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Molecular cloning and biochemical characterization of indole-3-acetic acid methyltransferase from poplar

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Abstract

Indole-3-acetic acid (IAA) is the most active endogenous auxin and is involved in various physiological processes in higher plants. Concentrations of IAA in plant tissues are regulated at multiple levels including *de novo* biosynthesis, conjugation/deconjugation, and degradation. In this paper, we report molecular isolation and biochemical characterization of a gene *PtIAMT1* from poplar encoding IAA methyltransferase (IAMT), which plays a role in regulating IAA homeostasis. *PtIAMT1* was identified from the poplar genome based on sequence similarity to *Arabidopsis IAMT*. A full-length cDNA of *PtIAMT1* was cloned from poplar roots via RT-PCR. Recombinant PtIAMT1 expressed in *Escherichia coli* was purified to electrophoretic homogeneity. Enzyme assays combined with GC–MS verified that PtIAMT1 catalyzes formation of methyl indole-3-acetate using *S*-adenosyl-L-methionine (SAM) as a methyl donor and IAA as a methyl acceptor. PtIAMT1 had a temperature optimum at 25 °C and a pH optimum at pH 7.5. Its activity was promoted by K^+ but inhibited by Fe^{2+}, Cu^{2+} and Zn^{2+}. Under steady-state conditions, PtIAMT1 exhibited apparent K_m values of 23.1 μM and 30.4 μM for IAA and SAM, respectively. Gene expression analysis showed that *PtIAMT1* had the highest level of expression in stems, a moderate level of expression in young leaves, and a low level of expression in roots. Presence of *PtIAMT1* transcripts in several organs suggests that *PtIAMT1* is involved in development of multiple organs in poplar.

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

1. Introduction

Indole-3-acetic acid (IAA) (**1**) is the most abundant and most active endogenous auxin in higher plants. It modulates diverse aspects of plant growth and development ranging from embryogenesis, tropic responses to light and gravity, lateral and adventitious root formation, to induction of vascular differentiation (Taiz and Zeiger, 2006). Concentrations of IAA (**1**) in plant tissues are maintained through regulation of its *de novo* biosynthesis, IAA (**1**) degradation, and both conjugation and deconjugation of IAA (**1**) with amino acids, peptides, and sugars (Ljung et al., 2002). A novel enzyme, IAA methyltransferase (IAMT), was recently identified from *Arabidopsis*. *Arabidopsis* IAMT (AtIAMT) catalyzes formation of methyl indole-3-acetate (**2**) (MeIAA) using *S*-adenosyl-L-methionine (SAM) as a methyl donor and IAA (**1**) as a methyl acceptor (Zubieta et al., 2003). Exogenous MeIAA (**2**) is much more potent than exogenous IAA (**1**) in inhibiting hypocotyl elongation of dark-grown *Arabidopsis* seedlings (Qin et al., 2005), indicating IAA (**1**) activities can be effectively regulated by methylation.

The gene encoding AtIAMT, *At5g55250* or *IAMT1*, is involved in *Arabidopsis* leaf development (Qin et al., 2005). Expression of *IAMT1* is developmentally regulated in *Arabidopsis* leaves. Before the eighth true leaf emerges, *IAMT1* is expressed ubiquitously in rosette leaves. After emergence of the eighth true leaf, *IAMT1* expression gradually fades away from the leaf center to the leaf edges (Qin

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et al., 2005). Down-regulating *IAMT1* expression leads to dramatic epinastic leaf phenotypes (Qin et al., 2005), which is consistent with IAA (**1**) overproduction mutants. *IAMT1* over-expressors display leaf phenotypes opposite to those of *IAMT1* RNAi lines. Roles for *IAMT1* in developmental processes other than leaf development in *Arabidopsis* are not yet clear. Regardless, over-expression of *IAMT1* in *Arabidopsis* affects auxin responses, for example, by disrupting plant response to gravity (Qin et al., 2005).

AtIAMT was originally identified based on structural modeling using the three-dimensional structure of *Clarkia breweri* salicylic acid methyltransferase (CbSAMT) as a template (Ross et al., 1999; Zubieta et al., 2003). Both AtIAMT and CbSAMT belong to a protein family called SABATH (D'Auria et al., 2003). Other known members of the family include benzoic acid/salicylic acid methyltransferase (BSMT) (Chen et al., 2003a; Pott et al., 2004), benzoic acid methyltransferase (BAMT) (Murfitt et al., 2000), jasmonic acid methyltransferase (JMT) (Seo et al., 2001), farnesoic acid methyltransferase (Yang et al., 2006) and nitrogen methyltransferases involved in caffeine biosynthesis (Kato et al., 2000; Yoneyama et al., 2006). Continued characterization of SABATH proteins with novel biochemical activities raises an intriguing question about the evolutionary trajectory of these enzymes. Because IAA (**1**) is universal plant hormone and methylation of IAA (**1**) catalyzed by IAMT has important consequences on plant growth and development, we are interested in understanding whether IAMTs in different plant species are evolutionarily conserved. In this paper, we report the molecular isolation and biochemical characterization of an *IAMT* gene from poplar (*Populus trichocarpa*, Torr. & Gray), a perennial woody species. A high degree of sequence similarity between PtIAMT1 and AtIAMT, as well as comparable kinetic properties between the two, implies that IAMT is a conserved enzyme. Gene expression analysis suggests *PtIAMT1* is involved in development of multiple organs in poplar.

2. Results and discussion

2.1. Identification and sequence analysis of poplar IAMT

To identify the putative poplar *IAMTs*, the protein sequence of AtIAMT, which is the only reported IAMT in plants (Zubieta et al., 2003), was used to blast search the sequenced poplar genome (Tuskan et al., 2000). Gene fgenesh1_pg.C_LG_I002776 was identified to be the most similar to AtIAMT. The protein encoded by fgenesh1_pg.C_LG_I002776 displayed IAMT activity (see Section 2.2). Because biochemical activity for all poplar SABATH proteins were not analyzed, it was not possible to rule out the possibility that other poplar SABATH proteins might have IAMT activity. Thus, fgenesh1_pg.C_LG_I002776 was named *PtIAMT1* (Fig. 1).

PtIAMT1 is localized on poplar chromosome 1 and encodes a protein of 385 amino acids. PtIAMT1 and AtIAMT are 76% identical at the amino acid sequence level. Both genes contain three introns and four exons, with positions of introns conserved in the two genes. The SAM binding site in CbSAMT has been determined (Zubieta et al., 2003). Sequence comparison revealed that amino acids comprising the SAM binding site in PtIAMT1 are identical to those of CbSAMT (Fig. 2a). In CbSAMT, Trp226 is one of the amino acids that form the salicylic acid binding site. In AtIAMT, Trp226 is replaced by a Gly residue. The loss of Trp226 at the active site location in AtIAMT creates a large and spacious pocket for recognizing and binding to the indole-ring of IAA (Zubieta et al., 2003). In PtIAMT1, Trp226 is also replaced with a Gly (Fig. 2a), providing further evidence to support an important role of Trp226 for binding with IAA.

PtIAMT1 is the second *IAMT* gene to be identified in plants. To understand the evolutionary relationship between PtIAMT1 and other known SABATH proteins including AtIAMT, a phylogenetic tree was constructed consisting PtIAMT1, AtIAMT, CbSAMT, *Arabidopsis* jasmonic acid methyltransferase (AtJMT), *Arabidopsis* benzoic acid/salicylic acid methyltransferase (AtBSMT1), *Arabidopsis* farnesoic acid methyltransferase (AtFAMT), snapdragon benzoic acid methyltransferase (AmBAMT), and coffee caffeine synthase (CCS1). PtIAMT1 is most related to AtIAMT (Fig. 2b), implying the presence of *IAMT* predated the divergence of poplar and *Arabidopsis* linages.

2.2. Purification of recombinant PtIAMT1 and determination of its substrate specificity

A *PtIAMT1* full-length cDNA was amplified from poplar root tissues by RT-PCR, cloned into the vector of

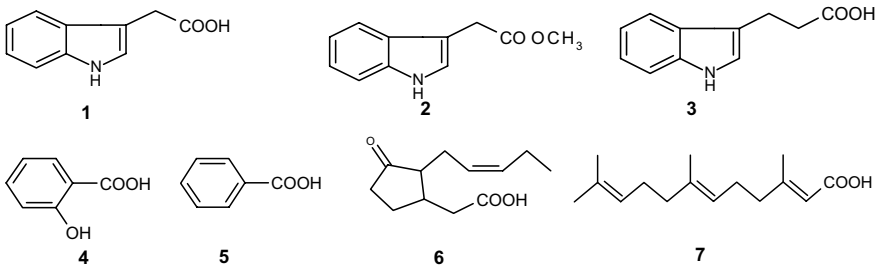

Fig. 1. Structures of compounds **1**–**7**.

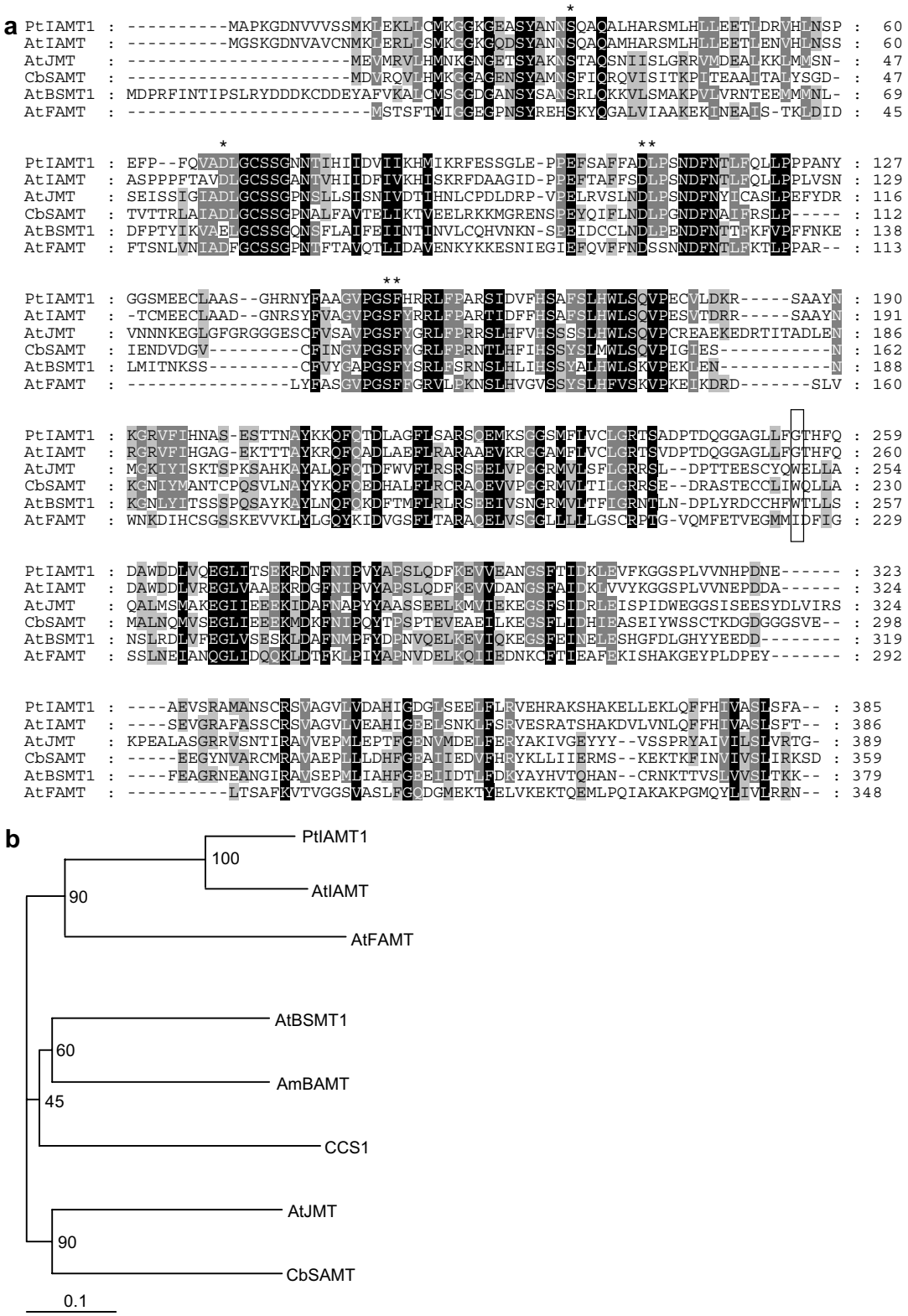

Fig. 2. Sequence analysis of PtIAMT1. (a) Alignment of deduced amino acid sequences of PtIAMT1 with AtIAMT and other representative carboxyl SABATH enzymes including CbSAMT (AF133053), *Arabidopsis* jasmonic acid methyltransferase (AtJMT, At1g19640), *Arabidopsis* benzoic acid/salicylic acid methyltransferase (AtBSMT1, At3g11480) and *Arabidopsis* farnesoic acid methyltransferase (AtFAMT, At3g44860), using the ClustalX program. Conserved or semi-conserved amino acids in four or more sequences are shown in white letters on black or grey backgrounds, respectively. Amino acids indicated with asterisks are SAM binding residues. Amino acids (positions Gly 255 of PtIAMT1 and Trp 226 of CbSAMT) critical for determining substrate specificity of SABATH enzymes are marked with a frame. (b) A neighbor joining phylogenetic tree based on protein sequence alignment of PtIAMT1, AtIAMT, AtJMT, AtBSMT1, AtFAMT, AmBAMT (Q9FYZ9) and CCS1 (BAC43760) using the ClustalX program. Branches were drawn to scale with the bar indicating 0.1 substitutions per site.

pCRT7/CT-TOPO, and fully sequenced. PtIAMT1 was expressed in *Escherichia coli* in its native form. The crude protein extract of PtIAMT1 showed IAMT activity (data not shown).

To purify PtIAMT1 expressed in *E. coli*, *PtIAMT1* full-length cDNA in pCRT7/CT-TOPO was subcloned into the vector of pET100/D-TOPO. In the latter vector, *PtIAMT1* coding sequence is fused to an N-terminal sequence containing codons for six histidine residues. His-tagged PtIAMT1 was expressed in *E. coli* then purified using Ni-NTA agarose to electrophoretic homogeneity (Fig. 3a).

Purified PtIAMT1 recombinant protein was assayed with IAA (**1**), indole-3-butyric acid (**3**), salicylic acid (**4**), benzoic acid (**5**), jasmonic acid (**6**) and farnesoic acid (**7**). PtIAMT1 had the highest level of catalytic activity with IAA (**1**), exhibiting a specific activity of 60 pkat/mg protein. It also exhibited activity with indole-3-butyric acid (**3**), another naturally occurring auxin. Specific activity of PtIAMT1 with indole-3-butyric acid (**3**) was about 11% (6.6 pkat/mg protein) of IAMT activity. PtIAMT1 had no activity with other compounds tested that are known substrates of other SABATH proteins.

To determine the chemical structure of the PtIAMT1 product, the compound produced from the PtIAMT1 enzyme assay using IAA (**1**) as a substrate was extracted with hexane and analyzed using GC–MS. As shown in Fig. 3b, the product had an identical retention time and mass spectrum to that of the authentic MeIAA (**2**) standard, confirming that PtIAMT1 catalyzes formation of MeIAA (**2**) using SAM as a methyl donor and IAA (**1**) as a methyl acceptor.

2.3. Biochemical properties of PtIAMT1

Under steady-state conditions, PtIAMT1 exhibited apparent K_m values of 23.1 μM and 30.4 μM for IAA (**1**) (Fig. 3c) and SAM (Fig. 3d), respectively. Calculated catalytic efficiency (K_{cat}/K_m) of PtIAMT1 is 4782 s^{-1} M^{-1}. Kinetic properties of PtIAMT1 are comparable to those of AtIAMT, which displayed a K_m value of 13 μM for IAA (**1**) and a catalytic efficiency of 2200 s^{-1} M^{-1} (Zubieta et al., 2003). Taken together, the phylogenetic, structural and biochemical evidence suggests that IAMT is a conserved enzyme.

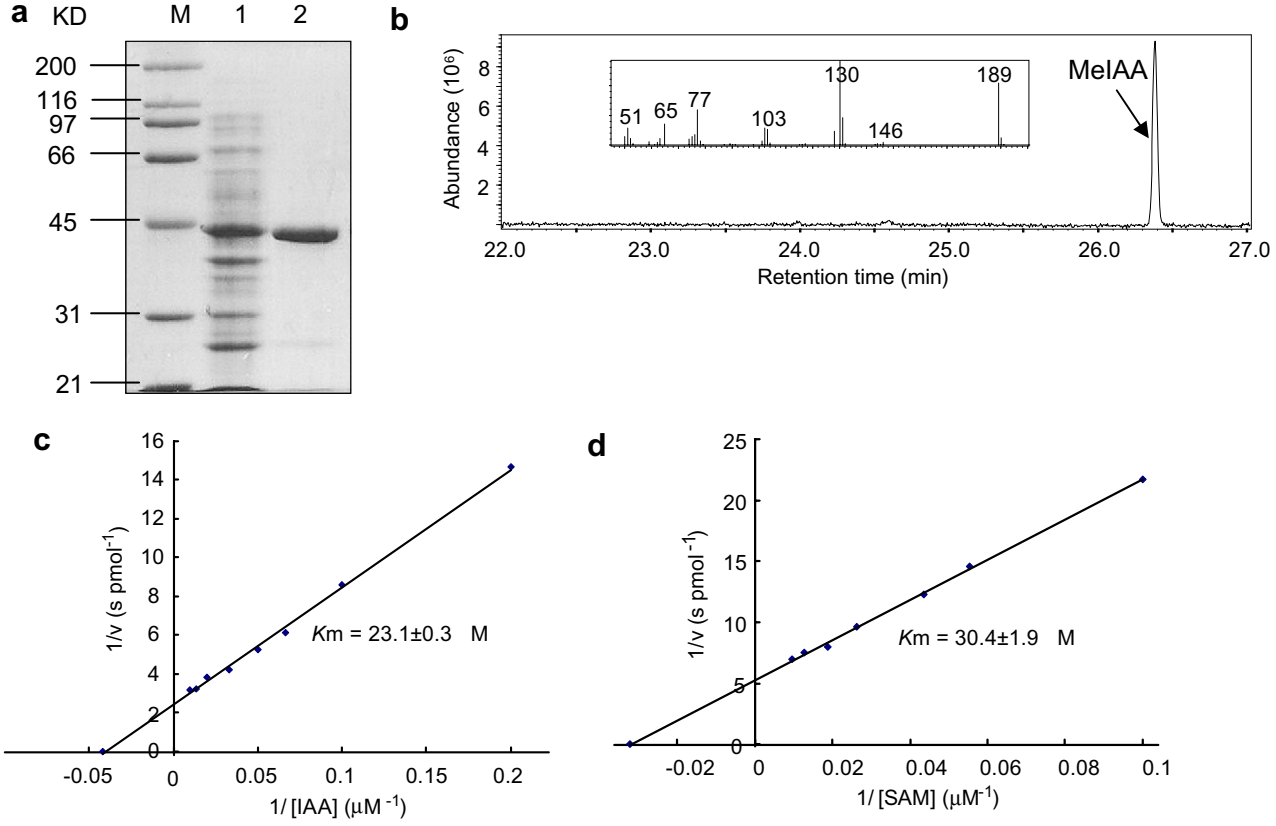

Fig. 3. Biochemical characterization of PtIAMT1. (a) SDS–PAGE of purified recombinant PtIAMT1 protein. His-tagged PtIAMT1 expressed in *E. coli* was purified as described in Section 3. Lane M contained protein molecular weight markers. Lane 1 contained crude extract, and lane 2 contained 1 μg of purified PtIAMT1 protein. The gel was stained with Coomassie Blue. (b) Identification by GC–MS of the product of the enzyme assay catalyzed by recombinant PtIAMT1 protein using IAA (**1**) as substrate. The product had a retention time of 26.4 min. Inset shows mass spectrum of the assay product. Both retention time and mass spectrum match those of authentic MeIAA (**2**). (c) Steady-state kinetic measurements of PtIAMT1 using IAA (**1**) as the substrate. One example of a Lineweaver–Burk plot is shown. (d) Steady-state kinetic measurements of PtIAMT1 using SAM as the substrate. One example of the Lineweaver–Burk plot is shown. In both (c) and (d), K_m values shown were not absolute. However, they were apparent under our limited reaction conditions.

PtIAMT1 had an optimal temperature at 25 °C (Fig. 4a). Activity at 4 °C and 40 °C was about 40% of the maximal activity. The optimum pH for PtIAMT1 was determined to be pH 7.5. At pH 6.5, the enzyme had ~34% of its maximal activity, whereas at pH 9.0, the enzyme displayed ~40% of the maximal activity (Fig. 4b). PtIAMT1 activity can also be affected by metal ions. K^+ stimulated PtIAMT1 activity by more than one fold, suggesting that the presence of K^+ in reaction solution may induce protein conformational changes and/or activation of PtIAMT1 active site leading a stimulatory effect on PtIAMT1 activity. Ca^{2+}, NH_4^+ and Na^+ had a mild stimulation on PtIAMT1 activity. By contrast, Mg^{2+} had a mild inhibitory effect and Mn^{2+} decreased the specific activity of PtIAMT1 by ~46%. Strong inhibitory effects on PtIAMT1 activity were observed with Cu^{2+}, Fe^{2+}, and Zn^{2+} (Fig. 4c). The partial or complete inhibition of PtIAMT1 activity by Mn^2, Cu^{2+}, Fe^{2+}, and Zn^{2+} suggests that these metal ions may induce protein conformational changes and/or inhibition of PtIAMT1 active site limiting PtIAMT1 catalytic ability.

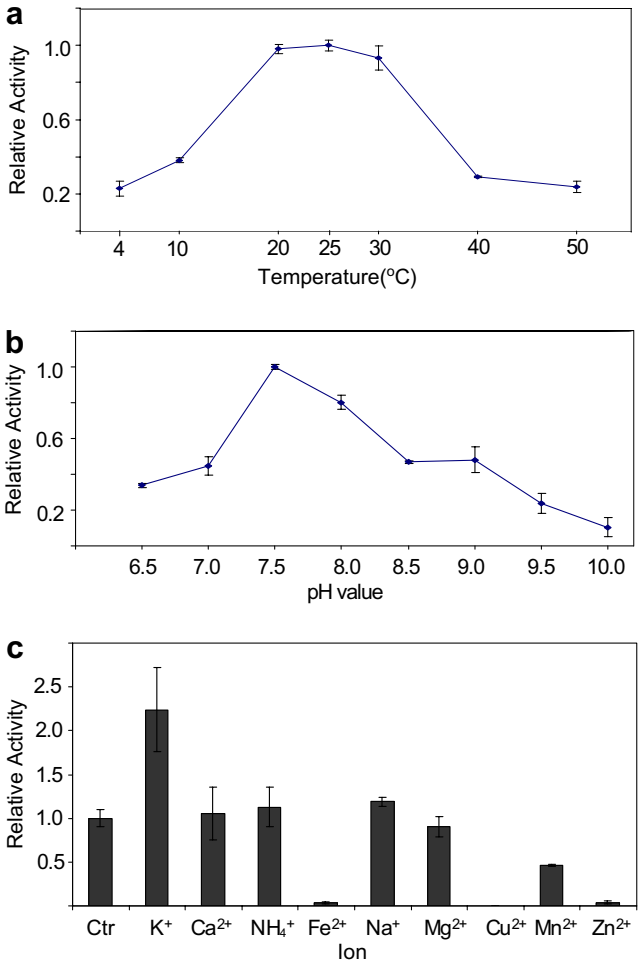

Fig. 4. Biochemical properties of PtIAMT1. (a) Optimal temperature of PtIAMT1. Level of PtIAMT1 activity at 25 °C was arbitrarily set at 1.0. (b) Effects of buffer pH on activity of PtIAMT1. Level of PtIAMT1 activity in the buffer of pH 7.5 was arbitrarily set at 1.0. (c) Effects of metal ions on activity of PtIAMT1. Metal ions were added to reactions in the form of chloride salts at 5 mM final concentrations. Level of PtIAMT1 activity without any metal ion added as control (Ctr) was arbitrarily set at 1.0. In all three panels, actual specific activity for 1.0 is 60 pkat/mg protein.

2.4. Gene expression analysis of *PtIAMT1*

To identify plant tissues where *PtIAMT1* was expressed, total RNA was isolated from newly emerged leaves (young leaves), old leaves (5 cm in length), stems and roots of 1-year-old poplar trees and used for semi-quantitative RT-PCR analysis. The highest level of *PtIAMT1* transcripts was observed in stems. Young leaves also showed an abundant expression of *PtIAMT1*, whereas no expression of *PtIAMT1* was detected in old leaves. A low level of expression of *PtIAMT1* was also detected in roots (Fig. 5).

2.5. Biological roles of *PtIAMT1*

Presence of *PtIAMT1* transcripts in leaves, roots and stems (Fig. 5) suggests involvement of this gene in development of these organs in poplar. In leaves, *PtIAMT1* showed an expression pattern similar to *AtIAMT1*. Both genes displayed high expression levels in young leaves than old leaves (Qin et al., 2005, Fig. 5), implying that *PtIAMT1* has a role similar to *AtIAMT1* in leaf development. *PtIAMT1* may also be involved in root development. IAA (**1**) is an important signal regulating root architecture. Although the mode of action is not well understood, the concentrations of IAA (**1**) appear important (Wang et al., 2003). *PtIAMT1* may have a role in poplar root development by regulating IAA (**1**) concentrations. As a perennial woody species, poplar differs from annuals such as *Arabidopsis* by displaying a number of distinctive developmental and anatomical characteristics. Of these, wood formation is arguably the most important. Wood formation in poplar is regulated by IAA (**1**) (Moyle et al., 2002). A high-level expression of *PtIAMT1* in poplar stems indicates that this gene may play a role in wood formation by regulating IAA (**1**) activities.

2.6. Concluding remarks

Pathways that regulate IAA activity in plants are complicated and imperfectly understood (Leyser, 2002). Identi-

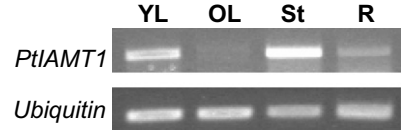

Fig. 5. Semi-quantitative RT-PCR analysis of *PtIAMT1* expression. Young leaves (YL), old leaves (OL), stems (St) and roots (R) were colleted from 1-year-old poplar trees grown in a greenhouse. Total RNA was extracted and used for RT-PCR analysis. PCR with primers for *Ubiquitin* was used to judge equality of concentration of cDNA templates in different samples.

fication of IAMT that appears to be evolutionarily conserved in different plant species adds a new layer of complexity to this network. Although physiological functions of MeIAA generally have been inconclusive, biochemical and gene expression evidence suggests an important role of *PtIAMT1* in poplar development likely through IAA methylation. Our lab has begun to generate transgenic poplar trees including both *PtIAMT1* over-expressors and RNAi lines that will help reveal biological roles of this gene.

3. Experimental

3.1. Plant material and chemicals

The female black cottonwood (*P. trichocarpa*) clone 'Nisqually-1', previously employed for whole genome sequencing (Tuskan et al., 2000), was used for gene cloning and expression analysis of *PtIAMT1*. Tissues used for gene expression analysis, including young leaves, old leaves, stems and roots, were collected from 1-year-old poplar trees grown in a greenhouse.

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

3.2. Database search and sequence analysis

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

Multiple protein sequence alignments were made using ClustalX software (Thompson et al., 1997), and displayed using GeneDoc (http://www.psc.edu/biomed/genedoc/). Phylogenetic trees were constructed using ClustalX program and viewed using TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

3.3. Cloning full-length cDNA of PtIAMT1

Root tissues of plants grown on tissue medium were collected from poplar seedlings at the six-leaf stage. Total RNA was extracted using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA) with DNA contamination removed using an on-column DNase treatment (Qiagen, Valencia, CA). After purification, total RNA (1.5 μg) was reverse-transcribed into first-strand cDNA in a 15 μL reaction volume using the first-strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ) as previously described (Chen et al., 2003b). *PtIAMT1* full-length cDNA was amplified using forward primer 5′-TGGCTCCTAAAGGTGACAATGTTGTTG-3′ and reverse primer 5′-CCCTTGTTCTCAAGCAAAAGAAAGAGA-3′ corresponding with the beginning and end of *PtIAMT1* coding region, respectively. The PCR was conducted using the following program: 94 °C for 2 min followed by 30 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. Products of PCR were separated on 1.0% agrose 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.

3.4. Purification of PtIAMT1 expressed in E. coli

Two primers, the forward primer 5′-CACCATGGCTCCTAAAGGTGACAATGTTG-3′ and the reverse primer 5′-CCCTTGTTCTCAAGCAAAAGAAAGAGA-3′, were used to subclone *PtIAMT1* cDNA in pCRT7/CT-TOPO into the vector of pET100/D-TOPO (Invitrogen, Carlsband, CA). To express the PtIAMT1 protein, the protein expression construct was transformed into the *E. coli* strain BL21 (DE3) CodonPlus (Stratagene, La Jolla, CA). Protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at a concentration of 500 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 the manufacturer instructions (Invitrogen, Carlsband, CA). Protein purity was verified by SDS–PAGE and protein concentrations were determined by the Bradford assay (Bradford, 1976).

3.5. Radiochemical IAMT activity assay (standard IAMT assay)

Radiochemical IAMT assays were performed with a 50 μL volume containing 50 mM Tris–HCl, pH 7.5, 1 mM IAA, and 3 μM ^{14}C-SAM with a specific activity of 51.4 mCi/mmol (Perkin–Elmer, Boston, MA). The assay was initiated by addition of SAM, maintained at 25 °C for 30 min, and stopped by addition of EtOAc (150 μL). After phase separation by 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). Radioactivity counts in the organic phase indicated the amount of synthesized MeIAA.

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

3.6. Determination of kinetic parameters of PtIAMT1

Increases in reaction rate by increasing concentrations of SAM and IAA (**1**) were evaluated through the radio-

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

3.7. Optimal temperature for PtIAMT1 activity

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

3.8. pH optimum for PtIAMT1 activity

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

3.9. Effectors

To examine effects of metal ions on PtIAMT1 activity, standard IAMT assays were performed in the independent presence of each of the following salts at 5 mM final concentration: KCl, $CaCl_2$, NH_4Cl, NaCl, $MgCl_2$, $MnCl_2$, $CuCl_2$, $FeCl_2$ and $ZnCl_2$. Results presented are the average of three independent assays.

3.10. Product identification

A reaction containing 150 μg purified PtIAMT1, 1 mM IAA and 600 μM unlabelled SAM was incubated in a 1 mL reaction containing 50 mM Tris–HCl, pH 7.5 at 25 °C for 4 h. The reaction product was extracted with hexane (1.5 mL), concentrated under N_2 gas and analyzed by Shimadzu GC (GC-17A)-MS (QP 5050A) system (Columbia, MD). A DB-5 column (30 m × 0.25 i.d. × 0.25 μm) was used with He as carrier gas at a flow rate of 1 mL min^{-1}. As a control, a similar reaction was performed, except that PtIAMT1 protein was denatured by boiling at 100 °C for 10 min before added to the assay. MeIAA (**2**) authentic standard was dissolved in EtOH at the concentration of 0.5 μg mL^{-1} and 1 μg MeIAA (**2**) was injected into the GC in a split (1/30) mode. The GC temperature program was as follows: 2 min at 80 °C followed by a ramp of 8 °C min^{-1} to 320 °C. Identity of the product was confirmed by comparison of GC retention times and mass spectra with that of the authentic standard.

3.11. Semi-quantitative RT-PCR analysis of PtIAMT1 expression

Total RNA extraction from young leaves, old leaves, stems and roots of 1-year-old poplar trees and subsequent first-strand cDNA synthesis were performed as described in Section 3.3. For PCR analysis in different organs, primers were designed to amplify a *PtIAMT1* fragment of 566 bp as follows: forward primer 5′-AGAAACCCTAGATAG-GGTGCAC-3′ and reverse primer 5′-CCAAGCAGA-CAAGAAACATGGA-3′. Two primers used for PCR amplification of *Ubiquitin* were designed as previously described (Kohler et al., 2004): forward primer 5′-CAG-GGAAACAGTGAG GAAGG-3′ and reverse primer 5′-TGGACTCACGAGGACAG-3′. Initially, PCR analysis was performed with *Ubiquitin*-specific primers using 0.1 μL, 0.2 μL, 0.5 μL and 1.0 μL cDNA. The program used to amplify *Ubiquitin* was as follows: 94 °C for 2 min followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, with a final extension at 72 °C for 10 min. Amplified products were separated on 1.0% agarose gel. Gels were stained with ethidium bromide, visualized under UV-light, and quantified using the Bio-Rad Quantity One software (Bio-Rad, Hercules, CA). Analysis showed amounts of amplified products with the *Ubiquitin*-specific primers increased linearly with increasing amounts of template cDNA. Therefore, 0.2 μL cDNA was chosen as the optimal template concentration for PCR analysis with *PtIAMT1*-specific primers. The program used to amplify the *PtIAMT1* fragment was as follows: 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. All PCRs were replicated twice using first-strand cDNA made from two independent RNA preparations.

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