Phytochemistry 70 (2009) 32–39

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Two poplar methyl salicylate esterases display comparable biochemical properties but divergent expression patterns

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ARTICLE INFO

Article history:
Received 3 June 2008
Received in revised form 27 October 2008
Available online 10 January 2009

Keywords:
Black cottonwood
Populus trichocarpa
Methyl esterase
SABP2
Methyl salicylate
Salicylic acid
Gene family
Molecular modeling

ABSTRACT

Two genes encoding proteins of 98% sequence identity that are highly homologous to tobacco methyl salicylate (MeSA) esterase (SABP2) were identified and cloned from poplar. Proteins encoded by these two genes displayed specific esterase activities towards MeSA to produce salicylic acid, and are named PtSABP2-1 and PtSABP2-2, respectively. Recombinant PtSABP2-1 and PtSABP2-2 exhibited apparent Km values of 68.2 ± 3.8 μM and 24.6 ± 1 μM with MeSA, respectively. Structural modeling using the three-dimensional structure of tobacco SABP2 as a template indicated that the active sites of PtSABP2-1 and PtSABP2-2 were highly similar to that of tobacco SABP2. Under normal growing conditions, *PtSABP2-1* showed the highest level of expression in leaves and *PtSABP2-2* was most highly expressed in roots. In leaf tissues of poplar plants under stress conditions, the expression of *PtSABP2-1* was significantly down-regulated by two stress factors, whereas the expression of *PtSABP2-2* was significantly up-regulated by four stress factors. The plausible mechanisms leading to these two highly homologous MeSA esterase genes involved in divergent biological processes in poplar are discussed.

1. Introduction

Plants respond to pathogen infection by activating a variety of defense mechanisms (Dangl and Jones, 2001). At the infection site and surrounding area, cells usually undergo programmed cell death, which leads to the formation of necrotic lesions (Mittler et al., 1996). In addition to local responses, plants frequently develop resistance in other parts of the plant. This resistance, called systemic acquired resistance (SAR), is broad-spectrum and long-lasting (Ryals et al., 1996). Salicylic acid (SA) (**1**) (Fig. 1) is a critical signal for activating both local and systemic defenses (Sticher et al., 1997). After pathogen invasion, the endogenous levels of SA (**1**) in the infection site and systemic tissues increase, which induces the expression of defense-associated genes including those encoding pathogenesis-related (PR) proteins (Lamb and Dixon, 1997). In recent years, molecular genetic and biochemical studies led to identification of a number of key components of the SA (**1**) signaling pathway (Durrant and Dong, 2004). The SA-binding protein 2 (SABP2) is one of them.

SABP2 was originally identified from tobacco using a biochemical approach. It is present in low abundance in tobacco cells and can specifically bind SA (**1**) with high affinity (Kumar and Klessig, 2003). The gene encoding SABP2 was cloned and its deduced protein sequence showed high similarity to α/β fold hydrolases (Kumar and Klessig, 2003). A genetic study demonstrated that *SABP2* is a vital component of the SA (**1**) signaling pathway. When *SABP2* expression was silenced in tobacco, several SA-dependent responses, including development of local resistance to tobacco mosaic virus (TMV) and induction of PR gene expression, were impeded (Kumar and Klessig, 2003; Kumar et al., 2006). The three-dimensional structure of SABP2 was recently determined, which showed typical structural features of an α/β fold hydrolase superfamily (Forouhar et al., 2005). Further biochemical study demonstrated that SABP2 has strong esterase activity using methyl salicylate (MeSA) (**2**) as substrate (Forouhar et al., 2005). MeSA (**2**) is an ubiquitous compound in plants. It is produced from SA (**1**) by the action of SA methyltransferase (SAMT) (Ross et al., 1999). The hydrophobic nature of MeSA (**2**) makes itself a diffusible intercellular signal transducer. A recent study demonstrated that MeSA (**2**) is a mobile signal for SAR in tobacco (Park et al., 2007). Produced from SA (**1**) at the local infection site by the action of SAMT, MeSA (**2**) is readily translocated through the phloem. In systemic tissues, MeSA (**2**) is perceived by SABP2 and converted back to SA (**1**), which is the active signal (Park et al., 2007).

The role of the SA-dependent signaling pathway in plant defenses has been relatively well studied in a variety of annual species, such as tobacco (Malamy et al., 1990), Arabidopsis (Cao et al.,

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doi:10.1016/j.phytochem.2008.11.014

1994; Clarke et al., 2000) and rice (Chern et al., 2005; Iwai et al., 2007). In contrast, little is yet known about the role of the SA (**1**) signaling pathway in plant defenses in perennial woody species. Such species are long lived and therefore, more prone to attacks by pathogens during their life cycle (Rinaldi et al., 2007). SA (**1**) treatment can induce expression of defense genes in trees such as pine (Davis et al., 2002), suggesting that the SA-dependent signaling pathway is also important for defense responses in perennial woody species.

We have undertaken a project to study plant defense mechanisms against biotic stresses in perennial woody species using *Populus* as a model. There are about 40 species within the genus *Populus*. Members of the *Populus* are among the fast-growing tree species in the world. That is one important reason why *Populus* has been considered a candidate as bioenergy crop. Because of its small genome, relative ease of genetic manipulation, and rapid growth, *Populus* has also been established as a model for forest tree genetics and genomics (Jansson and Douglas, 2007). Black cottonwood (*Populus trichocarpa*, Torr. & Gray) is the first tree species whose genome has been fully sequenced (Tuskan et al., 2006), which provides unprecedented opportunities for the study of tree biology and physiology. Despite the many advantages of using poplar as a tree model as well as a bioenergy crop, poplar trees are susceptible to many pathogens (Ostry and McNabb, 1985). Elucidating the natural defense mechanisms of poplar is therefore important for both basic and applied sciences. The current study concerns the SA (**1**) signaling pathway in poplar.

Previous studies showed that some common characteristics of the SA (**1**) signaling pathway are shared by poplar and some herbaceous plants. For example, SA (**1**) treatment can induce expression of defense genes in both poplar (Koch et al., 2000) and tobacco (Uknes et al., 1993). However, poplar also has distinct features. For example, the basal levels of SA (**1**) in poplar are much higher than those in tobacco and Arabidopsis (Koch et al., 2000), posing a question on the uniqueness of the molecular mechanism of the SA (**1**) signaling pathway in poplar. In this paper, we were particularly interested in determining whether a key component of the SA (**1**) signaling pathway, SABP2, is conserved in poplar. Gene cloning, biochemical function characterization and expression analysis of two SABP2 genes from black cottonwood are reported.

2. Results

2.1. Identification and sequence analysis of putative poplar SABP2 genes

When the protein sequence of tobacco SABP2 (Kumar and Klessig, 2003) was used to blast search the sequenced poplar genome (Tuskan et al., 2006) using e-10 as a cut-off E-value, 30 proteins significantly homologous to SABP2 were identified. Two proteins, estExt_fgenesh4_pm.C_LG_VII0354 and eugene3.00070971, are most similar to tobacco SABP2 with an E-value lower than e-100. The two proteins displayed specific MeSA esterase activity (see Section 2). Thus, estExt_fgenesh4_pm.C_LG_VII0354 and eugene3.00070971 were named PtSABP2-1 and PtSABP2-2, respectively.

Both PtSABP2-1 and PtSABP2-2 are localized on chromosome VII. They are about 34 kbs from each other. Both genes contain two introns and three exons. Sequence comparison showed that the first introns of the two genes are 100% identical and the second introns 97% identical (data not shown). The promoter regions of the two genes were also compared. Significant sequence similarity was detected in the regions from start codon to about 300 bps upstream of the start codon. In contrast, the promoter regions between 300 and 800 bps upstream of the start codon of the two genes share no significant sequence similarity (see Supplementary data).

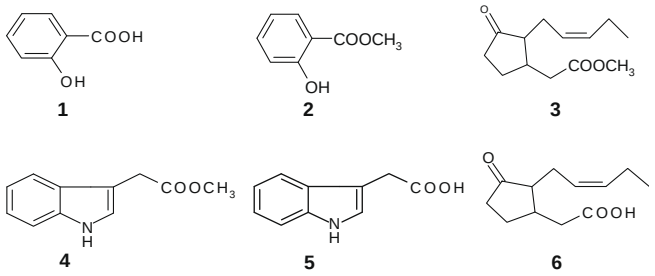

Fig. 1. Structures of compounds **1**–**6**.

Full-length cDNAs of PtSABP2-1 and PtSABP2-2 were cloned via RT–PCR from poplar leaf and root cDNAs, respectively. Both PtSABP2-1 and PtSABP2-2 encode a protein of 263 amino acids. Whereas PtSABP2-1 and PtSABP2-2 are 98% identical to each other, their sequences are 77% identical to that of tobacco SABP2 (Fig. 2). The putative pIs of PtSABP2-1, PtSABP2-2 and NtSABP2 are 5.01, 4.78 and 5.27, respectively.

2.2. PtSABP2-1 and PtSABP2-2 display MeSA esterase activity

Recombinant proteins of PtSABP2-1 and PtSABP2-2 were expressed in *Escherichia coli* from their corresponding full-length cDNAs. In the vector pET100/D-TOPO, the full-length cDNAs of PtSABP2-1 and PtSABP2-2 were fused to an N-terminal sequence containing codons for six histidine residues. His-tagged PtSABP2-1 and PtSABP2-2 were expressed in *E. coli* then purified using Ni-NTA agarose to near electrophoretic homogeneity (see Supplementary data).

Purified PtSABP2-1 and PtSABP2-2 were tested for esterase activity using MeSA as substrate. While the amounts of MeSA (**2**) decreased, the amounts of SA (**1**) increased in the same reaction, indicating that both PtSABP2-1 and PtSABP2-2 (not shown) catalyzed the hydrolysis of the methyl group of MeSA (**2**) to form SA (**1**). Both of PtSABP2-1 and PtSABP2-2 had the optimum pH at 7.0 (see Supplementary data). Under steady-state conditions, PtSABP2-1 exhibited an apparent Km value 68.2 ± 3.8 μM with MeSA and a V_{max} value of 4.2 ± 0.04 pmol S^{-1} (Fig. 3A). PtSABP2-2 had a Km value 24.6 ± 1 μM with MeSA (**2**) and a V_{max} value of 0.35 ± 0.03 pmol S^{-1} (Fig. 3B).

2.3. Substrate specificity analysis of PtSABP2-1 and PtSABP2-2

In *in vitro* enzyme assays, tobacco SABP2 displayed esterase activity with multiple substrates, including MeSA (**2**), MeJA (**3**) and MeIAA (**4**), with different specific activities (Forouhar et al., 2005). For comparison, PtSABP2-1 and PtSABP2-2 were also analyzed for esterase activity with MeJA (**3**) and MeIAA (**4**). At 10 μM and 100 μM concentrations of substrates, PtSABP2-1 and PtSABP2-2 showed no or very low activity with MeJA (**3**) and MeIAA (**4**) (Fig. 4). At 1 mM concentration of substrates, PtSABP2-1 and PtSABP2-2 showed significant activity with MeJA (**3**). However, the esterase activity of PtSABP2-1 and PtSABP2-2 with MeJA (**3**) is only 9% and 14% of their corresponding activities with MeSA (**2**), respectively (Fig. 4). These results indicate that the two poplar SABP2 proteins are highly specific for MeSA (**2**) among the three substrates tested at both physiologically relevant (10 μM) and irrelevant (1 mM) concentrations.

2.4. Structural features of PtSABP2-1 and PtSABP2-2

The crystal structure of tobacco SABP2 has been experimentally determined (Forouhar et al., 2005). Since tobacco SABP2 and the

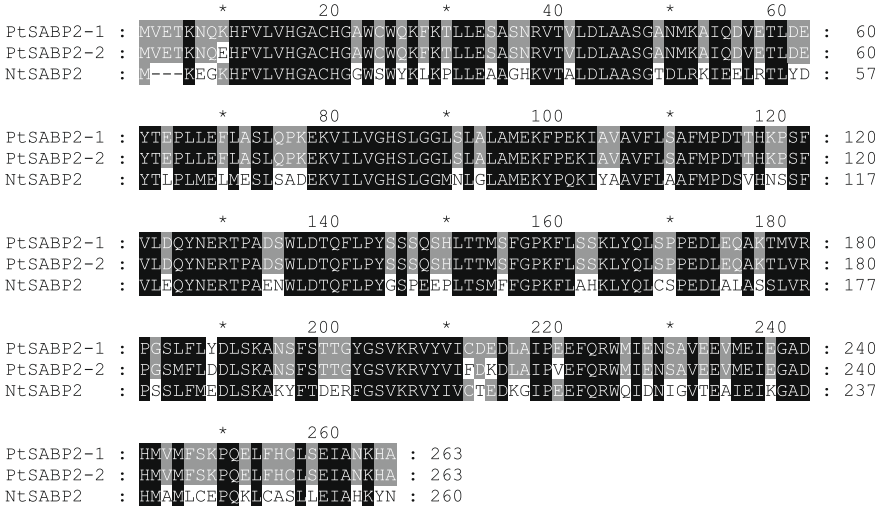

Fig. 2. Multiple sequence alignment of deduced amino acid sequences of PtSABP2-1 and PtSABP2-2 and the protein sequence of tobacco SABP2 (NtSABP2).

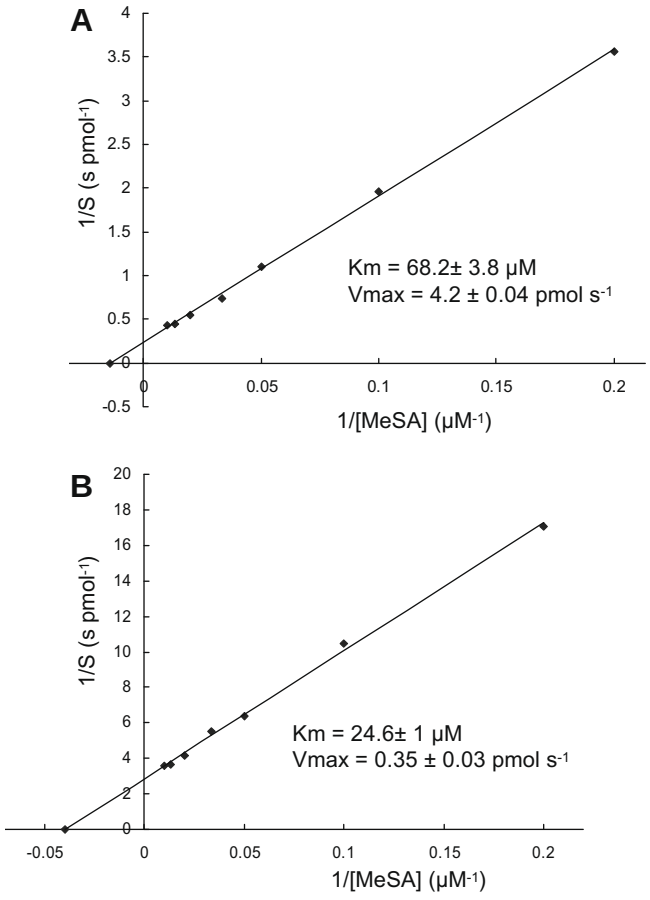

Fig. 3. Biochemical properties of PtSABP2-1 and PtSABP2-2. Steady-state kinetics of PtSABP2-1 and PtSABP2-2 was measured using MeSA (**2**) as substrate. Examples of the Lineweaver–Burk plot for PtSABP2-1 (A) and PtSABP2-2 (B) were shown.

two poplar SABP2s possess 77% sequence identity, reliable three-dimensional models of PtSABP2-1 and PtSABP2-2, including their active sites, were constructed using tobacco SABP2 structure as a template (Fig. 5). The overall structures of PtSABP2-1 and PtSABP2-2 are very similar to that of tobacco SABP2, consistent with their high sequence conservation. In the active site region, PtSABP2-1 has only one substitution with respect to tobacco SABP2: G212 of tobacco SABP2 is replaced by A215 in PtSABP2-1 (Fig. 5A). The extra methyl group in PtSABP2-1 resulted from the G to A change is not expected to affect the binding of SA, in agreement with the kinetic studies. PtSABP2-2 has two substitutions: G212 and L181 of tobacco SABP2 are respectively replaced by A215 and M184 in PtSABP2-2 (Fig. 5B). The introduction of a bulkier Met side chain in PtSABP2-2 probably has an impact on the observed lower Km value of this enzyme (Fig. 3).

2.5. Expression analysis of PtSABP2-1 and PtSABP2-2

RT–PCR analysis was performed to determine the expression patterns of *PtSABP2-1* and *PtSABP2-2*. Gene specific primers were designed to discriminate the transcripts of the two genes (Fig. 6A). Total RNA was isolated from young leaves, old leaves, stems, and roots of one year-old poplar trees and used for semi-quantitative RT–PCR analysis. Gene expression analysis (Fig. 6B) showed that *PtSABP2-1* had the highest level of expression in leaves and a low level of expression in stems and roots. In contrast, *PtSABP2-2* had the highest level of expression in roots, a moderate level of expression in stems and a low level of expression in leaves.

To understand the potential roles of *PtSABP2-1* and *PtSABP2-2* in plant defense, expression of the two genes under stress conditions was also analyzed. Poplar plants were either physically injured or treated with SA (**1**), MeJA (**3**), or a fungal elicitor alamethicin. Leaves of the treated poplar plants and appropriate control plants were collected for extraction of RNAs, which were used RT-PCR analysis. The expression of *PtSABP2-1* (**1**) was not significantly affected by the stress factors tested, except for 2 h wounding and SA (**1**) treatment for 24 h, which significantly suppressed the expression of *PtSABP2-1* (Fig. 6C). In contrast, the expression of *PtSABP2-2* was significantly up-regulated by a number of treatments, including 24 h wounding, 2 h SA (**1**) treatment, 4 h MeJA (**3**) treatment and 2 h alamethicin treatment. Treatments by wounding, SA (1), and MeJA (**3**) at other time points did not significantly affect the expression of *PtSABP2-2* (Fig. 6C).

3. Discussion

SAR has been shown to be activated by a vascular-mobile signal that moves throughout the plant from the infected leaves (Durrant and Dong, 2004). The nature of the mobile signal for SAR, however,

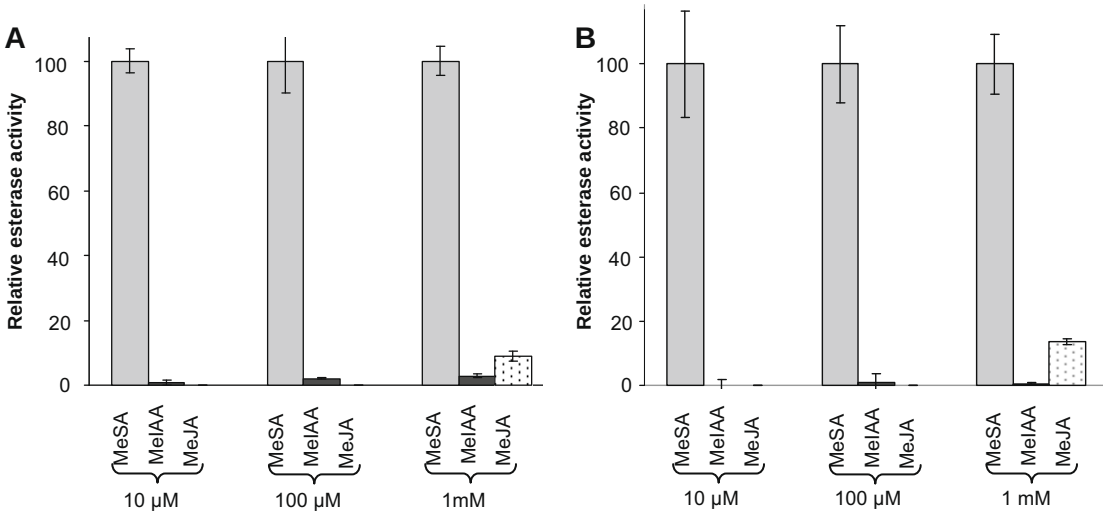

Fig. 4. Substrate specificity of PtSABP2-1 and PtSABP2-2. Relative methyl esterase activity of PtSABP2-1 (A) and PtSABP2-2 (B) was measured with MeSA (**2**), MeIAA (**4**), and MeJA (**3**) at three different concentrations (10 μM, 100 μM, and 1 mM). The activity with MeSA (**2**) at each of the substrate concentrations was set at 100%. The activity ratios between MeSA (**2**) and the other substrates are indicated.

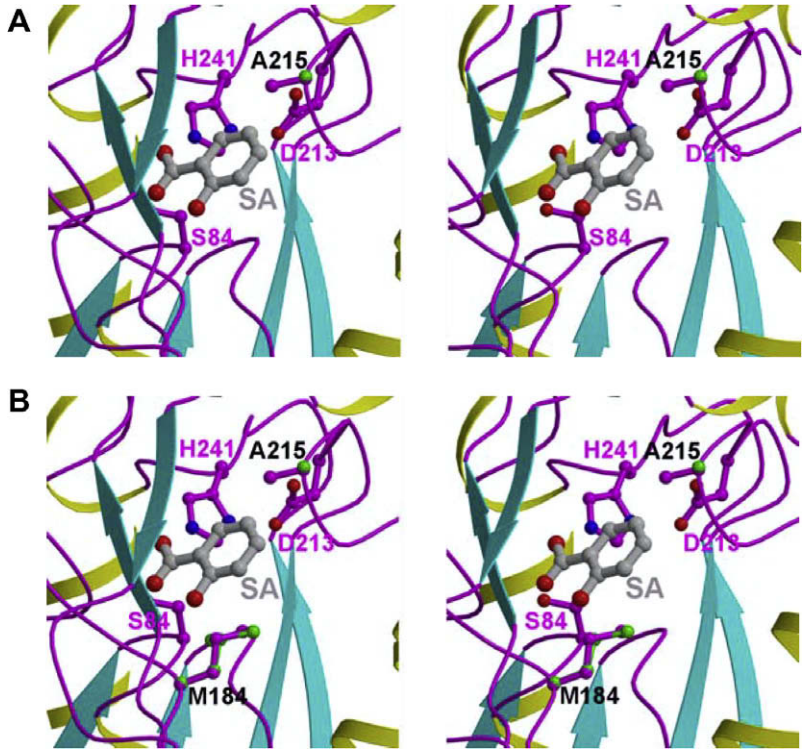

Fig. 5. Active sites of PtSABP2-1 and PtSABP2-2. (A) Stereo-view representation of the active site of PtSABP2-1; the SA (light grey) and the side chains of the catalytic triads, S84, D213, and H241 (all in magenta) are shown as ball-and-stick models. A215 (magenta) is labeled in black so as to distinguish it from the corresponding residue G212 of tobacco SABP2, which is depicted in green ball; and (B) stereo-view representation of the active site of PtSABP2-2. The two substitutions, A215 and M184, in the active site of PtSABP2-2, corresponding to G213 and L181 (both shown in green) of tobacco SABP2, are shown in ball-and-stick models and labeled in black.

has been a subject of controversy. SA (**1**) has been demonstrated to be essential for the activation of SAR (Gaffney et al., 1993). SA (**1**) itself, however, has been disassociated as the mobile signal (Vernooij et al., 1994). MeSA, the methyl ester of SA (**1**), was recently demonstrated to be a mobile signal for SAR in tobacco (Park et al., 2007). With MeSA (**2**) being the inactive mobile signal, it is critical that MeSA (**2**) is converted back to SA (**1**) at the site of action. This reaction is catalyzed by MeSA esterase. Prior to this study, the only MeSA esterase that has been functionally characterized is SABP2 from tobacco (Forouhar et al., 2005). Here, we showed that poplar, a woody perennial species, contains two functional *SABP2* genes. PtSABP2-1 and PtSABP2-2 were identified from the poplar genome based on their high sequence similarity to tobacco SABP2 (Fig. 2). In *in vitro* biochemical studies, like tobacco SABP2, both PtSABP2-1 and PtSABP2-2 displayed highly specific esterase activity towards MeSA (**2**) (Figs. 3 and 4), supporting that MeSA (**2**) is the in vivo substrate. Whether PtSABP2-1 and PtSABP2-2 accept substrates other than MeSA (**2**) in planta, however, remains to be determined. The Km value of tobacco SABP2 was reported to be 8.6 μM (Forouhar et al., 2005). The Km values

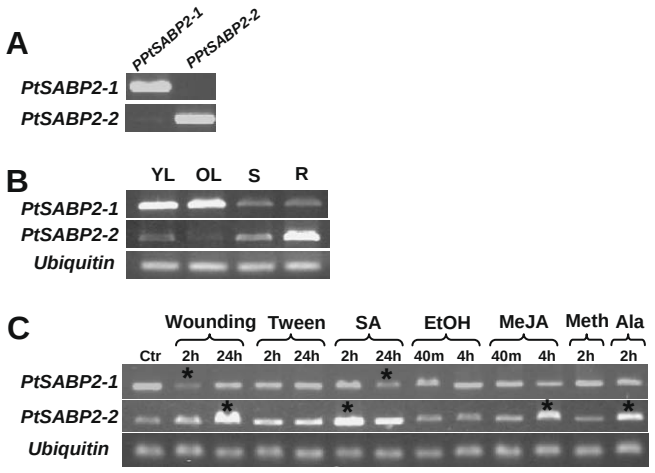

Fig. 6. Expression analysis of *PtSABP2-1* and *PtSABP2-2*. (A) Plasmids *PPtSABP2-1* and *PPtSABP2-2* containing full-length cDNAs of *PtSABP2-1* and PtSABP2-2, respectively, were used as templates in PCRs with gene-specific primers for PtSABP2-1 and PtSABP2-2; (B) tissue-specific expression of PtSABP2-1 and PtSABP2-2. Young leaves (YL), old leaves (OL), stems (S), and roots (R) were colleted from one year-old poplar trees grown in the greenhouse; and (C) expression of PtSABP2-1 and PtSABP2-2 in leaves of poplar plants that were either physically injured (wounding, 2 h and 24 h), or treated with one of the following chemicals: salicylic acid (SA) (**1**), methyl jasmonate (MeJA) (**3**) and alamethicin (Ala). Treatments with 0.1% tween (Tween), ethanol (EtOH) and methanol (Meth) were used as controls for SA (**1**), MeJA (**3**) and Ala treatments, respectively. Bands marked with a asterisk (*) indicate that the gene showed significant difference in expression levels in treated vs. control plants ($P < 0.05$). In both (B) and (C), PCR with primers for *Ubiquitin* was used to judge equality of the concentration of cDNA templates in different samples.

of PtSABP2-1 and PtSABP2-2 are about 10 times and three times higher than that of tobacco SABP2. It will be interesting to determine whether the endogenous levels of MeSA (**2**) in poplar tissues under stresses are higher than that in tobacco tissues under stresses. The overall three-dimensional structures and active sites of the three proteins are highly conserved (Fig. 5). Despite their similar biochemical properties, the two poplar *SABP2* genes display divergent expression patterns. They showed different tissue-specificity under normal growing conditions and different responses to stress factors (Fig. 6), suggesting that these two genes have divergent biological roles.

Studying the potential involvement of SABP2s in poplar-pathogen interactions will be important for elucidation of the biological roles of poplar SABP2s, especially in the context of a SA metabolism that is somehow different from that in tobacco. Whereas the SA (**1**) levels in healthy leaves of tobacco are low, in healthy tissues of poplar they are high (Koch et al., 2000; Morse et al., 2007). In poplar, overexpressing a bacterial SA hydroxylase gene *nahG* in poplar did not cause a significant reduction of the SA (**1**) levels (Morse et al., 2007), which is different from the study with tobacco (Delaney et al., 1994). How PtSABP2-1 and PtSABP2-2 function in poplar with respect to SA metabolism as compared with that in tobacco remains to be determined. Poplar mosaic virus (PopMV) is the best characterized tree virus (Cooper, 1993). The infection dynamics of PopMV has been investigated using different genotypes of *Populus* (Smith and Campbell, 2004). The poplar-PopMV system, therefore, presents a good model for determining whether PtSABP2s function in SAR in this perennial woody species. Such study will provide further evidence on whether SABP2-dependent SAR mediated by SA (**1**) is a conserved defense pathway.

In addition to its involvement in SAR, MeSA (**2**), the substrate of SABP2, has been implicated in a number of other biological/ecological processes. MeSA (**2**) is often emitted as a volatile compound from the plants that are being challenged by stress factors, including insect feeding (Chen et al., 2003a), virus infection (Shulaev et al., 1997), and elicitor treatment (Chen et al., 2003a). MeSA (**2**) produced under such stress conditions has been suggested to have a number of biological roles. For example, insect-induced volatile MeSA (**2**) has been suggested to be involved in attracting natural enemies of the feeding insects (Dicke et al., 1990). Virus-induced air-borne MeSA (**2**) has been suggested to act as a signal to activate defense responses in nearby healthy plants (Shulaev et al., 1997). In all these cases, if SA (**1**) is the active signal, then inactive MeSA (**2**) would need to be converted back to SA (**1**). If true, it would suggest that SABP2 has multiple biological functions in addition to its role in SAR, which is supported by induction of expression of poplar *SABP2* genes by multiple stress factors (Fig. 6).

The presence of two MeSA esterase genes with a same biochemical function but seemingly divergent biological roles is intriguing. It is tempting to speculate that this reflects the specific nature of natural defenses of perennial woody species. Due to their long generation time, perennial woody species can not match the evolutionary rates of microbial pathogens that go through several generations every year (Rinaldi et al., 2007). Therefore, positive adaptation of defense mechanism may be necessary for perennial woody species to survive. Overrepresentation of defense-related genes, such as NBS-LRR in the first sequenced tree genome, supports this notion (Tuskan et al., 2006). The presence of two *SABP2* genes with divergent expression patterns may provide poplar trees an advantage in defense against pathogens.

4. Concluding remarks

The mechanism that leads to functional divergence of PtSABP2-1 and PtSABP2-2 is also intriguing. The two SABP2s are highly homologous to each other, suggesting they are the consequence of gene duplication. Gene duplication may result from whole genome duplication, segmental duplication of chromosomes, or local duplication caused by unequal crossover (Zhang, 2003; Yang et al., 2006). *PtSABP2-1* and *PtSABP2-2* are localized on chromosome VII and are 30 kbs from each other. They are therefore likely the consequence of local gene duplication. The high sequence similarity between the two genes, even in introns, implies that the duplication event occurred relatively recently. The promoter regions of the two genes, however, show relatively high degree of divergence, suggesting that promoter divergence is probably a major determinant of functional divergence of *PtSABP2-1* and *PtSABP2-2*.

5. Experimental

5.1. Plant material and chemicals

The female black cottonwood clone 'Nisqually-1', which was used for whole genome sequencing (Tuskan et al., 2006), was used for gene cloning and expression analysis in this study. The tissues used for gene expression analysis at normal growing conditions, including young leaves, old leaves, stems, and roots, were collected from one year-old poplar trees grown in the greenhouse. Leaf tissues used for gene expression analysis under stress conditions were collected from poplar plants at the eight-leaf stage that were vegetatively propagated grown on MS medium. Poplar plants were either physically injured or treated with several chemicals, including SA (**1**), methyl jasmonate (MeJA) (**3**) or a fungal elicitor alamethicin. For physical injury, leaves were cut with a sterile razor blade to produce three lateral incisions on each side of the midvein and the wounded leaves were collected at 2 h and 24 h after wounding. For SA (**1**) treatment, poplar plants were sprayed with either 5 mM SA (**1**) solution in 0.1% Tween (pH 7.0) or 0.1% Tween only as control. Leaves were collected at 2 h and 24 h after initiation of the

treatment. For MeJA (**3**) treatment, poplar plants were placed in a 1 L glass jar containing a cotton tip applied with 1 μl MeJA (**3**) dissolved in EtOH (200 μl) ethanol. Poplar plants placed in another 1 L glass jar containing only EtOH (200 μl) were used as control. The glass jar was quickly sealed. Leaves were collected after 40 min and 4 h after initiation of the treatment. For alamethicin treatment, leaves were cut-off from the base of the petiole and the detached leaves then placed apright in a small glass beaker containing 10 ml of 5 μg/ml alamethicin (dissolved 1000-fold in water from a 5 mg/ml stock solution in 100% MeOH). As a control, detached leaves were submerged in 0.1% MeOH in H_2O. Only the petiole of each leaf was submerged in the solution. The glass beaker was then sealed with Saran wrap and placed in a growth chamber. Leaves were collected 2 h after the treatment. Two replicates were performed for each treatment. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

5.2. Database search and sequence analysis

To identify putative poplar *SABP2*-like esterase genes, the protein sequence of tobacco *SABP2* (accession: AY485932) was used as a query to search the genome sequence database of poplar (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) using the BlastP algorithm (Altschul et al., 1990). Two poplar genes encoding proteins with the highest level of sequence similarities (77%) to tobacco SABP2 were chosen for further analysis.

Nucleic acid and protein sequence alignments were made using the ClustalX program (Thompson et al., 1997), and displayed using GeneDoc (http://www.psc.edu/biomed/genedoc/).

5.3. Cloning full-length cDNA of PtSABP2-1 and PtSABP2-2

Total RNA was extracted from the leaf and root tissues using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and DNA contamination was removed with an on-column DNase (Qiagen, Valencia, CA, USA) treatment. 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, USA) as previously described (Chen et al., 2003b). *PtSABP2-1* full-length cDNA was amplified with the leaf cDNA using the forward primer 5′-CACCATGGTAGAGACCAAGAATCAGA-3′ and the reverse primer 5′-CAAACCACTATAAGTAAGGGTGCTGA-3′. *PtSABP2-2* full-length cDNA was amplified with the root cDNA using the forward primer 5′-CACCATGGTAGAGACCAAGAATCAGG-3′ and the reverse primer 5′-TATAACTAAGGGTGCTGATAAGGACA-3′. PCR was set as follows: 94 °C for 2 min followed by 30 cycles at 94 °C for 30 s, 60 °C for 45 s and 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. The PCR product was separated on 1.0% agrose gel. The target band was sliced from the gel and purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The PCR product was cloned into pET100/D-TOPO vector using the protocol recommended by the vendor (Invitrogen, Carlsband, CA, USA). The cloned cDNAs in pET100/D-TOPO vector were fully sequenced.

5.4. Purification from E. coli-expressed recombinant proteins

To express PtSABP2-1 and PtSABP2-2, the corresponding protein expression constructs were transformed into *E. coli* strain BL21 (DE3) CodonPlus (Stratagene, La Jolla, CA, USA). Protein expression was induced by IPTG for 18 h at 22 °C and cells were lysed by sonication. His-tagged PtSABP2-1 and PtSABP2-2 proteins were purified from *E. coli* cell lysate using Ni-NTA agarose following the manufacturer's instruction (Invitrogen, Carlsband, CA, USA). The purity of the protein was verified by SDS–PAGE and the concentration of the protein was determined by the Bradford assay (Bradford, 1976).

5.5. MeSA esterase activity assay

In a single assay, the reaction was performed in 90 μl volume containing 50 mM Tris–HCl, pH 7.5, 4 μg purified enzyme, and 600 μM MeSA (**2**) at 25 °C. The individual assays were terminated by addition of 10 μl 2 N HCl at one of the following time points: 0 h, 1 h, 2 h, and 4 h after initiation of the reaction. Then individual assays were divided into two halves. The first half (50 μl) was extracted with EtOH (100 μl) and the organic phase analyzed for the amount of MeSA (**2**) using GC–MS as previously described (Zhao et al., 2008). The second half was used for identification and quantification of SA (**1**). Aliquots (50 μl) of crude assays were quantitatively transferred to scintillation vials and dried down in a He stream. The dried samples were dissolved in 500 μL of silylation–grade CH_3CN followed by addition of 500 μL *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce Chemical Co., Rockford, IL, USA), and then heated for 1 h at 70 °C to generate trimethylsilyl (TMS) derivatives. After 2 days, 1 μL aliquots were injected into a ThermoFisher DSQII GC–MS, fitted with an Rtx-5MS (crosslinked 5% PH ME Siloxane) 30 m × 0.25 mm × 0.25 μm film thickness capillary column (Restek, Bellefonte, PA, USA). The standard quadrupole GC–MS was operated in electron impact (70 eV) ionization mode, with six full-spectrum (70–650 Da) scans per second. Gas (helium) flow was set at 1.1 mL/min with the injection port configured in the splitless mode. The injection port and detector temperatures were set to 220 °C and 300 °C, respectively. The initial oven temperature was held at 50 °C for 2 min and was programmed to increase at 20 °C/min to 325 °C and held for another 11.25 min, before cycling back to the initial conditions. The SA (**1**) peak was quantified by extracting 267 m/z to minimize integration of co-eluting metabolites. Peaks were quantified by area integration and the concentrations were derived from an external calibration curve of amount of SA (**1**) injected versus peak area integration of the extracted m/z. Final values are an average of three independent measurements.

5.6. Determination of relative specific activity of PtSABP2-1 and PtSABP2-2 with three substrates

A two-step radiochemical esterase assay was performed to determine the substrate specificity of PtSABP2-1 and PtSABP2-2 following a protocol previously reported (Forouhar et al., 2005). The reaction of the first step was performed with a 40 μl volume containing 50 mM Tris–HCl, pH 7.5, one of the three substrates (MeSA, MeJA and methyl indole-3-acetate (MeIAA)) at 10 μM, 100 μM or 1 mM concentrations and 0.5 μg purified PtSABP2-1 or PtSABP2-2 for 30 min at 25 °C. After that, the samples were boiled for 5 min to stop the reaction and denature the enzyme. The second step reaction started with the addition of 3 μM ^{14}C-adenosyl-L-methionine (SAM) with a specific activity of 51.4 mCi/mmol (Perkin Elmer, Boston, MA, USA), 120 μM SAM and purified methyltransferase (SAMT from Arabidopsis (Chen et al., 2003a), indole-3-acetic acid (**5**) methyltransferase from poplar (Zhao et al., 2007), or jasmonic acid (**6**) methyltransferase from Arabidopsis (Seo et al., 2001)). The reaction was allowed to proceed for 30 min at 25 °C. Radiolabeled products were extracted and radioactivity counted using a liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA) as previously described (D'Auria et al., 2002). Three independent assays were performed for each substrate.

5.7. pH optimum for PtSABP2-1 and PtSABP2-2 activities

Both PtSABP2-1 and PtSABP2-2 activities were determined in 50 mM Bis–Tris propane buffer for the pH range across 6.0–10.0 using the radiochemical assay described above. Data presented are the average of three independent assays.

5.8. Determination of kinetic parameters of PtSABP2-1 and PtSABP2-2

The increase in reaction rate with increasing concentrations of MeSA (**2**) was evaluated with the radiochemical assay described above and was found to obey Michaelis–Mention kinetics. Appropriate enzyme concentrations and incubation times were determined in time-course assays so that the reaction velocity was linear during the assay period. To determine the Km for MeSA (**2**), the concentrations of MeSA (**2**) were independently varied in the range from 5 μM to 100 μM. Lineweaver–Burk plots were made to obtain apparent Km values and maximum velocity values, as previously described (Chen et al., 2003a). Final values are an average of three independent measurements.

5.9. Molecular modeling

The tobacco SABP2 and the two poplar MeSA esterases, PtSABP2-1 and PtSABP2-2, share approximately 77% identity with no gap in their sequence alignment (Fig. 2). This allowed us to use the crystal structure of tobacco SABP2 in a complex with SA (**1**) at 2.1 Å resolution (PDB accession code 1Y7I, Forouhar et al., 2005) as a reliable template to generate structural models for both PtSABP2-1 and PtSABP2-2. Using the XtalView program (McRee, 1999), the models were built manually and were subject to two cycles of refinement by CNS, primarily for energy minimization of both the protein and the bound SA (**1**) ligand (Brünger et al., 1998).

5.10. Determination of gene expression using semi-quantitative RT–PCR

Total RNA extraction from young leaves, old leaves, stems, and roots of one year-old poplar trees and leaves of poplar plants treated with various stress factors and subsequent first-strand cDNA synthesis were performed essentially the same as described for full-length cDNA cloning. Forward primer 5′-ACAATGGTAGAGACCAAGAATCAGA-3′ and reverse primer 5′-TGGTATCGCTAAATCTTCATCGC-3′ were used as gene-specific primers for PtSABP2-1. Forward primer 5′-ACAATGGTAGAGACCAAGAATCAGG-3′ and reverse primer 5′-ACTGGTATCGCTAAATCTTTGTCAA-3′ were used as gene-specific primers for PtSABP2-2. The two primers used for the PCR amplification of *Ubiquitin* were designed as previously described (Kohler et al., 2004): forward primer 5′-CAGGGAAACAGTGAGGAAGG-3′ and reverse primer 5′-TGGACTCACGAGGACAG-3′. Initially, PCR was performed with PtSABP2-specific primers using 0.1 μL, 0.2 μL, 0.5 μL and 1.0 μL cDNA from each sample as template. The PCR program was set as follows: 94 °C for 2 min followed by 30 cycles (PtSABP2-1) or 32 cycles (PtSABP2-2) at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. Amplified products were separated on a 1.0% agarose gel and stained with ethidium bromide. Gels were visualized under UV-light and quantified using the Bio-Rad Quantity One software (Bio-Rad, Hercules, CA, USA). Analysis showed that the amounts of amplified products with gene-specific primers increased linearly with increasing amounts of template cDNA. Therefore, 0.2 μL cDNA was chosen as the optimal amount of template for final data collection. For the *Ubiquitin* gene, PCR was performed in separate tubes under conditions similar to those for PtSABP2 genes except that the reactions were performed for 25 cycles. The levels of the *Ubiquitin* gene were used for normalization. All PCRs were replicated three times using the first-strand cDNA made from two independent RNA preparations. The *t*-test using the SPSS software was performed to identify significant differences in expression levels in control and treated samples.

Acknowledgements

We are grateful to Byung-Guk Kang for providing poplar plants for gene expression analysis. This research was supported in part by the DOE Office Biological and Environmental Research – Genome to Life Program through the BioEnergy Science Center (BESC), and by the Tennessee Agricultural Experiment Station.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.11.014.

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