

**Biochemistry and Evolution of Phytohormone-methylating SABATH
Methyltransferase in Plants**

**A Dissertation Presented for the
Doctor of Philosophy
Degree**

The University of Tennessee, Knoxville

Minta Chaiprasongsuk

May 2016

ACKNOWLEDGEMENTS

This research would not have been possible without the support of several peoples. I want to acknowledge these people who have made my PhD study happen. First, I want to express my deepest gratitude to my advisor Dr. Feng Chen for his guidance, understanding and encouragement through my PhD study. He has been training me to be a responsible researcher with a positive attitude toward strenuous research efforts. I also sincerely appreciate my co-advisors, Dr. Max Cheng, Dr. Hem Bhandari and Dr. Hong Guo for their advice and feedback. I want to thank the Royal Thai Government Scholarship for funding support. Without this support I would not have made it this far. I also want to thank my present and past lab members for their continual professional support and personal friendship: Dr. Hao Chen, Dr. Xinlu Chen, Dr. Gitika Shrivastava, Lori Osburn, Ayla Norris, Dr. Jingyu Lin, Dr. Nan Zhao, Dr. Yifan Jiang, Dr. Guanglin Li, Dr. Jianyu Fu, Wangdan Xiong and Chi Zhang. I want to express my deepest gratitude for Dr. Robert Orr and his wife Donna Orr for both academic suggestions and support while I am in USA. Without their support I would have had a hard time surviving in the USA. I want to express my thanks to all my friends and coworkers at the University of Tennessee who supported and made this study possible. Finally, I owe special thanks to my family, who have given me unconditional love, support, encouragement, care and patience. They were always there whenever I need them, in all the hardships and shared the many uncertainties, challenges, and frustrations. Without their continued support and counsels, I could not complete this process.

ABSTRACT

Known members of Phytohormone-methylating compounds are plant synthesis compounds that serve as attractants of other living organisms beneficial to the plants or as defense against other biotic as well as abiotic agents. To increase their fitness and survival in a stressful environment plants produce distinct sets of phytohormone-methylating compounds. Plant genomes can encode the necessary enzymes to acquire the ability to make new specialized compounds during evolution. This dissertation aims to investigate the biochemical and biological functions and evolution of SABATH genes in different lineages of plants. Black cottonwood, *Brachypodium* and Norway spruce genome were used as the model for dicotyledon, monocotyledon and gymnosperm for evolution study. Using a comparative genomic approach 28, 12 and 10 SABATH genes were identified in black cottonwood, *Brachypodium* and Norway spruce, respectively. In order to understand biochemical and biological of SABATH from dicotyledon plant, selected enzyme members from black cottonwood were encoded and only one protein was determined to methylate phytohormone. PtJMT1 displayed activity with JA over BA. Site-mutation of Ser-153 and Asn-361 were construct in order to reveal a key residue that determine the relative activity of the enzyme with JA and BA. Homology-based structural modeling indicated that substrate alignment, in which Asn-361 is involved, plays a role in determining the substrate specificity of PtJMT1. The expression of PtJMT1 was induced by plant defense signal molecules methyl jasmonate and salicylic acid and a fungal elicitor alamethicin, suggesting that PtJMT1 may have a role in plant defense against biotic stresses. In order to understand the evolution of phytohormone-methylating enzymes entire SABATH genes in *Brachypodium* and Norway spruce were encoded and recombinant proteins were tested with phytohormone compounds for methyltransferase activities. In *Brachypodium* three proteins were

determined to methylate phytohormone. These three enzymes (BdSABATH5, BdSABATH6 and BdSABATH8) have IAMT activity. All three members also had JMT activity, with BdSABATH5 and 6 having higher activity with JA than with IAA. In Norway spruce, five proteins were determined to methylate phytohormone. Three JMTs (PaJMT, PaJMT2, and PaJMT3), one IAMT (PaIAMT) and one SAMT (PaSAMT) enzymes from Norway spruce.

TABLE OF CONTENTS

CHAPTER I Introduction: The Phytohormone-methylating SABATH Methyltransferase	
in Plants	1
Content.....	2
Reference.....	19
Appendix	30
CHAPTER II Molecular and Biochemical Characterization of the Jasmonic Acid	
Methyltransferase Gene from Black Cottonwood (Populus	
trichocarpa).....	36
Abstract.....	37
Introduction.....	39
Results.....	41
Discussion.....	46
Concluding remarks.....	48
Experimental design.....	49
References.....	55
Appendix.....	61
CHAPTER III Enzyme Promiscuity-based Recent Evolution of an Ancient Biochemical	
Function in the SABATH Family of Methyltransferases.....	74
Abstract.....	75
Introduction.....	77
Results.....	79
Discussion.....	83

Concluding remarks.....	85
Material and method.....	86
References.....	89
Appendix.....	92
CHAPTER IV Evolution of Phytohormone-methylating Enzymes: Implications from the Characterization of the SABATH family of Methyltransferases from Norway spruce.....	103
Abstract.....	104
Introduction.....	105
Results.....	107
Discussion.....	110
Concluding remarks.....	111
Material and method.....	112
References.....	116
Appendix.....	121
CHAPTER V Conclusions and Perspectives	129
Conclusions.....	130
Perspectives and implications for future research.....	133
References.....	135
VITA.....	136

LIST OF TABLES

Table 1.1 SABATH genes in plants.....	30
Table 2.1 SABATH genes in the genome of <i>Populus trichocarpa</i>	61
Table 2.2 Primers used for cloning of full-length cDNAs.....	62
Table 3.1 SABATH genes in the genome of <i>Brachypodium distachyon</i>	92
Table 3.2 Screening of relative activities of BdSABATHs with 5 substrates	93
Table 4.1 SABATH genes in the genome of <i>Picea albies</i>	121
Table 4.2 Screening of relative activities of PaSABATHs with 5 substrates	122
Table 4.3 Michaelis-Menten kinetic parameters for Norway spruce enzymes (PaSABATH10, PaSABATH4 and PaSABATH5) with jasmonic acid (JA).....	123

LIST OF FIGURES

Figure 1.1 MeIAA biosynthesis.....	33
Figure 1.2 MeSA biosynthesis.....	34
Figure 1.3 MeJA biosynthesis.....	35
Figure 2.1 Structures of compounds.....	63
Figure 2.2 Identification of the catalytic product of PtJMT1.....	64
Figure 2.3 Biochemical properties of PtJMT1.....	65
Figure 2.4 Substrate specificities of PtJMT1 and its mutants.....	67
Figure 2.5 A Structure-based alignment.....	68
Figure 2.6 Homology models of the active site of wild-type PtJMT1 and the mutant of PtJMT1 (N361S) complexed with <i>S</i> -adenosyl-l-homocysteine (AdoHcy or SAH) and jasmonic acid (1) (JA) or benzoic acid (2) (BA).....	69
Figure 2.7 Semi-quantitative RT-PCR analysis of PtJMT1 expression.....	70
Figure 2.8 Expression of PtJMT1 under stress conditions.....	71
Figure 2.9 Phylogenetic analysis of PtJMT1 with other known SABATH proteins.....	72
Figure 3.1 Phylogenetic analysis of SABATH genes from plants.....	94
Figure 3.2 Exon and intron of SABATH genes from <i>Brachypodium</i>	96
Figure 3.3 SABATH gene locations on <i>Brachypodium</i> chromosomes	97
Figure 3.4 Relative enzyme activity of sorghum ortholog gene (Sb05g027465) with 5 substrate.	98
Figure 3.5 Phylogenetic analysis of 12 othologs genes from 4 different species of <i>Brachypodium</i> with known SABATH enzymes.....	99

Figure 3.6 Relative enzyme activities of SABATHs from different species of <i>Brachypodium</i> with 5 substrates.....	101
Figure 4.1 Exon and intron of SABATHs genes in Norway spruce.....	124
Figure 4.2 Biochemical properties of PaJMT.....	125
Figure 4.2 Detailed biochemical characterization of PaJMT.....	126
Figure 4.4 Phylogenetic analysis of SABATHs Family in Norway spruce.....	127

CHAPTER I

Introduction: The Phytohormone-methylating SABATH Methyltransferase in Plants

Content

Plant secondary metabolites

Compounds known as plant secondary metabolites are involved in complex processes in plant metabolism. Some secondary metabolites serve as attractants for pollinators and seed dispersing animals; some help plants defend against insects and pathogens; others are involved in survival under abiotic stress (Zhao et al., 2005). Secondary metabolites are very specific to plant species, i.e., a plant species produces a unique secondary metabolite profile that is related to the specific physiological and ecological processes of the species.

Secondary metabolite biosynthesis genes make up a large portion of plant genomes. 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). Presently, only a small number of these genes have been functionally characterized. The biochemical functions of the majority of the genes involved in secondary metabolism are unclear. Functional characterization of these genes may provide specific details about their evolution and functions in different plant species. This fundamental information may provide key knowledge and tools for the development of sustainable agricultural systems, for effective pest management and abiotic stress resistance.

Hydroxylation, glycosylation, methylation and acylation are examples of enzymatic reactions involved in the biosynthesis of most secondary metabolites (Noel et al., 2005). Methyltransferases enzymes are one of the methylation enzymes that transfer 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 known as the most widely used methyl donor.

Most types of plant metabolites of both primary and secondary metabolism, such as DNA, protein, lipid and small molecular metabolites, are known to be methylated.

Types of small molecule methyltransferases in plants

Small molecule methyltransferases (MTs) can be divided into different types based on the chemical nature of substrates. The types are listed as follows: carbon MT (CMT), nitrogen MT (NMT), sulfur MT (SMT) and oxygen MT (OMT). Recently, a large variety of enzymes that methylate 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.

Type I OMTs

Type I OMTs is a large group of proteins that is involved in benzene ring methylation substrate and include proteins such as phenylpropanoids, flavonoids, isoflavonoids, alkaloids, eugenol, chavicol, coumarins, orcinols, and stilbenes. These proteins have a molecular mass of approximately 40 kDa. For example, Isoflavonoid OMTs (IOMT) catalyzed 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 structures of several Type I OMTs have been solved, such as the chalcone O-methyltransferase (ChOMT) and the isoflavone O-methyltransferase (IOMT) from alfalfa (Zubieta et al., 2001).

Type II OMTs

Type II OMTs contain a family of proteins with a molecular mass of 23-28 kDa whose known substrate are CoA-esters of phenylpropanoids. Their main role is related in lignin biosynthesis. This type of OMTs requires a divalent cation to mediate the methyl group transfer. For example, Caffeoyl Coenzyme A 3-O-methyltransferase (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 studied (Ferrer et al., 2005); it shows high structural similarity to the animal catechol OMT.

Type III OMTs (SABATHs)

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 the methyl group from SAM to the carboxyl group or the nitrogen group of the substrate, forming 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. Their crystal structure has been resolved with *clarkia breweri* Salicylic acid methyltransferase (CbSAMT) (Zubieta et al., 2003).

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), benzoic acid (BA; Dudareva et al., 2000; Murfitt et al., 2000) and farnesoic acid (FA; 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 a naturally occurring auxin in seeding plants. It is a general phytohormone that plants produce for their growth and development processes. Moreover, it is also involved in plant interactions with the environment (Cohen et al., 2003). 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. At present, the physiological effects of IAA in plants have been well studied. 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).

IAA activity is modulated at three different interdependent levels: homeostasis, polar transport, and auxin responses. There is a variety of mechanisms plants use to regulate IAA concentration, including biosynthesis, conjugation/deconjugation, degradation, and down-stream signaling transduction (Ljung et al., 2002; Woodward and Bartel, 2005). Low levels of IAA

exists as free IAA, and most IAA molecules exist as IAA-ester or IAA amide conjugates in plants. Ester-type conjugates comprise one of the groups of conjugates that has been identified in a variety of plant species, which include the carboxyl group of IAA which is linked to sugars, such as glucose; and amide-type conjugates. Another group is the carboxyl group which 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. 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).

The conjugation of IAA has been reported as methyl ester of IAA (MeIAA) which was characterized by IAA-methyltransferase (IAMT1) from *Arabidopsis thaliana* (*A. thaliana*), (Qin et al., 2005). There is evidence that IAMT1 is involved in *Arabidopsis* leaf development. Down-regulating IAMT1 expression leads to dramatic epinastic leaf phenotypes. Auxin overproduction mutants have epinastic leaves. Overexpression of IAMT1 displays leaf phenotypes opposite to those of IAMT1 RNAi lines. These findings suggest that IAMT1 has a role in regulating IAA homeostasis. Furthermore, the application of exogenous MeIAA appears similar but more strongly inhibits hypocotyl and root elongation than using 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 supported by the identification of several methyl esterases in *Arabidopsis* (Yang et al., 2008).

Other research has found that IAMTs are conserved among plant species such as rice, *Arabidopsis*, and poplar (Zhao et al, 2008). In monocotyledon plants, there is only the report of

OsIAMT from rice that has been cloned and characterized. Zhao also found that OsIAMT1 exhibited expression in multiple tissues of rice. It showed high levels of expression in roots and panicles, while PtIAMT1 showed high levels of expression in stems, moderate levels of expression in young leaves and low levels of expression in roots. So, high levels of expression of IAMT may play an important role in regulating IAA activities in multiple tissues (Zhao, 2008). The IAMT mechanism is still unclear because MeIAA is not an active auxin and is more nonpolar than IAA. However, IAMT is the enzyme that transfer the methyl group to IAA and produce MeIAA. (Figure 1.1) MeIAA could easy diffuse across membranes, thus it is possible to transport IAA in MeIAA form to neighboring cells or distant targets (Yang et al., 2008).

SA and MeSA: biosynthesis, metabolism and biological function

SA is one of the phenolic compounds naturally present in higher plants. The highest contents of salicylic acid in the free form as well as in the form of its glycoside have been found and reported in many families of plants. It is an essential plant signaling molecule, involved in a variety 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, research was conducted on tobacco leaves treated with SA or acetyl salicylic acid (aspirin). The leaves exhibited enhanced resistance to TMV infection and increased pathogenesis-related (PR) protein accumulation (White, 1979; Antoniwand et al., 1980). Exogenous SA treatment increases the resistance ability of 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). A large number of mutants and transgenic experiments

was used to demonstrate how SA is essential in plant defense. Constitutively expressed systemic acquired resistance (SAR) accumulated high levels of SA in several *Arabidopsis* mutants (Bowling et al., 1994; Bowling et al., 1997). The *nahG* gene of *Pseudomonas putida* overexpressed in *Arabidopsis* and tobacco are examples of SA function in plant defense (Seskar et al., 1998). This enzyme encodes salicylate hydroxylase, which converts SA to the biologically inactive catechol. When these transgenic plants were infected by pathogens, SA cannot accumulate, resulting in failure of inducing PR gene expression and SAR (Seskar et al., 1998). Moreover, SA is also considered as the endogenous long distance signal molecule which accumulates in a non-infected region to induce plant defense response, which is supported by the detection of SA in the phloem of pathogen-infected tobacco and cucumbers (Mettraux et al., 1990; Rasmussen et al., 1991). 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).

The application of exogenous SA could enhance protection against several types of abiotic stresses such as high or low temperature and heavy metals (Eszter et al., 2007; Dorothea et al., 2005). 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. 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).

In contrast, 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. MeSA as a constituent of floral flavor which can attract insect pollinators has been reported in many publications (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 pathogens in healthy tissues of the infected plant and neighboring uninfected plants (Seskar et al., 1998; Shulaev et al., 1997). Recently, MeSA has been proven an endogenous signal inducing SAR (Park et al., 2007).

MeSA is also reported to be involved in tritrophic interactions. The concentration of MeSA was detected to be highly increased in 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 preferred the MeSA-containing volatile blends, and MeSA attracted predatory mites in an amount-dependent way (De Boer and Dicke, 2004). These findings 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 1.2), 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 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

The Jasmonic acid (JA)-signaling pathway plays critical roles in many biological processes in plants. JA is best known for its role in wound-signaling. When plants are attacked by insects, JA production is promoted, which leads to the induction of various defenses. 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).

MeJA is a component of floral scents of many species, and it has been suggested to be the signal for talking trees. MeJA is made from JA by the action of SAM-dependent methyltransferase (Figure 1.3). 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 in plant defense mechanisms (Seo et al., 2001). It is expressed under normal conditions. Interestingly, overexpressing JMT in Arabidopsis leads to an elevated level of methyl jasmonate but does not change the level of free JA.

Transgenic plants were more resistant to a virulent fungus *B. cinerea* than were wild-type plants (Seo et al., 2001). The biological roles of such modification are not completely understood. However, methyl jasmonate is hydrophobic and easier for cell-to-cell transport and, therefore, has been suggested to act as an internal signaling molecule

To date there are AtJMT from *Arabidopsis*, PtJMT from cottonwood, SlJMT from tomato, FvJMT from strawberry and BjJMT from *Brassica juncea* that have been characterized (Seo et al., 2001; Zhao et al., 2013; Tieman et al., 2010; Preu et al. 2014; Meur et al., 2015). These findings have shown JMT only from dicotyledon plants. However, no other JMT in monocotyledon and gymnosperm has been reported. Information about the 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 us to understand the evolution of substrate specificity of the SABATH family. Further investigation in this subject will help identify key residues responsible for substrate specificity of the SABATH family.

Unknown functional enzyme from SABATH sequences from many plants

Genomics projects with various plant species display the existence of a large number of *SABATH* genes in plants (D'Auria, 2003). *Arabidopsis*, black cottonwood, and rice contain 24, 28, and 41 *SABATH* genes (Chen et al., 2003; D'Auria, 2003, Zhao et al., 2013, Zhao et al., 2008). The *Arabidopsis* *SABATH* genes are scattered on all five chromosomes with some of them localized in clusters (D'Auria, 2003). Six of 24 *AtSABATH* genes have been biochemically characterized. There are five biochemical activities, including JMT, BSMT, IAMT, FAMT and GAMT. AtJMT is the first JMT gene to be characterized (Seo et al., 2001). Another JMT gene is

from cottonwood PtJMT. This PtJMT1 using JA and BA as substrate were 175 μ M and 341 μ M, respectively. Moreover, these two JMT are orthologs and closely related. Based on the identification of these SABATH genes and hypotheses, 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 display BMT and SAMT as the enzyme has both activities (Chen et al., 2003). AtBSMT gene cloning from Arabidopsis displays the correlated emission patterns of methyl salicylate and methyl benzoate from plants and expression patterns of *AtSABATH* genes under various conditions (Chen et al., 2003). However, AtIAMT was identified 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 assay, and individual SABATH proteins were tested with a pool of chemicals including known substrates of SABATH proteins and compounds that were 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 functions of unknown SABATH genes, a combination of molecular genetics, biochemistry, and functional genomics is required. Another JMT from

poplars that has been identified and characterized in the same case. Only few genes cluster and homolog with AtJMT have been selected for studying. This is because SABATH genes from black cottonwood have 28 gene members (Zhao et al., 2013). The study of the evolution of this gene family is a challenge when unknown proteins have yet to be identified and characterized.

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 monocotyledon 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 the previous section, the Arabidopsis genome contains 24 SABATH genes. So far, only 6 of them have been biochemically characterized. Determination of functions of unknown SABATH genes in Arabidopsis and other plants species remains a challenging task. Moreover, another JMT from cottonwood is also from dicotyledon plants. 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 from various

plant species need to be isolated and characterized. Such analysis, especially identification of orthologous SABATH genes from diverse plant species, will help us to understand the evolution of SABATH genes within and among plant species. Moreover, complete SABATH genes in monocotyledon and gymnosperm will help us to understand their evolution more clearly and answer some of the question addressed earlier.

Gene discovery as a tool for studying the comparative functional genomic approach of the SABATH family

Gene discovery using functional genomics and comparative genomics is effective. Functional genomics is the study of the functions of genes at a large scale, for example, microarray analysis can help to identify the candidate genes. Comparative functional genomics is the study of the functions of genes at a large scale based on genomic comparison of a variety of plant species. The genomes of three plant species, including Norway spruce, *Brachypodium*, and black cottonwood have been fully sequenced (Nystedt, Street et al., 2013; The International *Brachypodium* Initiative, 2010; Tuskan et al., 2006). Genome sequencing for many other plant species is also available i.e. *Arabidopsis*, rice, sorghum, maize and poplar, have been fully sequenced (The *Arabidopsis* Genome Initiative, 2000; Goff et al., 2002; Initiative, 2010; Paterson et al., 2009; Schnable et al., 2009; Tuskan et al., 2006). This research finding provides unprecedented opportunities for cross-species comparison of gene families.

In this dissertation, 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 Norway spruce and *Brachypodium*. Then the phylogenetic analysis among moss, Norway spruce, rice, sorghum, maize, *Brachypodium*, Colorado blue columbine, tomato, black

cottonwood, Arabidopsis, and soybean SABATH genes will be performed to identify the orthologous genes. Selected 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.

Black cottonwood (*Populus trichocarpa*), purple false brome (*Brachypodium distachyon*) and Norway spruce (*Picea albies*) as plant models

In this dissertation, Black cottonwood, purple false brome and Norway spruce are used as plant models. Black cottonwood was chosen as a model plant species for the isolation and functional characterization of JMT. Black cottonwood has significant impact as a model of perennial woody species. It has a small genome size (about 480 Mbp) when compared with other tree species. To date the genome of this tree has been completely sequenced, making it available to establish the various functional genomics tools in tree species (Tuskan et al., 2006). The black cottonwood also has rapid growth and prolific sexual reproduction. Moreover, the ease of gene cloning and poplar transformation are significant characteristics to consider as a model woody tree for studying the physiology and ecology of forest trees (Bradshaw et al., 2000). As a potential bioenergy crop, the increased yield of propagation in this species is a desirable characteristic (Davis, 2008). Genetic improvement of poplars for higher biomass yield is our goal. However, in order to improve resistance to biotic stress, more understanding of the natural defense mechanisms of tree species is needed. This aspect of fundamental study about their defense mechanisms is highly important and will help us understand the evolution of plant defenses in perennial woody species. In addition, this also has practical application for improving woody crops. Previous research has found that many hybrid poplars are more

susceptible to pathogens (Newcombe, 1998). In some areas, herbivorous insects cause serious loss in poplar plantations (Ostry et al. 1988). A better understanding of defense responses in woody species can help us improve plantations.

The purple false brome (*Brachypodium distachyon*) is known as a potential grass model because of its small genome size and its close relationship to wheat and other temperate grass species. The purple false brome life cycle is very short and has the additional advantage of completing the whole cycle in one month. It is advantageous to study the whole system of this plant, taking less time to do and having less complexity of genome size when compared to the genomes of rice, sorghum or wheat. *Brachypodium distachyon* is the most beneficial organism in which to study the mechanism of secondary metabolite biosynthesis in the SABATH family. Moreover, the entire genome sequence has been released. It raises interesting questions: how many genes are involved in methyltransferases, and how they are conserved to other plant species? It is also intriguing to ask how JMTs with different degrees of biochemical functions respond with substrate. Furthermore, the purple false brome has been proposed as a bridge between rice (*Oryza sativa* L.) and temperate cereal crops for genomics research (Draper et al. 2001). *B. distachyon* has a number of advantages as a model species, such as annual growth habit, self-fertility, short generation time, and a small genome (355 Mb) (The International *Brachypodium* Initiative, 2010), and it has five pairs of readily identifiable chromosomes. The biological features in *B. distachyon* are not found in rice but are important for temperate cereals. Crop characteristics include freezing tolerance, vernalization requirement and host resistance to specific pathogens or insect pests (Draper et al., 2001). In addition, although *B. distachyon* accessions have varying vernalization requirements and different reactions to several plant pathogens (Draper et al., 2001; Routledge et al., 2004; Allwood et al., 2006), genetic variation in

response to other biotic or abiotic stresses has not been reported. Such information could help facilitate utilization of *B. distachyon* resources in crop breeding.

Norway spruce (*Picea abies* (L.) Karst) was chosen as a model plant species for the isolation and functional characterization of JMT. Conifers are long-lived organisms that must defend themselves against a myriad of insects, pathogens and herbivores. Conifers depend in large part on the production of volatile compounds both for chemical and physical defenses. *Picea abies* is a species of conifer that survives at higher latitudes in Norway (Parducci, Jorgensen et al. 2012), but based on the present day population genetic structure, it does not seem that these populations have contributed extensively to their re-colonization of Scandinavia. Extensive information has been accumulated on the genetic control of commercially important traits in spruce due to the efforts on genetic improvement. Norway spruce has been used to study the transient transcript accumulation of monoterpenoid synthases and diterpenoid synthases in stem tissues after MeJA induction (Faldt, Martin et al. 2003). According to Nystedt, Street et al. (2013), Norway spruce is a fully sequenced genome that will accelerate the investigation of gymnosperm biology and will also provide broader genetic and evolutionary insight. The extensive amount of genomic information combined with well-studied biology makes Norway spruce a useful model for studying the molecular biology and biochemistry of the SABATH family in gymnosperms. Only one gene of the SABATH family has been functionally characterized from gymnosperm. It is IAMT from white spruce (*Picea glauca*) PgIAMT (Nan et al., 2009). Functional studies of SABATH genes in gymnosperms may provide important information into the evolution of the SABATH family as well as the biological processes that they control.

For maximum benefit from fully sequenced genomes of black cottonwood, *Brachypodium* and Norway spruce. **I propose to use the comparative functional genomics approach to identify the SABATH families in black cottonwood, *Brachypodium* and Norway spruce. The investigation of the biochemical and biological functions and evolution is used to select the members of the SABATH family. The goal of this PhD research is to increase the understanding of function and evolution in 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 black cottonwood, *Brachypodium* and Norway spruce. In the objective 2, black cottonwood JMT will be cloned and its biochemical and biological functions will be studied. In the objective 3, IAMT and JMT from *Brachypodium* will be studied. Phylogenetic and structural analysis will be performed to understand the evolution of the SABATH family. Objective 4 is JMT from *Brachypodium* and sorghum will be studied in order to understand promiscuous enzymes of SABATH gene families in monocotyledon plants. In objective 5, Norway spruce JMT will be cloned and its biochemical will be studied in order to understand JMTs in gymnosperm. Phylogenetic and structural analysis will be performed to understand the evolution of gymnosperm and angiosperm in SABATH gene families.

References

- Aimé S, Cordier C, Alabouvette C, Olivain C** (2008) Comparative analysis of PR gene expression in tomato inoculated with virulent *Fusarium oxysporum* f. sp. *lycopersici* and the biocontrol strain *F. oxysporum* Fo47. *Physiol. Mol. Plant Path.* **73**: 9–15.
- Allwood JW, Ellis DI, Heald JK, Goodacre R, Mur LAJ** (2006) Metabolomic approaches reveal that phosphatidic and phosphatidyl glycerol phospholipids are major discriminatory non-polar metabolites in responses by *Brachypodium distachyon* to challenge by *Magnaporthe grisea*. *Plant J.* **46**: 351–368
- Altschul SF, Stephen F, Gish W, Miller W, Myers EW, Lipman DJ** (1990) Basic local alignment search tool. *J Mol Bio* **215** (3): 403-410
- Ashihara H, Sano H, Crozier A** (2008) Caffeine and related purine alkaloids: Biosynthesis, catabolism, function and genetic engineering. *Phytochemistry* **69**: 841-856
- Arnold TM, Appel H, Patel V, Stocum E, Kavalier A, Schultz JC** (2004) Carbohydrate translocation determines the phenolic content of *Populus* foliage: a test of the sink-source model of plant defense. *New Phytologist* **164**(1): 157–164
- Barkman TJ, Martins TR, Sutton E, Stout JT** (2007) Positive selection for single amino acid change promotes substrate discrimination of a plant volatile-producing enzyme. *Mol. Biol. Evol.* **24**, 1320–1329
- Bradford MM** (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254

- Bradshaw HD, Ceulemans R, Davis J, Stettler R** (2000) Emerging model systems in plant biology: Poplar (*Populus*) as a model forest tree. *Journal of Plant Growth Regulation* **19**: 306-313
- Chen F, D'Auria JC, Tholl D, Ross JR, Gershenzon J, Noel JP, Pichersky E** (2003a) An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J.* **36**: 577-588
- Chen F, Tholl D, D'Auria JC, Farooq A, Pichersky E, Gershenzon J** (2003b) Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. *Plant Cell* **15**: 481-494
- Cheong JJ, Choi YD** (2003) Methyl jasmonate as a vital substance in plants. *Trends Genet* **19**: 409-13
- Cohen JD, Slovin JP, Hendrickson AM** (2003) Two genetically discrete pathways convert tryptophan to auxin: more redundancy in auxin biosynthesis *TRENDS in Plant Science* **8**: 197-199
- Creelman RA, Mullet JE** (1995) Jasmonic acid distribution and action in plants – regulation during development and response to biotic and abiotic stress. *Proc Natl Acad Sci USA* **92**: 4114-4119
- Davis JM** (2008) Genetic Improvement of Poplar (*Populus* spp.) as a Bioenergy Crop. In: Vermerris W, editor. *Genetic Improvement of Bioenergy Crops*, Springer: New York , p 397-419
- Draper J, Mur LAJ, Jenkins G, Ghosh-Biswas GC, Bablak P, Hasterok R, Routledge APM** (2001) *Brachypodium distachyon* , a new model system for functional genomics in grasses. *Plant Physiology* **127**: 1539–1555

- D'Auria JC, Chen F, Pichersky E** (2003) The SABATH family of methyltransferases in *Arabidopsis thaliana* and other plant species. *Rec. Adv. Phytochem.* **37**: 95–125
- Dudareva N, Murfitt LM, Mann CJ, Gorenstein NL, Kolosova N, Kish CM, Bonham C, Wood K** (2000) Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers. *Plant Cell* **12**:949–961
- Effmert U, Saschenbrecker S, Ross J, Negre F, Fraser CM, Noel JP, Dudareva N, Piechulla B** (2005) Floral benzenoid carboxyl methyltransferases: from *in vitro* to *in planta* function. *Phytochemistry* **66**: 1211–1230
- Fukami H, Asakura T, Hirano H, Abe K, Shimomura K, Yamakawa T** (2002) Salicylic acid carboxyl methyltransferase induced in hairy root cultures of *Atropa belladonna* after treatment with exogeneously added salicylic acid. *Plant Cell Physiol* **43** (9): 1054-1058.
- Goff, SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, Hadley D, Hutchison D, Martin C, Katagiri F, Lange BM, Moughamer T, Xia Y, Budworth P, Zhong J, Miguel T, Paszkowski U, Zhang S, Colbert M, Sun WL, Chen L, Cooper B, Park S, Wood TC, Mao L, Quail P, Wing R, Dean R, Yu Y, Zharkikh A, Shen R, Sahasrabudhe S, Thomas A, Cannings R, Gutin A, Pruss D, Reid J, Tavtigian S, Mitchell J, Eldredge G, Scholl T, Miller RM, Bhatnagar S, Adey N, Rubano T, Tusneem N, Robinson R, Feldhaus J, Macalima T, Oliphant A, Briggs S** (2002) A Draft Sequence of the Rice Genome (*Oryza sativa* L. ssp. *japonica*) *Science* **5**: 92-100
- Izawa T, Takahashi Y, Yano M** (2003) Comparative biology comes into bloom: genomic and genetic comparison of flowering pathways in rice and *Arabidopsis*. *Current Opinion in Plant Biology* **6**: 113-120

- Kato M, Mizuno K, Crozier A, Fujimura T, Ashihara H** (2000) Plant biotechnology: caffeine synthase gene from tea leaves. *Nature* **406**: 956-95
- Katoh K, Standley DM** (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability (Outlines version 7). *Molecular Biology and Evolution* **30**:772-780
- Knudsen JT, Tollsten L, Bergstrom LG** (1993) Floral scents – a checklist of volatile compounds isolated by headspace techniques. *Phytochemistry* **33**: 253–280
- Koo YJ, Kim MA, Kim EH, Song JT, Jung C, Moon JK, Kim JH, Seo HS, Song SI, Kim JK, Lee JS, Cheong JJ, Choi YD** (2007) Overexpression of salicylic acid carboxyl methyltransferase reduces salicylic acid-mediated pathogen resistance in *Arabidopsis thaliana*. *Plant Mol Biol* **64**: 1–15
- Köllner TG, Lenk C, Zhao N, Seidl-Adams I, Gershenzon J, Chen F, Degenhardt J** (2010) Herbivore-Induced SABATH Methyltransferases of maize that methylate anthranilic acid using S-Adenosyl-L-Methionine. *Plant Physiol.* **153**(4): 1795–1807
- Liechti R, Gfeller A, Farmer EE** (2006) *Jasmonate Biochemical Pathway*. *Science Signaling* **322**: 1-2
- Ljung K, Hull AK, Kowalczyk M, Marchant A, Celenza J, Cohen JD, Sandberg G** (2002) Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. *Plant Mol Biol* **49**: 249–272
- Mizuno K, Kato M, Irino F, Yoneyama N, Fujimura T, Ashihara H** (2003) The first committed step reaction of caffeine biosynthesis: 7-methylxanthosine synthase is closely homologous to caffeine synthases in coffee (*Coffea Arabica L.*) *Febs Letters* **547**: 56–60

- Murfitt LM, Kolosova N, Mann CJ, Dudareva N** (2000) Purification and characterization of S-adenosyl-l-methionine: benzoic acid carboxyl methyltransferase, the enzyme responsible for biosynthesis of the volatile ester methyl benzoate in flowers of *Antirrhinum majus*. Arch Biochem Biophys **382**: 145–151
- Murata J, Roepke J, Gordon H, De Luca V** (2008). The Leaf Epidermome of *Catharanthus roseus* Reveals Its Biochemical Specialization. The Plant Cell **20**(3): 524–542
- Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Hagen G, Alonso JM, Cohen JD, Farmer EE** (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development **132**: 4107–4118
- Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JDG** (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science **312**: 436–439
- Negre F, Kolosova N, Knoll J, Kish CM, Dudareva N** (2002) Novel S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase, an enzyme responsible for biosynthesis of methyl salicylate and methyl benzoate, is not involved in floral scent production in snapdragon flowers. Archives of Biochemistry and Biophysics **406**: 261–270
- Negre F, Kish CM, Boatright J, Underwood B, Shibuya K, Wagner C, Clark DG, Dudareva N** (2003) Regulation of methyl benzoate emission after pollination in snapdragon and petunia flowers. Plant Cell **15**: 2992–3006
- Newcombe G** (1998) A review of exapted resistance to diseases of Populus. European Journal of Forest Pathology **28**: 209–216
- Ogawa M, Herai Y, Koizumi N, Kusano T, Sano H** (2001) 7-Methylxanthine methyltransferase of coffee plants. J Biol Chem. **276**: 8213–8218

- Ostry ME, Wilson LF, McNabb HS Jr, Moore LM** (1988) A guide to insect, disease, and animal pests of poplars. Agriculture Handbook, 677. Washington, D.C.: US Department of Agriculture
- Paterson AH, Bowers JE, Bruggmann R, Dubchak I, Grimwood J, Gundlach H, Haberer G, Hellsten U, Mitros T, Poliakov A, Schmutz J, Spannagl M, Tang H, Wang X, Wicker T, Bharti AK, Chapman J, Feltus FA, Gowik U, Grigoriev IV, Lyons E, Maher CA, Martis M, Narechania A, Otiillar RP, Penning BW, Salamov AA, Wang Y, Zhang L, Carpita NC, Freeling M, Gingle AR, Hash CT, Keller B, Klein P, Kresovich S, McCann MC, Ming R, Peterson DG, Mehboob-ur-Rahman, Ware D, Westhoff P, Mayer KF, Messing J, Rokhsar DS** (2009) The *Sorghum bicolor* genome and the diversification of grasses. *Nature* **457**: 551-556
- Pott M, Pichersky E, Piechulla B** (2002) Evening specific oscillations of scent emission, SAMT enzyme activity, and SAMT mRNA in flowers of *Stephanotis floribunda*. *J Plant Physiol*, **159**: 925–934
- Pott M, Hippauf F, Saschenbrecker S, Chen F, Ross J, Kiefer I, Slusarenko A, Noel JP, Pichersky E, Effmert U, Piechulla B** (2004) Biochemical and structural characterization of benzenoid carboxyl methyltransferases involved in floral scent production in *Stephanotis floribunda* and *Nicotiana suaveolens*. *Plant Physiol*. **135**:1946–1955
- Preuß A, Augustin C, Figueroa CR, Hoffmann T, Valpuesta V, Sevilla JF, Schwab W** (2014) Expression of a functional jasmonic acid carboxyl methyltransferase is negatively correlated with strawberry fruit development. *Journal of Plant Physiology* **171** (15): 1315–1324

- Qin G, Gu H, Zhao Y, Ma Z, Shi G, Yang Y, Pichersky E, Chen F, Liu M, Chen Z, Qu LJ** (2005) Regulation of auxin homeostasis and plant development by an indole-3-acetic acid carboxyl methyltransferase in Arabidopsis. *Plant Cell* **17**: 2693-2704
- Ross JR, Nam KH, D'Auria JC, Pichersky E** (1999) S-adenosyl-l-methionine: salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. *Arch Biochem Biophys* **367**: 9–16
- Routledge APM, Shelley G, Smith JV, Draper J, Mur LAJ, Talbot NJ** (2004). Magnaporthe grisea interactions with the model grass *Brachypodium distachyon* Closely resemble those with rice (*Oryza sativa*). *Mol. Plant Pathol.* **5**, 253–265
- Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD** (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. *Proc Natl Acad Sci USA* **98**: 4788–4793
- Schnable PS, Ware D, Fulton RS, Stein JC, Wei F, Pasternak S, Liang C, Zhang J, Fulton L, Graves TA, Minx P, Reily AD, Courtney L, Kruchowski SS, Tomlinson C, Strong C, Delehaunty K, Fronick C, Courtney B, Rock SM, Belter E, Du F, Kim K, Abbott RM, Cotton M, Levy A, Marchetto P, Ochoa K, Jackson SM, Gillam B, Chen W, Yan L, Higginbotham J, Cardenas M, Waligorski J, Applebaum E, Phelps L, Falcone J, Kanchi K, Thane T, Scimone A, Thane N, Henke J, Wang T, Ruppert J, Shah N, Rotter K, Hodges J, Ingenthron E, Cordes M, Kohlberg S, Sgro J, Delgado B, Mead K, Chinwalla A, Leonard S, Crouse K, Collura K, Kudrna D, Currie J, He R, Angelova A, Rajasekar S, Mueller T, Lomeli R, Scara G, Ko A, Delaney K, Wissotski M, Lopez G, Campos D, Braidotti M, Ashley E, Golser W, Kim H, Lee S, Lin J, Dujmic Z, Kim W, Talag J, Zuccolo A, Fan C, Sebastian A, Kramer M, Spiegel L, Nascimento L, Zutavern**

- T, Miller B, Ambroise C, Muller S, Spooner W, Narechania A, Ren L, Wei S, Kumari S, Faga B, Levy MJ, McMahan L, Buren PV, Vaughn MW, Ying K, Yeh CT, Emrich SJ, Jia Y, Kalyanaraman A, Hsia AP, Barbazuk WB, Baucom RS, Brutnell TP, Carpita NC, Chaparro C, Chia JM, Deragon JM, Estill JC, Fu Y, Jeddeloh JA, Han Y, Lee H, Li P, Lisch DR, Liu S, Liu Z, Nagel DH, McCann MC, SanMiguel P, Myers AM, Nettleton D, Nguyen J, Penning BW, Ponnala L, Schneider KL, Schwartz DC, Sharma A, Soderlund C, Springer NM, Sun Q, Wang H, Waterman M, Westerman R, Wolfgruber TK, Yang L, Yu Y, Zhang L, Zhou S, Zhu Q, Bennetzen JL, Dawe RK, Jiang J, Jiang N, Presting GG, Wessler SR, Aluru S, Martienssen RA, Clifton SW, McCombie WR, Wing RA, Wilson RK (2009) The B73 Maize Genome: Complexity, Diversity, and Dynamics. *Science* **20**: 326 (5956), 1112-1115**
- Taiz L, Zeiger E (2006) Plant Physiology, 4th ed. Sinauer Associates, Sunderland, MA, pp. 467-508**
- Thomma BPHJ, Eggermont K, Penninckx IAMA, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF (1998) Separate Jasmonate-Dependent and Salicylate-Dependent Defense-Response Pathways in Arabidopsis and Salicylate-Dependent Defense-Response Microbial Pathogens. *Proc Natl Acad Sci USA*. **95**:151070-15111**
- Tieman D, Zeigler M, Schmelz E, Taylor MG, Rushing S, Jones JB, Klee HJ (2010) Functional analysis of a tomato salicylic acid methyl transferase and its role in synthesis of the flavor volatile methyl salicylate. *Plant J*. **62**, 113–123**
- Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, Schein J, Sterck L, Aerts A, Bhalerao RR, Bhalerao RP, Blaudez D, Boerjan W, Brun A, Brunner A, Busov V, Campbell M, Carlson J, Chalot**

- M, Chapman J, Chen GL, Cooper D, Coutinho PM, Couturier J, Covert S, Cronk Q, Cunningham R, Davis J, Degroeve S, Dejardin A, Depamphilis C, Detter J, Dirks B, Dubchak I, Duplessis S, Ehlting J, Ellis B, Gendler K, Goodstein D, Gribskov M, Grimwood J, Groover A, Gunter L, Hamberger B, Heinze B, Helariutta Y, Henrissat B, Holligan D, Holt R, Huang W, Islam-Faridi N, Jones S, Jones-Rhoades M, Jorgensen R, Joshi C, Kangasjarvi J, Karlsson J, Kelleher C, Kirkpatrick R, Kirst M, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leple JC, Locascio P, Lou Y, Lucas S, Martin F, Montanini B, Napoli C, Nelson DR, Nelson C, Nieminen K, Nilsson O, Pereda V, Peter G, Philippe R, Pilate G, Poliakov A, Razumovskaya J, Richardson P, Rinaldi C, Ritland K, Rouze P, Ryaboy D, Schmutz J, Schrader J, Segerman B, Shin H, Siddiqui A, Sterky F, Terry A, Tsai CJ, Uberbacher E, Unneberg P (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* **313**: 1596-1604
- The Arabidopsis Genome Initiative** (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408** (6814): 796–815
- The International *Brachypodium* Initiative** (2010) Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature* **463** (7282): 763–768
- Thompson JD, Gibson, T.J, Plewniak F, Jeanmougin F, Higgins DG** (1977) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **24**: 4876-4882
- Uefuji H, Ogita S, Yamaguchi Y, Koizumi N, Sano H** (2003). Molecular Cloning and Functional Characterization of Three Distinct *N*-Methyltransferases Involved in the Caffeine Biosynthetic Pathway in Coffee Plants. *Plant Physiology* **132**(1): 372–380

- Varbanova M, Yamaguchi S, Yang Y, McKelvey K, Hanada A, Borochoy R, Yu F, Jikumaru Y, Ross J, Cortes D, Ma CJ, Noel JP, Mander L, Shulaev V, Kamiya Y, Roderick S, Weiss D, Pichersky E** (2007) Methylation of gibberellins by *Arabidopsis* GAMT1 and GAMT2. *Plant Cell* **19**: 32–45
- Vijayan P, Shockey J, Levesque CA, Cook RJ, Browse J** (1998) A role for jasmonate in pathogen defense of *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **95**:7209–7214
- Woodward AW, Bartel B** (2005) Auxin: regulation, action, and interaction. *Ann Bot* **95**:707–735
- Wu Q, Presig CL, VanEtten HD** (1997) Isolation of the cDNAs encoding (+)6 α -hydroxymaackiain 3-O-methyltransferase, the terminal step for the synthesis of the phytoalexin pisatin in *Pisum sativum*. *Plant Molecular Biol* **35**: 551–560
- Yang Y, Yuan JS, Ross J, Noel JP, Pichersky E, Chen F** (2006) An *Arabidopsis thaliana* methyltransferase capable of methylating farnesoic acid. *Arch Biochem Biophys* **448**: 123–132
- Yang Y, Xu R, Ma C, Vlot AC, Klessig DF, Pichersky E** (2008) Inactive methyl indole-3-acetic acid ester can be hydrolyzed and activated by several esterases belonging to the *AtMES* esterase family of *Arabidopsis*. *Plant Physiol.* **147(3)**: 1034–1045
- Yoneyama N, Morimoto H, Ye CX, Ashihara H, Mizuno K, Kato M** (2006) Substrate specificity of N-methyltransferase involved in purine alkaloids synthesis is dependent upon one amino acid residue of the enzyme. *Molecular Genetics and Genomics* **275** (2): 125–135
- Zhao J, Davis LC, Verpoorte R** (2005) Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol Adv* **23**:283–333

- Zhao N, Guan J, Lin H, Chen F** (2007) Molecular cloning and biochemical characterization of indole-3-acetic acid methyltransferase from poplar. *Phytochemistry* **68**: 1537–1544
- Zhao N, Ferrer JL, Ross J, Guan J, Yang Y, Pichersky E, Noel JP, Chen F** (2008) Structural, biochemical, and phylogenetic analyses suggest that indole-3-acetic acid methyltransferase is an evolutionarily ancient member of the SABATH family. *Plant Physiol* **146**: 455 - 467
- Zhao N, Boyle B, Duval I, Ferrer JL, Lin H, Seguin A, Mackay J, Chen F** (2009). SABATH methyltransferases from white spruce (*Picea glauca*): gene cloning, functional characterization and structural analysis. *Tree Physiology* **79**: 947-957
- Zhao N, Guan J, Ferrer J-L, Engle N, Chern M, Ronald P, Tschaplinski TJ, Chen F** (2010) Emission, biosynthesis and regulation of insect-induced methyl salicylate and methyl benzoate from rice. *Plant Physiol Biochem* **48**: 279–287
- Zhao N, Yao J, Chaiprasongsuk M, Li G, Guan J, Tschaplinski TJ, Guo H, Chen F** (2013) Molecular and biochemical characterization of the jasmonic acid methyltransferase gene from black cottonwood (*Populus trichocarpa*). *Phytochemistry* **94**: 74-81

Appendix

Table 1.1 SABATH genes in plants

Gene	Host plant	Reference
Salicylic acid methyltransferase (CbSAMT)	<i>Clarkia breweri</i>	(Ross, Nam 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)
Salicylic acid methyltransferase (HcSAMT)	<i>Hoya carnosa</i>	(Effmert et al., 2005)
Salicylic acid methyltransferase (DwSAMT)	<i>Datura wrightii</i>	(Barkman et al., 2007)
Salicylic acid methyltransferase (SISAMT)	<i>Solanum lycopersicum</i>	(Tieman et al., 2010; Ament et al., 2010)
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., 2003a)
Benzoic acid:salicylic acid methyltransferase (AIBSMT)	<i>Arabidopsis (lyrata)</i>	(Chen et al., 2003a)
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)
Benzoic acid:salicylic acid methyltransferase (OsBSMT)	<i>Oryza sativa</i>	(Koo et al., 2007; Zhao et al., 2010)
Indole-3-acetic acid methyltransferase (AtIAMT1)	<i>Arabidopsis thaliana</i>	(Qin et al., 2005)
Indole-3-acetic acid methyltransferase (PtIAMT1)	<i>Populus trichocarpa</i>	(Zhao et al., 2007)
Indole-3-acetic acid methyltransferase (OsIAMT1)	<i>Oryza sativa</i>	(Zhao et al., 2008)
Indole-3-acetic acid methyltransferase (PgIAMT1)	<i>Picea glauca</i>	(Zhao et al., 2009)
Jasmonic acid methyltransferase (AtJMT)	<i>Arabidopsis thaliana</i>	(Seo et al., 2001)
Jasmonic acid methyltransferase (SIJMT)	<i>Solanum lycopersicum</i>	(Tieman et al., 2010)
Jasmonic acid methyltransferase (PtJMT)	<i>Populus trichocarpa</i>	(Zhao et al., 2013)

Table 1.1 Continues

Gene	Host plant	Reference
Jasmonic acid methyltransferase (FvJMT)	<i>Fragaria vesca</i>	(Preuß et al. 2014)
Jasmonic acid methyltransferase (BjJMT)	<i>Brassica juncea</i>	(Meur et al., 2015)
Farnesoic acid methyltransferase (AtFAMT)	<i>Arabidopsis thaliana</i>	(Yang et al., 2006)
Gibberellic acid methyltransferase (AtGAMT1)	<i>Arabidopsis thaliana</i>	(Varbanova et al., 2007)
Gibberellic acid methyltransferase (AtGAMT2)	<i>Arabidopsis thaliana</i>	(Varbanova et al., 2007)
Antranilic acid methyltransferase (ZmAAMT1-3)	<i>Zea mays</i>	(Kollner, Lenk et al. 2010)
caffeine synthase 1 (CaCCS1)	<i>Coffea arabica</i>	(Mizuno et al., 2003)
xanthosine methyltransferase (CaXMT1)	<i>Coffea arabica</i>	(Ogawa et al., 2001)
3,7-dimethylxanthine N-methyltransferase (CaDXMT1)	<i>Coffea arabica</i>	(Uefuji et al., 2003)
7-methylxanthine N-methyltransferase (CaMXMT1/CTS1)	<i>Coffea arabica</i>	(Ogawa et al., 2001)
7-methylxanthine N-methyltransferase (CaMXMT2)	<i>Coffea arabica</i>	(Uefuji et al., 2003)
theobromine synthase 2 (CaCTS2)	<i>Coffea arabica</i>	(Mizuno et al., 2003)
caffeine synthase (CsTCS1)	<i>Camellia sinensis</i>	(Kato et al., 2000)
caffeine synthase (CsTCS2)	<i>Camellia sinensis</i>	(Kato et al., 2000)
7-methylxanthosine synthase 1 (CaCmXRS1)	<i>Coffea arabica</i>	(Mizuno et al., 2003)
caffeine synthase (TcBCS1)		(Yoneyama et al., 2006)
caffeine synthase (TcBCS2)	<i>Theobroma cacao</i> <i>Theobroma cacao</i>	(Ashihara et al. 2008)
theobromine synthase (CpPCS1)	<i>Camellia ptilophylla</i>	(Yoneyama et al., 2006)
theobromine synthase (CpPCS2)	<i>Camellia ptilophylla</i>	(Yoneyama et al., 2006)
theobromine synthase (CiICS1)	<i>Camellia irrawadiensis</i>	(Yoneyama et al., 2006)
theobromine synthase (CiICS2)	<i>Camellia irrawadiensis</i>	(Yoneyama et al., 2006)

Table 1.1 Continues

Gene	Host plant	Reference
loganic acid methyltransferase (CrLAMT)	<i>Catharanthus roseus</i>	(Murata et al., 2008)
carboxyl methyltransferase (BonBMT)	<i>Bixa orellana</i>	(Bouvier et al., 2003)
cinnamate/p-coumarate carboxyl methyltransferase 1 (ObCCMT1)	<i>Ocimum basilicum</i>	(Kapteyn et al., 2007)
cinnamate/p-coumarate carboxyl methyltransferase 2 (ObCCMT2)	<i>Ocimum basilicum</i>	(Kapteyn et al., 2007)
cinnamate/p-coumarate carboxyl methyltransferase 3 (ObCCMT3)	<i>Ocimum basilicum</i>	(Kapteyn et al., 2007)

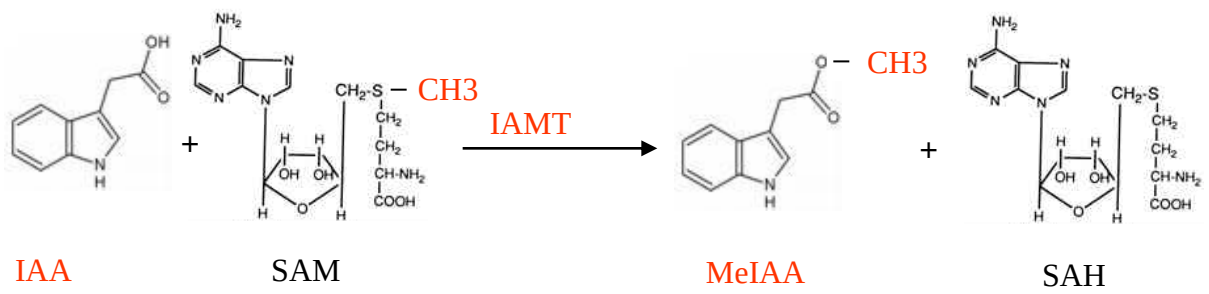


Figure 1.1 MeIAA biosynthesis

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

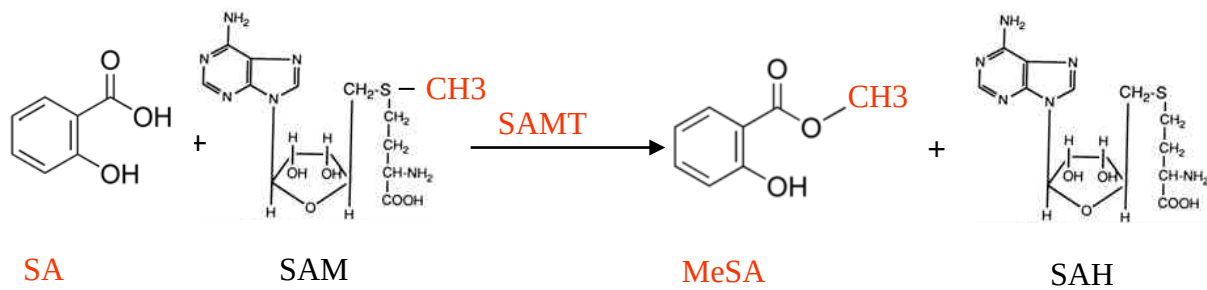


Figure 1.2 MeSA biosynthesis

MeSA: Methyl Jasmonate

SAMT: salicylic acid methyltransferase

SA: salicylic acid

SAM: S-adenosyl-L-methionine

SAH: S-adenosyl-L-homocysteine

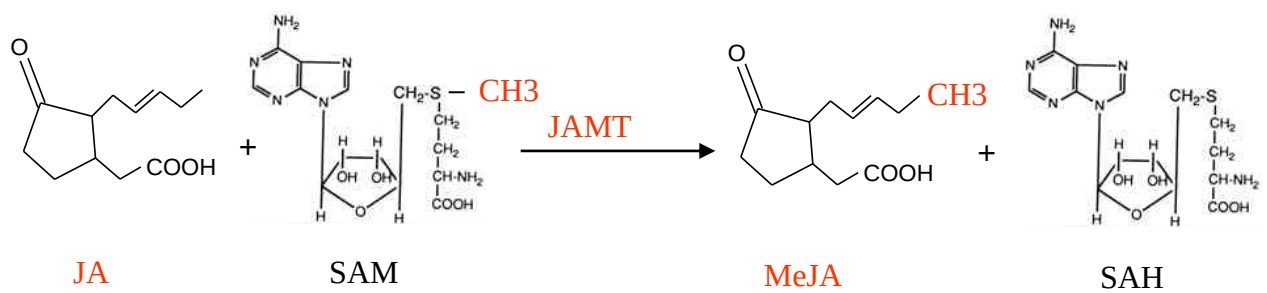


Figure 1.3 MeJA biosynthesis

MeJA: Methyl Jasmonate

JAMT: jasmonic acid methyltransferase

JA: jasmonic acid

SAM: S-adenosyl-L-methionine

SAH: S-adenosyl-L-homocysteine

CHAPTER II

Molecular and Biochemical Characterization of the Jasmonic Acid Methyltransferase Gene from Black Cottonwood (*Populus trichocarpa*)

Adapted from:

Zhao N, Yao J, Chaiprasongsuk M, Li G, Guan J, Tschaplinski TJ, Guo H, Chen F (2013) Molecular and biochemical characterization of the jasmonic acid methyltransferase gene from black cottonwood (*Populus trichocarpa*). *Phytochemistry* 94: 74-81.

Abstract

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

PtJMT1 may have a role in plant defense against biotic stresses. Phylogenetic analysis suggests that PtJMT1 shares a common ancestor with the *Arabidopsis* JMT, and functional divergence of these two apparent JMT orthologs has occurred since the split of poplar and *Arabidopsis* lineages.

Introduction

Methyl jasmonate (2) (MeJA) (Figure 4.1) is a naturally occurring compound known to be produced by many plants; it is involved in diverse biological processes (Cheong and Choi, 2003). It has also been shown to play important roles in regulating plant defenses against insect herbivory (Creelman et al., 1992, Howe, 2005 and Koo et al., 2009). When released as an insect-induced airborne signal, MeJA (2) can prime the defense of neighboring plants of insect-infected plants for inter-plant communications (Reymond and Farmer, 1998 and Kessler and Baldwin, 2001). As a constituent of floral scents of many plants (Knudsen et al., 1993), MeJA (2) may be involved in attracting pollinators. Furthermore, it has been shown to be involved in regulating plant growth and reproductive development (Creelman and Mullet, 1995 and Nojavan-Asghari and Ishizawa, 1998).

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

evolutionarily ancient member of the SABATH family conserved in seed plants. Other members, such as SAMTs, may have evolved from IAMT (Zhao et al., 2008). The evolutionary relationship between JMT and other members of the SABATH family, however, has not been extensively studied.

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

In this study, black cottonwood (*Populus trichocarpa*) was chosen as a model plant species for the isolation and functional characterization of JMT. Due to the relative ease of genetic manipulation and the availability of a fully-sequenced genome (Bradshaw et al., 2000 and Tuskan et al., 2006), black cottonwood has become an important model for studying tree physiology and ecology. It has also been used as a model plant for the study of the plant

SABATH family of methyltransferases: The IAMT gene has been isolated and characterized from this plant (Zhao et al., 2007). Herein is reported the molecular cloning and biochemical functional characterization of the JMT gene from black cottonwood (PtJMT1), which is the apparent ortholog of AtJMT.

Results

Identification of the jasmonic acid carboxyl methyltransferase gene from poplar

Using the protein sequence of *C. breweri* SAMT (CbSAMT), the prototype SABATH (Ross et al., 1999) as query, the blast search analysis of the genome of *P. trichocarpa* led to the identification of 28 SABATH proteins designated PtSABATH1 to PtSABATH28 (Table 2.1). PtSABATH3 was most similar to Arabidopsis JMT (AtJMT) with a 57% sequence identity. To determine whether PtSABATH3 function as JMT, a full-length cDNA of PtSABATH3 was amplified from poplar tissues using RT-PCR, cloned into a protein expression vector, and expressed in *Escherichia coli* to produce recombinant enzyme. Recombinant PtSABATH3 was tested for methyltransferase activity with JA (1), BA (3), salicylic acid (4) (SA), indole-3-acetic acid (5) and gibberellic acid (6) (structures 3–6 not shown). PtSABATH3 showed the highest level of specific activity with JA (1). Methyl transfer catalyzed by PtSABATH3 using JA (1) as substrate was demonstrated to occur to the carboxyl group (Figure 2.2). PtSABATH3 also showed activity with BA (3), which was about 22% of that with JA (1). It did not show activity with other carboxylic acids tested. Therefore, PtSABATH3 was renamed as PtJMT1.

To obtain information about whether the close homologs of PtJMT1 have JMT activity, PtSABATH2 and PtSABATH4 were analyzed. These two genes were selected for analysis

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

Biochemical properties of PtJMT1

Escherichia coli-expressed PtJMT1 was purified and then subject to detailed biochemical characterization. Under pseudo Michaelis–Menten conditions, PtJMT1 exhibited apparent *K_m* values $175.7 \pm 15.8 \mu\text{M}$ and $341.3 \pm 31.2 \mu\text{M}$ for JA (1) and BA (3), respectively (Figure 2.3A and B). The *K_{cat}* for JA (1) and BA (3) was 0.013 S^{-1} and 0.003 S^{-1} , respectively. For thermostability, PtJMT1 was 100% stable for 30 min at 4 °C. It lost about 10%, 20% and 40% of its maximal activity when incubated at 37 °C, 42 °C and 50 °C, respectively for 30 min. When incubated at 60 °C for 30 min, PtJMT1 lost all of its activity (Figure 2.3C). The optimum pH of PtJMT1 was 6.5. At pH 6.0, pH 7.0, pH 8.0 and pH 8.5, the enzyme displayed about 80%, 50%, 20% and 5% of its maximal activity, respectively (Figure 2.3D). Certain ions can affect PtJMT1 activity. K^{+} significantly stimulated PtJMT1 activity. NH_4^{+} and Na^{+} had a mild stimulation on PtJMT1 activity. Ca^{2+} and Mg^{2+} had a mild inhibitory effect. Mn^{2+} decreased the activity of PtJMT1 by 40%, and Cu^{2+} , Fe^{2+} , Fe^{3+} and Zn^{2+} all had a strong inhibitory effect on PtJMT1 activity (Figure 2.3E).

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

To understand the structural basis underlying the different specific activities of PtJMT1 with JA (1) and BA (3), in particular why PtJMT1 showed higher specific activity with JA (1) than the former, (3), two amino acids, Ser-153 and Asn-361, of PtJMT1 were selected for site-directed mutagenesis studies. As an active site residue, Ser-153 was selected because previous studies have shown that the residue at this position is important for determining the activity of SABATH proteins in methylating JA (1) or SA/BA (4/3). For example, a Tyr to Ser mutation at this position of CbSAMT showed increased activity with JA (1) (Zubieta et al., 2003). For the S153Y mutant of PtJMT1, its activities with JA (1) and BA (3) were reduced to about 3% and 38% of its activities with the wild type enzyme (Figure 4.4). Asn-361 is another active site residue. It was noticed that several enzymes using BA (3) as the most preferred substrate, but which have no activity with JA (1), have a Ser at this position (Figure 4.5). To test the importance of this variation, a N361S mutant was generated. The activities of the N361S mutant using JA (1) and BA (3) as substrate were about 84% and 140% of the activities of the wild type enzyme (Figure 2.4). In contrast, the S153Y/N361S double mutant showed activities that were very similar to those of the S153Y single mutant (Figure 2.4). Because the N361S mutant exhibited specific activities that were comparable to those of the wild type enzyme, it was further examined for its kinetic parameters. The apparent K_m values of the N361S mutant using JA (1) and BA (3) as substrate were determined to be $310.3 \pm 12.6 \mu\text{M}$ and $63.1 \pm 13.6 \mu\text{M}$, respectively. Its corresponding K_{cat} values for using JA (1) and BA (3) as substrates were determined to be 0.015 S^{-1} and 0.028 S^{-1} , respectively. All three mutants were also tested for

methyltransferase activity with SA (4), indole-3-acetic acid (5) and gibberellic acid (6), but did not show any activity.

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

Based on a previous study, the relative reduction of specific activity of the PtJMT1 S153Y mutant with JA (1) is probably because this change occludes much of the active site near the SAM/SAH binding site, which makes the binding of JA (1) in the mutant less sterically favorable than the wild type enzymes (Zubieta et al., 2003). To further understand the structural basis underlying the specific activity changes involving N361S, homology-based structural models of the complexes of PtJMT1 and its N361S mutant with substrate JA (1) (Figure 2.6A and B) and BA (3) (Figure 2.6C and D) were created. The alignment between the carboxylate of BA (3) and SAH did not change significantly in going from the wild-type (Figure 2.6C) to the mutated enzyme (Figure 2.6D). By contrast, a significant change was observed in the alignment between the carboxylate of JA (1) and SAH in going from wild-type (Figure 2.6A) to the mutant (Figure 2.6B). The homology modeling and docking calculations suggest that the reduced specific activity of N361S mutant with JA (1) (Figure 2.4) was partly due to the relatively poor alignment of JA (1) in the active site.

Expression of PtJMT1 in different tissues

To determine in what plant tissues PtJMT1 is expressed, total RNA was isolated from young leaves, old leaves, stems and roots of one-year-old poplar trees and used for gene expression analysis by semi-quantitative RT-PCR. PtJMT1 expression was observed in all four tissues examined with the highest level of expression detected in stems (Figure 2.7).

Expression of PtJMT1 in poplar leaves under stress conditions

To determine the expression patterns of PtJMT1 under stress conditions, black cottonwood seedlings were treated with three biotic stress factors: SA (4), MeJA (2) and a fungal elicitor alamethicin. Leaf tissues were collected for gene expression analysis (Figure 2.8). SA treatments at both 2 h and 24 h caused significant induction of PtJMT1 expression. At 24 h, the expression increased one fold. For MeJA (2), while the shorter time treatment (40 min) did not change the expression of PtJMT1, 4 h treatment led to more than one fold induction. The treatment by alamethicin for 2 h also led to significant induction in *PtJMT1* expression (Figure 2.8).

Phylogenetic analysis of PtJMT1 with other known SABATH proteins

To understand the evolutionary relatedness between PtJMT1 with other known SABATH proteins, a phylogenetic tree was reconstructed using a number of biochemically characterized MTs (Figure 2.9). The SABATH proteins analyzed include PtJMT1, PtSABATH2, PtSABATH4, AtJMT, tomato JMT (SlJMT), CbSAMT, IAMTs from Arabidopsis, poplar and rice, farnesoic acid methyltransferase from Arabidopsis (AtFAMT) and two gibberellic acid methyltransferases from Arabidopsis, selected characterized SAMTs from a variety of plant species and three caffeine synthases (CaXMT1, CaDXMT1, CaS1). A SABATH protein from the moss *Physcomitrella patens* (PpSABATH1, Zhao et al., 2012) was used as an outgroup (Figure 2.9). Phylogenetic analysis showed that PtJMT1 was most closely related to AtJMT, while the tomato JMT showed closer relatedness to BAMT and BSMTs than to PtJMT1 and AtJMT (Figure 2.7).

Discussion

In this article, the isolation, biochemical characterization, structure–function analysis and expression analysis of the PtJMT1 gene from black cottonwood is reported encoding jasmonic acid methyltransferase. PtJMT1 is the third plant JMT gene, after AtJMT from Arabidopsis (Seo et al., 2001) and the tomato JMT (Tieman et al., 2010), to be isolated and biochemically characterized. Phylogenetic analysis suggests that PtJMT1 and AtJMT are apparent orthologs, implying the existence of a JMT gene in the last common ancestor of the poplar and Arabidopsis lineages. In contrast, the tomato JMT gene shows a closer relatedness to BSMTs/SAMTs than to PtJMT1 and AtJMT (Figure 2.9). Because the tomato genome has recently been fully sequenced (Tomato Genome Consortium, 2012), the tomato genome sequence (<http://solgenomics.net/tools/blast/index.pl>) was searched for all putative SABATH genes. This analysis did not identify an apparent ortholog of PtJMT1 and AtJMT in tomato, which implies independent evolution of JMT in poplar/Arabidopsis and tomato lineages.

Despite their close evolutionary relatedness, PtJMT1 and AtJMT displayed striking differences in substrate specificity. While both enzymes showed the highest level of catalytic activity with JA (1), PtJMT1 and AtJMT showed different degrees of promiscuity. PtJMT1 can also use BA (3) as a substrate, while AtJMT does not (Seo et al., 2001). The tomato JMT also has the highest level of relative activity with JA (1). It can also use BA (3) as a substrate, resembling PtJMT1. This raises an intriguing question about substrate specificity evolution of JMT and JMT-related enzymes. From previous studies, IAMT was found to be conserved in rice, Arabidopsis, poplar and white spruce, indicating that IAMT is conserved in seed plants (Zhao et al., 2008). It was hypothesized that JMT and SAMT/BAMT may have evolved from IAMTs.

However, the relationship between JMT and SAMT/BAMT is not clear. A previous study suggested that the ancestor of SAMT and BSMT may have BAMT activity (Chen et al., 2003a). Because both PtJMT1 and the tomato JMT exhibit activity with BA (3), it is tempting to speculate that JMT evolved from BAMT. If this hypothesis is proven, it would suggest that the specific activity of AtJMT for JA (1) but not BA (2) represents a selection for specificity. The evolution of specificity can be achieved by changes of a few amino acids. As shown in our site-directed mutagenesis studies, the relative specificity of PtJMT1 using JA (1) and BA (2) as substrates can be readily reversed by the change of one of the two amino acids tested (Figure 4.4). In the case of SAMT/BSMT, a positive selection of a single amino acid appears to play a critical role in the relative specificity with SA (4) and BA (2) (Barkman et al., 2007). It will be interesting to determine whether positive selection is also a mechanism responsible for the evolution of JMT and BAMT. Continued isolation and functional characterization of JMT, SAMT, and IAMT genes from plants of key phylogenetic positions will open new avenues for the understanding of the trajectory and mechanisms underlying substrate specificity evolution of the SABATH family.

In a previous study (Yao et al. 2011), it was demonstrated that a good alignment between the transferable methyl group of SAM and the lone pair of electrons on the oxygen of the substrate carboxylic acids is of importance for the efficient methyl transfer. For CbSAMT, a good alignment could be achieved in the reactant complex of this enzyme containing SA (4) and SAM, but not in the complex containing 4-hydroxybenzoate (4HA) and SAM. These results are consistent with experimental observation that for CbSAMT the activity on 4HA is only 0–0.8% of that on SA (4) (Zubieta et al., 2003). In this study, the result of the homology-based structural

modeling of the PtJMT1 mutant N361S reinforces this notion: the N361S mutant showed reduced catalytic efficiency (K_{cat}/K_m) with JA (1) than the wild type enzyme, which could be partly explained by the changed alignment of JA (1) in the active site (Figure 2.6). While the structural basis for the increased activity of the N361S mutant with BA (2) is unclear, this result does suggest that the residue at the 361 position of PtJMT1 plays an important role in determining its relative specific activities towards JA (1) and BA (2).

In addition to being a model tree species, poplar is also considered an important bioenergy crop (Davis, 2008). Improving poplar trees for increased resistance to biotic stresses is integral to the genetic improvement of poplar trees for higher biomass yield. Understanding natural defense mechanisms of poplar trees will be of profound importance for such endeavors. The expression of PtJMT1 was induced by a number of stress factors tested (Figure 2.8), suggesting that this gene has an important role in regulating the defense response of poplar trees against biotic stresses. It will be useful to elucidate the molecular mechanisms underlying PtJMT1-mediated plant resistance. At the same time, PtJMT1 may be used as an important gene for rationally designed genetic engineering of poplar trees for improved resistance against biotic stresses.

Concluding Remarks

PtJMT1 is the third S-adenosyl-l-methionine: jasmonic acid carboxyl methyltransferase gene, after the Arabidopsis gene AtJMT and tomato JMT, to be isolated and biochemically characterized. Among all SABATH proteins in poplar and Arabidopsis, PtJMT1 is most similar to AtJMT, implying that the genes encoding them are orthologs. JA (1) is the most preferred

substrate for PtJMT1, which also displayed activity with BA (3). It is not yet clear which of the two enzymes, PtJMT1 or AtJMT, represents the ancestral state of substrate specificity of JMT. Site-directed mutagenesis studies identified two amino acids in the active site of PtJMT1 that are important for determining the relative specificity between JA (1) and BA (3). The expression of PtJMT1 was induced by a number of stress factors, suggesting that this gene has a role in the defense of poplar trees against biotic stresses.

Experimental Design

Sequence retrieval and analysis

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

Plant material and chemicals

The female black cottonwood (*P. trichocarpa*) clone ‘Nisqually-1’, which was used for whole genome sequencing, was used for gene cloning and expression analysis of PtJMT1. Young leaves (newly-emerged leaves), old leaves (5 cm in length), stems and roots were

collected from one-year-old poplar trees and used for examining tissue specificity of PtJMT1 expression. To examine PtJMT1 expression under stress conditions, leaf tissues were collected from poplar plants at the eight-leaf stage that were vegetatively propagated grown on MS medium. These plants were treated with either alamethicin for 2 h, SA (4) for 2 h and 24 h, or MeJA (2) for 40 min and 4 h, as previously described (Zhao et al., 2009). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise noted.

Cloning full-length cDNA of PtSABATH genes

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

Purification of recombinant PtJMT1

The full-length cDNA of PtJMT1 in pEXP5/CT TOPO was subcloned into a protein expression vector pET32a. To express recombinant PtJMT1 in *E. coli*, the protein expression construct was transformed into *E. coli* strain BL21 Codon Plus (Stratagene, La Jolla, CA). Protein expression was induced by isopropyl β-d-1-thiogalactopyranoside (IPTG) for 18 h at

room temperature, and the cells were lysed by sonication. His-tagged PtJMT1 protein was purified from the *E. coli* cell lysate using Ni-NTA agarose following the manufacturer instructions (Invitrogen, Carlsband, CA). Protein purity was verified by SDS-PAGE, and protein concentrations were determined by the Bradford assay (Bradford, 1976).

Radiochemical activity assay

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

Determination of kinetic parameters of PtJMT1

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

pH optimum for PtJMT1 activity

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

Effectors

A standard radiochemical activity assay was carried out in the presence of one of the following cations present at a final concentration of 5 mM: K^+ , Ca^{2+} , NH_4^+ , Na^+ , Mg^{2+} , Mn^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} , Zn^{2+} . Results presented are an average of three independent assays.

Semi-quantitative RT-PCR analysis of *PtJMT1* expression

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

Quantitative RT-PCR

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

Site-mutagenesis of *PtJMT1*

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

TTAATTTCTAACCATTGAAATGACCAAGCTGGTGTACTTG-3'. The full length of mutant was amplified using this reverse primer and the *PtJMT1* forward primer. The double mutant S153Y/N361S was constructed on the basis of these two mutants using the same method.

Structural modeling

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

Statistical analysis

Statistical analysis of *PtJMT1* expression in control and treated plants was conducted using SAS (Version 9.2) (SAS Institute, NC) based on three biological replicates and three technical replicates. Levels of significance were calculated using Student's *t*-test at $p < 0.05$. Sequences were deposited in GenBank (<http://www.ncbi.nlm.nih.gov>) with the accession numbers KC894590 (PtJMT1), KC894591 (PtSABATH4) and KC894592 (PtSABATH2). The homology model of PtJMT1 was deposited in the PMBD protein model database (<http://www.caspar.it/PMDB/>) with the accession number of PM0079066.

References

- Altschul SF, Stephen F, Gish W, Miller W, Myers EW, Lipman DJ** (1990) Basic local alignment search tool. *J Mol Bio* **215** (3): 403-410
- Barkman TJ, Martins TR, Sutton E, Stout JT** (2007) Positive selection for single amino acid change promotes substrate discrimination of a plant volatile-producing enzyme. *Mol. Biol. Evol.* **24**, 1320–1329
- Bradford MM** (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254
- Bradshaw HD, Ceulemans R, Davis J, Stettler R** (2000) Emerging model systems in plant biology: Poplar (*Populus*) as a model forest tree. *Journal of Plant Growth Regulation* **19**: 306-313
- Chen F, D'Auria JC, Tholl D, Ross JR, Gershenzon J, Noel JP, Pichersky E** (2003a) An *Arabidopsis* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J* **36**: 577-588
- Chen F, Tholl D, D'Auria JC, Farooq A, Pichersky E, Gershenzon J** (2003b) Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. *Plant Cell* **15**, 481–494
- Cheong JJ, Choi YD** (2003) Methyl jasmonate as a vital substance in plants. *Trends Genet.* **19**, 409–413
- Creelman RA, Tierney ML, Mullet JE** (1992) Jasmonic acid methyl Jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. *Proc. Natl. Acad. Sci. USA* **89**, 4938–4941

- Creelman RA, Mullet JE** (1995) Jasmonic acid distribution and action in plants – regulation during development and response to biotic and abiotic stress. *Proc Natl Acad Sci USA* **92**: 4114-4119
- D'Auria JC, Chen F, Pichersky E** (2003) The SABATH family of MTs in *Arabidopsis thaliana* and other plant species. In *Recent Advances in Phytochemistry*, Vol. 37. (Romeo, J.T., ed). Oxford: Elsevier Science Ltd., pp. 253–283
- D'Auria JC, Chen F, Pichersky E** (2002) Characterization of an acyltransferase capable of synthesizing benzylbenzoate and other volatile esters in flowers and damaged leaves of *Clarkia breweri*. *Plant Physiol.* **130**, 466–476
- Davis JM** (2008) Genetic improvement of poplar (*Populus* spp.) as a bioenergy crop. In: Vermerris, W. (Ed.), *Genetic Improvement of Bioenergy Crops*. Springer, New York, pp. 397–419
- Howe GA** (2005) Jasmonates as signals in the wound response. *J. Plant Growth Regul.* **23**, 223–237
- Kessler A, Baldwin IT** (2001) Defensive function of herbivore induced plant volatile emissions in nature. *Science* **291**, 2141–2144
- Knudsen JT, Tollsten L, Bergstrom LG** (1993) Floral scents – a checklist of volatile compounds isolated by headspace techniques. *Phytochemistry* **33**, 253–280
- Kohler A, Blaudez D, Chalot M, Martin F** (2004) Cloning and expression of multiple metallothioneins from hybrid poplar. *New Phytol.* **164**, 83–93
- Koo AJ, Gao X, Jones AD, Howe GA** (2009) A rapid wound signal activates the systemic synthesis of bioactive jasmonates in *Arabidopsis*. *Plant J.* **59**, 974–986

- Murfitt LM, Kolosova N, Mann CJ, Dudareva N** (2000) Purification and characterization of S-adenosyl-L-methionine: benzoic acid carboxyl methyltransferase, the enzyme responsible for biosynthesis of the volatile ester methyl benzoate in flowers of *Antirrhinum majus*. *Arch. Biochem. Biophys.* **382**, 145–151
- Nojavan-Asghari M, Ishizawa K** (1998) Inhibitory effects of methyl jasmonate on the germination and ethylene production in cocklebur seeds. *J. Plant Growth Regul.* **17**, 13–18
- Qin G, Gu H, Zhao Y, Ma Z, Shi G, Yang Y, Pichersky E, Chen F, Liu M, Chen Z, Qu LJ** (2005) Regulation of auxin homeostasis and plant development by an indole-3-acetic acid carboxyl methyltransferase in *Arabidopsis*. *Plant Cell* **17**: 2693-2704
- Reymond P, Farmer EE** (1998) Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* **1**, 404–411
- Ross JR, Nam KH, D'Auria JC, Pichersky E** (1999) S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. *Arch Biochem Biophys* **367**: 9–16
- Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD** (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. *Proc Natl Acad Sci USA* **98**: 4788–4793
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG** (1997) The Clustalx windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **24**, 4876–4882
- Tieman D, Zeigler M, Schmelz E, Taylor MG, Rushing S, Jones JB, Klee HJ** (2010) Functional analysis of a tomato salicylic acid methyl transferase and its role in synthesis of the flavor volatile methyl salicylate. *Plant J.* **62**, 113–123

- Tomato Genome Consortium** (2012) The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* **485**, 635–641
- Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, Schein J, Sterck L, Aerts A, Bhalerao RR, Bhalerao RP, Blaudez D, Boerjan W, Brun A, Brunner A, Busov V, Campbell M, Carlson J, Chalot M, Chapman J, Chen GL, Cooper D, Coutinho PM, Couturier J, Covert S, Cronk Q, Cunningham R, Davis J, Degroeve S, Dejardin A, Depamphilis C, Detter J, Dirks B, Dubchak I, Duplessis S, Ehlting J, Ellis B, Gendler K, Goodstein D, Gribskov M, Grimwood J, Groover A, Gunter L, Hamberger B, Heinze B, Helariutta Y, Henrissat B, Holligan D, Holt R, Huang W, Islam-Faridi N, Jones S, Jones-Rhoades M, Jorgensen R, Joshi C, Kangasjarvi J, Karlsson J, Kelleher C, Kirkpatrick R, Kirst M, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leple JC, Locascio P, Lou Y, Lucas S, Martin F, Montanini B, Napoli C, Nelson DR, Nelson C, Nieminen K, Nilsson O, Pereda V, Peter G, Philippe R, Pilate G, Poliakov A, Razumovskaya J, Richardson P, Rinaldi C, Ritland K, Rouze P, Ryaboy D, Schmutz J, Schrader J, Segerman B, Shin H, Siddiqui A, Sterky F, Terry A, Tsai CJ, Uberbacher E, Unneberg P, et al.** (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* **313**: 1596-1604
- Varbanova M, Yamaguchi S, Yang Y, McKelvey K, Hanada A, Borochoy R, Yu F, Jikumaru Y, Ross J, Cortes D, Ma CJ, Noel JP, Mander L, Shulaev V, Kamiya Y, Rodermeel S, Weiss D, Pichersky E** (2007) Methylation of gibberellins by *Arabidopsis* GAMT1 and GAMT2. *Plant Cell* **19**: 32–45

- Yang Y, Yuan JS, Ross J, Noel JP, Pichersky E, Chen F** (2006) An *Arabidopsis thaliana* methyltransferase capable of methylating farnesoic acid. *Arch Biochem Biophys* **448**: 123-132
- Yao J, Xu Q, Chen F, Guo H** (2011) QM/MM free energy simulations of salicylic acid methyltransferase: the effects of transition state stabilization on substrate specificity. *J. Phys. Chem. B* **115**, 389–396
- Zhao N, Guan J, Lin H, Chen F** (2007) Molecular cloning and biochemical characterization of indole-3-acetic acid methyltransferase from poplar. *Phytochemistry* **68**, 1537–1544
- Zhao N, Ferrer JL, Ross J, Guan J, Yang Y, Pichersky E, Noel JP, Chen F** (2008) Structural, biochemical, and phylogenetic analyses suggest that indole-3-acetic acid methyltransferase is an evolutionarily ancient member of the SABATH family. *Plant Physiol* **146**: 455 - 467
- Zhao N, B Boyle, I Duval, JL Ferrer, H Lin, A Seguin, J Mackay and F Chen** (2009) SABATH methyltransferases from white spruce (*Picea glauca*): gene cloning, functional characterization and structural analysis. *Tree Physiology* **79**: 947-957
- Zhao N, Guan J, Ferrer JL, Engle N, Chern M, Ronald P, Tschaplinski TJ, Chen F** (2010) Emission, biosynthesis and regulation of insect-induced methyl salicylate and methyl benzoate from rice. *Plant Physiol Biochem* **48**: 279–287
- Zhao N, Ferrer JL, Moon HS, Kapteyn J, Zhuang X, Hasebe M, Stewart Jr NC, Gang DR, Chen F** (2012) A SABATH methyltransferase from the moss *Physcomitrella patens* catalyzes S-methylation of thiols and has a role in detoxification. *Phytochemistry* **81**, 31–41

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

Appendix

Table 2.1 *SABATH* genes in the genome of *Populus trichocarpa*

Gene	Locus name	Size of encoded Protein
<i>PtSABATH1</i>	Potri.001G359000	385
<i>PtSABATH2</i>	Potri.005G045900	372
<i>PtSABATH3</i>	Potri.005G230100	369
<i>PtSABATH4</i>	Potri.007G021300	364
<i>PtSABATH5</i>	Potri.007G021400	409
<i>PtSABATH6</i>	Potri.007G089500	345
<i>PtSABATH7</i>	Potri.008G136200	373
<i>PtSABATH8</i>	Potri.008G136300	359
<i>PtSABATH9</i>	Potri.010G104700	331
<i>PtSABATH10</i>	Potri.010G104800	353
<i>PtSABATH11</i>	Potri.012G049900	364
<i>PtSABATH12</i>	Potri.012G052400	364
<i>PtSABATH13</i>	Potri.014G168200	368
<i>PtSABATH14</i>	Potri.015G041900	442
<i>PtSABATH15</i>	Potri.017G121600	354
<i>PtSABATH16</i>	Potri.017G121800	370
<i>PtSABATH17</i>	Potri.017G122000	370
<i>PtSABATH18</i>	Potri.017G122700	352
<i>PtSABATH19</i>	Potri.017G122900	380
<i>PtSABATH20</i>	Potri.019G016200	364
<i>PtSABATH21</i>	Potri.019G022000	367
<i>PtSABATH22</i>	Potri.019G022100	193
<i>PtSABATH23</i>	Potri.019G022200	311
<i>PtSABATH24</i>	Potri.019G022400	367
<i>PtSABATH25</i>	Potri.019G022600	375
<i>PtSABATH26</i>	Potri.T072400	364
<i>PtSABATH27</i>	Potri.T138800	364
<i>PtSABATH28</i>	Potri.T139200	364

Table 2.2 Primers used for cloning of full-length cDNAs

Gene	Forward primer	Reverse primer
<i>PtSABATH2</i>	ATGGAGAAAGAGAGCTCAAA	GGAAAAAGAGACAACTCCAAATGCTG
<i>PtSABATH3</i>	ATGGAAGTAATGCAAGTGCTTCACATG	TTAATTCTAACCATTGAAATGACCAAGTT
<i>PtSABATH4</i>	ATGGAGGTTGCTCAAGTGCTTCACAT	TCATCCCTTTCTAGTCACGGAAACAGT

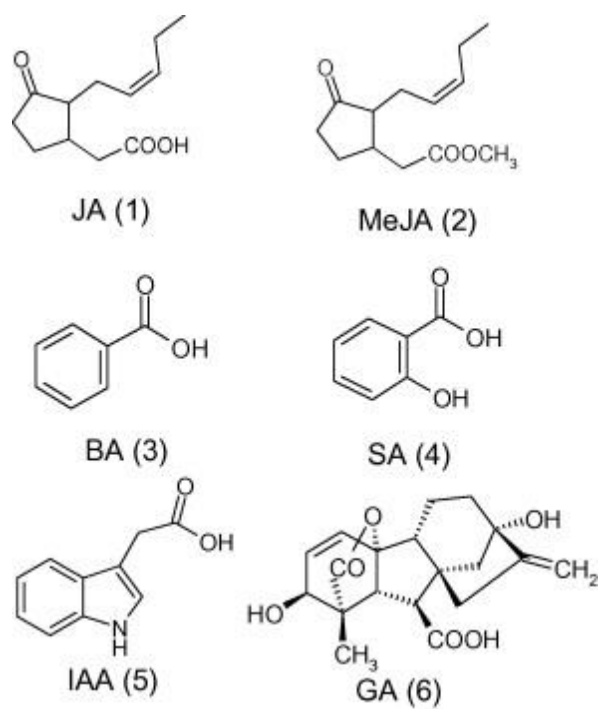


Figure 2.1 Structures of compounds: JA (1), MeJA (2), BA (3), SA (4), IAA (5), and GA (6).

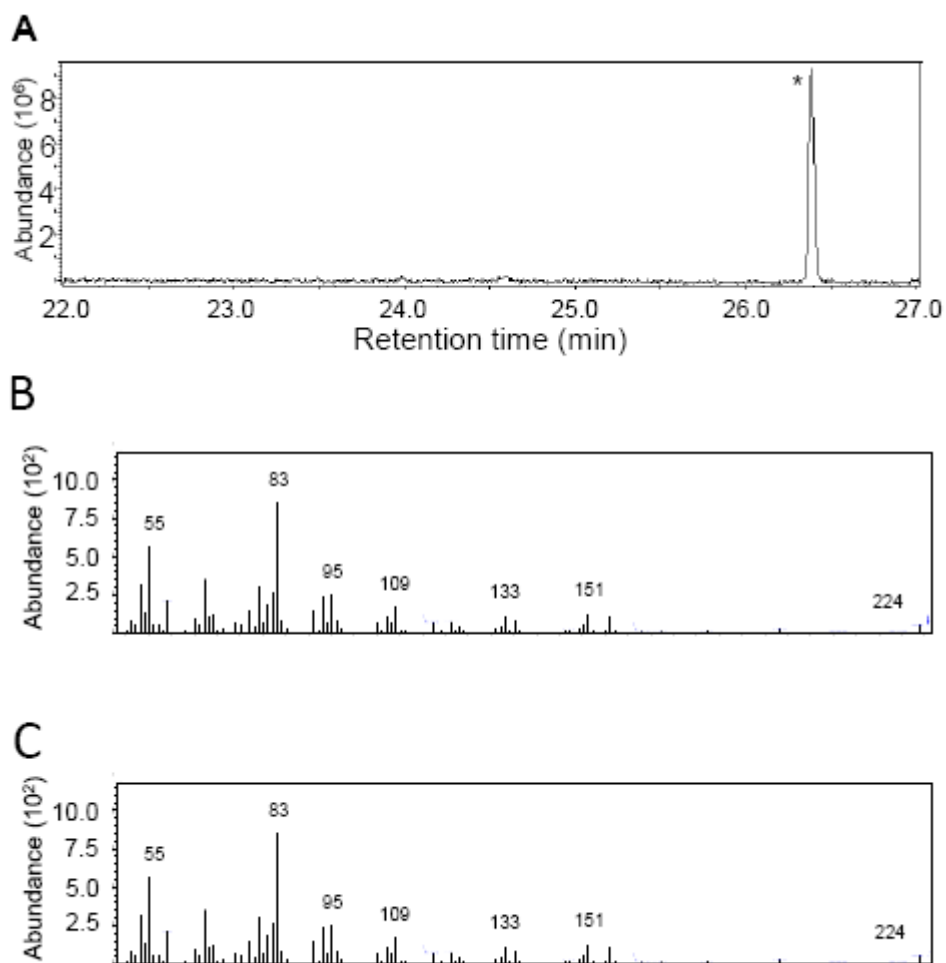


Figure 2.2 Identification of the catalytic product of PtJMT1. (A) GC chromatogram of the product formed by the action of PtJMT1 proteins using jasmonic acid as substrate. (B). Mass spectrum of the peak in (A). (C). Mass spectrum of authentic methyl jasmonate.

Figure 2.3 Biochemical properties of PtJMT1. (A) Steady-state kinetic measurements of PtJMT1 using jasmonic acid (1) as substrate. (B) Steady-state kinetic measurements of PtJMT1 using benzoic acid (3) as substrate. (C) Thermostability of PtJMT1. The activity of PtJMT1 incubated at 4 °C for 30 min (0.71 ± 0.01 pmol/s) was arbitrarily set at 1.0. (D) pH effect on PtJMT1 activity. Level of PtJMT1 activity in the buffer of pH 6.5 (0.84 ± 0.04 pmol/s) was arbitrarily set at 1.0. (E) Effects of metal ions on activity of PtJMT1. Metal ions were added to reactions in the form of chloride salts at a final concentration of 5 mM. Level of PtJMT1 activity without any metal ion added as control (0.74 ± 0.04 pmol/s) was arbitrarily set at 1.0. Jasmonic acid (1) was used as the substrate for enzyme assays in (C) to (E). Each value is an average of three independent measurements.

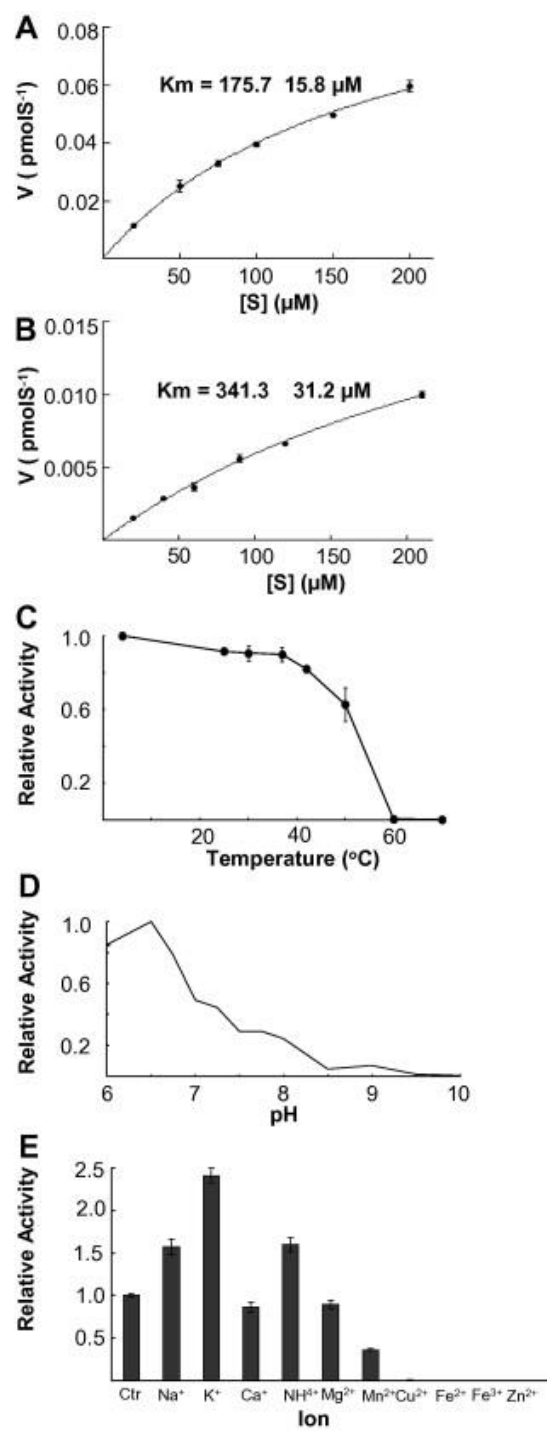


Figure 2.3 Biochemical properties of PtJMT1.

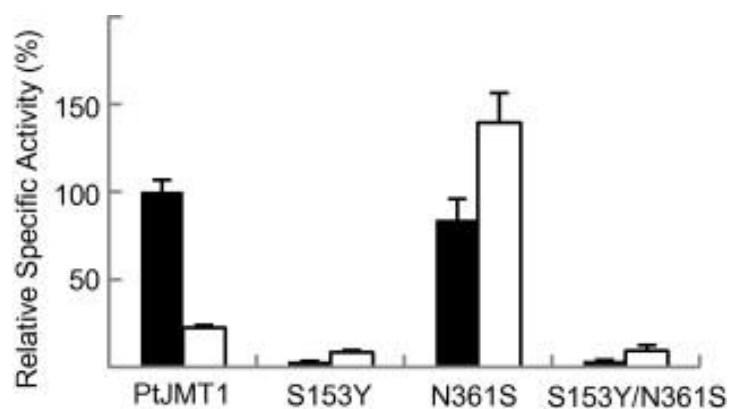


Figure 2.4 Substrate specificities of PtJMT1 and its mutants. The relative specific activities of two mutants of PtJMT1, S153Y and N361S, and double mutant S153Y/N361S for using jasmonic acid (1) (black bars) and benzoic acid (3) (white bars), were compared to those of PtJMT1. The PtJMT1 activity with jasmonic acid (1) (0.72 ± 0.02 pmol/s) was arbitrarily set at 1.0. Each value is an average of three independent measurements.

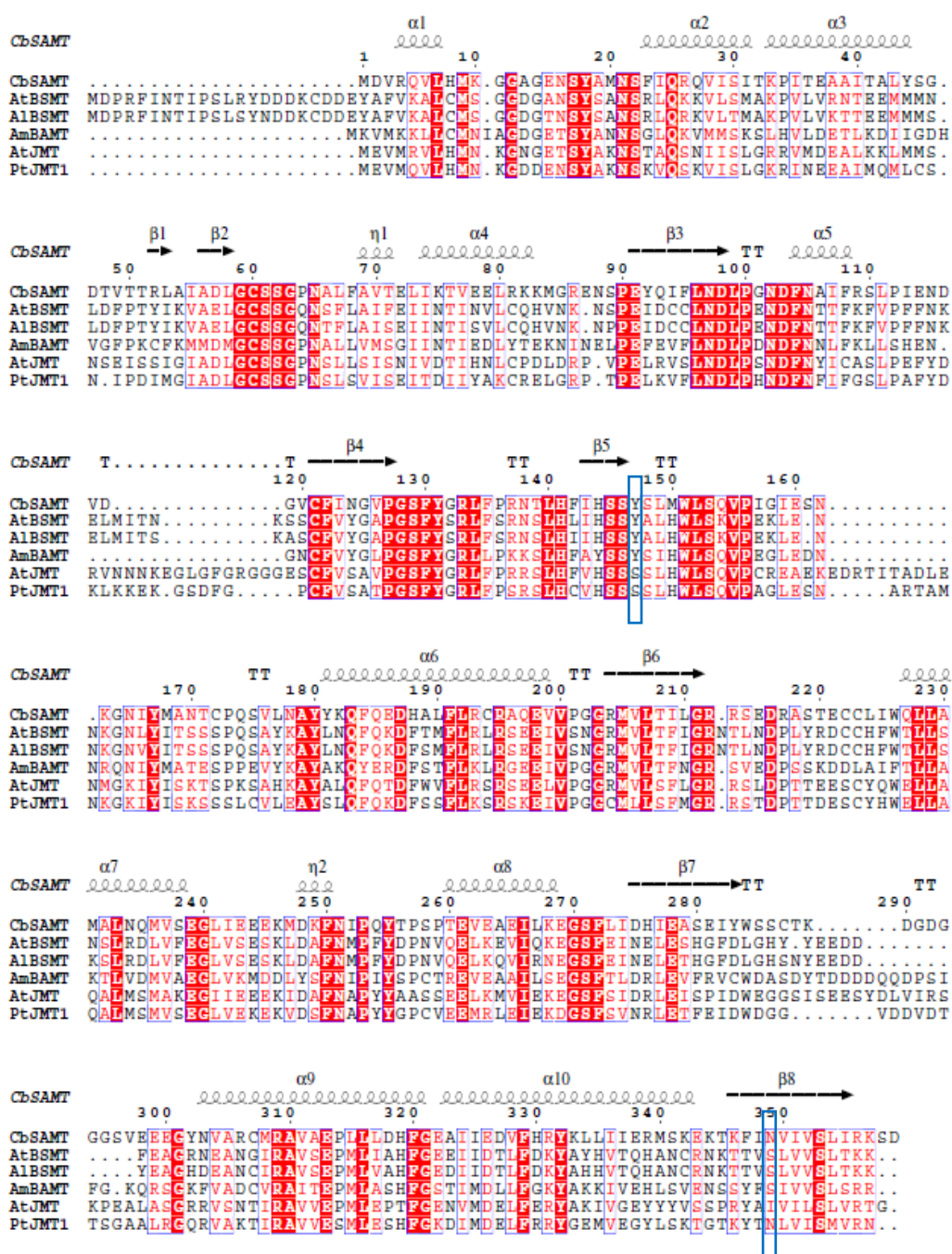


Figure 2.5 A. Structure-based alignment. Sequence alignment of poplar jasmonic acid methyltransferase (PtJMT1) and representative SABATH enzymes including *Clakia breweri* SAMT (CbSANT), *Antirrhinum majus* BMT (AmBAMT), *Arabidopsis thaliana* BSMT (AtBSMT) and *Arabidopsis thaliana* JMT (AtJMT).

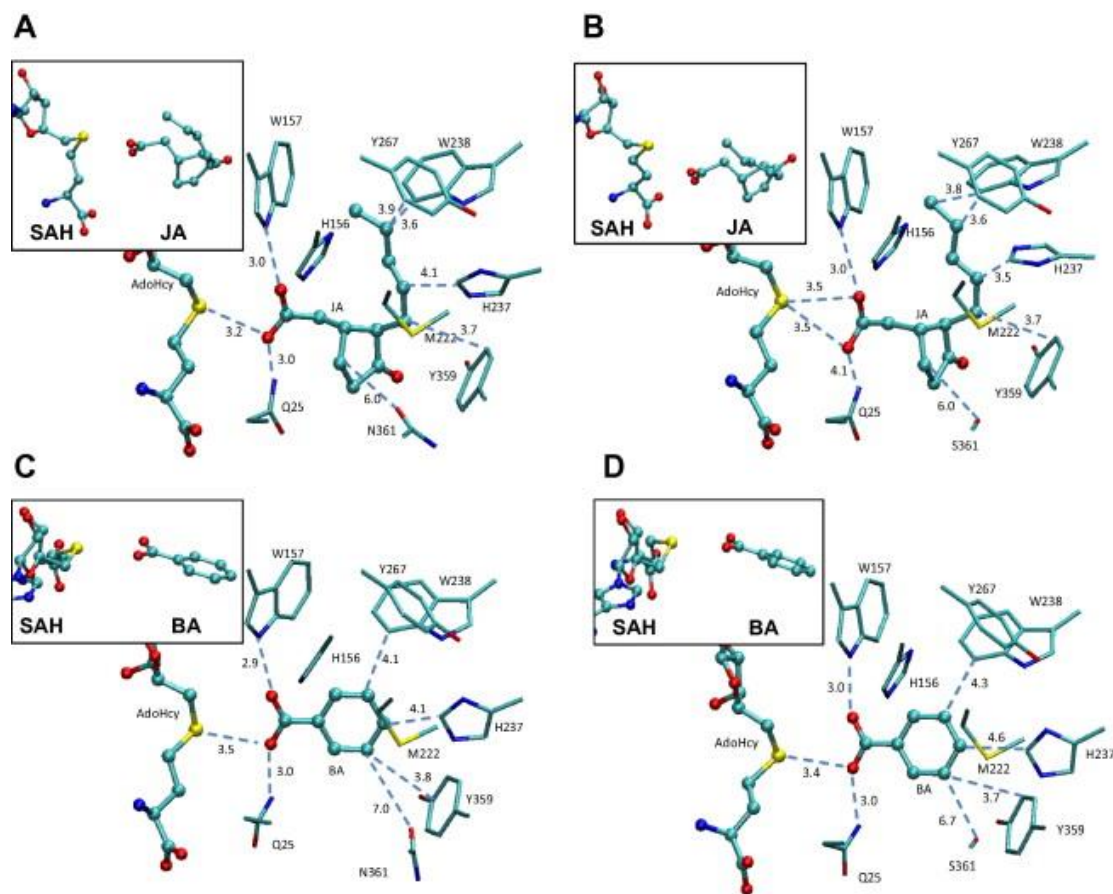


Figure 2.6 Homology models of the active site of wild-type PtJMT1 and the mutant of PtJMT1 (N361S) complexed with *S*-adenosyl-L-homocysteine (AdoHcy or SAH) and jasmonic acid (1) (JA) or benzoic acid (2) (BA). PtJMT1 and its mutant (N361S) are shown in sticks. SAH, JA and BA (3) are shown in balls and stick models. Some distances are given. The relative orientation of SAH and the carboxylate group in each structure is shown on the top (left). (A) The wild-type PtJMT1 complexed with SAH and JA (1). (B) The PtJMT1 mutant (N361S) complexed with SAH and JA (1). (C) The wild-type PtJMT1 complexed with SAH and BA (3). (D) The PtJMT1 mutant (N361S) complexed with SAH and BA (3).

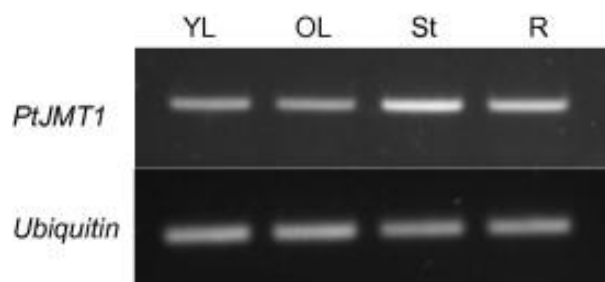


Figure 2.7 Semi-quantitative RT-PCR analysis of *PtJMT1* expression. Young leaves (YL), old leaves (OL), stems (St) and roots (R) were collected from 1-year-old poplar trees grown in a greenhouse. Total RNA was extracted and used for RT-PCR analysis. PCR with primers for a ubiquitin gene (Ubiquitin) was used to judge equality of concentration of cDNA templates in different samples.

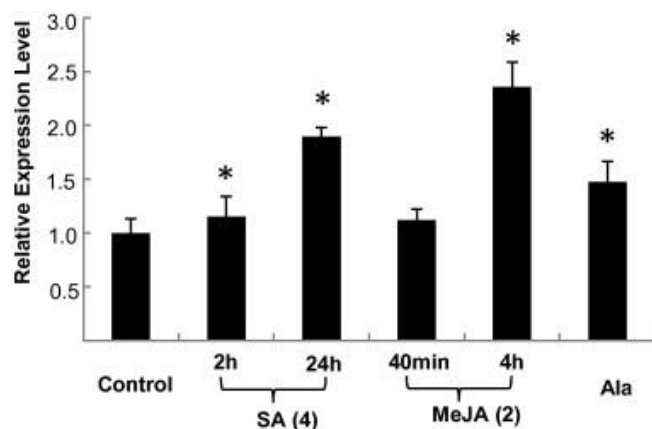


Figure 2.8 Expression of *PtJMT1* under stress conditions. Seedlings of black cottonwood were treated with salicylic acid (3) (SA) for 2 h and 24 h, methyl jasmonate (2) (MeJA) for 40 min and 4 h, alamethicin (Ala) for 2 h. Leaves were collected and used for quantitative RT-PCR analysis. The expression level of *PtJMT1* in untreated control was arbitrarily set at 1.0. Three biological replicates and three technical replicates were performed for gene expression analysis. The expression levels were presented as mean + SE. Bars marked with asterisk (*) indicate that *PtJMT1* showed a significant difference in expression levels in treated vs. control plants ($p < 0.05$).

Figure 2.9 Phylogenetic analysis of PtJMT1 with other known SABATH proteins.

CbSAMT, *C. breweri* SAMT (Accession # AF133053); AmSAMT, *Antirrhinum majus* SAMT (Accession # AF515284); HcSAMT, *Hoya carnosa* SAMT (Accession # AJ863118); AmBAMT: *A. majus* BAMT (Accession # AF198492); NsBSMT, *Nicotiana suaveolens* BSMT (Accession # AJ628349); AtBSMT, *Arabidopsis thaliana* BSMT (Accession # BT022049); AlBSMT, *Arabidopsis lyrata* BSMT (Accession # AY224596); AbSAMT, *Atropa belladonna* SAMT (Accession # AB049752); OsBSMT1, *Oryza sativa* BSMT1 (Accession # XM467504); SlSAMT, *Solanum lycopersicum* SAMT (Accession # NM001247880); AtJMT, *Arabidopsis thaliana* JMT (Accession # AY008434); SlJMT, *Solanum lycopersicum* JMT (EST cLEI13O14); AtFAMT, *Arabidopsis thaliana* FAMT (Accession # AY150400); AtIAMT1, *Arabidopsis thaliana* IAMT1 (Accession # AK175586); PtIAMT1, *Populus trichocarpa* IAMT1 (fgenes4_pg.C_LG_I002808); OsIAMT1, *Oryza sativa* IAMT1 (Accession # EU375746); AtGAMT1, *Arabidopsis thaliana* GAMT1 (At4g26420); AtGAMT2, *Arabidopsis thaliana* GAMT2 (At5g56300); Cas1, *Coffea arabica* caffeine synthase 1 (Accession # AB086414); CaXMT1, *Coffea arabica* xanthosine methyltransferase 1 (Accession # AB048793); CaDXMT1, *Coffea arabica* 3,7-dimethylxanthine methyltransferase 1 (Accession # AB084125); PtSABATH2, *Populus trichocarpa* SABATH2 (Accession # KC894592); PtSABATH4, *Populus trichocarpa* SABATH4 (Accession # KC894591). PpSABATH1, *P. patens* SABATH1 (gw1.47.108.1). PpSABATH1 was used as outgroup. Branches are drawn to scale with the bar indicating 0.1 substitutions per site.

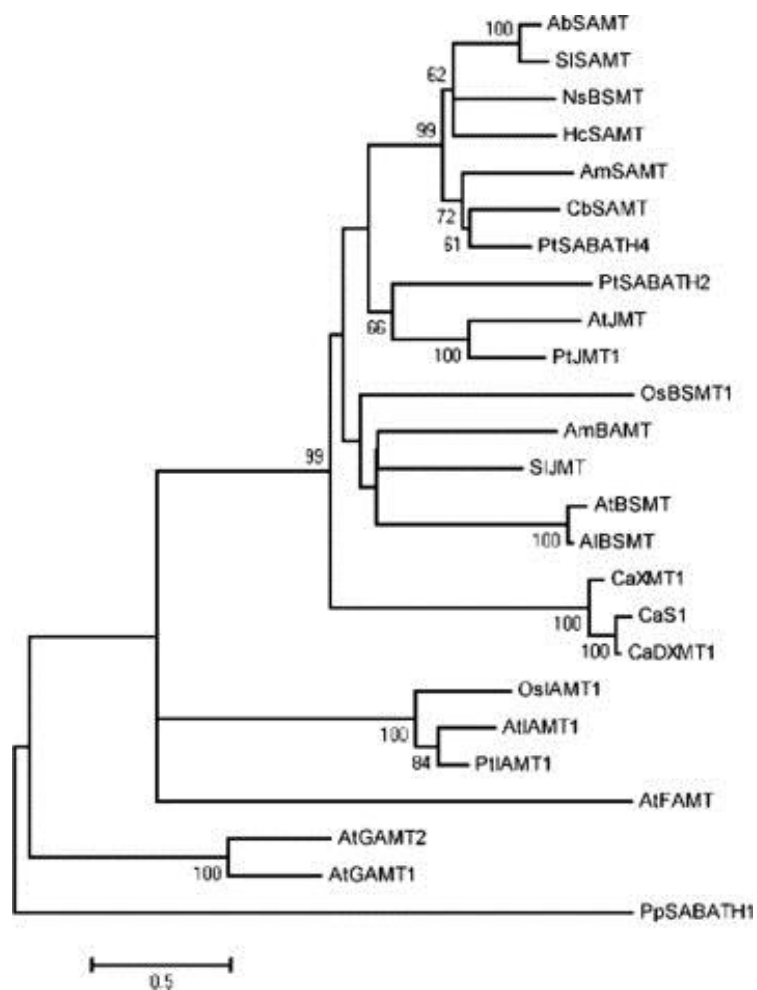


Figure 2.9 Phylogenetic analysis of PtJMT1 with other known SABATH proteins.

CHAPTER III

Enzyme Promiscuity-based Recent Evolution of an Ancient Biochemical Function in the SABATH Family of Methyltransferases

Minta Chaiprasongsuk, Ping Qian, Hong Guo, and Feng Chen. Enzyme Promiscuity-based Recent Evolution of an Ancient Biochemical Function in the SABATH Family of Methyltransferases. Drafted

Abstract

Gene duplication followed by functional divergence is the primary mechanism for the creation of genetic novelty. Through this process, one copy of a duplicated gene can retain its original function while another copy may acquire a novel, adaptive function. For enzymes, promiscuity is believed to be the engine for the creation of such novel catalytic functions through gene duplication followed by functional divergence. In this study, we present a case where enzyme promiscuity serves as a foundation for the recreation of a lost ancient activity. The study was conducted with the SABATH family of methyltransferases from plants. The known members of the SABATH family include indole-3-acetic acid methyltransferase (IAMT) and jasmonic acid methyltransferase (JMT). IAMT has been suggested to be an evolutionary ancient member of the SABATH family, and JMT evolved from IAMT. In this study, we found that the genome of *Brachypodium distachyon* lacked an apparent ortholog of IAMT. Instead, three members of the BdSABATH family (BdSABATH5, 6 and 8) were found to have IAMT activity. All three members also had JMT activity, with BdSABATH5 and 6 having higher activity with JA than with IAA. Selected SABATH genes in the same clade as BdSABATH5, 6 and 8 from sorghum were experimentally determined to encode JMT. Therefore, it was concluded that BdIAMT in *Brachypodium* evolved from an ancestral JMT. While JMT earlier evolved from IAMT, this suggests that the promiscuity of JMT with IAA provides the raw material for the recent evolution of IAMT in *B. distachyon*. The orthologs of BdSABATH5, 6 and 8 were

isolated from three *Brachypodium* species. Molecular evolution analysis suggested that *Brachypodium* SABATH8 is under purifying selection. The stabilizing selection also is shown between BsSABATH5 and BpSABATH5. This event is also shown in BrSABATH5 and BpSABATH5. The implications of this study for the interpretation of phylogenetics are also discussed.

Introduction

Gene duplication is a major mechanism for the generation of new genetic material (Ohno 1970, Ober, 2010). After duplication, one copy of the duplicated gene usually retains the original (or ancestral) function, while the other copy may shift away from the ancestral function and acquire a new function. The continued process of gene duplication followed by functional divergence produces gene families with individual members having divergent functions. In gene families of enzymes, individual members may use different substrates to catalyze the production of different metabolites. It has been a longstanding interest to understand how an enzyme evolves novel catalyze activity (Ober, 2010). Many case studies support that enzyme promiscuity, which is defined as the ability of an enzyme to catalyze a fortuitous side reaction in addition to its main reaction (Khersonsky and Tawfik, 2010; Aharoni et al., 2005), holds the key to the evolution of novel catalytic activity for the relaxed duplicated copy of the genes (Khersonsky and Tawfik, 2010).

Gene loss is another critical mechanism for the evolution of gene families. The observed differences in gene repertoires between genomes, particularly among eukaryotes, are believed to be largely accounted for heavily by lineage-specific gene loss (Aravind et al. 2000). With the loss of a gene encoding enzyme, the ability to catalyze a specific biochemical reaction is lost. Such a lost activity may be reinvented through the reiteration of the process of gene duplication and functional divergence. One particular question we are interested in is, what if the lost activity is the ancestral activity? Could it be reinvented by a reversing process? And if so, what is the role of promiscuity?

To answer the above questions, we chose to study the SABATH family of methyltransferases in plants. The majority of the known members of the SABATH family catalyze the methylation of carboxylic acids, especially phytohormones. These include indole-3-acetic acid methyltransferase (IAMT), GAMT, JMT, SAMT, BAMT (Qin et al., 2005; Zhao et al., 2008; Varbanova et al., 2007; Ross et al., 1999; Zhao et al., 2010; Chen et al., 2003; Seo et al., 2001.; Zhao et al., 2013; Murfitt et al., 2000). A subset of the SABATH family catalyzes nitrogen-methylation for the production of caffeine (Kato et al. 2000; McCarthy and McCarthy, 2007). A recent study showed that a SABATH gene from the moss *Physomitrella patens* catalyzes S-methylation of thiols. These diverse substrate specificities exhibited by the SABATH family clearly illustrate the process of gene duplication and functional divergence. While our understanding of the role of promiscuity in this process is limited, a number of SABATH genes having both SAMT and BAMT activities do support the role of promiscuity. In understanding the evolutionary trajectory of the SABATH family in terms of catalytic activities, previous studies suggested three key observations. One is that the ancestral activity of the SABATH family in lower plants was as a sulfur-methyltransferase, and carboxyl-methyltransferases had evolved from a S-methyltransferase activity. The second is that N-methyltransferase in the SABATH family have independently evolved multiple times. The third observation is that IAMT is an ancestral activity. IAMT genes have been shown to be conserved at least in seed plants (Zhao et al, 2008). This is sensible because IAA is a phytohormone and plays critical roles in many biological processes from lower to higher plants. Methylation of IAA by IAMT has been demonstrated to deactivate IAA (Qui et al., 2005; Yang et al., 2006). GA is another critical plant hormone. Similarly, the function of GAMT can be critical for plants in which GA is a critical plant hormone. So far, GAMT has only been identified in *Arabidopsis*. Its apparent ortholog is

absent in rice. This suggests that the orthologous GAMT, if indeed conserved, may have been lost in certain plant lineages.

With a relatively comprehensive knowledge of IAMT, the goal of this study is to determine whether the orthologous IAMT is lost in certain plants, and if so, whether the IAMT activity has been reinvented. If the IAMT activity has been reinvented, then what is the role of promiscuity in this evolutionary process. In this study, we found that the IAMT gene is absent in certain monocots plant while they are conserved in gymnosperms and dicots. The presence of an IAMT gene in rice and its absence in *Brachypodium* suggests that the loss of orthologous IAMT in *Brachypodium* is a relatively recent event. This serves as a nice system to address the above questions.

Results

Analysis of the SABATH family: IAMT ortholog is absent in certain monocots plants

To understand the evolution of the SABATH family, SABATH family genes were obtained for sequenced plants including *Physcoitrella patens*, *Amborella trichopoda*, *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, *Aquilegia coerulea*, *Solanum lycopersicum*, *Populus trichocarpa*, *Araidopsis thaliana* and *Glycine max*. Phylogenetic analysis was performed. Specifically, we examined the clade containing the known IAMTs from Arabidopsis, rice, black cottonwood and white spruce. This clade contains one or multiple genes for all the dicots, *Amborella*. For monocots, orthologous genes were detected for *Zostera*, *Phoenix*, *Elaeis*, and rice, but absent in sorghum and *Brachypodium* (Figure 3.1). The results suggests the loss of the orthologous IAMT in these monocot plants. Of all the monocots plants that were analyzed, *Brachypodium* is most closely related to rice. The presence of IAMT in rice

and its absence in *Brachypodium* suggests that the loss in IAMT in *Brachypodium* occurred after the split of rice and *Brachypodium*. Therefore, it was a relatively recent event, and we further determined that *Brachypodium* is an ideal system for us to answer the fundamental question we set to address.

The SABATH family in *Brachypodium distachyon*

The absence of an orthologous IAMT gene in *Brachypodium* prompted us to ask whether this ancestral activity is lost. For that reason, the entire SABTATH family in *Brachypodium* was characterized. The SABATH family in *Brachypodium* contains 12 members. Genes designated BdSABATH1 to BdSABATH12 are shown in Table 3.1. BdSABATH10 and BdSABATH12 are both pseudogenes. Ten of the 12 BdSABATH genes appear to be full-length intact genes. The size of these ten proteins ranges from 301 to 383 amino acid. Full-length cDNAs for all these putative full-length genes were cloned, expressed in *E. coli* and tested individually with selected known substrate of the SABATH family. Recombinant enzymes from BdSABATH 5, 6 and 8 showed IAMT activity. BdSABATH5 and 6 are localized on *Brachypodium* chromosome 2 and encode proteins of 356 and 361 amino acids. BdSABATH 8 is localized on *Brachypodium* chromosome 3 and encodes a protein of 356 amino acids. These three genes contain 3 introns and 4 exons. (Figure 3.2)

Substrate preference of BdSABATH 5, 6 and 8

Recombinant BdSABATH5 was tested for methyltransferase activity with JA, BA, salicylic acid (SA), indole-3-acetic acid (IAA) and gibberellic acid (GA). BdSABATH5 showed the highest level of specific activity with JA. Methyl transfer catalyzed by BdSABATH5 using JA as substrate was demonstrated to occur to the carboxyl group. BdSABATH5 also showed

activity with IAA and BA, which were about 11.19 % and 2.44 % with JA relatively. It did not show activity with other carboxylic acids tested. Recombinant BdSABATH6 show the highest level of specific activity with JA (1). BdSABATH6 also showed activity with IAA (5), BA (3) and SA (4), which were 22.41, 5.29% and 2.74% with JA, respectively. However, BdSABATH8 displayed the highest level of catalytic activity with IAA (5) and 35.74 % with BA (3).

Orthologs of BdSABATH5, 6 and 8 in two other plant species

There are 6 and 7 ortholog genes from rice and sorghum in the same clade with BdSABATH5, 6 and 8. However, there is no SABATH from the dicotyledon plant cluster in this clade. In order to obtain more information about these three genes in *Brachypodium*, the orthologs from sorghum and rice were selected for this study.

Identification of JMT from sorghum and rice

The presence of closely related IAMT and JMT genes in *Brachypodium* intrigued us to inquire about the activity of the immediate ancestor of these three duplicated genes. To gain insight into this question, we examine the apparent orthologs of BdSABATH 5, 6 and 8 from rice and sorghum. There are 6 genes and 7 genes from rice and sorghum, respectively, in this clade. SABATH from rice and sorghum as following Os06g02790, Os06g020969, Os06g021020 and Sb0527465.1 were chosen for experimental analysis (Figure 3.1). Recombinant Sb0527465.1 was tested for methyltransferase activity with JA, BA, salicylic acid (SA), indole-3-acetic acid (IAA) and gibberellic acid (GA). Sb0527465.1 enzyme showed the highest level of specific activity with JA. Methyl transfer catalyzed by Sb0527465.1 using JA as substrate was demonstrated to occur in the carboxyl group. Sb0527465.1 also showed activity with IAA, BA, and SA, which were 42.80 %, 32.36 % and 25.68 % with JA respectively. This recombinant

enzyme also used GA as the substrate at 7.78 % with JA. In contrast, the selected members from rice had no JMT activity (Figure 3.4)

Molecular evolution of BdSABATH5, 6 and 8 from four species of *Brachypodium*.

In order to understand the divergence variation along these three genes in *Brachypodium* from other monocotyledon plants, the orthologues in 4 different species of *Brachypodium*, *B. distachyon* (Bd), *B. pinnatum* (Bp), *B. rupestre* (Br) and *B. sylvaticum* (Bs), were analyzed. Twelve genes from *Brachypodium* have been identified. The phylogenetic tree was constructed to understand the relationship among these genes and known SABATH genes in plants (Figure 3.5). The phylogenetic tree shows twelve genes from *Brachypodium* are closely related to each other and cluster with PtJMT from black cottonwood and AtJMT from Arabidopsis. Similar to the first phylogenetic tree shown in Figure 3.1, BsSABATH8, BpSABATH8, BrSABATH8 and BdSABATH8 show identical protein sequences in all four species of *Brachypodium*. Moreover, these recombinant enzymes have IAMT activity. This finding indicates a purifying selection of IAMT genes in different species of *Brachypodium*. The loss of orthologs of IAMT in *Brachypodium* suggest that IAMT from *Brachypodium* occurred after the split of rice and *Brachypodium*. Moreover, the IAMT from *Brachypodium* shows promiscuous activity of IAA over JA. This could be another reason why IAA from *Brachypodium* have evolved independently from other IAMT of other monocotyledon, dicotyledon and gymnosperm plants. However, orthologs of BrSABATH5 and BpSABATH5 show identical protein sequences of JMT from these two species. This pronounced similarity indicates the stabilizing selection of these two genes. While BdSABATH5 and BrSABATH5 are more closely related, their recombinant enzymes indicate activity of JA as shown in Figure 3.6. Moreover, orthologs of BrSABATH6 and

BpSABATH6 show identical protein sequence of JMT from these two species. This also indicates the stabilizing selection between these genes. However, BsSABATH6 and BdSABATH6 are more closely related. These four recombinant enzymes have activity with JA over IAA (Figure 3.6).

Discussion

In this study, we found 3 of 12 SABATH genes with IAMT activity. These three genes, BdSABATH 5, 6 and 8, encode protein 356, 361 and 356 amino acid (Table 3.1). BdSABATH8 is more closely related to BdSABATH6 and BdSABATH6 than to the IAMT proteins from the other plants, suggesting these three evolved independently (Figure 3.1). BdSABATH5 and BdSABATH6 are more similar to each other (83.19%) than to BdSABATH8 (79.67%), while BdSABATH6 is similar to BdSABATH8 at 75.88%. BdSABATH5 and BdSABATH6 are also located on the same chromosome 2, while BdSABATH8 is located on chromosome 3. (Table 2.2 and Figure 3.3). The similarity of these three genes from *Brachypodium*, suggests they resulted from gene duplications.

Gene duplication is a major mechanism for the generation of new genetic material. One common fate of most duplicated copies is to become psudeogenes (which are not expressed or do not have any functions) as is the case in this study. Two of the 12 SABATH genes from *Brachypodium* which did not express that are BdSABATH10 and BdSABATH12, while another 5 genes have no functions. This occurrence can be caused by genetic disablements. Normally an organism will turn off the function of duplicated genes to reduce a fitness cost because duplicated genes usually generate functional redundancy (Zhang 2003). However, some duplicated genes will not become pseudogenes because the extra amount of gene products they

provide is beneficial. For example, multiple genes code for rRNAs and histones, both of which are in high demand in cells (Zhang 2003). For our study these three duplicated SABATH genes may have a high demand in *Brachypodium* for their development. Other than becoming pseudogenes or being conserved for original functions, duplicated genes may also be stably maintained through functional divergence. However, our report shows the genome of *Brachypodium distachyon* lacks an apparent ortholog of IAMT. Instead, three members of the BdSABATH family (BdSABATH5, 6 and 8) were found to have IAMT activity. All three members also have JMT activity, with BdSABATH5 and 6 having higher activity with JA than with IAA. These results present a case where enzyme promiscuity serves a foundation for the recreation of a lost ancient activity.

To determine the activity of the immediate ancestor of these three duplicated genes, we selected SABATH genes in the same clade as BdSABATH5, 6 and 8 from sorghum for study. After determining the selected gene from sorghum this enzyme can encode JMT that is similar to BdSABATH5 and 6. Therefore, it is concluded that BdIAMT in *Brachypodium* evolved from ancestral JMT. While JMT earlier evolved from IAMT, this suggests that the promiscuity of JMT with IAA provides the raw material for the recent evolution of IAMT in *B. distachyon*. These three genes from *Brachypodium* and one gene from sorghum appear polyphyletic to IAMTs isolated from poplar (Zhao et al., 2007) and white spruce (Zhao et al., 2009) and JMTs isolated from *Arabidopsis* (D'Auria et al. 2003) and poplar (Zhao et al., 2013). This indicates that the selected clade evolved independently from other IAMTs and JMTs.

The orthologs of BdSABATH5, 6 and 8 were isolated from other three *Brachypodium* species in order to gain information about SABATH genes and study the ancient functions of these three genes. Twelve genes from different *Brachypodium* have previously been identified

and characterized. Molecular evolution analysis suggests that *Brachypodium* SABATH8 is under purifying selection. These four SABATH8 recombinant enzymes show activity with IAA over JA. Stabilizing selection also shown between BsSABATH5 and BpSABATH5. This is also shown in BrSABATH6 and BpSABATH6. Both SABATH5 and SABATH6 enzymes from all four *Brachypodium* species show activity with JA over IAA (Figure 3.6). This finding of recombinant enzyme activity indicates the presence of promiscuous enzymes from SABATH genes.

Concluding Remarks

BdIAMTs have a promiscuity biochemical function and appear polyphyletic to IAMTs isolated from gymnosperm, basal angiosperm, monocotyledon, and dicotyledon plants. SABATHs genes from *Brachypodium* were found to be more closely related to each other than to JMT from other plants, implying that they resulted from relatively recent gene duplications. JA was the most preferred substrate for BdSABATH5 and BdSABATH6

BdSABATH8 preferred IAA over the JA substrate. Interestingly, these gene duplications of BdJMTs were all functional instead of having genetic disablement mechanisms to reduce fitness cost. This indicates BdJMTs are very beneficial to *Brachypodium*.

Selected SbSABTH enzymes of sorghum were determined to encode JMT. This could indicate that BdIAMT in *Brachypodium* evolved from an ancestral JMT. JMT earlier evolved from IAMT, suggesting that the promiscuity of JMT with IAA provides the raw material for the recent evolution of IAMT in *Brachypodium distachyon*.

The orthologs of BdSABATH5, 6 and 8 were isolated from three *Brachypodium* species. Molecular evolution analysis suggested that *Brachypodium* SABATH8 is under purifying

selection. Stabilizing selection was shown between BsSABATH5 and BpSABATH5. This event was also present in BrSABATH5 and BpSABATH5.

Materials and Methods

Plant materials and chemicals

The purple false brome (*Brachypodium distachyon*), previously employed for whole genome sequencing (The International *Brachypodium* Initiative, 2010), was used for gene cloning and expression analysis of *BdSABATH*. Tissues used for gene expression analysis, including young leaves, old leaves, stems and roots, were collected from plants grown one month in a growth chamber. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Database search and sequence analysis

In order to identify putative *BdSABATH* genes, BLAST searches were conducted on the Phytozome plant genome database and website (<http://www.phytozome.net/>) using *C. breweri* SAMT (CbSAMT), comparing the similarities (Altschul et al., 1990). A plant methyltransferase gene encoding a protein with the highest level of sequence similarity to this protein was chosen for further analysis. For phylogenetic tree construction, multiple protein sequence alignments were constructed using MAFFT version 7 (Katoh and Standley, 2013). The FastTree program was used to construct the phylogenetic tree.

Cloning full-length cDNA of MT

Total RNA was extracted using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA) with DNA contamination removal using an off-column DNase treatment (Qiagen, Valencia, CA). After purification, total RNA (1.5 µg) was reverse-transcribed into the 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). *MT* full-length cDNA was amplified using forward primer and reverse primer corresponding with the beginning and end of the *MT* 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 MT expressed in *E. coli*

MTs were subcloned from cDNA from pCRT7/CT-TOPO into the vector of pET100/D-TOPO (Invitrogen, Carlsband, CA). To express the MTs 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 μM 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 manufacturer instructions (Invitrogen, Carlsband, CA). Protein purity was verified by SDS-PAGE, and protein concentrations were determined by the Bradford assay (Bradford, 1976).

Radiochemical MT activity assay

Radiochemical MT assays were performed with a 50 μ L volume containing 50 mM Tris-HCl, pH 7.5, 1 mM substrate 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 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). The amount of radioactivity in the organic phase indicated the amount synthesized in the methylated substrates. Three independent assays were performed for each compound.

Reference

- Aravind L, Watanabe H, Lipman DJ, Koonin EV** (2000) Lineage-specific loss and divergence of functionally linked genes in eukaryotes. *Proc. Natl. Acad. Sci. USA.* **97**:11319–11324
- Aharoni A, Gaidukov L, Khersonsky O, McQ Gould S, Roodveldt C, Tawfik DS** (2005) The 'evolvability' of promiscuous protein functions. *Nature Genetics* **37** (1): 73–76
- Chen F, D'Auria JC, Tholl D, Ross JR, Gershenzon J, Noel JP, Pichersky E** (2003) An *Arabidopsis* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J* **36**: 577-588
- Kato M, Mizuno K, Crozier A, Fujimura T, Ashihara H** (2000) Caffeine synthase gene from tea leaves. *Nature* **406**: 956–957
- Khersonsky O, Tawfik DS** (2010) Enzyme promiscuity: a mechanistic and evolutionary perspective. *Annual review of biochemistry* **79**: 471-505
- Mccarthy AA, Mccarthy JG** (2007) The structure of two N-methyltransferases from the caffeine biosynthetic pathway. *Plant physiol.* **144** (2): 879-889
- Murfitt LM, Kolosova N, Mann CJ, Dudareva N** (2000) Purification and characterization of S-adenosyl-l-methionine: benzoic acid carboxyl methyltransferase, the enzyme responsible for biosynthesis of the volatile ester methyl benzoate in flowers of *Antirrhinum majus*. *Arch Biochem Biophys* **382**: 145–151
- Ohno S** (1970) Evolution by gene duplication. New York: Springer. pp.1-160

- Ober D** (2010) Gene Duplications and the time thereafter-examples from plant secondary metabolism. *Plant Biol.* **12**: 570-577
- Pichery E** (1990) Nomad DNA—a model for movement and duplication of DNA sequences in plant genomes. *Plant Mol.Biol.* **15**:437-448
- Qin G, Gu H, Zhao Y, Ma Z, Shi G, Yang Y, Pichersky E, Chen F, Liu M, Chen Z, Qu LJ** (2005) Regulation of auxin homeostasis and plant development by an indole-3-acetic acid carboxyl methyltransferase in *Arabidopsis*. *Plant Cell* **17**: 2693-2704
- Ross JR, Nam KH, D'Auria JC, Pichersky E** (1999) S-adenosyl-l-methionine: salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. *Arch Biochem Biophys* **367**: 9–16
- Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD** (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. *Proc Natl Acad Sci USA* **98**: 4788–4793
- Varbanova M, Yamaguchi S, Yang Y, McKelvey K, Hanada A, Borochoy R, Yu F, Jikumaru Y, Ross J, Cortes D, Ma CJ, Noel JP, Mander L, Shulaev V, Kamiya Y, Roderick S, Weiss D, Pichersky E** (2007) Methylation of gibberellins by *Arabidopsis* GAMT1 and GAMT2. *Plant Cell* **19**: 32–45
- Yang Y, Yuan JS, Ross J, Noel JP, Pichersky E, Chen F** (2006) An *Arabidopsis thaliana* methyltransferase capable of methylating farnesoic acid. *Arch Biochem Biophys* **448**: 123-132
- Zhao N, Ferrer JL, Ross J, Guan J, Yang Y, Pichersky E, Noel JP, Chen F** (2008) Structural, biochemical, and phylogenetic analyses suggest that indole-3-acetic acid

methyltransferase is an evolutionarily ancient member of the SABATH family. *Plant Physiol* **146**: 455 - 467

Zhao N, Guan J, Ferrer JL, Engle N, Chern M, Ronald P, Tschaplinski TJ, Chen F (2010)

Emission, biosynthesis and regulation of insect-induced methyl salicylate and methyl benzoate from rice. *Plant Physiol Biochem* **48**: 279–287

Zhao N, Yao J, Chaiprasongsuk M, Li G, Guan J, Tschaplinski TJ, Guo H, Chen F (2013)

Molecular and biochemical characterization of the jasmonic acid methyltransferase gene from black cottonwood (*Populus trichocarpa*). *Phytochemistry* **94**: 74-81

Appendix

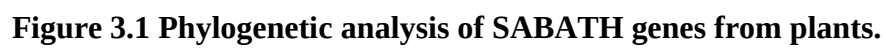
Table 3.1 SABATH genes in the genome of *Brachypodium distachyon*

Gene	Brachy ID	Chr	Chromosome Location	Protein size
BdSABATH1	Bradi1g44620	1	42774707 - 42776496	377
BdSABATH2	Bradi1g43080	1	40575772 - 40579333	382
BdSABATH3	Bradi1g42760.1	1	40083986 - 40086439	383
BdSABATH4	Bradi2g47550	2	47943065 - 47944525	381
BdSABATH5	Bradi2g60180	2	57571431 - 57573486	356
BdSABATH6	Bradi2g60170.1	2	57566656 - 57568430	361
BdSABATH7	Bradi3g05550	3	3916112 - 3917898	360
BdSABATH8	Bradi3g57790	3	57469278 - 57470901	356
BdSABATH9	Bradi4g00890	4	462739 - 463700	301
BdSABATH10	Bradi4g08320.1	4	7346399 - 7349675	202
BdSABATH11	Bradi4g16077	4	16752864 - 16756878	345
BdSABATH12	Bradi4g16110	4	16783707 - 16790252	357

Table 3. 2 Screening of relative activities of BdSABATHs with 5 substrates

	BdSABATH5	BdSABATH6	BdSABATH8
JA	100.00	100.00	35.74
BA	2.44	5.29	0.00
SA	0.00	2.74	0.00
IAA	11.19	21.41	100.00
GA3	0.00	0.00	0.00

Figure 3.1 Phylogenetic analysis of SABATH genes from plants. *Physcoitrella patens*, *Amborella tricapoda*, *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor*, *Aquilegia coerulea*, *Solanum lycopersicum*, *Populus trichocarpa*, *Arabidopsis thaliana* and *Glycine max*. And other known IAMT enzymes from *Picea*, *Zostera*, *Phoenix*, and *Elaeis*.



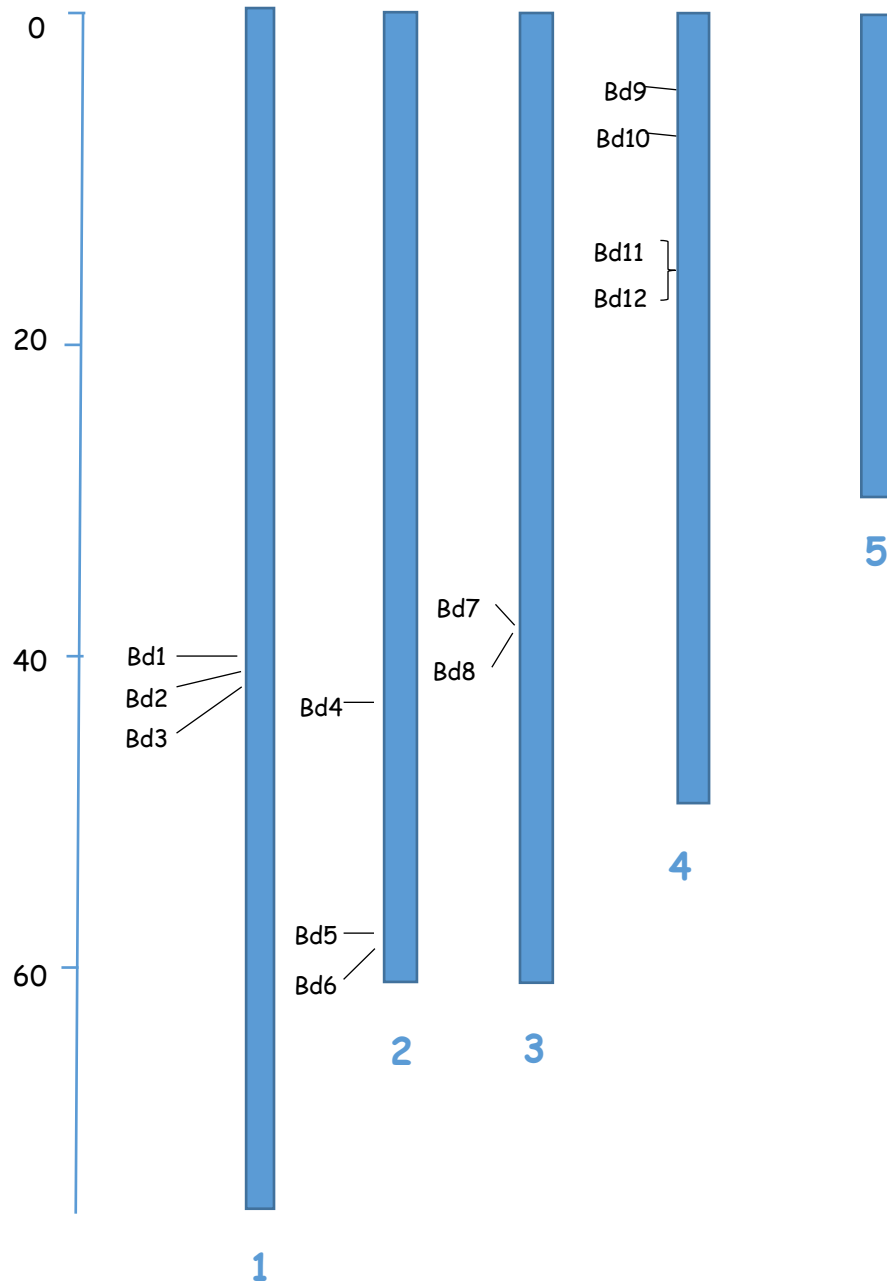
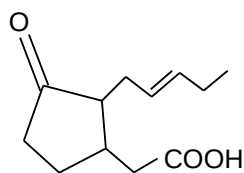
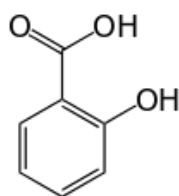


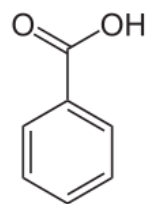
Figure 3.3 SABATH gene locations on *Brachypodium* chromosomes. Bd1 to Bd12 are BdSABATH1 to BdSABATH12 genes from *Brachypodium*. Numeric 1 to 5 in blue color represent the chromosome 1 to chromosome 5 in *Brachypodium*.



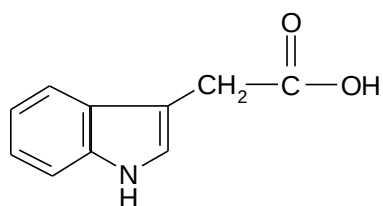
100%
Jasmonic acid



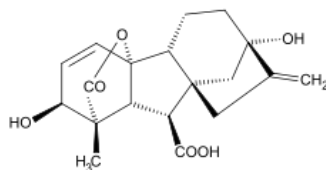
32.36%
Benzoic acid



25.68%
Salicylic acid



42.80%
Indole-3-acetic acid



7.78%
Gibberellic acid

Figure 3.4 Relative enzyme activity of sorghum ortholog gene (Sb05g027465) with 5 substrate. Activity with the favored substrate was used to normalize all other activities for each enzyme, which range from 0 to 100. Sb05g027465 show high relative preference jasmonic acid (JA) than indole acetic acid (IAA).

Figure 3.5 Phylogenetic analysis of 12 orthologs genes from 4 different species of *Brachypodium* with known SABATH enzymes. CbSAMT, *C. breweri* SAMT (Accession # AF133053); AmBAMT: *A. majus* BAMT (Accession # AF198492); NsBSMT, *Nicotiana suaveolens* BSMT (Accession # AJ628349); AtBSMT, *Arabidopsis thaliana* BSMT (Accession # BT022049); AtJMT, *Arabidopsis thaliana* JMT (Accession # AY008434); AtFAMT, *Arabidopsis thaliana* FAMT (Accession # AY150400); *Arabidopsis thaliana* IAMT1 (Accession # AK175586); PtIAMT1, *Populus trichocarpa* IAMT1 (fgenesh4_pg.C_LG_I002808); OsIAMT1, *Oryza sativa* IAMT1 (Accession # EU375746); AtGAMT1, *Arabidopsis thaliana* GAMT1 (At4g26420); AtGAMT2, *Arabidopsis thaliana* GAMT2 (At5g56300); Cas1, *Coffea arabica* caffeine synthase 1 (Accession # AB086414); CaXMT1, *Coffea arabica* xanthosine methyltransferase 1 (Accession # AB048793); CaDXMT1, *Coffea arabica* 3,7-dimethylxanthine methyltransferase 1 (Accession # AB084125); Branches are drawn to scale with the bar indicating 0.1 substitutions per site.

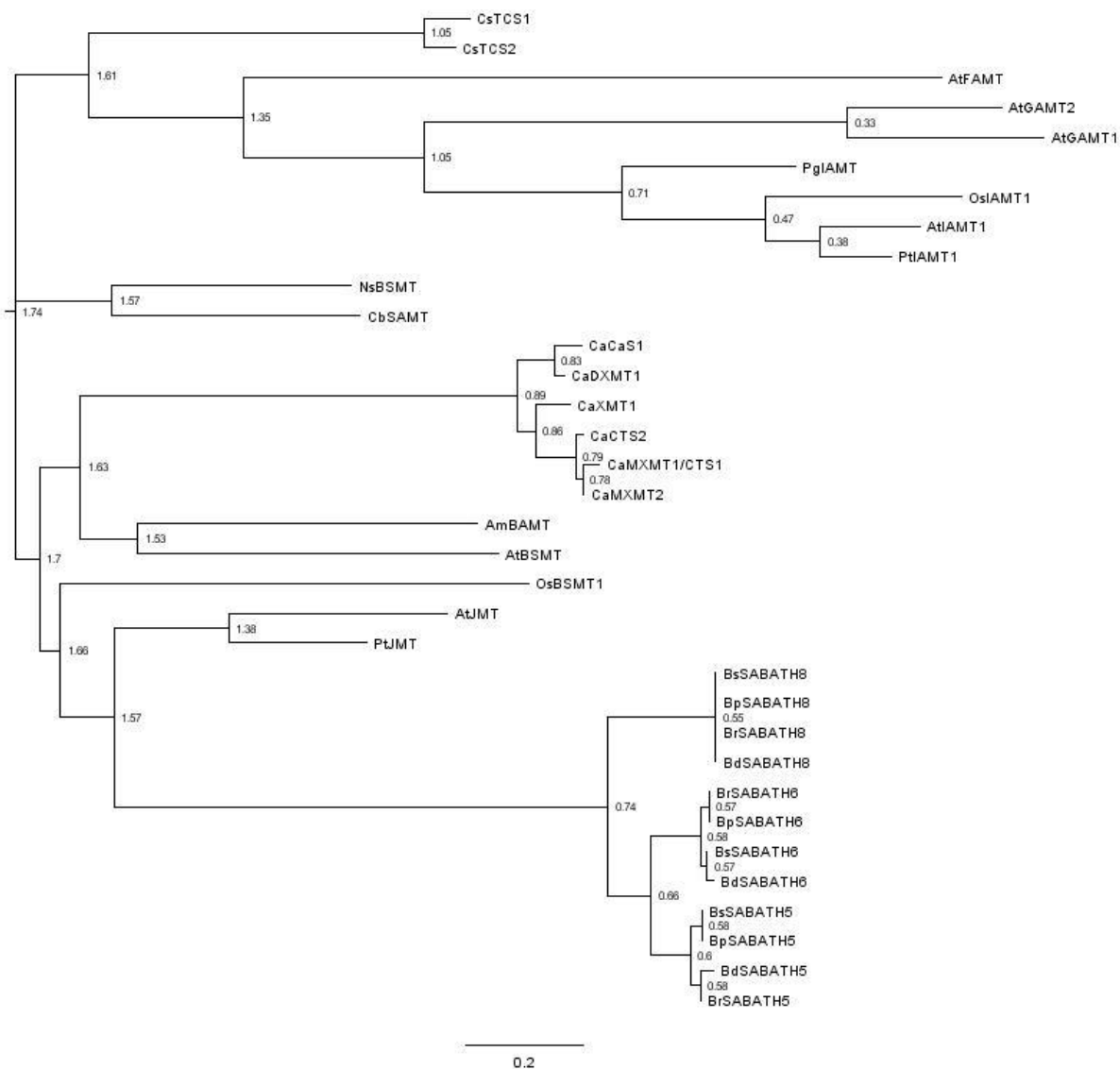


Figure 3.5 Phylogenetic analysis of 12 orthologous genes from 4 different species of *Brachypodium* with known SABATH enzymes.

Figure 3.6 Relative enzyme activities of SABATHs from different species of *Brachypodium* with 5 substrates. *B. distachyon* (Bd), *B. pinnatum* (Bp), *B. rupestre* (Br) and *B. sylvaticum* (Bs). Activity with the favored substrate was used to normalize all other activities for each enzyme, which range from 0 to 100. Most SABATH5 and SABATH6 show high relative preference for jasmonic acid (JA) than indole acetic acid (IAA). Most SABATH8 show high relative preference for indole acetic acid (IAA) than jasmonic acid (JA). Numeric structures are as follow: 1, jasmonic acid; 2, salicylic acid; 3, benzoic acid; 4, indole acetic acid; 5, gibberellic acid.

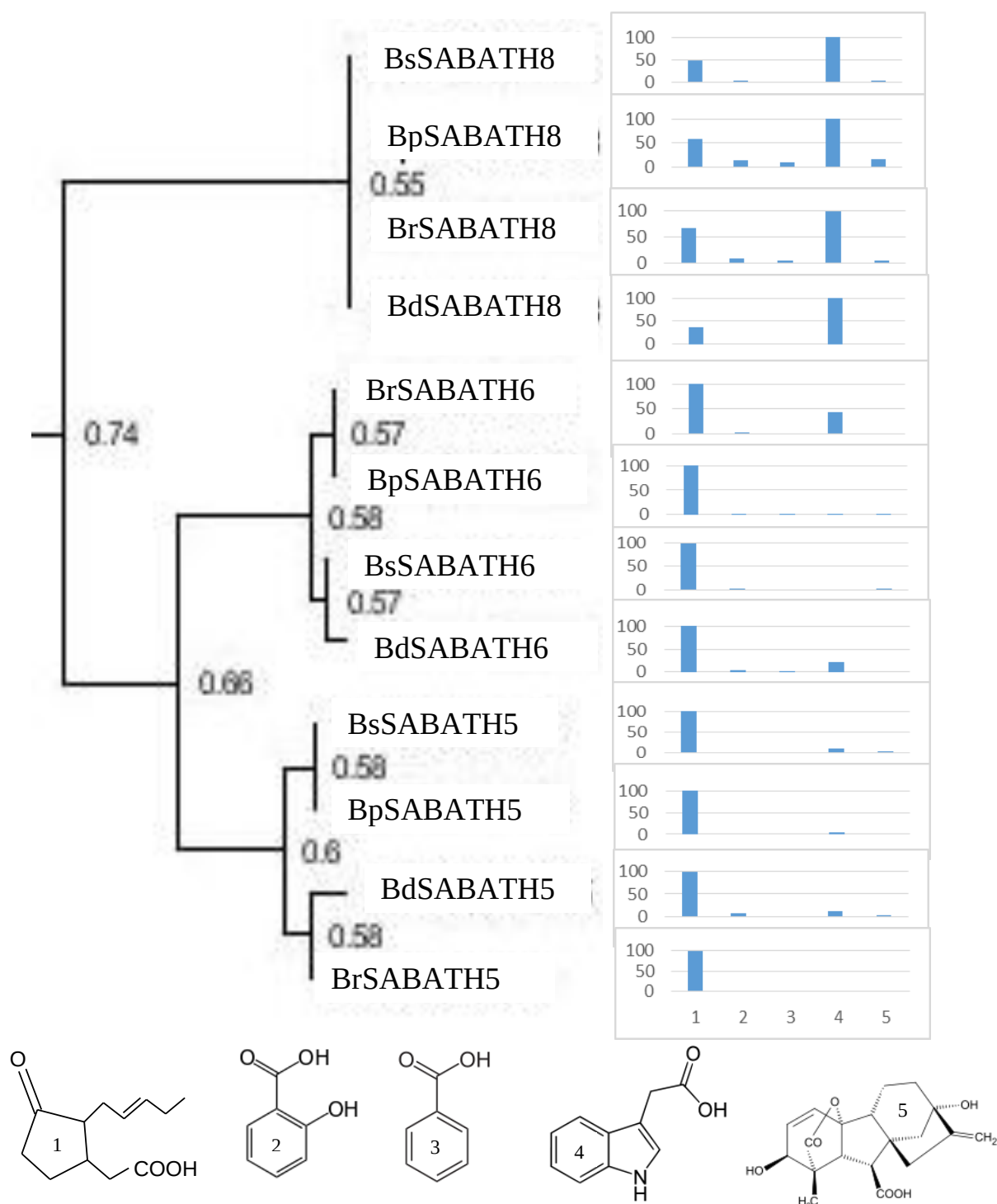


Figure 3.6 Relative enzyme activities of SABATHs from different species of *Brachypodium* with 5 substrates.

CHAPTER IV

Evolution of Phytohormone-methylating Enzymes: Implications from the Characterization of the SABATH family of Methyltransferases from Norway spruce

Minta Chaiprasongsuk, Ping Qian, Hong Guo, and Feng Chen. Evolution of Phytohormone-methylating Enzymes: Implications from the Characterization of the SABATH family of Methyltransferases from Norway spruce. Drafted

Abstract

Phytohormone-methylating compounds are plant synthesis compounds that serve as attractants of other living organisms beneficial to the plants or as defense against other biotic as well as abiotic agents. To increase their fitness and survival in a stressful environment plants produce distinct sets of phytohormone-methylating compounds. Plant genomes can encode the necessary enzymes to acquire the ability to make new specialized compounds during evolution. In this study, we present a case independent evolution of phytohormone methylating enzymes via the study of Norway spruce SABATH genes. Herein is reported the isolation and biochemical characterization of JMT and SAMT from Norway spruce. The three JMTs from Norway spruce are most closely related to each other than to other SABATH proteins from the same plants, suggesting that these three genes resulted from relatively recent gene duplications. PaJMTs appear polyphyletic to JMTs isolated from angiosperms. This indicates JMTs in gymnosperms and angiosperms evolved independently. Phylogenetic analysis suggests that PaJMTs are closely related to AtGMT1 and AtGMT2. This is an example of the ability of the Norway spruce to produce different phytohormone-methylating compounds that are already present in other plant lineages.

Introduction

Phytohormones are chemicals produced by plants and function at extremely low concentrations (Cohen and Bandurski, 1982). They regulate many biological processes ranging from plant growth and development to plant interactions with the environment (Gray, 2004). A number of phytohormones are carboxylic acids. These include IAA, GA, ABA, SA and JA. For each of these five phytohormones, their ester forms via the methylation of carboxyl group have been identified in plants (Schmelz et al., 2004). In general, carboxyl methylation is believed to deactivate a phytohormone. For methyl IAA and methyl GA, demethylation has been shown to activate the hormone (Qin et al., 2005; Zhao et al., 2008; Varbanova et al., 2007; Yang et al., 2008). For SA and JA, the biosynthesis of their corresponding methyl esters leads to novel functions. Both MeSA and MeJA can function as a volatile compound. In addition, both have been suggested to function as a mobile signal for long-distance transport. Similarly, demethylation of MeSA and MeJA will lead to rapid accumulation for SA and JA needed for specific biological activities (Soe et al. 2001; Wasternack 2007; Van Poecke et al. 2001; Van den Boom et al. 2004).

The enzymes that catalyze the methylation of IAA, GA, SA and JA have been identified from various plants (Qin et al., 2005; Zhao et al., 2008; Varbanova et al., 2007; Ross et al., 1999; Zhao et al., 2010; Chen et al., 2003; Seo et al., 2013, Zhao et al.). They have been named IAMT, GAMT, SAMT and JMT respectively. In contrast, it remains elusive whether MeABA identified in plants are products of enzymatic reactions. IAMT, GAMT, SAMT and JMT are all SAM-dependent methyltransferases, catalyzing the transfer of a methyl group from SAM to the carboxyl group of IAA, GA, SA and JA to form their corresponding methyl esters. Interestingly,

IAMT, GMT, SAMT and JMT are homologous enzymes, belonging to the same protein family called the SABATH (D'Auria et al., 2003). The SABATH genes form a mid-sized gene family in several plants that have been studied. The Arabidopsis, poplar and rice genomes contain 24, 28 and 41 SABATH genes respectively. In addition to the above enzymes, the SABATH family also contains enzymes that methylate benzoic acid (Murfitt et al., 2000), farnesoic acid (Yang et al., 2006), cinnamate/p-coumarate (Kapteyn et al., 2007), anthranilic acid (Eilert U., and B. Wolters, 1989; Tobias et al., 2010), nicotinic acid (Upmeier et al., 1998) and nitrogen-methyltransferase involved in caffeine biosynthesis (Kato et al. 2000; McCarthy and McCarthy, 2007). The SABATH family renders a useful system for understanding the structural basis underlying substrate specificity discrimination of a protein family because of a wide range of substrates of varying structure (Pott et al., 2004).

For the SABATH proteins that methylate phytohormones, we have been interested in characterizing their biological functions. In addition, we have been interested in understanding the functional evolution of the SABATH family, particularly related to the enzymes methylating hormones. In the pursuit of these questions, we have made two important findings. The first is that IAMT appears to be conserved in seed plants (Zhao et al, 2008). IAMTs from Arabidopsis, rice, poplar tree and white spruce form a monophyletic clade (Zhao et al, 2009). The second finding is that the SABATH proteins methylating phytohormone have not evolved in mosses, as shown in *Physcomitrella*. The *P. patens* genome contains four SABATH genes, one of which was shown to have a thiol methylating property. None of the four SABATH genes can methylate hormone carboxylic acids (Zhao et al, 2012). The second finding suggests that such enzymes evolved in a later stage of plant development.

In order to gain further insight into the evolution of the SABATH proteins methylating phytohormes, in this study we elected to characterize the SABATH family in Norway spruce (*Picea abies* (L.) Karst). Therefore, there are two justifications for this study. The first justification is the lack of knowledge about the SABTH family in a wide range of taxa. Except one IAMT genes isolated from white spruce and four genes isolated from moss, all other SABATH genes that have been characterized were from angiosperms. Therefore, the study of the SABTH family in Norway spruce will add to the body of knowledge of this family in gymnosperms. The second justification is the availability of whole genome sequence for this plant (Tuskan et al., 2006). This will enable the identification and characterization of the entire SABATH family in this plant.

Results

The SABATH Gene Family in Norway spruce

In order to gain the complete SABATH gene family from the fully sequenced Norway spruce genome sequencing project (Tuskan et al., 2006), *C. breweri* SAMT (CbSAMT) (Ross et al., 1999) was used as a query to conduct blast search analysis of the genome sequence database of Norway spruce using the BLASTP algorithm (Altschul et al., 1990). This blast search led to the identification of 10 SABATH proteins designated PaSABATH 1-10 (Table 4.1). The size of these ten proteins ranges from 379 to 398 amino acid. Sequence similarity ranges from 49% to 91%. These genes are distributed on contig MA_3356 to MA_10435381 (Table 4.1). Is there any tandem duplication? Most Norway spruce genes have 4 exons and 3 introns that are PaSABATH 1-3 and PaSABATH 6-9. The only three genes from Norway spruce have 3 exons and 2 introns are PaSABATH4, PaSABATH5 and PaSABATH10 (Table. 4.1 and Figure. 4.1). No

PaSABATH genes contain more than 4 introns as similar to AtSABATH genes (D'Auria et al. 2003).

Phylogenetic Analysis of SABATHs Family in Norway spruce

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 Spruce SABATH proteins and known SABATH proteins from other plants was constructed (Figure 4.4). When only SABATHs from Norway spruce are considered, PaSABATHs group into one big clade containing all of the 10 PaSABATHs, subdivided into 3 subclades. Subclade I contains known JMT from Arabidopsis, strawberry, and *Populus trichocarpa*. Subclade II contains SABATHs from the tea plant. Subclade III contains all of the genes of the PaSABATH and is closely related to known Arabidopsis proteins that are AtGMT1 and AtGMT2.

Clade II contains SAMT in a variety of plants, and Clade III contains the SABATH gene of coffee caffeine synthase and from other SABATH members of the coffee plant. Notably, PgIAMT1, OsIAMT1, PtIAMT1 and AtIAMT1 form a monophyletic group, which is closely related to SABATHs identified in the Norway spruce (PaSABATH1) (Figure 4.4).

Biochemical Characterization of individual SABATH proteins from Norway spruce

In conducting the cloning of full-length cDNAs, the cDNAs were first expressed in *E. coli* to produce recombinant proteins. Then individual recombinant proteins were tested with five substrates: jasmonic acid (JA), benzoic acid (BA), salicylic acid (SA), indole-3-acetic acid (IAA) and gibberellic acid (GA). PaSABATH1 showed the highest level of specific activity with IAA. Methyl transfer catalyzed by PaSABATH2 using SA as substrate was demonstrated to occur to

the carboxyl group. PaSABATH4, 5 and 10 showed the highest level of specific activity with JA (Table 4.2).

Detailed biochemical properties of three JAMTs

To determine the pH optimum of the enzymatic assays, PaJMT was assayed with JA at buffers with differing pH values between pH 6.5 to pH 10.0. The optimal pH was determined to be pH 7. At pH 6.5, and the enzyme showed 90% of maximal activity. At pH 9.0, the activity was 5% of the maximum (Figure 4.2A). As observed for other SABATH proteins that have been biochemically characterized, PaJMT activity can be affected by metal ions. K^+ and Mn^{2+} stimulated PaJMT activity by more than 1.1 and 1.05 -fold. Inclusion of Mg^{2+} resulted in an approximately 10% reduction while Ca^{2+} , NH_4^+ 20 reduction in PaJMT activity. In contrast Na^{2+} , Fe^{2+} , and Zn^{2+} all had a strong inhibitory effect on PaJMT activity, reducing PaJMT activity by more than 95% (Figure 4.2B). For thermostability, PaJMT was 100% stable for 30 min at 4 °C. It increased about 10% and 70% of its maximal activity when incubated at room temperature (RT) and 37 °C. It lost about 10% and 60% of its maximal activity when incubated at 42 °C and 50 °C, respectively for 30 min. When incubated at 60 °C for 30 min, PtJMT1 lost almost all of its activity (Figure 4.2C).

All three JAMT proteins (PaSABA10, PaSABATH4, and PaSABATH5) are purified using Ni-NTA agarose. A purified enzyme for each recombinant protein was used for determination of general catalytic properties and kinetic parameters. (Table 4.3). The kinetic parameter for PaSABATH10 with JA as substrate is about twofold higher than PaSABATH5. While the kinetic parameter for PaSABATH4 with JA as substrate is about threefold higher than PaSABATH10. Moreover, these three enzymes used jasmonic acid as a substrate. They were

subjected to detailed biochemical characterization under pseudo Michaelis–Menten conditions, PaSABATH10 exhibited apparent K_m values $13.96 \pm 12.39 \mu\text{M}$ for JA (Figure 4.3A). The K_{cat} of PaSABATH10 for JA was 0.001732 S^{-1} (Table 4.3). When PaSABATH4 exhibited apparent K_m values $148.2 \pm 450.4 \mu\text{M}$ (Figure 4.3B), the K_{cat} of PaSABATH4 for JA was 0.000379 S^{-1} (Table 4.3). When PaSABATH5 exhibited apparent K_m values $6.415 \pm 2.403 \mu\text{M}$ (Figure 4.3C), the K_{cat} of PaSABATH5 for JA was 0.014758 S^{-1} (Table 4.3).

Discussion

In this study, we found that five of the 10 SABATH family genes in Norway spruce encode enzymes capable of methylating four phytohormones: IAA, GA, SA, JA. Based on their most preferred substrates, this five genes were designated as PaIAMT, PtSAMT, PaJMT, PaJMT2 and PaJMT3. Because multiple IAMTs and SAMTs have been characterized for detailed biochemical activities, this paper focused on our detailed biochemical analysis of three JMTs. PaJMT, PaJMT2, and PaJMT3 encode protein 366, 382, and 400 amino acid. These three enzymes are identical from 37% to 40% at the amino acid sequence level with AtGMT1 and AtGMT2. All three enzymes are most active with JA. However, PaJMT3 is slightly methylating GA (2.89%). (Table 4.2)

PaIAMT forms a monophyletic clade with IAMTs from Arabidopsios, rice, black cottonwood and white spruce, suggesting that IAMT is conserved in seed plants. In contrast, PaSAMT are in different clades with SAMT from Arabidopsis, rice and other plants, consisting with a previous notion that SAMT have evolved multiple times in seed plants. The three JMTs from Norway spruce are more closely related to each other than to other SABATH proteins from the same plants, suggesting that these three genes resulted from relatively recent gene

duplications. PaJMTs appear polyphyletic to JMTs isolated from *Arabidopsis* (D'Auria et al. 2003) and black cottonwood (Zhao et al., 2013). This indicates JMTs in gymnosperms and angiosperms evolved independently.

Based on the phylogenetic relatedness of phytohormone-methylating enzymes, we speculate that IAMT is the most ancient activity. SAMT and JMT evolved from IAMT, multiple times. The structural analysis results is necessary to govern substrate specificity.

It will be interesting to determine the biological activities of IAMT, SAMT and JMTs in Norway spruce. In white spruce, IAMT was suggested to be involved in multiple biological processes including embryogenesis and xylem formation (Zhao et al. 2009). In comparison to angiosperms, our knowledge of the functions of MeSA and MeJA for gymnosperms is very limited. MeSA is produced in many species for diverse purposes. However, MeSA is released in Norway spruce saplings after MeJA treatment in a diurnal rhythm at rates of less than $20 \text{ ng} \times \text{h}^{-1} \text{g} \times \text{needle fresh weight}^{-1}$ (Martin et al. 2003). It will also be interesting to determine the biochemical and biological functions of the remaining five SABATH genes in this plant. It is noted that the size of the SABATH family compared to *Arabidopsis*, poplar and rice is significantly smaller.

Concluding Remarks

PaJMT, PaJMT2 and PaJMT3 were the first JMTs to have been characterized in Gymnosperms. These three JMTs genes from the Norway spruce are more closely related to each other than to the JMTs from other plants, implying that they resulted from relatively recent gene duplications. JA is the most preferred substrate for PaJMT, PaJMT2 and PaJMT3. Interestingly, these gene duplications of PaJMTs are all functional instead of having genetic disablement

mechanisms to reduce their fitness cost. This indicates PaJMTs are very beneficial to the Norway spruce. PaJMTs appear polyphyletic to JMTs isolated from angiosperms, indicating JMTs in gymnosperms and angiosperms evolved independently. Phylogenetic analysis suggested that PaJMTs are closely related to AtGMT1 and AtGMT2. The ability of the Norway spruce to produce different phytohormone-methylating compounds that are already present in other plant lineages. This indicate the independent evolution of PaJMTs from Norway spruce that evolved differently from Arabidopsis JMT and black cottonwood JMT. Furthermore, this result could be used to reveal the evolution of SABATH genes.

Material and Methods

Plant materials and chemicals

The Norway spruce (*Picea albies*), previously employed for whole genome sequencing was used for gene cloning and expression analysis of PaSABATHs. Tissues used for gene expression analysis, including needles, stems, young male cones and young female clones, were collected from trees. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Database search and sequence analysis

In order to identify putative PaSABATHs genes, BLAST searches were performed on the spruce genome project database and website (<http://congenie.org/start/>) using OsSAMT protein sequencing from *Oryza sativa* (accession: NP_001047944). The BlastP algorithm was used to compare similarities (Altschul et al., 1990). A plant methyltransferase gene encoding a protein with the highest level of sequence similarity to this protein was chosen for further analysis. For the phylogenetic tree construction, multiple protein sequence alignments were constructed using

MAFFT version 7 (Katoh and Standley, 2013). The FastTree program will be used to construct the phylogenetic tree.

Cloning full-length cDNA of PaSABATH

Total RNA was extracted using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA) with DNA contamination removed using an off-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). *MT* full-length cDNA was amplified using forward primer and reverse primer corresponding with the beginning and end of the *SABATH* 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 PaSABATH expressed in *E. coli*

PaSABATHs were subcloned from cDNA from pCRT7/CT-TOPO into the vector of pET100/D-TOPO (Invitrogen, Carlsband, CA). To express the MTs 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 µM for 18 h at 25 °C, with cells lysed by sonication. His-tagged

PaSABATH proteins were purified from the *E. coli* cell lysate using Ni-NTA agarose following manufacturer instructions (Invitrogen, Carlsband, CA). Protein purity was verified by SDS-PAGE and protein concentrations were determined by the Bradford assay (Bradford, 1976).

Radiochemical MT activity assay

Radiochemical MT assays were performed with a 50 μ L volume containing 50 mM Tris-HCl, pH 7.5, 1 mM substrate, 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 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). The amount of radioactivity in the organic phase indicated the amount of synthesis of the methylated substrates. Three independent assays were performed for each compound.

Determination of kinetic parameters of SABATH methyltransferase

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 MTs, to determine a K_m value for SAM, concentrations of SAM were independently varied from 3 to 120 μ M, while substrates were held constant at 1 mM. To determine the K_m for substrates, concentrations substrates were independently varied from 10 to 200 μ M, while SAM was held constant at 200 μ M. Assays were conducted at 25 $^{\circ}$ C for 30 min. The kinetic parameters K_m and V_{max} were calculated with GraphPad Prism 5 software for Windows (GraphPad Software Inc.), using standard settings for non-linear regression curve fitting the Michaelis-Menten equation.

pH optimum for MT activity

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

References

- Altschul SF, Stephen F, Gish W, Miller W, Myers EW, Lipman DJ** (1990) Basic local alignment search tool. *J Mol Bio* **215** (3): 403-410
- Chen F, D'Auria JC, Tholl D, Ross JR, Gershenzon J, Noel JP, Pichersky E** (2003) An *Arabidopsis* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J* **36**: 577-588
- Cohen JD, Bandurski RS** (1982) Chemistry and physiology of the bound auxins. *Ann. Rev. Plant Physiol* **33**: 403–430
- D'Auria JC, Chen F, Pichersky E** (2003) The SABATH family of MTs in *Arabidopsis thaliana* and other plant species. In *Recent Advances in Phytochemistry*, Vol. 37. (Romeo, J.T., ed.). Oxford: Elsevier Science Ltd., pp. 253–283
- Eilert U, Wolters B** (1989). Elicitor induction of S-adenosyl-L-methionine: Anthranilic acid N-methyltransferase activity in cell suspension and organ cultures of *Ruta graveolens* L. *Plant Cell, Tissue and Organ Culture* **18**: 1-18
- Faldt, J, Martin D, Miller B, Rawat S, Bohlmann J** (2003) Traumatic resin defense in Norway spruce (*Picea abies*): methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. *Plant Mol Biol* **51**(1): 119-133
- Gray WM** (2004) Hormonal regulation of plant growth and development. *Plos Biol.* **2**(9): 1270-1273

- Kapteyn J, Qualley AV, Xie Z, Fridman E, Dudareva N, Gang DR** (2007) Evolution of Cinnamate/p-Coumarate carboxyl methyltransferases and their role in the biosynthesis of methylcinnamate. *The Plant Cell*, **19**: 3212–3229
- Kato M, Mizuno K, Crozier A, Fujimura T, Ashihara H** (2000) Caffeine synthase gene from tea leaves. *Nature* **406**, 956–957
- Köllner TG, Lenk C, Zhao N, Seidl-Adams I, Gershenzon J, Chen F, Degenhardt J** (2010) Herbivore-Induced SABATH Methyltransferases of maize that methylate anthranilic acid using S-Adenosyl-l-Methionine. *Plant Physiol.* **153**(4): 1795–1807
- Martin DM, Gershenzon J, Bohlmann J** (2003) Induction of volatile terpene biosynthesis and diurnal emission by methyl Jasmonate in foliage of Norway spruce. *Plant Physiol.* **132**:1586-1599
- McCarthy AA, McCarthy JG** (2007) The Structure of Two N-Methyltransferases from the Caffeine Biosynthetic Pathway. *Plant Physiol.* **144**(2): 879–889
- Murfitt LM, Kolosova N, Mann CJ, Dudareva N** (2000) Purification and characterization of S-adenosyl-l-methionine: benzoic acid carboxyl methyltransferase, the enzyme responsible for biosynthesis of the volatile ester methyl benzoate in flowers of *Antirrhinum majus*. *Arch Biochem Biophys* **382**: 145–151
- Nystedt B, Street NR, Wetterbom A, Zuccolo A, Lin YC, Scofield DG, Vezzi F, Delhomme N, Giacomello S, Alexeyenko A, Vicedomini R, Sahlin K, Sherwood E, Elfstrand M, Gramzow L, Holmberg K, Hallman J, Keech O, Klasson L, Koriabine M, Kucukoglu M, Kaller M, Luthman J, Lysholm F, Niittyla T, Olson A, Rilakovic N, Ritland C, Rossello JA, Sena J, Svensson T, Talavera-Lopez C, Theissen G, Tuominen H, Vanneste K, Wu ZQ, Zhang B, Zerbe P, Arvestad L, Bhalerao R, Bohlmann J, Bousquet J, Gil**

- RG, Hvidsten TR, de Jong P, MacKay J, Morgante M, Ritland K, Sundberg B, Thompson SL, Van de Peer Y, Andersson B, Nilsson O, Ingvarsson PK, Lundeberg J and Jansson S** (2013) The Norway spruce genome sequence and conifer genome evolution. *Nature* **497**(7451): 579-584
- Parducci L, T Jorgensen, MM Tollefsrud, E Elverland, T Alm, SL Fontana, KD Bennett, J Haile, I Matetovici, Y Suyama, ME Edwards, K Andersen, M Rasmussen, S Boessenkool, E Coissac, C Brochmann, P Taberlet, M Houmark-Nielsen, N. K. Larsen, L Orlando, MT Gilbert, KH. Kjaer, IG Alsos, E Willerslev** (2012) Glacial survival of boreal trees in northern Scandinavia. *Science* **335**(6072): 1083-1086
- Pott MB, F Hippauf, S Saschenbrecker³, F Chen, J Ross, I Kiefer, A Slusarenko, JP Noel, E Pichersky, U Effmert, B Piechulla** (2004) Biochemical and Structural Characterization of Benzenoid Carboxyl Methyltransferases Involved in Floral Scent Production in *Stephanotis floribunda* and *Nicotiana suaveolens*. *Plant Physiol.* **135**: 1946-1955
- Qin G, Gu H, Zhao Y, Ma Z, Shi G, Yang Y, Pichersky E, Chen F, Liu M, Chen Z, Qu L-J** (2005) Regulation of auxin homeostasis and plant development by an indole-3-acetic acid carboxyl methyltransferase in *Arabidopsis*. *Plant Cell* **17**: 2693-2704
- Ross JR, Nam KH, D'Auria JC, Pichersky E** (1999) S-adenosyl-l-methionine: salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. *Arch Biochem Biophys* **367**: 9–16
- Schmelz EA, Engelberth J, Tumlinson JH, Block A and Alborn HT** (2004) The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites. *The Plant Journal* **39**, 790–808

- Upmeyer B, W Gross, S Ko"ster, W Barz** (1998) Purification and properties of S-adenosyl-l-methionine:Nicotinic acid-N-methyltransferase from cell suspension cultures of *Glycine max* L. Archives of Biochemistry and Biophysics **262**(2): 445–454
- Van den Boom CEM, Van Beek TA, Posthumus MA, De Groot A, Dicke M** (2004) Qualitative and quantitative variation among volatile profiles induced by *Tetranychus urticae* feeding on plants from various families. J Chem Ecol **30**:69–89
- Van Poecke RMP, Posthumus MA, Dicke M** (2001) Herbivoreinduced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: chemical, behavioral, and gene-expression analysis. J Chem Ecol **27**:1911–1928
- Varbanova M, Yamaguchi S, Yang Y, McKelvey K, Hanada A, Borochoy R, Yu F, Jikumaru Y, Ross J, Cortes D, Ma CJ, Noel JP, Mander L, Shulaev V, Kamiya Y, Roderick S, Weiss D, Pichersky E** (2007) Methylation of gibberellins by *Arabidopsis* GAMT1 and GAMT2. Plant Cell **19**: 32–45
- Wasternack C** (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot **100**:681–697
- Yang Y, Yuan JS, Ross J, Noel JP, Pichersky E, Chen F** (2006) An *Arabidopsis thaliana* methyltransferase capable of methylating farnesoic acid. Arch Biochem Biophys **448**: 123-132
- Zhao N, Ferrer JL, Ross J, Guan J, Yang Y, Pichersky E, Noel JP, Chen F** (2008) Structural, biochemical, and phylogenetic analyses suggest that indole-3-acetic acid methyltransferase is an evolutionarily ancient member of the SABATH family. Plant Physiol **146**: 455 - 467

- Zhao N, Boyle B, Duval I, Ferrer JL, Lin H, Seguin A, Mackay J, Chen F** (2009). SABATH methyltransferases from white spruce (*Picea glauca*): gene cloning, functional characterization and structural analysis. *Tree Physiology* **79**: 947-957
- Zhao N, Guan J, Ferrer JL, Engle N, Chern M, Ronald P, Tschaplinski TJ, Chen F** (2010) Emission, biosynthesis and regulation of insect-induced methyl salicylate and methyl benzoate from rice. *Plant Physiol Biochem* **48**: 279–287
- Zhao N, Ferrer JL, Moon Hs, Kapteyn J, Zhuang X, Hasebe M, Stewart CN JR, Gang Dr, Chen F** (2012) A SABATH Methyltransferase from moss *Physcomitrella patens* catalyzes S-methylation of thiols and has a role in detoxification. *Phytochemistry*. **81**:31-41
- Zhao N, Yao J, Chaiprasongsuk M, Li G, Guan J, Tschaplinski TJ, Guo H, Chen F** (2013) Molecular and biochemical characterization of the jasmonic acid methyltransferase gene from black cottonwood (*Populus trichocarpa*). *Phytochemistry* **94**: 74-81

Appendix

Table 4.1 SABATH genes in the genome of *Picea albies*

Name	Gene ID	Contig	Strand	Location	Exon	Protein size
PaSABATH1	MA_98251g0010	MA_98251	+	4376-8059	4	398
PaSABATH2	MA_3356g0010	MA_3356	+	6419-7884	4	394
PaSABATH3	MA_10433097g0010	MA_10433097	+	2164-3688	4	379
PaSABATH4	MA_128083g0020	MA_128083	+	9232-10613	3	391
PaSABATH5	MA_9561g0010	MA_9561	+	22915-24314	3	382
PaSABATH6	MA_10435381g0020	MA_10435381	+	2911-7051	4	390
PaSABATH7	MA_48038g0020	MA_48038	+	5952-7431	4	382
PaSABATH8	MA_13253g0010	MA_13253	+	34123-39548	4	392
PaSABATH9	MA_25646g0010	MA_25646	+	5401-7126	4	390
PaSABATH10	MA_10259819g0010	MA_10259819	+	4110-5493	3	400

Table 4.2 Screening of relative activities of PaSABATHs with 5 substrates

	PaSABATH1	PaSABATH2	PaSABATH4	PaSABATH5	PaSABATH10
Benzoic acid	0.76	10.02	0.16	1.42	0.77
Salicylic acid	0.00	100.00	0.00	0.27	1.72
Jasmonic acid	0.76	0.00	100.00	100.00	100.00
Indole-3-acetic acid	100.00	0.05	0.00	2.72	10.82
Gibberellic acid	0.00	0.00	0.00	0.54	2.83

Table 4.3 Michaelis-Menten kinetic parameters for Norway spruce enzymes (PaSABATH10, PaSABATH4 and PaSABATH5) with jasmonic acid (JA)

	PaSABATH10	PaSABATH4	PaSABATH5
K_m (JA), μM	11.8	31.68	6.42
K_{cat} (JA), s^{-1}	$1.732 \cdot 10^{-3}$	$3.79 \cdot 10^{-4}$	$1.4758 \cdot 10^{-2}$
K_{cat}/K_m (JA), $\text{s}^{-1}\text{xM}^{-1}$	$1.47 \cdot 10^{-4}$	$1.19 \cdot 10^{-5}$	$2.29 \cdot 10^{-3}$

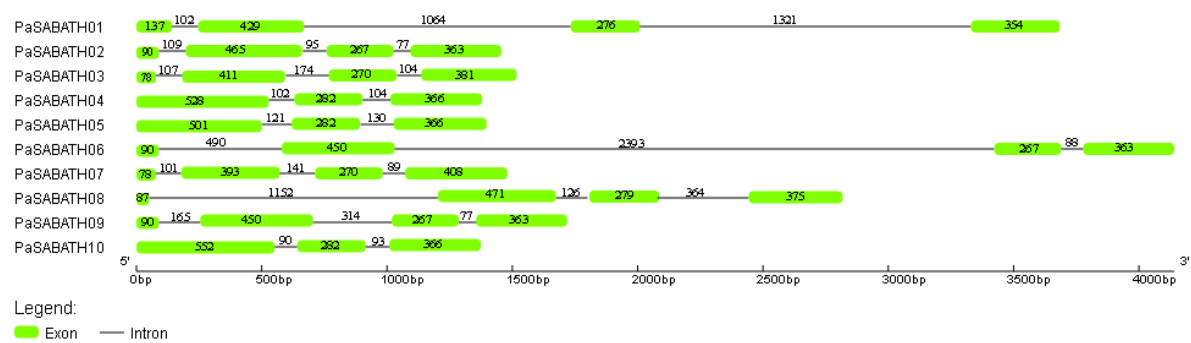


Figure 4.1 Exon and intron of SABATHs genes in Norway spruce.

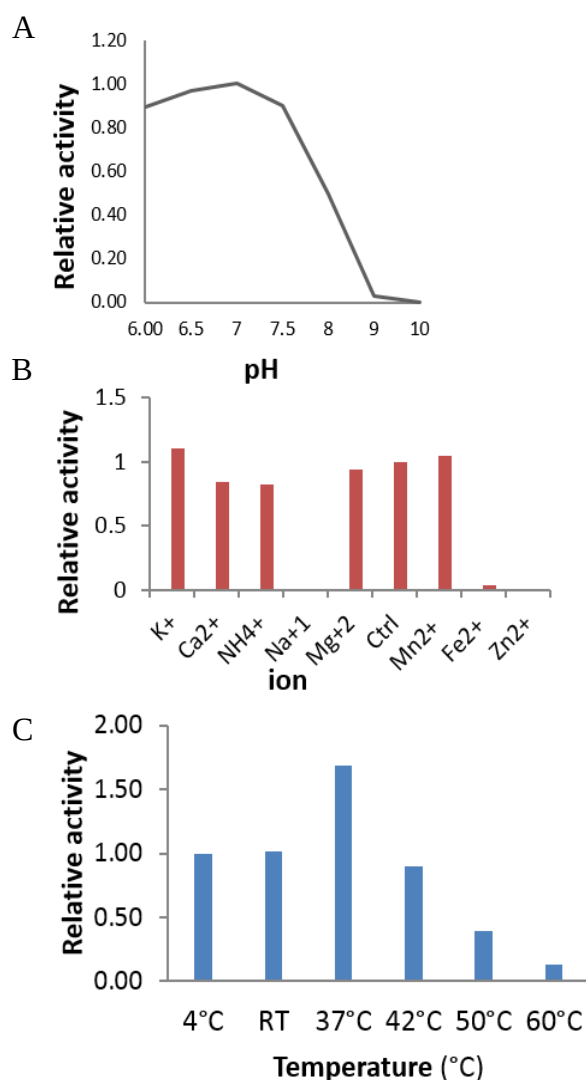


Figure 4.2. Biochemical properties of PaJMT. (A) pH effect on PaJMT activity. Level of PaJMT activity in the buffer of pH 7 was arbitrarily set at 1.0. (B) Effects of metal ions on activity of PaJMT. Metal ions were added to reactions in the form of chloride salts at a final concentration of 5 mM. Level of PaJMT activity without any metal ion added as control was arbitrarily set at 1.0. (C) Thermostability of PaJMT. The activity of PaJMT1 incubated at 4 °C for 30 min was arbitrarily set at 1.0. Jasmonic acid (1) was used as the substrate for enzyme assays in (A) to (C). Each value is an average of three independent measurements.

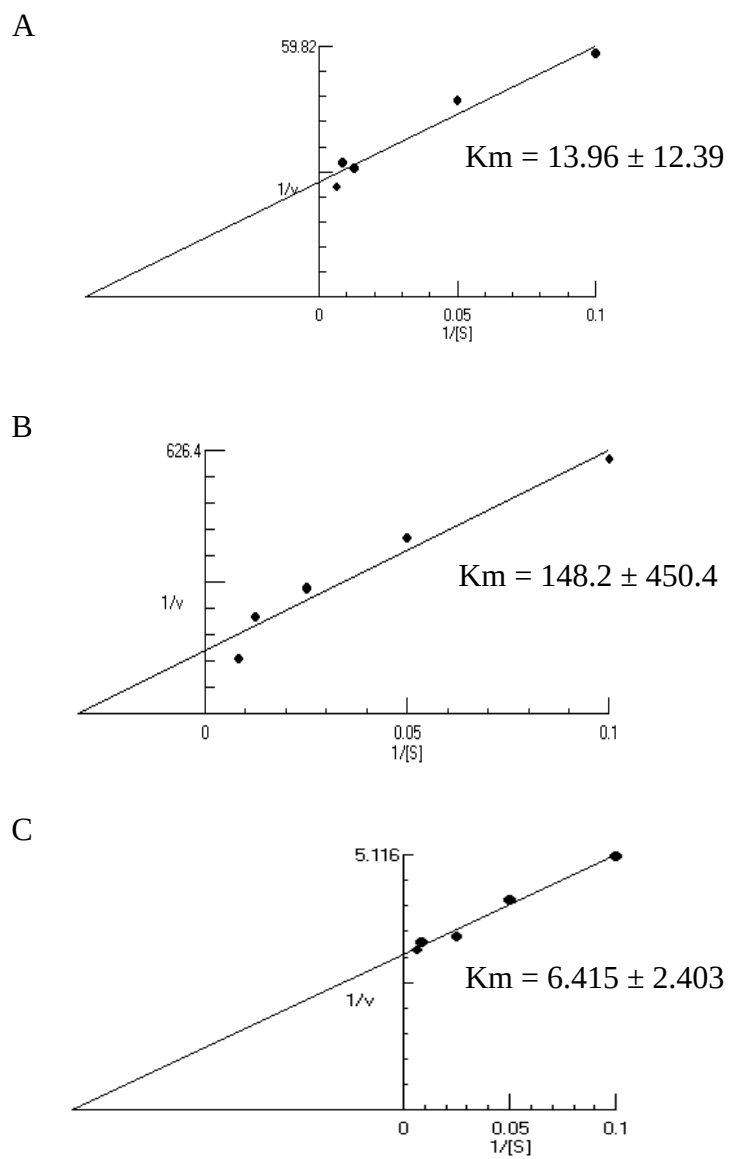


Figure 4.3. Detailed biochemical characterization of PaJMTs. Kinetics parameter of PaSABATH10, PaSABATH4, and PaSABATH5. (A) Steady-state kinetic measurements of PaSABATH10 using jasmonic acid as substrate. (B) Steady-state kinetic measurements of PaSABATH4 using jasmonic acid as substrate. (C) Steady-state kinetic measurements of PaSABATH5 using jasmonic acid as substrate.

Figure 4.4. Phylogenetic Analysis of SABATHs Family in Norway spruce with known proteins. CbSAMT, *C. breweri* SAMT (Accession # AF133053); AmSAMT, *Antirrhinum majus* SAMT (Accession # AF515284); HcSAMT, *Hoya carnosa* SAMT (Accession # AJ863118); AmBAMT: *A. majus* BAMT (Accession # AF198492); NsBSMT, *Nicotiana suaveolens* BSMT (Accession # AJ628349); AtBSMT, *Arabidopsis thaliana* BSMT (Accession # BT022049); AlBSMT, *Arabidopsis lyrata* BSMT (Accession # AY224596); AbSAMT, *Atropa belladonna* SAMT (Accession # AB049752); OsBSMT1, *Oryza sativa* BSMT1 (Accession # XM467504); SlSAMT, *Solanum lycopersicum* SAMT (Accession # NM001247880); AtJMT, *Arabidopsis thaliana* JMT (Accession # AY008434); SlJMT, *Solanum lycopersicum* JMT (EST cLEI13O14); AtFAMT, *Arabidopsis thaliana* FAMT (Accession # AY150400); AtIAMT1, *Arabidopsis thaliana* IAMT1 (Accession # AK175586); PtIAMT1, *Populus trichocarpa* IAMT1 (fgenes4_pg.C_LG_I002808); OsIAMT1, *Oryza sativa* IAMT1 (Accession # EU375746); AtGAMT1, *Arabidopsis thaliana* GAMT1 (At4g26420); AtGAMT2, *Arabidopsis thaliana* GAMT2 (At5g56300); Cas1, *Coffea arabica* caffeine synthase 1 (Accession # AB086414); CaXMT1, *Coffea arabica* xanthosine methyltransferase 1 (Accession # AB048793); CaDXMT1, *Coffea arabica* 3,7-dimethylxanthine methyltransferase 1 (Accession # AB084125); PtSABATH2, *Populus trichocarpa* SABATH2 (Accession # KC894592); PtSABATH4, *Populus trichocarpa* SABATH4 (Accession # KC894591). PpSABATH1, *P. patens* SABATH1 (gw1.47.108.1). PpSABATH1 was used as outgroup. Branches are drawn to scale with the bar indicating 0.1 substitutions per site.

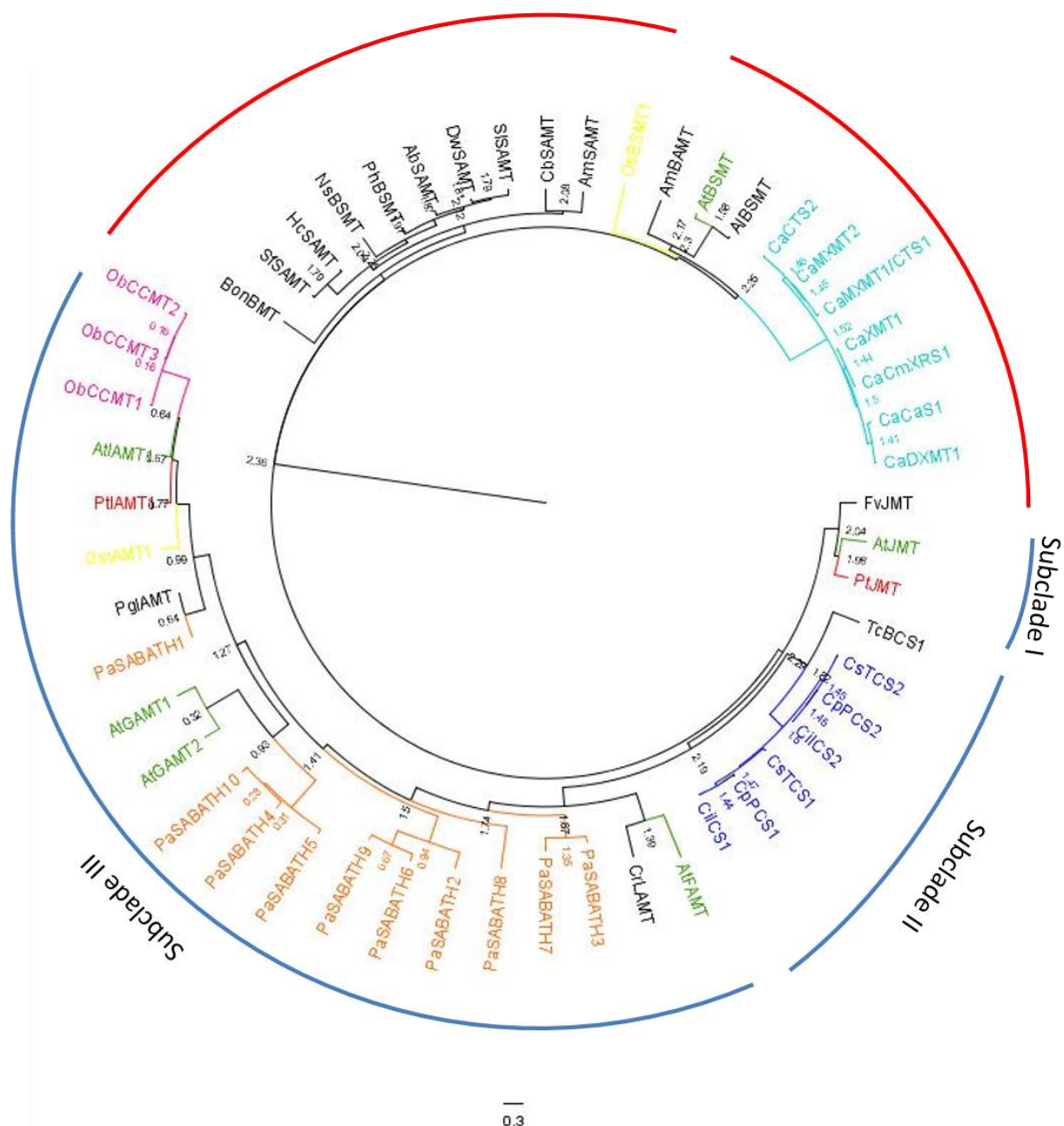


Figure 4.4. Phylogenetic Analysis of SABATHs Family in Norway spruce

CHAPTER V

Conclusions and Perspectives

Conclusions

In this dissertation, comparative functional genomics approaches were employed to study Norway spruce, *Brachypodium* and black cottonwood of SABATH gene families. Norway spruce, *Brachypodium* and black cottonwood were used as a model plant for gymnosperm, monocotyledon, and dicotyledon plants, respectively. One black cottonwood, three *Brachypodium* genes and four Norway spruce genes were identified with SABATH activities, and biochemical functions of these genes were studied. JMT from black cottonwood was identified and characterized in terms of biochemical and biological functions. The evolution of the functions of the SABATH gene family were investigated. Moreover, *Brachypodium* enzymes showed promiscuity based on their biochemical function. Furthermore, the phyto-hormone methylating enzymes in Norway spruce were surveyed using phylogenetic and enzyme structure analysis to understand the relationship of SABATH gene families between gymnosperm and angiosperm evolution. This research resulted in the following conclusions.

First, genome analysis revealed that the black cottonwood genome contains 28 SABATH genes. PtJMT1 is the third S-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase gene, after the Arabidopsis gene AtJMT and tomato JMT, to be isolated and biochemically characterized. Among all SABATH proteins in poplar and Arabidopsis, PtJMT1 is most similar to AtJMT, implying that the genes encoding them are orthologs. JA (1) is the most preferred substrate for PtJMT1, which also displayed activity with BA (3). It is not yet clear which of the two enzymes, PtJMT1 or AtJMT, represents the ancestral state of substrate specificity of JMT. Site-directed mutagenesis studies identified two amino acids in the active site of PtJMT1 that are important for determining the relative specificity between JA (1) and BA (3). The expression of

PtJMT1 was induced by a number of stress factors, suggesting that this gene has a role in the defense of black cottonwood trees against biotic stresses.

Second, genome analysis revealed that the *Brachypodium* genome contains 12 SABATH genes. BdIAMTs have a promiscuity biochemical function and appear polyphyetic to IAMTs isolated from gymnosperm, basal angiosperm, monocotyledon, and dicotyledon plants. SABATH genes from *Brachypodium* are more closely related to each other than to JMT from other plants, implying that they resulted from relatively recent gene duplications. JA is the most preferred substrate for BdSABATH5 and BdSABATH6. BdSABATH8 preferred IAA over JA substrate. Interestingly, these gene duplications of BdJMTs are all functional instead of having genetic disablement mechanisms to reduce a fitness cost. This indicates BdJMTs are very beneficial to *Brachypodium*.

Third, selected SbSABTH enzymes of sorghum were determined to encode JMT. This could indicate that BdIAMT in *Brachypodium* evolved from an ancestral JMT. While JMT earlier evolved from IAMT, this suggests that the promiscuity of JMT prefer JA over IAA provides the raw material for the recent evolution of IAMT in *Brachypodium distachyon*.

Fourth, the orthologs of BdSABATH5, 6 and 8 were isolated from three *Brachypodium* species. Molecular evolution analysis suggested that *Brachypodium* SABATH8 is under purifying selection. The stabilizing selection also is shown between BsSABATH5 and BpSABATH5. This event is also shown in BrSABATH5 and BpSABATH5.

Fifth, 10 SABATH genes were identified in the Norway spruce genome. There were three JMTs from Norway spruce, namely *PaJMT*, *PaJMT2*, and *PaJMT3*. These three JMTs genes from Norway spruce were more closely related to each other than to JMT from other

plants, implying that they resulted from relatively recent gene duplications. JA is the most preferred substrate of these three enzymes. PaJMTs could be generated from gene duplication. However, these three enzymes are all functional instead of having genetic disablement mechanisms to reduce fitness cost, indicating PaJMTs are very beneficial to the Norway spruce. PaJMTs appear polyphyletic to JMTs isolated from angiosperms, further indicating that JMTs in gymnosperms and angiosperms evolved independently. Phylogenetic analysis suggests that PaJMTs are closely related to AtGMT1 and AtGMT2. This is an example of the ability of the Norway spruce to produce different phytohormone-methylating compounds that are already present in other plant lineages and could be used to reveal the evolution in SABATH genes.

Overall, in this dissertation, the comparative functional genomics approach was used to identify the SABATH families in black cottonwood, *Brachypodium*, and Norway spruce. The biochemical and biological functions of selected members were studied in order to increase the knowledge of evolutionary aspects of the SABATH family.

Perspectives and Implications for Future Research

Despite the substantial progress made in this research, there are still many questions remaining about the functions and evolution of the SABATH families of black cottonwood, *Brachypodium* and Norway spruce. Further experiments could be extended from the following perspectives.

Biochemical functions of SABATH genes

Black cottonwood, *Brachypodium*, and Norway spruce contain 28, 12, and 10 SABTH genes, respectively. The biochemical function of one black cottonwood PtJMT1 highly prefer JA than BA. The selected number of PtSABATH genes provided extinguish JMT function different from AtJMT. However, the further study of complete set of genes from black cottonwood will provide clear information for evolution aspects of SABATH gene. The expression of PtJMT1 was induced by a number of stress factors, suggesting that this gene has a role in the defense of black cottonwood trees against biotic stresses. In order to understand biological function of this gene, PtJMT1 overexpression in transgenic plant is necessary for solving this question.

The biochemical function of four Norway spruce SABATH genes have determined the substrates and detail biochemical functions. In Norway spruce, there are three JMT genes in this taxa that are very different from the earlier study shown in black cottonwood and Arabidopsis that report a single JMT gene (Seo et al., 2001; Zhao et al. 2013). In this dissertation, PaJMTs genes resulted from relatively recent gene duplications. Moreover, PaJMTs appear polyphyletic to JMTs isolated from angiosperms. The further reconstruction of ancestral metabolic enzymes from this finding could reveal the molecular mechanisms of gene duplication and independent evolution of the SABATH family. Gene expression analysis in different organs of the Norway

spruce may provide more knowledge of biological functions of PaJMTs. As also shown in *Brachypodium*, there are two JMTs from this taxa. Identification of the entire set of SABATHs in these two species showed extinguished sets of phytohormone-methylating enzymes. In Norway spruce, JMTs were closely related to GMT from Arabidopsis. Interestingly, JMTs from *Brachypodium* were involved in promiscuous activity with JA over IAA molecule. The dual activities of these three enzymes is intriguing in evolution aspects. Dual activity of SABATH has been identified in plants such as AtBSMT, NsBSMT and OsBSMT Arabidopsis, rice, and Australian Tobacco (Chen et al. 2003; Pott et al. 2004; Zhao et al.2010,). It is not surprising that all these enzymes have both SAMT and BAMT functions, because SA and BA are structurally very similar. PtJBMT is another dual activity that has been studied and has huge differences in the substrate structure (Zhao et al. 2013). However BdJMTs are the first JMT that have dual activity of JA and IAA that are also largely different in their structures. How BdJMTs catalyze these two structurally different substrates is still unknown. The determined three-dimensional structure is necessary for solving this question. Since BdSABATH and SbSABATH are the results of promiscuous activity of JMT enzymes, this provide a powerful set of genes to study gene duplication and evolution of the SABATH family. Moreover, the biological function of MeJA in Norway spruce and *Brachypodium* are still unknown. Ortholog genes from BdJMT in different species provide further information in gene duplication events of *Brachypodium*. However, the reconstruction of ancestral metabolic enzymes from this result could reveal the molecular mechanisms of gene duplication and independent evolution of the SABATH family.

References

- Chen F, D'Auria JC, Tholl D, Ross JR, Gershenzon J, Noel JP, Pichersky E** (2003) An *Arabidopsis* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J* **36**: 577-588
- Pott MB, Hippauf F, Saschenbrecker S, Chen F, Ross J, Kiefer I, Slusarenko A, Noel JP, Pichersky E, Effmert U, Piechulla B** (2004) Biochemical and Structural Characterization of Benzenoid Carboxyl Methyltransferases Involved in Floral Scent Production in *Stephanotis floribunda* and *Nicotiana suaveolens*. *Plant Physiol.* **135**: 1946-1955
- Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD** (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. *Proc Natl Acad Sci USA* **98**: 4788–4793
- Zhao N, Guan J, Ferrer JL, Engle N, Chern M, Ronald P, Tschaplinski TJ, Chen F** (2010) Emission, biosynthesis and regulation of insect-induced methyl salicylate and methyl benzoate from rice. *Plant Physiol Biochem* **48**: 279–287
- Zhao N, Yao J, Chaiprasongsuk M, Li G, Guan J, Tschaplinski TJ, Guo H, Chen F** (2013) Molecular and biochemical characterization of the jasmonic acid methyltransferase gene from black cottonwood (*Populus trichocarpa*). *Phytochemistry* **94**: 74-81

VITA

Minta Chaiprasongsuk was born in Udonthani, Thailand on January 7, 1977. She moved to Bangkok, Thailand in 1999, where she finished her early education. She received her Bachelor of Science degree in Botany from Mahidol University in Bangkok, Thailand in 2002. She graduated Master of Science degree in Botany from Kasetsart University in 2007. Soon after, she became a lecturer at Kasetsart University. She then moved to Knoxville, Tennessee to join Dr. Feng Chen's lab in the fall of 2010, where she studied Biochemistry and evolution of the phytohormone-methylating enzymes in SABATH family of plants. She received her PhD in the Plants, Soils, and Insects major, Plant Molecular Genetics Concentration in 2016.