GAMT2 (At5g56300); Cas1, *Coffea arabica* caffeine synthase 1 (AB086414); CaXMT1, *Coffea arabica* XMT1 (AB048793); CaDXMT1, *Coffea arabica* DXMT1 (AB084125). Branches were drawn to scale with the bar indicating 0.1 substitutions per site.

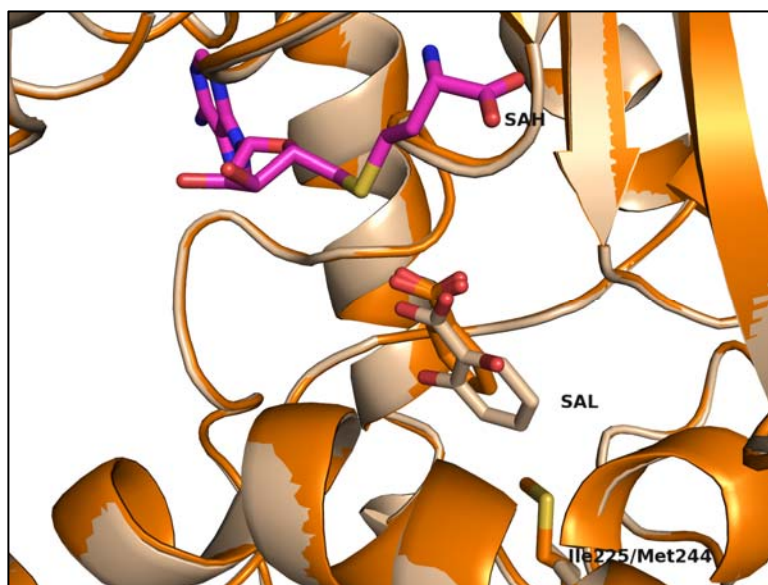


Figure 4.5. Homology model of OsBSMT1 active site (orange), calculated with Modeller (Sali and Blundell, 1993) based on CbSAMT structure (wheat). Secondary structure is represented as ribbons. In this figure, the N-terminal loop-helices (residues 1-30) are omitted to make a better view of the substrate 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 wheat 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>).

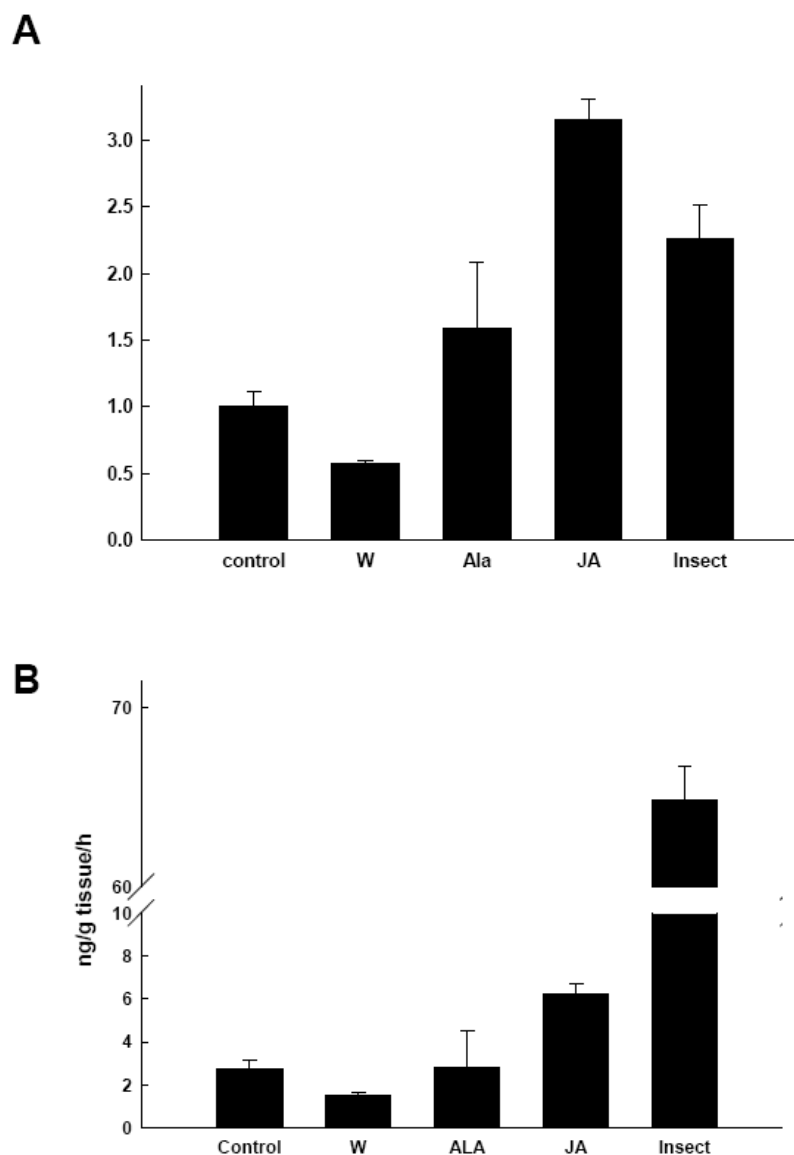


Figure 4.6. Effects of stress factors on expression of *OsBSMT1* (A) and emission of MeSA and MeBA (B). Wild type Nipponbare plants were treated with ala, BTH, physical wounding, jasmonic acid and FAW. Aerial parts of the plants were collected and used for quantitative real-time PCR analysis. Additional plants treated with same conditions were also used for headspace collection. Volatiles were analyzed using GC-MS.

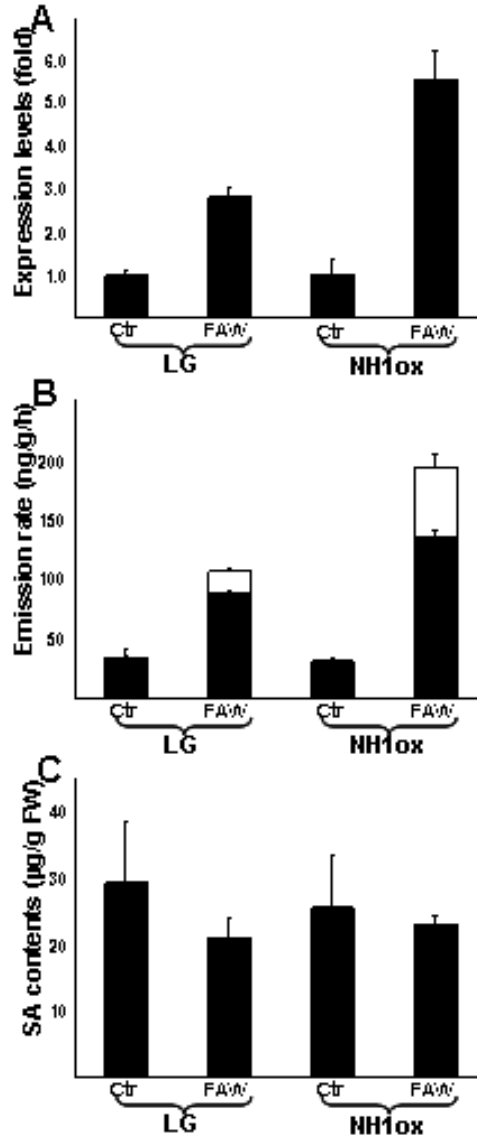


Figure 4.7. Comparison of *OsBSMT1* expression, emission of MeSA and MeBA, and contents of SA. A. Expression of *OsBSMT1* in aerial parts of the untreated (Ctr) and FAW-treated (FAW) treated LG and NH1ox plants analyzed using quantitative real-time PCR. B. Emission of MeSA (black bars) and MeBA (open bars) from untreated (Ctr) and FAW-treated (FAW) treated LG and NH1ox plants based on headspace analysis. C. Contents of SA in aerials parts of the untreated (Ctr) and FAW-treated (FAW) treated LG and NH1ox plants.

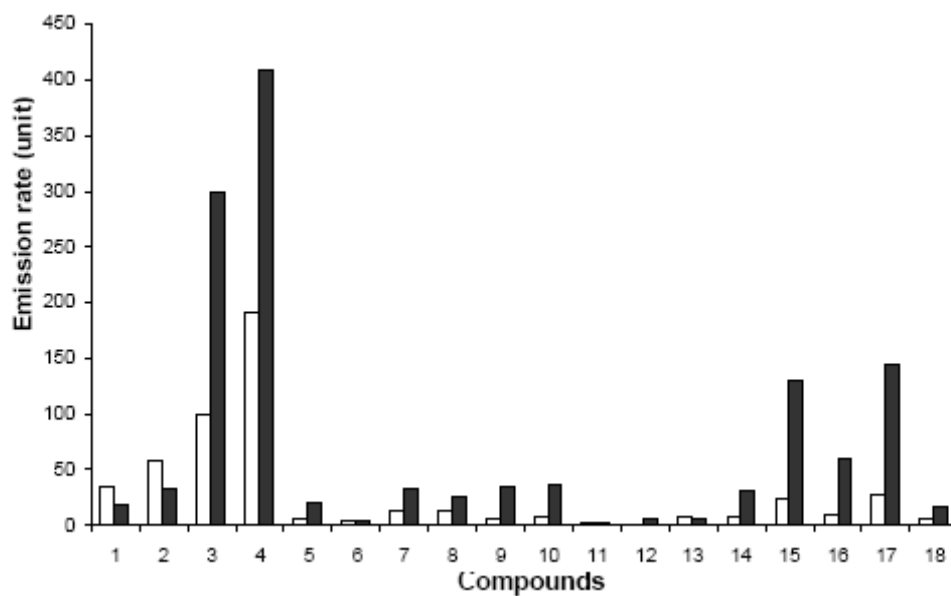


Figure 4.8. Emission rates of other representative volatiles from untreated and FAW-treated LG (open bar) and NH1ox plants (black bars). 1, Nonanal; 2, decanal; 3, linalool; 4, (E)- β -caryophyllene; 5, zingiberene.

Chapter V. A Novel SABATH Methyltransferase from Poplar Having Carboxyl Methyltransferase Activity with both Jasmonic Acid and Benzoic Acid

Adapted from:

Nan Zhao, Ju Guan, and Feng Chen. A Novel SABATH Methyltransferase from Poplar Having Carboxyl Methyltransferase Activity with both Jasmonic Acid and Benzoic Acid. Drafted

Abstract

The JA signaling is an important pathway. JMT is an important enzyme that converts JA to MeJA. I used a comparative genomics approach to identify JMT gene from poplar. Genome analysis revealed that the poplar genome contains 33 SABATH-like genes. Phylogenetic analysis of all poplar SABATH genes with all SABATH genes identified in the Arabidopsis and rice genomes identify that PtSABATH9 is the ortholog of AtJMT. More than half of these genes are expressed in more than one organs. PtSABATH9 was cloned and expressed in *E. coli*. Recombinant PtSABATH9 was purified to near homogeneity through two-steps of ion-change chromatography. Purified PtSABATH9 showed the methyltransferase activity with both jasmonic acid and benzoic acid. The enzyme was named poplar JBMT. PtJBMT optimum temperature, optimum pH 6.5, Enzyme activity can be affected by the addition of certain metal ions in the reaction buffer. Under steady-state conditions, PtJBMT1 exhibited K_m value of $134.2 \pm 13.4 \mu\text{M}$ with JA and 295.9 ± 7.5 with BA. PtJBMT1 appears to have a level of expression in leaves, stems and roots of young seedlings. In the leaves of young seedlings, the expression of PtJBMT1 was slightly induced by wounding and MeJA treatment. The potential biological functions of PtJBMT1 are discussed. Site-directed mutagenesis revealed a key residue that determines the relative activity of the enzyme with JA and BA.

Key words: Jasmonic acid; Benzoic acid; Methyltransferase; Methyl jasmonate;

SABATH family; Poplar

Introduction

The jasmonic acid (JA)-signaling pathway plays critical roles in many biological processes in plants. JA is best known as a wound signaling. When plants are attacked by insects, JA production is promoted, which lead to the induction of various defenses (Vijayan et al., 1998; Liechti and Farmer, 2006). In addition to insect defense, the JA signaling pathway is also critical for controlling plant defenses against pathogens (Vijayan et al., 1998; Liechti and Farmer, 2006). It plays a critical role in the basal resistance of the plant to fungal pathogens such as *Botrytis cinerea* (Thomma et al., 1998). When a plant is attacked by a pathogen, the JA-mediated signaling pathway will be activated. Such activation induces the expression of a large number of defense pathways, for example, the induced expression of pathogenesis-related genes (Seo et al., 2001). In addition, JA is an important hormone for developmental programs, including root growth (Henkes et al., 2008), flower development (Nagpal et al., 2005), and seed germination (Creelman and Mullet, 1995).

JA is synthesized from the LOX pathway. MeJA is a methyl ester of JA. MeJA is a component of floral scents of many species. MeJA has been suggested to be the signal for talking tree. MeJA is made from JA by the action of SAM-dependent methyltransferase. JMT was first isolated from Arabidopsis (Seo et al., 2001). JMT appears to be related to SAMT and BAMT. Together, they belong to a protein family called SABATH (D'Auria, 2003). JMT appears to have a role on plant defense (Seo et al., 2001). It expresses in normal conditions. Interestingly, overexpressing JMT in

Arabidopsis led to the elevated level of methyl jasmonate but did not change the level of free JA. The transgenic plants were more resistant to a virulent fungus *B. cinerea* than wild type plants (Seo et al., 2001). The biological roles of such modification have not been completely understood. However, because methyl jasmonate is hydrophobic and easier for cell-to-cell transport, it has been suggested to act as an internal signaling molecule (Seo et al., 2001).

In addition to AtJMT, another JMT has been isolated from Brassica (Song et al., 2000). No other JMT has been reported, despite the role of JA pathway has been suggested to be important for many plant species. Isolation and characterization of JMT from other plant species will provide important information regarding the role of JMT. Additionally, identification of JMT from other plant species will help understand the evolution of substrate specificity of the SABATH family. In a previous study, site-directed mutagenesis studies showed that JMT can be derived from SAMT. Further investigation in this subject will help identify key residues responsible for substrate specificity of the SABATH family.

In this chapter, I am interested in studying JMT in a perennial species. Poplar is becoming an important model for tree physiology and ecology. It is one important candidate as a bioenergy crop. Because of its small genome, easy to be genetically manipulated, especially with a fully sequenced genome, the poplar is becoming the tree “*Arabidopsis*” (Bradshaw et al., 2000). As other plants, tree species have evolved

sophisticated mechanisms to defense against biotic stresses. Understanding natural defense of tree species is fundamentally important, which will help us understand the evolution of plant defense in perennial woody species. In addition, this has a practical meaning. Many hybrid poplars are susceptible to pathogens (Newcombe, 1998). In some area, herbivorous insects could also cause severe damage to poplar plantation (Ostry et al., 1988). It is therefore important to understand how poplar tree defense themselves against biotic stresses. It has also become a model for studying plant defense response against pathogens, virus and herbivore insects (Arnold et al., 2004). Compared to annual plants, some defense mechanisms are common, and some mechanisms for specific to perennial wood species (Shatters et al., 2004). In my previous studies, it is shown that poplar contains an IAMT gene, which also belongs to the SABATH family, is conserved with Arabidopsis and rice IAMTs (zhao et al., 2007). In this chapter, I identified all SABATH genes from the poplar genome. Gene expression analysis was performed to understand their involvement in biology. Phylogenetic analysis led to the identification of a poplar ortholog. This gene, PtSABATH9, cloned and characterized. Different from AtJMT, the poplar gene showed activity with both JA and BA. Expression of poplar JMT and SAMT under certain stress conditions was analyzed. Site-directed mutagenesis led to the identification of key residues.

Materials and methods

Sequence retrieval and analysis

The protein sequence of *C. breweri* SAMT (accession: AF133053) was used initially as a query sequence to search against the poplar genome database (<http://www.tigr.org/tdb/e2k1/osa1>) using the BlastP algorithm (Altschul et al., 1990). The newly identified SABATH-like sequences were used reiteratively to search the same sequence database. The cutoff e-value was set to be e^{-6} . The chromosome locations of the poplar *SABATH* genes were generated by Map Viewer (<http://www.ncbi.nlm.nih.gov/mapview/static/MVstart.html>). Multiple protein sequence alignments were constructed using ClustalX software (Thompson et al., 1997), and displayed using GeneDoc (<http://www.psc.edu/biomed/genedoc/>). The phylogenetic trees were produced using Paup3.0 and viewed using the TreeView software (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

Plant material and chemicals

The female black cottonwood (*Populus trichocarpa*) clone ‘Nisqually-1’, which was used for whole genome sequencing, was used for gene cloning and expression analysis of *SABATH* genes. The tissues used for gene expression analysis, including young leaves, old leaves, stems, and roots, were collected from one year-old poplar trees grown in the green house. The tissues used for gene expression analysis at stress conditions were collected from poplar seedlings at tissue culture medium grown in the green house. The poplar seedlings were treated with Ala for 2 hours, SA, wounding

for 2 hours and 24 hours, and treated with methyl jasmonate (MeJA) for 40 min and 4 hours. For the SA treatment, poplar seedlings at tissue culture medium were sprayed with 5mM SA, 0.1% Tween X-100 (0.069g SA was dissolved in 1ml Ethanol, and then was diluted 100-fold in 100ml water, add 100ul Tween X-100). For the alamethicin treatment, poplar seedlings were grown in the jar containing 10 ml of 5 ug ml⁻¹ alamethicin (5mg ml⁻¹ stock solution in 100% methanol was dissolved 1000-fold in water). For the wounding treatment, two incisions were made on every one leaf by the sterile razor blade. For MeJA treatment, 1ul MeJA was dissolved in 200 ul ethanol, and was sprayed into the filter paper which was placed in the jar. All the treatment was carried out in the jar, and the jar was sealed. All chemicals were purchased from Sigma-Aldrich (St. Louis, MR) unless otherwise noted.

Gene expression analysis via RT-PCR

Semi-quantitative RT-PCR expression analysis of the poplar genes were performed essentially as previously described (Chen et al., 2003). The primer sequences and sizes of the products are shown in Table 5.2. All primers were tested with genomic DNA to confirm their effectiveness. Total RNA was extracted from the tissue using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and DNA contamination was removed with an on-column DNase (Qiagen, Valencia, CA) treatment. 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 (Chen et al., 2003). One µL of cDNA were

used in each PCR reaction under the following conditions: an initial denature at 95°C for 2 min followed by 30 cycles of 95°C for 45 sec, 57 °C for 45 sec and 72 °C for 60 sec, and then followed by an extension step of 72 °C for 10 min.

Screen for substrate specificity

For the substrate screening, radiochemical assays were performed with a total volume of 50 µL containing 50 mM Tris-HCl pH 7.5 buffer, 5 µL protein, 0.5µl [¹⁴C]-SAM (specific activity 52.7 mCi/mmol, Perkin Elmer Instruments, Shelton, CT), and 1 µL of substrates. The reaction was incubated at room temperature for 30 min, after which the products of the assay were extracted with 150 µL ethylacetate and counted in scintillation counter. The substrates include salicylic acid, jasmonic acid, indole-3-acetic acid and benzoic acid.

Purification of recombinant PtJBMT

Total RNA extracted from leaf tissue was used for the gene cloning of PtJBMT. Full-length cDNA of PtJBMT was amplified using the forward primer 5'-ATGGAAGTAATGCAAGTGCTTCACATG -3' and the reverse primer 5'-TTAATTTCTAACCATTGAAATGACCAAGTT -3'. The PCR products were cloned into pEXP5/CT TOPO vector using the protocol recommended by the vendor (Invitrogen, Carlsband, CA). The cloned cDNAs in pEXP5/CT TOPO vector were sequenced using T7 primers.

To express PtJBMT protein in *E. coli*, the plasmid pTOPO-PtJBMT was transformed into *E. coli* strain BL21 DE3 codon plus (Invitrogen, Carlsband, CA). Protein expression was induced by IPTG for 18 hours at room temperature and the cells were lysed by sonication. Proteins expressed in pEXP5/CT TOPO vector were purified with FPLC system as previously described (Ross et al., 1999). The cell lysis was loaded onto the DE53 cellulose column (Whatman, Maidstone, England), which was installed in a Pharmacia FPLC apparatus and preequilibrated with buffer A (containing 50 mM Tris-HCL, pH 7.0, 10% glycerol). Flow rate was 1 ml/min. After the enzyme was loaded, the column was washed with 20 ml buffer A containing a linear gradient of KCL from 0 to 200mM, and eluted with a linear gradient from 200 to 400 mM KCL in buffer A. Fractions were collected and assayed for enzyme activity. The fractions with enzyme activity were pooled together and diluted threefold with buffer A, and then were loaded onto the HiTrap Q FF column (GE Health Life Sciences, San Francisco, CA), which was installed in a Pharmacia FPLC apparatus and preequilibrated with buffer A. Flow rate was 0.5 ml/min. After that, the column was washed with a linear gradient from 0 to 100 mM KCL and eluted with a linear gradient from 100 to 400 mM KCL in buffer A. Fractions were collected and enzyme activity was determined. Protein purity was verified by SDS-PAGE and protein concentration was determined by the Bradford assay.

Radiochemical activity assay

For the PtJBMT, radiochemical assay was performed with 50 µl volume

containing 50 mM Tris–HCl, pH 7.5, 1mM JA and 0.4 μ l ^{14} C-SAM (Perkin Elmer, Boston, MA). The assay was started by the addition of SAM and kept at room temperature for 30 min. The reaction was stopped by the addition of 150 μ l ethyl acetate, vortexed, and phase-separated by a 1 min centrifugation at 14,000g. The upper organic phase was counted using a liquid scintillation counter (Beckman Coulter, Fullerton, CA) as previously described (D’Auria et al., 2002). The amount of radioactivity in the organic phase indicated the amount of synthesized MeJA. Three independent assays were performed for each compound.

Determination of kinetic parameters of PtJBMT

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. For the PtJBMT, to determine a K_m value for SAM, concentrations of SAM were independently varied from 3 to 120 μ M, while JA was held constant at 1 mM. To determine the K_m for JA and BA, concentrations of JA and BA were independently varied from 10 to 200 μ M, while SAM was held constant at 200 μ M. Assays were conducted at 25 °C for 30 min. Lineweaver–Burk plots yielded apparent K_m values as previously described (Chen et al., 2003). Final values represent the average of three independent measurements.

pH optimum for PtJBMT activity

PtJBMT activities were determined in 50 mM Bis-Tris propane buffer for the pH

range from 6.5 to 10 using the standard radiochemical activity assay. The data presented are an average of three independent assays.

Effectors

A standard radiochemical activity assay was carried out in the presence of one of the following cations, present at a final concentration of 5 mM: K^+ , Ca^{2+} , NH_4^+ , Na^+ , Mg^{2+} , Mn^{2+} , Cu^{2+} , Fe^{2+} , Zn^{2+} . Results presented are an average of three independent assays.

Real-time quantitative RT-PCR

After treatments, leaf tissue was harvested for the total RNA extraction. Total RNA was extracted from the tissue using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and DNA contamination was removed with an on-column DNase (Qiagen, Valencia, CA) treatment. 1.5 μ g of total RNA was reverse transcribed into first strand cDNA in a 15 μ L reaction volume using the First-strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ) as previously described (Chen et al., 2003). Real-time quantitative PCR (RT-PCR) was performed on an ABI7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR green fluorescence dye (Bio-Rad Laboratories) as previously described (Yang et al., 2006). The gene-specific primers were designed as followed: forward primer 5'-CCCACCACGGACGAGAGT -3' and the reverse primer 5'-CCCCTCAGAAACCATGCTCAT -3. The two primers used for the PCR

amplification of *Ubiquitin* were designed as the internal control: forward primer 5'-CAGGGAAACAGTGAG GAAGG-3' and reverse primer 5'-TGGACTCACGAGGACAG-3'.

Site-mutagenesis of PtJBMT

In order to identify the function of key residue for JA activity, a serine (S) residue was changed to tyrosine (Y). PCR method was used to construct the mutant. Two primers were designed: Mutant forward primer: 5'-GTGTGCACTCTTCTTATAGTCTTCACTGGCTC- 3'; Mutant reverse primer: 5'-GAGCCAGTGAAGACTATAAGAAGAGTGCACAC -3'. These two primers were totally complementary, and the codon TAT in forward primer was designed to instead the TCT in normal PTJBMT. And then PtJBMT plasmid was used as template for PCR amplification. Two fragments were amplified, one is using the forward primer of PtJBMT and the Mutant reverse primer; another fragment is using the reverse primer of PtJBMT and the Mutant forward primer. Next, these two fragments were put together as templates for the second step PCR amplification. The primers for the full length cDNA amplification of PtJBMT were used for the second step PCR. The PCR products were cloned into pEXP5/CT TOPO vector and sequenced using T7 primers.

Results

Identification of the SABATH gene family in Poplar

To identify the complete *SABATH* gene family in the poplar genome, the protein

sequence of *C. breweri* SAMT (CbSAMT), the prototype SABATH (Ross et al., 1997), was initially used to search the genome sequence database of poplar using BlastP algorithm (Altschul et al., 1990). The new SABATH-like sequences detected were in turn used reiteratively to search the same sequence database. Through the exhaustive sequence search, 33 sequences encoding proteins significantly similar to known SABATHs were identified in the poplar genome (Table 5.1). It should be noted that among the 33 *PsSABATH* sequences, 10 of them appear to encode proteins shorter than 300 amino acid residues. Some of the shorter proteins may be due to inaccurate annotation (Rouze et al., 1999), and some of them may be pseudogenes. Even the genes annotated to encode proteins at the size of about 350 amino acid residues may be pseudogenes. Further full-length cDNA cloning efforts will clarify whether individual *PtSABATH* genes are intact genes or pseudogenes.

Phylogenetic analysis of PtSABATHs, AtSABATHs and OsSABATHs

To date, the complete gene family of SABATH had been identified in Arabidopsis and rice (D'Auria et al., 2003; Zhao et al., 2008). To understand the evolutionary relationships among the *PtSABATHs* and among the *PtSABATHs*, *AtSABATHs* and *OsSABATHs*, a phylogenetic tree containing all poplar, Arabidopsis and rice SABATHs was constructed (Figure 5.1). (The *OsSABATHs* are grouped into three clades (I, II and III). Clade I is the largest clade containing 23 genes. It can be further divided into two subclades. One subclade contains the majority of the genes on chromosome 6. The other subclade contains all *OsSABATH* genes localized on

chromosome 11. The analysis also revealed that most OsSABATHs and AtSABATHs cluster in species-specific distinct clades (Figure 5.1). All OsSABATHs and AtSABATHs are grouped into five clades (I, II, III, IV and V). Clade I is rice-specific and clades IV and V are Arabidopsis-specific. Two AtSABATHs are clustered with 10 OsSABATHs to form clade II. Three AtSABATHs are clustered with eight OsSABATHs to form clade III.)

Expression analysis of PtSABATH Genes

To obtain information on the biological processes in which *PtSABATHs* are involved, comprehensive gene expression analyses using semi-quantitative RT-PCR were performed for 27 *PtSABATH* genes, 6 genes are not involved because of their short sequence annotation. Gene expression analyses were performed with young leaves, stems and roots from one year-old poplar trees grown in the green house.

In these experiments, results for different genes in the same organ are directly comparable, because an identical aliquot of cDNA from the original RT reaction was used in each PCR procedure. To determine whether equal amounts of cDNA were used in the reactions involving different organs, I also performed RT-PCR with primers designed for ubiquitin. After RT-PCR, amplified fragments from mRNAs of 19 of the 27 *PtSABATH* genes were obtained from at least one organ (Figure 5.2). The expression of 17 genes was detected in leaves, 11 in stems, 13 in roots. 10 genes showed expression in all tissues examined. In contrast, 8 genes showed no expression

in any of the tissues examined.

Among them, *PtSABATH9* showed high levels of expression in multiple tissues. Together with the phylogenetic analysis, in which *PtSABATH9* was group with AtJMT. Therefore, full-length cDNA of *PtSABATH9* was amplified from appropriate tissues, cloned into the vector pEXP5/CT TOPO, and sequenced. The coding sequences of these genes were confirmed to be same as annotated (Figure 5.2).

Screen for substrate specificity of a selected group of PtSABATHs

In order to screen the substrate specificity of *PtSABATH9*, the plasmid pTOPO-PtJBMT was heterologously expressed in *E. coli* to produce recombinant proteins. Previously characterized SABATHs have been shown to methylate either a carboxyl group or the nitrogen group of the substrate. I therefore screened the activities of *PtSABATH9* with several well known substrates, salicylic acid, jasmonic acid, benzoic acid and Indole-3-acetic acid. Results showed *PtSABATH9* showed high activity toward jasmonic acid and low activity with benzoic acid. Therefore, *PtSABATH9* was named poplar jasmonic acid, benzoic acid MT (*PtJBMT*).

Biochemical properties of PtJBMT

To determine the PH optimum of the enzymatic assays, *PtJBMT* enzyme assay reacted in 50mM Bis-Tris buffer with PH ranging from 6.0 to 10. Results show that the optimum PH of *PtJBMT* is 6.5. At PH 6.0, it was 80% activity of the maximal

activity, at PH 7.0, it was 50%, at PH 8.0, it was about 20%, and at 8.5, it was only 5% activity of the maximum.

To identify the temperature effect on PtJBMT enzymatic activity, PtJBMT protein was incubated at temperature ranging from 4 °C to 70 °C respectively for 30 min, and chilled in ice. Then the enzyme treated in each temperature was used for the enzyme assay reaction. PtJBMT was 100% stable for 30 min at 4 °C, about 90% at 20 °C, 30 °C and 37 °C, the activity was still 80% when incubated for 30 min at 42 °C. When kept at 50 °C for 30 min, PtJBMT lose 40% of its activity. But at 60 °C it lost all of its activity.

Metal ions effect on PtJBMT activity. The enzyme assay reaction was performed with one of the next metal ions in the essay buffer at the final concentration of 5mM: K⁺, Ca⁺, NH₄⁺, Na⁺, Mg²⁺, Mn²⁺, Cu²⁺, Fe²⁺, Fe³⁺, Zn²⁺. K⁺ was found to stimulate JMT activity greatly. NH₄⁺ and Na⁺ have a little stimulation on PtJBMT activity. Ca²⁺ and Mg²⁺ have a mild inhibitory effect. Mn²⁺ decreased the specific activity by 40%, and Cu²⁺, Fe²⁺, Fe³⁺ and Zn²⁺ had a strong inhibitory effect on PtJBMT activity.

Kinetic parameters for PtJBMT were also determined. Under steady-state conditions, PtJBMT exhibited apparent Km values 41.36μM for SAM when JA was used as substrate. It also showed Km values of 134.15±13.4 μM and 295.9±7.5 μM

for JA and BA, respectively. The Kcat for JA was $13 \times 10^{-3} \text{ S}^{-1}$. The Kcat for BA was $3 \times 10^{-3} \text{ S}^{-1}$

Expression of PtJBMT1 in Poplar leaves under stress conditions

To determine the stress conditions in which PtJBMT was induced, the poplar seedling were treated with Alamethicin for 2 hours, SA and wounding for 2 hours and 24 hours, treated with methyl jasmonate for 40 min and 4 hours. The total RNA was isolated from leaves of these treated poplar seedling and used for the real-time quantitative RT-PCR experiments. As shown from the results, the expression of the PtJBMT gene showed slight influence by the treatments. After 2 hours of treatment, wounding, SA and Ala treatments increased the PtJBMT expression by approximately 1.2- 1.5 fold, and 40 minutes of treatment by MeJA increased the expression a little, up to 1.13 fold. After treatment for more time, the PtJBMT gene expression showed different induction patterns. SA treatment for 24 induced the expression to 1.9 fold, and MeJA treatment for 4 hours increased the expression to 2.4 fold. However, after 24 hour of wounding treatment, the expression of the PtJBMT gene was lower than the control (Figure 5.6).

Site-directed mutagenesis of PtJBMT

In order to identify the function of key residue for JA activity, a serine (S) residue was changed to tyrosine (Y). PCR method was used to construct the mutant. Results showed PtJBMT mutant showed increased relative activities with BA and

some substrates containing similar structure with BA. As shown in Table 5.3, for the PtJBMT mutant, the activity with BA is 3 fold higher than the one with JA. These suggest the serine residue may be important for the substrate specificity of PtJBMT.

Discussion

Poplar is the third plant species, after Arabidopsis and rice, in which the entire SABATH gene family has been identified (Tuskan et al., 2006). Expression analysis showed that the majority of the PtSABATH genes have specific expression patterns under normal conditions, supporting these gene products have diverse biological roles in the normal growing poplar tree. Many of SABATH gene products have been identified to have important role in plant growth and development as well as plant defense to the biotic stress. Indole-3-acetic acid MT (IAMT), a conserved member of SABATH family, has been identified from Arabidopsis, rice and poplar. All of these three genes inhibited expression in multiple tissues, suggesting IAMT may be involved in multiple biological functions. PtIAMT is the only SABATH gene identified from poplar to date. It showed expression in young leave and stem and was hypothesized to be important in leaf development and xylem formation (zhao et al., 2007). Expression analysis suggests 10 PtSABATH genes showed high level of expression in multiple tissues, among them, PtSABATH1 has been identified as PtIAMT before. Here, PtSABATH9 were identified as PtJBMT. However, I can not determine the substrates for the other PtSABATH genes. Except these 10 genes, 9 other SABATH members inhibited tissue-specific expression in at least one organ. 8

genes showed no expression in any of the tissues examined. Besides, 7 genes are not involved in the gene expression analysis because of their short sequence annotation. Those suggest many of PtSABATH genes might lose their biological functions. It will be interesting to examine the biological function of all other PtSABATHs.

Methyl jasmonate (MeJA) is reported as an important regulator of various developmental processes, such as seed germination, flowering, fruit development, and leaf senescence (Creelman and Mullet, 1995). In addition, MeJA has a role in plant defense response against insect herbivory (Creelman et al., 1992). Some evidence supports that MeJA, as an air-borne signal molecule, induces the SAR (Reymond and Farmer, 1998; Kessler and Baldwin, 2001). So far, two JMT genes have been isolated from *Arabidopsis* and *brassica*, the transgenic experiment exhibited AtJMT enhanced resistance against pathogen (Seo et al., 2001). Expression analysis showed PtJBMT also has high level of expression in multiple tissues under normal conditions, suggesting it is critical in poplar growth and development. In hybrid poplar, fresh hybrid poplar leaves was found to contain 1.3 μg of methyl jasmonate per gram of leaf tissue (Wilbert et al., 1998). This provides evidence that MeJA is constitutively made. The amount of jasmonic acid in the same tissues was determined to be about 2.6 μg per gram leaf tissue. MeJA may be hydrolyzed to form free jasmonic acid by the action of methyl jasmonate esterase. Like JA, SA is also a signaling molecule. The methyl ester of SA, MeSA, is also formed by the action of a SABATH protein, SAMT. MeSA recently was shown to be the mobile signal of SAR (Park et al., 2007). Therefore,

MeJA may play a similar function.

Methyl benzoic acid has been studied as a floral scent compound. It also often induced when plants are under biotic stress (D'Auria et al., 2002). For example, *Arabidopsis* plants emit MeSA when infested with insects (Chen et al., 2003). However, its role in such conditions has not been well studied. The floral scent BAMT appears to be highly specific for BA, having no activity with SA (Murfitt et al., 2000). Because of the high similarity in chemical structures of SA and BA, some SAMT can also use BA as substrate and some BAMT can use SA as substrate. While most of these activities were detected in in vitro assays, the study in *Arabidopsis* suggests that a same enzyme is responsible for the production of both MeSA and MeBA (Chen et al., 2003).

The unique feature of PtJBMT is its activity with both JA and BA. The catalytic efficiency of PtJBMT with JA is 4 times higher than that with BA. How this in vitro activity contribute the production of MeBA in planta remains to be determined. It is also interesting to think about substrate specificity evolution. In the rice genome, there is not a clear ortholog of *Arabidopsis* JMT. On the contrast, the *Arabidopsis* and poplar JMTs appear to be orthologs. It is needed to identify JMTs from other plant species in order to gain insight into the evolution of JMTs. It is intriguing how the JAMT and BAMT are evolved. "Promiscuous activities, or cross-reactivities, between different members of the same superfamily may provide important hints regarding

evolutionary and mechanistic relationships” (Roodveldt and Tawfik, 2005).

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Appendix

Table 5.1. Poplar SABATH gene

Gene	ID	Chr	Location	Size of Predicted Protein
PtMT1	fgenes4_pg.C_LG_I002808	1	LG_I:28894791-28898608	385
PtMT2	gw1.X.6373.1	5	LG_X:10922233-10924991	357
PtMT3	fgenes4_pg.C_LG_XIX000232	19	LG_XIX:2695373-2697587	367
PtMT4	eugene3.09930001		scaffold_993:1634-4046	364
PtMT5	eugene3.00071131	7	LG_VII:11066466-11069147	364
PtMT6	eugene3.00190163	19	LG_XIX:1952075-1954479	364
PtMT7	fgenes4_pg.C_scaffold_70000127		scaffold_70:871535-873729	359
PtMT8	estExt_fgenes4_pg.C_LG_XII0423	12	LG_XII:4251724-4254885	364
PtMT9	gw1.V.1425.1	5	LG_V:15969929-15974581	368
PtMT10	gw1.VIII.1099.1	8	LG_VIII:8990106-8991817	350
PtMT11	gw1.II.2664.1	2	LG_II:21702715-21710157	353
PtMT12	gw1.2047.2.1		scaffold_2047:1593-3392	351
PtMT13	fgenes4_pg.C_LG_VII001116	7	LG_VII:11052632-11054615	362
PtMT14	gw1.XV.887.1	15	LG_XV:3459911-3461430	333
PtMT15	eugene3.00002347	2	LG_II:21710051-21710583	146
PtMT16	fgenes4_pg.C_LG_VII000519	7	LG_VII:4156380-4158228	345
PtMT17	gw1.II.388.1	2	LG_II:21756435-21759146	327
PtMT18	gw1.XIX.648.1	19	LG_XIX:2722379-2724108	336
PtMT19	gw1.XIV.3807.1	14	LG_XIV:9448106-9450319	363
PtMT20	eugene3.113410001		scaffold_11341:112-1228	257
PtMT21	gw1.328.1.1		scaffold_328:12492-13481	243
PtMT22	gw1.XIX.476.1	19	LG_XIX:1960921-1961725	222
PtMT23	gw1.VIII.1912.1	8	LG_VIII:8982052-8987259	351
PtMT24	fgenes4_pg.C_scaffold_2047000002		scaffold_2047:5432-6116	188
PtMT25	eugene3.52250001		scaffold_5225:2661-3138	131
PtMT26	gw1.XIX.631.1	19	LG_XIX:2677601-2684272	305
PtMT27	eugene3.09900001		scaffold_990:1955-2999	236
PtMT28	gw1.XIX.624.1	19	LG_XIX:2633030-2635249	365
PtMT29	eugene3.00002344	2	LG_II:21700829-21701309	132
PtMT30	eugene3.00100985	10	LG_X:10920409-10921963	331
PtMT31	eugene3.11900001		scaffold_1190:6083-6842	93
PtMT32	eugene3.09930001		scaffold_993:1634-4046	364
PtMT33	gw1.5398.1.1		scaffold_5398:2342-3126	205

Table 5.2. Primers for RT-PCR Analysis of *PtSABATH* Gene Expression

Gene Name	Forward Primer Sequences (5'-3')	Reverse Primer Sequences (5'-3')
<i>PtSABATH1</i>	AGAAACCCTAGATAGGGTGAC	CCAAGCAGACAAGAAACATGGA
<i>PtSABATH2</i>	ATCGAGGCTTCACTTCTCCTCA	CTTAGAGAGCCAGTGTAGTGCAG
<i>PtSABATH3</i>	CACTGGCTTTCTAAGGTGCCAG	ACCAACATCGGTTCTGCAACT
<i>PtSABATH4</i>	CTCAACATTAATCAAAAGCCTGAG	TCCATTTTTTCAATGTCAAAGG
<i>PtSABATH5</i>	CAATCACCAGAATATCAGGTGC	CCAAACGATCAATGGTAAAGGAC
<i>PtSABATH6</i>	CTCAACATTAATCAAAAGCCTGAA	TCCATTTTTTCAATGTCAAAGC
<i>PtSABATH7</i>	GTTTCAGATTTTGGTCCCTGTTATA	AAGGGCAATGATCTCAAGGCA
<i>PtSABATH8</i>	GCAGGAGCTGTGCAGAATGTTA	ATTCCAAGCAGGAGAGCTAACG
<i>PtSABATH9</i>	TATGCCAAATGCAGAGAGTTGG	GACCTTTTCTTCTCGACGAGC
<i>PtSABATH10</i>	AAATCAGAGACTGATGCGCCTC	CCTCATTTGGTGCATTTGTGTAG
<i>PtSABATH11</i>	GAATACATTCTTCGAGTGGAA	GCAAGATCCAAAGAAGTCATTGA
<i>PtSABATH12</i>	TCAGAGGGGAGGGCTGGTTA	GATTCCGTCAGGAAGACCGAG
<i>PtSABATH13</i>	GACTTTTCCCAACGATTGCT	GGGAAAGCATGGATTCTTGGA
<i>PtSABATH14</i>	GAAGCCTCTCTTCACATTGTCAT	TTCATGTCCAAAGTGCTCCGA
<i>PtSABATH15</i>	TATCAGAGGGGAGTGTGAACG	AAGACCCGAAAAGCTCATAGCT
<i>PtSABATH16</i>	CCCTCACAGCTACACCAAGAAT	CTGGTGAATTCACATCACCCAG
<i>PtSABATH17</i>	GTTGGTGGAGATGGACCTCAA	TGTTCCATGCAGGAGACTTGC
<i>PtSABATH18</i>	GCGAAGGCAAGTCCTCGATC	CTCTTGAACAATTCATCCGTTATA
<i>PtSABATH19</i>	AGAAGAAGGTGATGTTAAAGGCA	GATCGGGATCTTAAAAATGCTG
<i>PtSABATH20</i>	GAAGGCATCAAAGAGACGCTT	CAAGATCCAAAGAAGTCATTGAA
<i>PtSABATH21</i>	GGAGTGCTGGTTATCGTGGA	TGCCGTCAGGAAGACCACAA
<i>PtSABATH23</i>	AAAATAGCCGCAAATGTTGCA	CCATCCCATCAGGGATGCTAT
<i>PtSABATH24</i>	GGTAGCATTCACTGCACAGGA	CTTAGAATGATGAACAGGTCCATG
<i>PtSABATH27</i>	TCAGTTCAGAGTGACATGAACAC	TCAATTAACGTTACGCTTGAG
<i>PtSABATH28</i>	GCGAGACCCAATACTCTCCTGT	CCAACATCGGTTCTGCAGCA
<i>PtSABATH30</i>	ATACTTTGCAGCTGGTGTGCTA	ATGATTGTTGAGCCTGGAAGAG
<i>PtSABATH32</i>	AATTCTTGTCGTCTATTGGTCGA	TGCCAGTTGTTGACAAAGTGTC

Table 5.3. Relative activities of PtJBMT and PtJBMT mutant with some common substrates

	PtJBMT	PtJBMT Mutant
JA	1	1
SA	0.5%	22.4%
BA	15.1%	336%
IAA	0.4%	0.0%
GA3	0.0%	0.0%
Anthranilic acid	0.1%	0.0%
2,4D	1.5%	0.0%
3Hydroxyl benzoic acid	0.4%	10.4%
4Hydroxyl benzoic acid	2.3%	62.4%

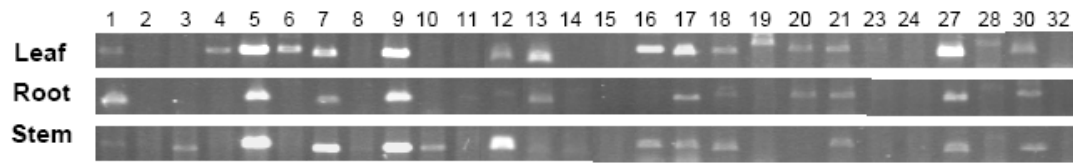


Figure 5.2. Expression analysis of poplar SABATH genes. Total RNA was extracted from leaves, stems, roots of two-week old seedling, panicles from four-month old plants, and germinating seeds, and used for RT-PCR. The numbers 1 to 32 on the top of the figure denotes *PtSABATH1* to *PtSABATH41*. The expression of an actin gene (A) was used as an internal control. PCR reactions using rice genomic DNA as template were performed to confirm the effectiveness of primers used in RT-PCR reactions. The nine highly-expressed genes whose full-length cDNAs were cloned are indicated with asterisks.

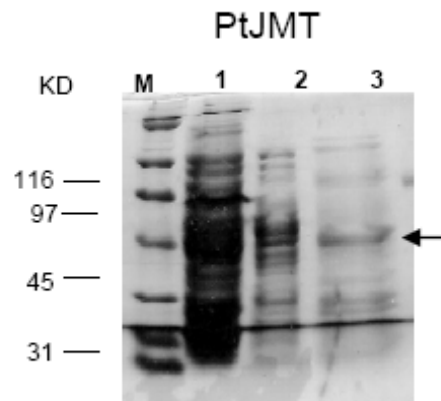


Figure 5.3. Protein purification of *PtJBMT1*. Protein expressed in *E. coli* was purified as described in Materials and methods. Lane M contained protein molecular weight markers. Lane 1 contained *PtJBMT1* crude extract, and lane 2 contained 1 μ g of DE53 fraction. Lane 3 contained 1 μ g of mono-Q fraction. The gel was stained with Coomassie Blue. Arrow indicates the target band for *PtJBMT1*.

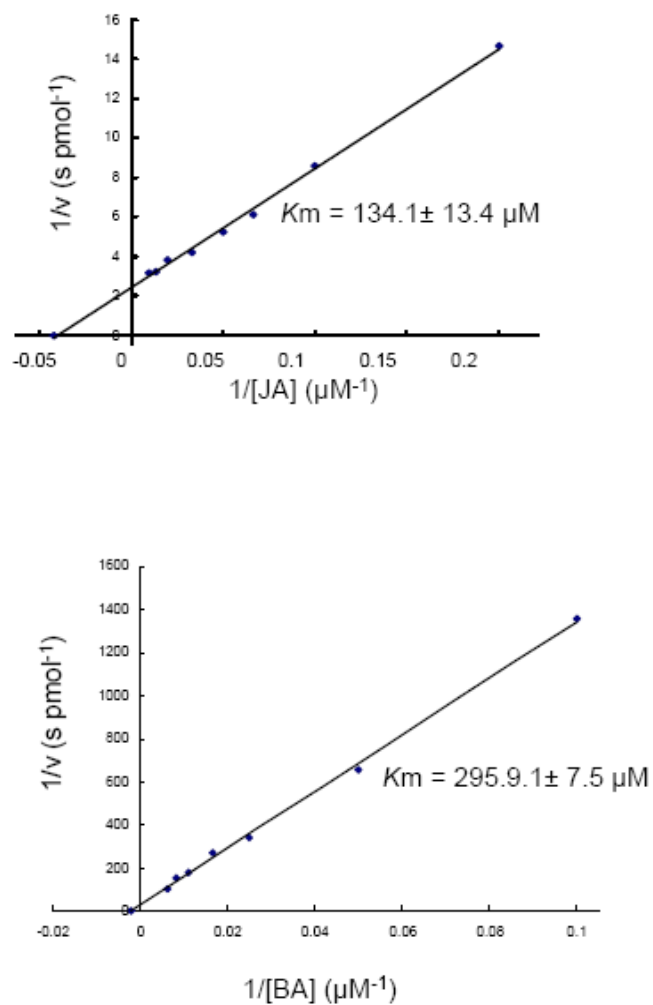


Figure 5.4. Biochemical properties of *PtJBMT*. (a) Steady-state kinetic measurements of *PtJBMT* using JA as the substrate. One example of a Lineweaver-Burk plot is shown. (b) Steady-state kinetic measurements of *PtJBMT* using BA as the substrate. One example of the Lineweaver-Burk plot is shown. In both (a) and (b), K_m values shown were not absolute. However, they were apparent under the limited reaction conditions.

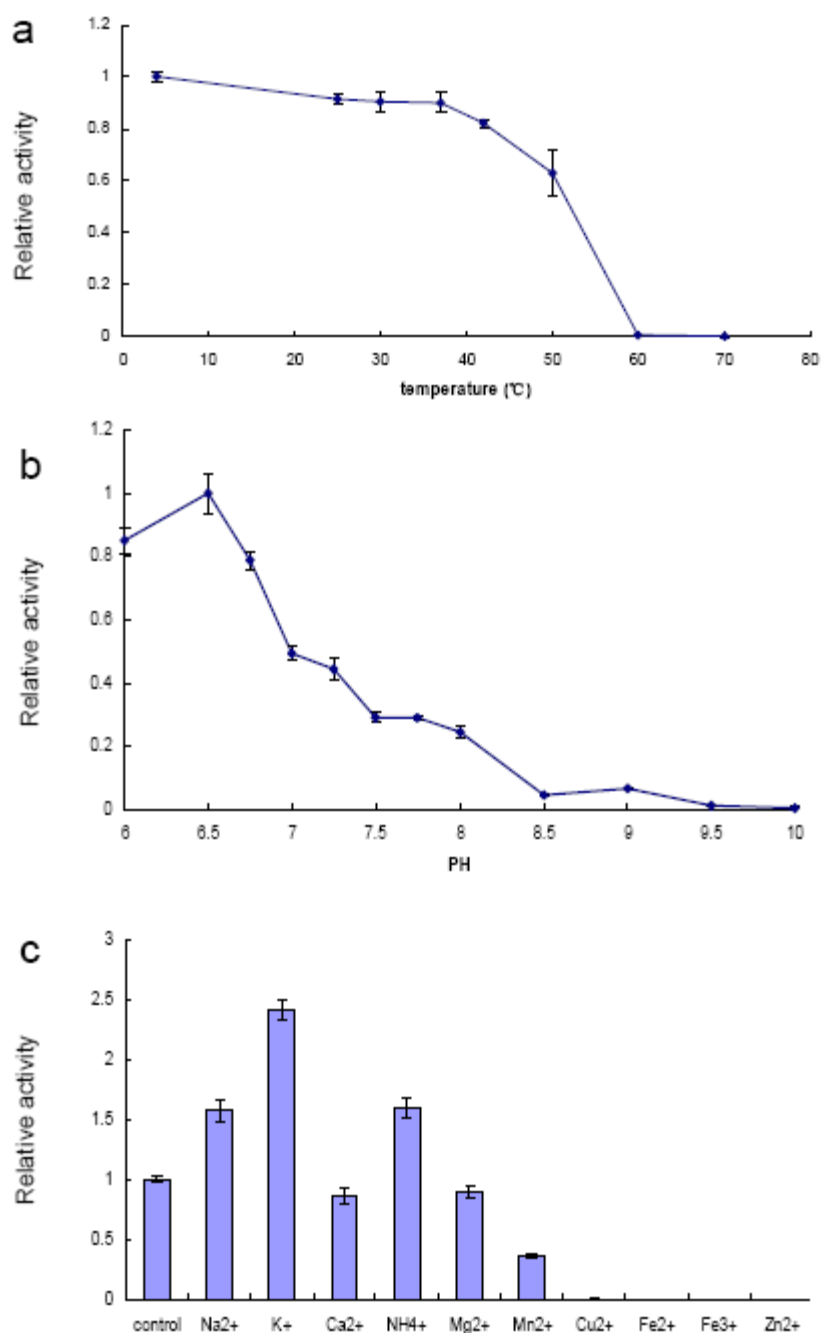


Figure 5.5. Biochemical properties of *PtJBMT*. (a) Effect of temperature on activity of *PtJBMT*. Level of *PtJBMT* activity at 4 °C was arbitrarily set at 1.0. (b) Effects of buffer pH on activity of *PtJBMT*. Level of *PtJBMT* activity in the buffer of pH 6.5 was arbitrarily set at 1.0. (c) Effects of metal ions on activity of *PtJBMT*. Metal ions

Figure 5.5. Continued

were added to reactions in the form of chloride salts at 5 mM final concentrations.

Level of PtJBMT activity without any metal ion added as control (Ctr) was arbitrarily set at 1.0. In all three panels, actual specific activity for 1.0 is 60 pkat/mg protein.

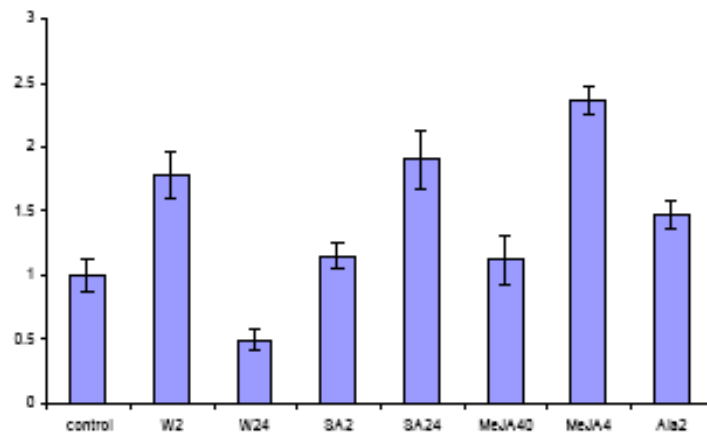


Figure 5.6. Expression of *PtJBMT1* under stress conditions. Wild type poplar were treated with physical wounding for 2h and 24h, SA for 2h and 24h, MeJA for 40 min and 4h, ALA for 2h. Aerial parts of the plants were collected and used for quantitative real-time PCR analysis. Expression of wild type poplar was arbitrarily set at 1.0.

Chapter VI. Conclusions and Perspectives

I. Conclusions

In the previous chapters, comparative genomic approaches were employed to identify *SABATH* genes from poplar and rice. The expression patterns of these genes in multiple tissues were analyzed using quantitative RT-PCR approach and some genes highly expressed in multiple tissues were cloned. Two rice genes and two poplar genes were identified with MT activities and biological functions of these genes were studied with respect to plant growth and development as well as plant interactions with the environment. The involvement of *OsBSMT1* with plant defense was surveyed, which helped to define the molecular mechanisms of rice defense against herbivorous insects as well as the role of *OsBSMT1* genes in the process. Moreover, the regulation of *OsBSMT1* in the indirect defense was studied using transgenic rice plants. The evolution of the function of *SABATH* genes and the *SABATH* gene family were investigated. In the end, I got the following ten conclusions.

First, 41 *SABATH* genes were identified in the rice genome. Expression analysis showed that more than half of the *OsSABATH* genes were expressed in one or multiple organs. Two genes including *OsIAMT1* and *OsSBMT1* displayed the highest level of catalytic activity towards IAA and SA, respectively.

Second, genome analysis revealed that the poplar genome contains 33 *SABATH* genes. Expression analysis showed that the majority of the *PtSABATH* genes have the

specific expression patterns under normal conditions (Figure 5.2), supporting that these gene products have diverse biological roles in the normal growing poplar tree. Two genes including *PtIAMT1* and *PtJBMT* were identified to have the highest level of catalytic activity with IAA and JA, respectively.

Third, *IAMT* may function as regulating the homeostasis of IAA in rice and poplar. Data showed *OsIAMT1* exhibited expression in multiple tissues. It showed high levels of expression in roots and panicles (Figure 3.6). And *PtIAMT1* showed high level of expression in stem, moderate level of expression in young leaf and low level of expression in root (Figure 2.5). As the most abundant naturally occurring auxin in higher plants, IAA exists in multiple plant tissues, involved in a diverse array of plant developmental processes. MeIAA is an inactive form of IAA, and can be hydrolyzed to free IAA by one or more plant esterases (Yang et al., 2008). Therefore, high level expression of *IAMT* may play a role of regulating IAA activities in multiple tissues.

Fourth, *OsBSMT1* is expressed in multiple tissues of rice plants grown under normal growing conditions (Zhao et al., 2008), suggesting that this gene has a role in the normal biology of healthy plants.

Fifth, *OsBSMT1* might have the defense functions. As shown in chapter III, rice plants treated with herbivorous insect fall armyworm emitted highly increased amount

of MeSA. Both microarray and quantitative real-time RT-PCR approaches showed, *OsBSMT1* gene was upregulated in insect-damaged rice plants. The specific induction of *OsBSMT1* by herbivory and enhanced emission of MeSA support that it has a specific role in plant response to insects. In the context of indirect plant defense, MeSA has been shown to be an active signal in attracting a number of carnivorous species (Dicke et al., 1990, De Boer and Dicke, 2004; Zhu and Park, 2005). The induced emission of MeSA in rice may play a similar role.

Sixth, *OsBSMT1* expression and emission of MeSA can be regulated through a crosstalk between SA signaling pathway and JA signaling pathway. 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. 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.

Seventh, *PtSABATH9* were identified as *PtJBMT*, which showed activities with both JA and BA. Expression analysis showed *PtJBMT* also has the high level of expression in multiple tissues under normal conditions, suggesting it is critical in poplar growth and development. The expression of the *PtJBMT* gene showed slight

influence by the treatments, indicating it has the defense role.

Eighth, site-directed mutagenesis of PtJBMT revealed a key serine residue that determines the relative activity of the enzyme with JA and BA, suggesting the change of specific residues can change the substrate specificity of SABATH enzyme. It provides important insights into the evolution of SABATH family genes.

Ninth, Comparative genome analysis suggested the rapid evolution of the SABATH gene family. Most of AtSABATHs, OsSABATHs and PtSABATHs evolved after the divergence between the herbaceous annual species and perennial woody species and after the split of monocot and dicot plants. As shown in figure 6.1, except for the IAMT, most of SABATHs are grouped into species specific clades.

Tenth, IAMT is likely an ancient member of the SABATH protein family, from which other SABATH MT activities, including JMT, SAMT and FAMT, may have emerged. As described in chapter III, structural, biochemical and phylogenetic evidence supports that IAMT is an evolutionarily ancient member of the SABATH family. In a recent study, I identified several IAMTs from other plant species. Phylogenetic analysis showed these IAMTs are grouped into one highly conserved clade (Figure 6.2).

Overall, in this dissertation, I use the comparative functional genomics approach

to identify the SABATH families in rice and poplar and further investigate the biochemical and biological functions and evolution of selected members of the SABATH family.

II. Perspectives

Despite the progress made, there are still many questions remained about function and evolution of the SABATH families in rice and poplar. Further experiments can be performed from the following perspectives.

Biochemical function of SABATH genes

Rice and poplar genomes contain 41 and 33 *SABATH* genes, respectively. The biochemical functions of two rice *SABATH* genes and two poplar *SABATH* genes have been identified. However, I can not determine the substrates for the rest of *SABATH* genes. Elucidating the biochemical functions of SABATH proteins will be critical for understanding their biological roles. Gene expression analysis showed 23 of the 41 *OsSABATH* genes expressed at least in one organ (Figure 3.6), and 19 of the 27 selected *PtSABATH* genes expressed at least in one organ (Figure 5.2), suggesting that these genes have a role in the normal biology of healthy plants. Genes that showed no expression in any of the tissues may be expressed under stress conditions. It will be interesting to examine how they contribute to the specific biology of plants. Moreover, identification of the complete family of *SABATH* genes may provide important insights into the evolution of the SABATH family. Phylogenetic analysis indicates most of *AtSABATHs*, *OsSABATHs* and *PtSABATHs* evolved after the divergence of these three lineages. It will be interesting to test whether some SABATHs catalyze divergent biochemical reactions that contribute to the unique biology and ecology of these plant species.

Biological functions of SABATH genes

The biological functions of *SABATH* genes in plants need to be further studied because of some unanswered questions. First, the majority of *SABATH* genes in rice and poplar await to be functionally characterized. However, gene expression analysis showed most of them expressed at least in one organ under the normal growth condition, suggesting they are critical in plant growth and development. It will be interesting to study the biological functions of these unidentified *SABATH* genes. Second, the role of IAMT is still controversial. MeIAA is regarded as inactive form of IAA, serves as regulating the IAA homeostasis in vivo. However, data showed most IAA molecules exist as IAA-ester or IAA-amide conjugates for storage or degradation (Ljung et al. 2002). The high levels expression of IAMT in multiple tissue indicated the methylation of IAA should be very critical for the plant growth and development. Does MeIAA only function as the storage form of IAA just like other IAA conjugation? A potential mechanism suggests that MeIAA is hydrophobic and easier for cell-to-cell transport, it may act as an internal signaling molecule. Further study will be needed to address the question. Third, as described in chapter IV and V, both *OsSBMT1* and *PtJBMT* showed high level of expression at multiple tissues under the normal condition, indicating they are critical in the plant growth and development. Rice is one of the plant species that contains higher levels of SA at tissues of plants grown under normal conditions (Silverman et al., 1995). MeJA is reported as an important regulator of various developmental processes, such as seed germination,

flowering, fruit development, and leaf senescence (Creelman and Mullet, 1995). These suggested the importance of MeSA and MeJA in plant normal developmental processes. However, the biological roles of them have not been completely understood. MeSA and MeJA has been suggested to act as internal signaling molecules under stress conditions, do they serve as the same functions at normal conditions? If so, some plant MeSA and MeJA esterases should be discovered under normal conditions. Fourth, what is the role of *OsBSMT1* in the indirect defense? MeSA is a frequent constituent of insect-induced plant volatiles. It has been detected in the headspace of insect-infested plants of lima bean (Arimura et al., 2002), *Arabidopsis* (Chen et al., 2003), tomato (Ament et al., 2004), and soybean (Zhu and Park, 2005). When tested in its pure, synthetic form, MeSA can attract several types of carnivores (Dicke et al., 1990, De Boer and Dicke, 2004; Zhu and Park, 2005). These suggest that MeSA is an active signal for indirect defense of tested plants. However, insect damaged rice released large amount of volatile organic compounds, and MeSA is just one of them. Does MeSA work singly or in combination with other volatiles in attracting the carnivorous enemies? In addition, MeSA has been detected at many insect-infested plants. The volatiles emitted in indirect defense have been proved to be diverse in term of plant species, insect order, and types of natural enemies (Yuan, 2007). Considering the important role of MeSA as the signaling molecule, is there the possibility that MeSA serves as signaling molecule to induce the emission of other volatiles? Fifth, another intriguing question is how MeSA emission was regulated. I have studied the regulation of MeSA production from the

substrate level and the signal transduction level. As described in chapter IV, the SA content is not important for the MeSA production. JA plays an essential role in insect-induced MeSA emission by cross-talk with SA signaling pathway. Next, further study the regulatory mechanism of these signaling pathways in plant defense responses should be focused on the transcriptional level. A microarray experiment has been performed to identify candidate genes for the volatiles production in fall armyworm damaged rice (Yuan et al., 2008). Result showed three WRKY transcription factor genes were upregulated. Some WRKY genes have been shown to be involved in plant defense (Wang et al., 2006). Therefore, isolation and characterization of these WRKY will help understand the regulatory mechanism of MeSA in plant indirect defense.

Genetic studies will be a good choice to study the biological functions of *SABATH* genes in plants. The transgenic plants with overexpression or knockout of specific *SABATH* gene will help to detect the biological function of this gene. By comparing the difference of phenotypes between the transgenic plants and wild type plants, the specific gene function could be determined.

Evolution of SABATH genes

Further understanding of the evolution of *SABATH* genes will help to elucidate the mechanisms of *SABATH* genes in plant growth and development as well as their interactions with the environment. I have achieved some improvement about the

evolution of *SABATH* family. As I presented at previous part, IAMT is likely an ancient member of the *SABATH* protein family, from which other *SABATH* MT activities, including JMT, SAMT and FAMT, may have evolved. However, some questions still need to be addressed. First, how old is the *SABATH* family? As I have mentioned in the chapter I, all characterized *SABATH* genes were isolated from angiosperm species. Are they present in gymnosperm and lower plants? I have identified three *SABATH* genes from the white spruce, a gymnosperm species. One of them showed activity with IAA and was named as spruce IAMT. Phylogenetic analysis showed spruce IAMT was grouped into one highly conserved clade with some IAMTs from other plant species (Figure 6.2). The identification of *SABATH* genes from white spruce suggests that the presence of *SABATH* genes predated the divergence of angiosperm and gymnosperm. It also implies that IAMT is the ancient member of *SABATH* family, at least in the plant species I detected till now. Whether *SABATH* genes present at lower plants needs to be further studied. Second, both of OsBSMT1 and PtJBMT have dual activities. The evolution of the dual activities is intriguing. For both of them, BA is not the major substrate. It is interesting to detect if there is the BAMT in rice and poplar. It is not surprising that OsBAMT1 has both SAMT and BAMT activities, because SA and BA have similar structures. There are several BSMTs identified in plants (Chen et al., 2003; Negre et al., 2002; Pott et al., 2004). The study with *Arabidopsis* BSMT showed the ancestor of AtBSMT1 may have higher activity with BA, i.e., the SAMT activity of AtBSMT1 evolved from BAMT activity (Chen et al., 2003). Therefore, OsBSMT1 may have a same

evolutionary trajectory. However, the structures of JA and BA are largely different. How PtJBMT catalyze these two structural different substrates is still unknown. The determined three-dimensional structure is useful for solving this question. Moreover, the biological functions of MeBA in rice and poplar are still unknown. MeBA is reported as the components of floral scents in the flowering plant species. I can not detect the emission of MeBA from poplar and rice under normal conditions, suggesting MeBA may lose the function in floral scents in these plant species. The low level of MeBA was released in the fall armyworm damaged rice. Whether it is involved in plant indirect defense remains to be determined. Third, site-directed mutagenesis of PtJBMT revealed the variation of specific residue can change the substrate selection of SABATH enzyme. This provides a powerful tool for us to study the evolution of SABATH family. Since IAMT is the ancient member of SABATH family, from which other SABATH members evolve, it will be accessibility to convert IAMT to other SABATHs, such SAMT and JMT by site-mutagenesis approach.

Overall, this research led to important understanding of SABATH gene family in plants. However, further studies need to be performed to identify the biochemical functions of the majority of *SABTAH* genes, the important functions of *SABATH* genes in plant development and defense as well as the evolution of the *SABATH* family in the plant kingdom.

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Appendix

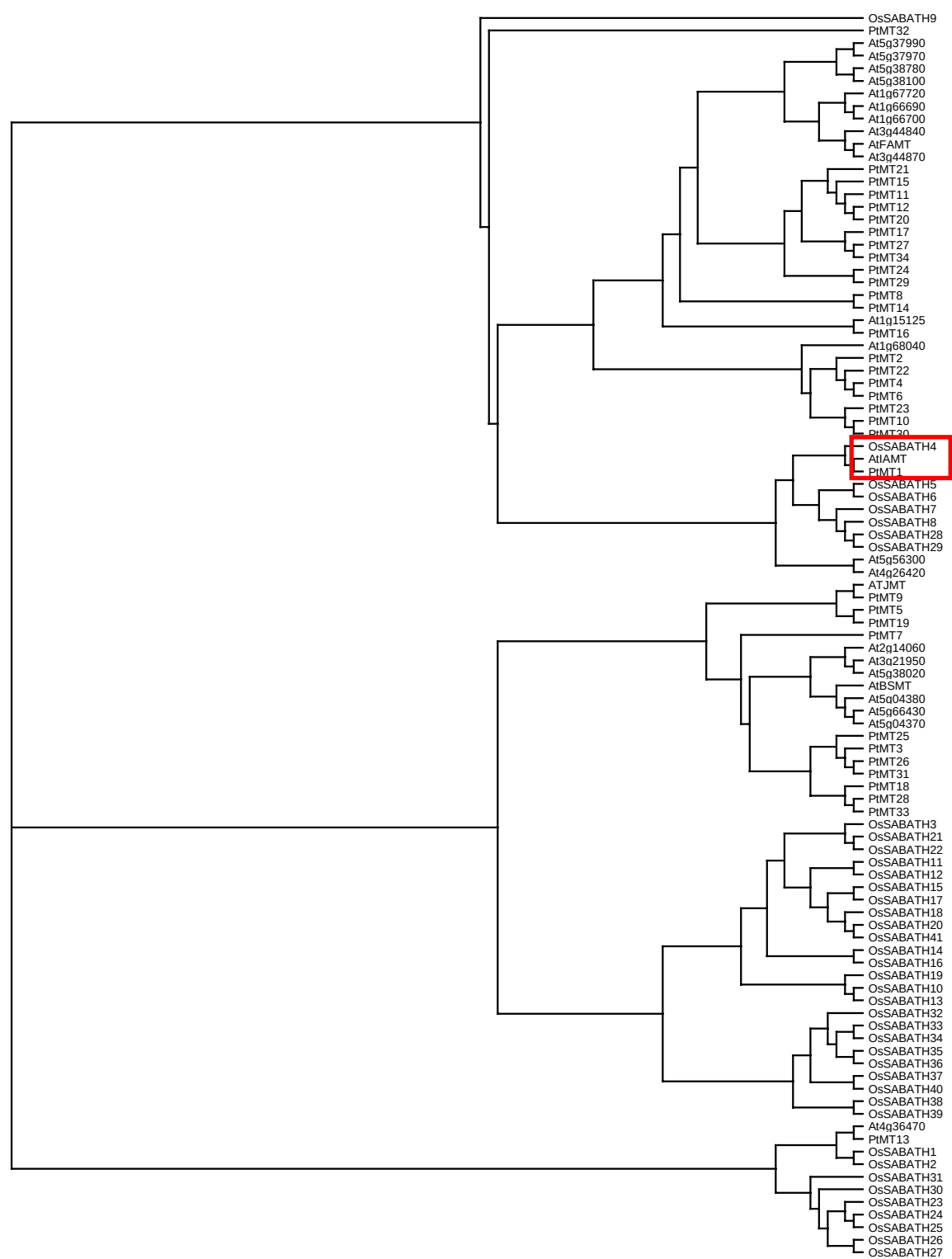


Figure 6.1. Phylogenetic analysis of Rice SABATHs, Poplar SABATHs and Arabidopsis SABATHs

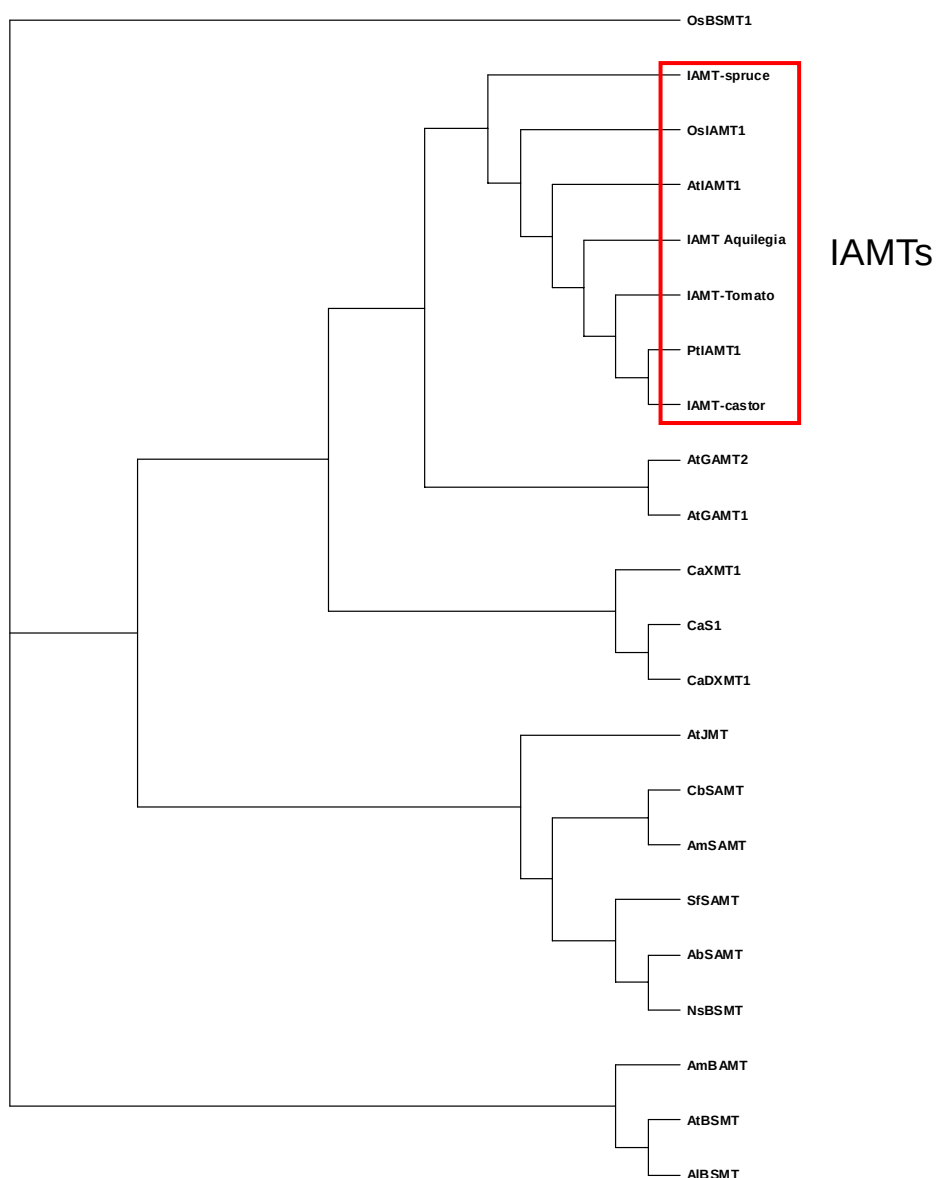


Figure 6.2. A neighbor-joining tree based on the degree of sequence similarity between some IAMTs and selected SABATHs from plants using the clustalX program. OsIAMT1, *Oryza sativa* IAMT1 (accession no.EU375746); PtIAMT1, *Populus trichocarpa* IAMT1 (fgenes4_pg.C_LG_I002808); AtIAMT1, *Arabidopsis thaliana* IAMT (accession no.AK175586); CbSMT, *Clarkia breweri* SMT (accession no.AF133053); AmSMT, *Antirrhinum majus* (snapdragon) SMT (accession

Figure 6.2. Continued

no.AF515284); SfSAMT, *Stephanotis floribunda* SAMT (accession no.AJ308570); AmBAMT, *Antirrhinum majus* BAMT (accession no.AF198492); NsBSMT, *Nicotiana suaveolens* BSMT (accession no.AJ628349); AtBSMT, *Arabidopsis thaliana* BSMT (accession no.BT022049); AlBSMT, *Arabidopsis lyrata* BSMT (accession no.AY224596); AbSAMT, *Atropa belladonna* SAMT (accession no.AB049752); PhBSMT, *Petunia hybrida* BSMT (accession no.AY233465); AtJMT, *Arabidopsis thaliana* JMT (accession no.AY008434); AtGAMT1, *Arabidopsis thaliana* GAMT1 (At4g26420); AtGAMT2, *Arabidopsis thaliana* GAMT2 (At5g56300); Cas1, *Coffea arabica* caffeine synthase 1 (accession no.AB086414); CaXMT1, *Coffea arabica* XMT1 (accession no.AB048793); CaDXMT1, *Coffea arabica* DXMT1 (accession no.AB084125). Branches were drawn to scale with the bar indicating 0.1 substitutions per site.

Vita

Nan Zhao was born in Tianjin, China, on December 18, 1978. After completing his early education in Tianjin, China, he entered the Nankai University, Tianjin, China in 1997, where he obtained his B.S. degree in 2001 and his M.S. degree in 2004. After graduation, he entered the Tasly Group, Tianjin, China in the fall of 2004, working as a technician to develop new biological products in Biotech-pharm Center. He married Jingyu Lin in July, 2005. Soon after, he became a Ph.D. student at University of Tennessee in August, 2005, where he is focused on the study of SABATH methyltransferases important for plant growth and defense using functional genomics. He received his PhD in 2008.