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# Molecular characterization of isopentenyltransferase (*BoAIPT1*) from *Bambusa oldhamii* expressed in *Escherichia coli*

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**ABSTRACT.** Cytokinins (CKs) are a group of phytohormones involved in stages of plant development, such as cell division and various other growth processes. The key enzyme in *de novo* biosynthesis of naturally occurring cytokinins is adenylate isopentenyltransferase (AIPT). This study amplified a PCR product, containing partial sequence encoding putative AIPT, using primers based on conserved protein sequences. Genomic DNA containing a full-length of AIPT was obtained by screening a genomic DNA library. This study used 5'-RACE and 3'-RACE, using gene specific primers, to clone and sequence cDNA encoding *BoAIPT1*. The open reading frame of *BoAIPT1* was 1035 bp in size, encoding 344-amino acids, with a molecular mass of 37.7 kDa. No introns were found by comparing AIPT from bamboo with AIPT sequences from other species. BoAIPT1 contains a putative ATP/GTP binding motif at the N-terminal as observed in other known AIPT. Recombinant BoAIPT1 expressed in *Escherichia coli* catalyzed the isopentenyl transfer reaction from dimethylallyldiphosphate (DMAPP) to the $N^6$ adenylate amino group. Real-time PCR analyses revealed BoAIPT1 expression increased simultaneously with green bamboo shoot growth, with the top green shoot section having the highest expression level.

**Keywords:** Adenylate isopentenyltransferase (AIPT); *Bambusa oldhamii*; Molecular cloning; Gene expression; *Escherichia coli.*

## INTRODUCTION

Cytokinins (CKs) are a class of phytohormones that are involved in a wide variety of plant growth (Tanaka et al., 2006; Sato et al., 2009) and development processes, including cell division, delay of senescence (Gan and Amasino, 1995; Kim et al., 2006), apical dominance shoot meristem function (Higuchi et al., 2004; Kurakawa et ai., 2007), and nutritional signaling (Sakabkibara, 2006; Argueso et al., 2009).

Depending on the structure of the side chain, cytokinins are classified into two groups, isoprenoid side chains and aromatic side chains (Mok and Mok, 2001). Natural isoprenoid CKs are classified into one of four basic molecules: isopentenyl adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DZ). Isoprenoid CKs are commonly found in higher plants, and are much more abundant than aromatic CKs. The metabolic pathway for isoprenoid CKs has been studied in *Arabidopsis* and in rice (Kakimoto, 2001; Takei et al., 2001; Takei et al., 2004; Sakamoto et al., 2006). Four factors regulate CK homeostasis in cells, including rate of *de novo* synthesis, rate of inter-conversion, rate of transport and rate of cytokinin conjugate breakdown (Sakakibara, 2006).

Two CK biosynthetic pathways have been described in plants, the adenylate pathway, and the tRNA pathway (Chen, 1997; Mok and Mok, 2001). In the adenylate pathway, isoprenoid CK biosynthesis is catalyzed by adenosine phosphate-isopentenyltransferase (AIPT; EC 2.5.1.27) using dimethylallyl diphosphate (DMAPP) and adenosine phosphate as substrates. AIPT transfers the isoprenyl group from dimethylallyldiphosphate (DMAPP) to the $N^6$-amino group of AMP, ADP, or ATP to produce iP-nucleotides. In *Arabidopsis*, prenyl side chains of iP-nucleotides undergo hydroxylation by cytochrome P450 monooxygenases. The CK-activating enzyme, LOG (Lonely Guy, Kuroha et al, 2009), has phosphoribohydrolase activity that releases a ribose 5'-monophosphate moiety from iP-nucleotides to produce active iP (Chen, 1997; Sakakibara, 2006). In the second pathway, tRNA-IPT catalyzes CK formation by isoprenylation of certain tRNA at an adenine adjacent to the 3'-end of the anticodon. Subsequent degradation of prenylated tRNA generates the CK nucleotide (Vreman and Skoog, 1972; Murai et al., 1994). Presently, the first pathway is more commonly accepted.

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AIPTs in higher plants such as *Arabidopsis thaliana*, rice, mulberry (*Morus alba*) and hops (*Humulus lupulus* L.) have been cloned and characterized (Sakano et al., 2004; Abe et al., 2007). In *Arabidopsis thaliana,* there are seven *AIPT* genes (*AtIPT1*, *AtIPT3*, *AtIPT4*, *AtIPT5*, *AtIPT6*, *AtIPT7,* and *AtIPT8*) (Takei et al., 2001). Using transient expression, four AtIPTs (AtIPT1, AtIPT3, AtIPT5, and AtIPT8) are localized in plastids (Kasahara et al., 2004). Farnesylation of AtIPT3 directs localization of the protein to the nucleus/cytoplasm (Galichet et al., 2001). Researchers have also identified AIPTs in some phytopathogenic microorganisms, such as *Agrobacterium tumefaciens* (Akiyoshi et al., 1984; Sakakibara et al., 2005). *AIPT* has been extensively studied in plants, and has recently been successfully applied to agriculture. Previous studies reported on transformation of the *AIPT* gene into broccoli, which produced a significant delay in senescence and yellowing of harvested flower heads and leaves (Chen et al., 2001; Chan et al., 2009).

The monocotyledonous plant, bamboo, *Bambusa oldhamii* (Family Poaceae) is found mainly in the tropics and the subtropics. Bamboo is often noted for its fast growth. During the rapid growth of bamboo, AIPT may play an important role in CK biosynthesis. We report here on the cloning of cDNA encoding *BoAIPT1* and analysis using real-time reverse transcription polymerase chain reaction (RT-PCR) to investigate *BoAIPT1* expression levels at different shoot growth stages to further understand properties of bamboo AIPT.

## MATERIALS AND METHODS

### Plant materials

Fresh *Bambusa oldhamii* was harvested from a bamboo farm in Taipei, Taiwan. Tissue samples were prepared from 15cm etiolated shoot (divided into top, middle, and base sections) and 30 cm green shoot (divided into top, middle, and base sections). The outermost sheath was removed and discarded, except the sheath of the etiolated shoot base, which was stored in liquid nitrogen. The shoots were diced and frozen in liquid nitrogen. All samples were stored at -80°C until used.

### Genomic DNA library screening

The genomic DNA library of *B. oldhamii* was constructed using the Lambda FIX® II/Xho I Partial Fill-In Vector Kit (Strategene, La Jolla, CA). The degenerate primers, IPT-F 5′-TGC C(G/T)C (A/T)CC A(C/A)C TGA TC(A/G) GC-3′ and IPT-R 5′-C(G/A)A GCT C(C/G)G GCA (C/G)GC C(G/T)A T-3′, were designed based on the conserved regions of AIPT genes from *Arabidopsis*, rice and hops. PCR was carried out under the following conditions: 95°C for 0.5 min, 48°C for 0.5 min, and 72°C for 0.5 min, with 30 cycles of reaction and a final extension for 5 min at 72°C.

A 254 bp BoAIPT1 fragment was used to generate a DIG-labeled probe (Roche, Germany) for screening the genomic DNA library. Plaques were blotted onto Hybond-$N^+$ membranes (Millipore, USA). Hybridization and detection were performed per manufacturer's instructions (Roche, Germany). Positive clone phage DNA was isolated (Lambda midi kit, QIAGENE) and sequenced.

### cDNA cloning of BoAIPT1

Bamboo shoots were frozen with liquid nitrogen and homogenized, and total RNA was extracted using the TRIZOL reagent (Invitrogen, USA). The BoAIPT1 specific primers, RP2F 5′-TGA CTG CTG CTT CCT GTG GG-3′ and RP2R 5′-CAG ATG CCT CGG GAG TAG TC-3′, were designed based on the coding region. Full length BoAIPT1 cDNA sequences were obtained by the RACE method (Clontech, USA).

### Expression of BoAIPT1 in *Escherichia coli*

The recombinant plasmid BoAIPT1 cDNA was subcloned into the pTrcHis A vector (Invitrogen, USA) using the N-terminal and C-terminal primer pairs: YEH2F 5′-ATC ACA GGA TCC ATG CCA CTC TAC TCT A-3′ (the *Bam*HI underlined) and YEH3R 5′-AGA GAA TTC TTA TAC TAT GGC GGC C-3′ (the *Eco*RI underlined). PCR was carried out at 95°C for 0.5 min, 52°C for 0.5 min, 72°C for 1 min, with 30 cycles of reaction and an extension for 5 min at 72°C. A hexahistidine tag was fused to the N-terminus. A single recombinant *E. coli* (BL21 DE3) colony was used to inoculate 1 L of LB (Luria-Bertani with 1M sorbitol and 2.5 mM betaine) medium, which contained 50 μg/ml of ampicillin. The cells were cultured at 37°C until $OD_{600}$ of 0.6 was reached. IPTG (isopropyl-β-D-thiogalactoside) was added to a final concentration of 0.3 mM, and the cells were further incubated at 18°C with vigorous (150 rpm) shaking overnight.

### Purification of the recombinant BoAIPT1 protein

An *E. coli* culture BL21 (DE3) harboring the expressing vector of BoAIPT1 was harvested at 6,500 *g* for 10 min, and resuspended in a 50 mM potassium phosphate buffer (KPB) pH 7.5, containing 0.15 M NaCl. A cell disrupter was used to disrupt cells on ice. Lysate was centrifuged at 40,000 *g* for 40 min at 4°C. The supernatant was then carefully removed and loaded onto a HisTrap™ FF 5ml column (GE Healthcare, USA). The column was eluted with 10, 50, 100, and 250 mM imidazole buffers. Recombinant protein was concentrated, and its buffer was exchanged with 50 mM KPB by Amicon Ultra (Millipore, Billerica, MA, USA) and stored at 4°C.

### BoAIPT1 activity assay

The protein concentration was assayed by the Bradford method (Protein Assay, Bio-Rad), and bovine serum albumin was used as the protein standard. The standard reaction buffer contained 2.0 μg of the purified recombi-

nant BoAIPT1, 100 mM Tris-HCl buffer, (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mg/ml bovine serum albumin, and, 1 mM DTT in a final volume of 100 µl.

Isopentenyl reactions were assayed at 30°C for 60 min, and terminated by boiling for 5 min. Half of the supernatant was removed and treated with calf intestine alkaline phosphatase (CAIP) buffer and incubated for 1 h at 37°C. This was then followed by centrifugation at 13,000 *g* for 1 min. Reaction products were profiled by reversed phase HPLC (Waters 1525 HPLC system with 2487 absorbance detector) using a Hypersil GOLD aQ column (4 × 250 mm, Thermo) with flow rate of 1.0 ml/min. The separation program was as follows: 100% buffer A (0.1 M acetic acid pH 3.3) for 15 min, followed by a linear gradient of 0-80% of buffer B (acetonitrile) for 35 min. UV absorbance was monitored at 280nm. Product retention times were compared to the standards purchased from Sigma.

### Preparation of total RNA

Frozen tissues were ground into powder using liquid nitrogen followed by total RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Genomic DNA was removed with TURBO DNA-free kits (Ambion, USA).

### Real-time RT-PCR

Total RNA, isolated from etiolated shoots, green shoots and sheath of etiolated shoot base, etiolated shoots and green shoots, was used as a template. Sequences of the primers for PCR are: RP2F 5′-TGA CTG CTG CTT CCT GTG GG-3′ and R257, 5′-GAG CTC CGG CAC GCC GAT-3′ for *BoAIPT1* and BoActinF1 5′-TGGCATCACACCTTCTACAA-3′ and BoActinR15′-ACCTGGATCTTCATGCTGCT-3′ for internal control *BoActin*. Complementary DNA strands were amplified using iScript One-Step RT-PCR Kit (Bio-Rad). Reverse transcription was carried out as follows: 50°C for 10 min for cDNA synthesis, and 95°C for 5 min for reverse transcriptase inactivation. Successive PCR was carried out for 40 cycles at 95°C for 10 sec, and 55°C for 30 sec in a thermal cycler (iQ5 Gradient Real Time PCR, Bio-Rad). All PCR products were subjected to verification using dissociation curves and ethidium bromide stained agarose gels to be single fragments. Subsequently, all fragments were cloned into pGEM-T vector for DNA sequencing. Sequence results showed all fragments were identical to the corresponding target gene region. The comparative $C_T$ method was applied for relative quantification of gene expression. This method establishes standard curves (iQ5 Gradient Real Time PCR, Bio-Rad) using arithmetic formulae. Relative fold expression changes were calculated as $2^{-\Delta\Delta C_T}$. To validate the experiments, the amplification efficiencies of the target (*BoAIPT1*) and reference (*BoActin*) were approximately equal to those obtained using the $\Delta\Delta C_T$ calculation.

## RESULTS

### Isolation and sequence analysis of BoAIPT1

Degenerate primers were designed for *BoAIPT1* gene according to conserved regions of AIPTs from other species (Kakimoto, 2001; Sakano et al., 2004; Sakamoto et al., 2006). A 254 bp DNA fragment was amplified by PCR using genomic DNA as a template. The deduced amino acid sequence was homologous to the corresponding regions of plant AIPTs. Thus, DNA was used as a hybridization probe to screen a bamboo shoot genomic library. One potential clone was selected and subjected to further analysis. Nucleotide sequence analysis revealed that the clones contained 11 kb, including an open reading frame that was designated BoAIPT1.

Gene specific primers were designed for 5′-RACE and 3′-RACE experiments to investigate transcription initiation sites and gene structure of *BoAIPT1*. This study used RACE to obtain a full-length cDNA of 1797 bp containing *BoAIPT1*. The sequence of this cDNA clone contained a 5′-noncoding region of 274 bp, an open reading frame of 1035 bp encoding BoAIPT1 with a molecular mass of 37,599 kDa and 344 amino acids, and a 3′ non-coding region of 488 bp. When comparing the *BoAIPT1* from cDNA with the genomic DNA, we found that the open reading frames consisted of one exon and no introns. Introns are absent in *AIPT* genes from other higher plants, including *OsIPT1~OsIPT8* (Sakamoto et al., 2006), *HlIPT* (Sakano et al., 2004) and *AtIPTs* (except *AtIPT8*) (Takei et al., 2001).

A multiple sequence alignment of BoAIPT1 and AIPT from other species indicated BoAIPT1 possessed an identity of 44-72 % (Figure 1). OsIPT5 and ZmIPT4 were the closest homologs (72%~69% identities) to BoAIPT1. The conserved ATP/GTP-binding sites (P-loop) motif ([A, G]-X4-G-K-[S, T]) in the N-terminal region of the AtIPTs and OsIPTs were also found in BoAIPT1. The ATP/GTP-binding motif is universally observed in ATP-consuming enzymes, including ATP binding cassette transporters (Kakimoto, 2001). A highly conserved $Asp^{82}$ is located downstream of the ATP/GTP-binding motif, which plays an essential role as a general base. There is a cluster of positively charged residues ($Lys^{83}$, $Arg^{218}$, $Arg^{261}$ and $Lys^{265}$) in BoAIPT1 at the open end of the ATP-binding channel (Chu et al., 2009).

### Cloning and purification of recombinant BoAIPT1 protein

The full-length cDNA of *BoAIPT1* was cloned into pTrcHisA in an attempt to produce recombinant His-tagged protein in *E. coli* BL21 (DE3). Our study performed purification of recombinant hexahistidine tagged BoAIPT1 using affinity chromatography. Protein supernatant was applied to a HisTrap ™ FF 5ml column (GE Healthcare, USA), eluted using different concentrations of imidazole buffers, and followed by an SDS-PAGE analy-

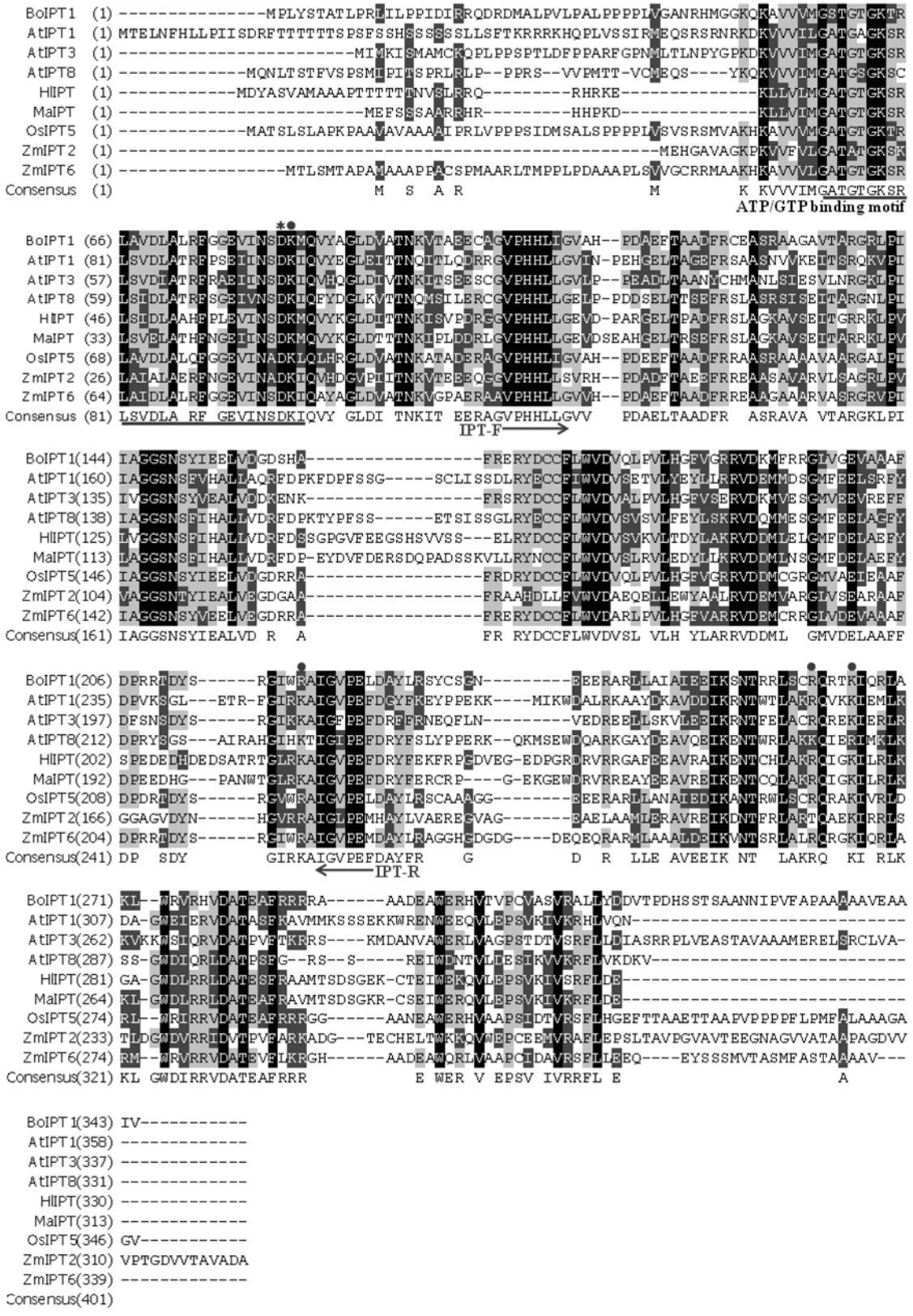


**Figure 1.** Multiple amino acid sequence alignments of BoIPT1 with AIPTs from other species. The sequence abbreviations and GenBank™ Accession numbers shown here are from *Bambusa oldhamii* (BoIPT1, HM044217), *Arabidopsis thaliana* (AtIPT1, AB061400; AtIPT3, AB061401; AtIPT8, AB062614), *Humulus lupulus* (HlIPT, Q5GHF7), *Malus hupehensis* (MhIPT, DQ792508), *Oryza sativa* (OsIPT5, BAC84458) and *Zea mays* (ZmIPT2, ABY78882; ZmIPT6, ABY78885). The conserved ATP/GTP binding motif, which can be converted in AIPT, is underlined. The degenerate primer pairs, IPT-F and IPT-R, are shown by arrows. The conserved $Asp^{62}$ is indicated by asterisk and dots represent the conserved positively charged residues.

sis (Figure 2A) and Western blot analysis (Figure 2B). A major band at about 40 kDa was seen in the Coomassie Brilliant Blue R-250 stained gel (Figure 2A, lane 2). This study also observed the presence of recombinant protein, detected by anti-His antibody, using Western blot (Figure 2B, lane 2). Molecular mass of the purified recombinant protein was estimated to be 40 kDa, which agreed with the molecular mass of BoAIPT1 (Figures 2A and B, Lane 6).

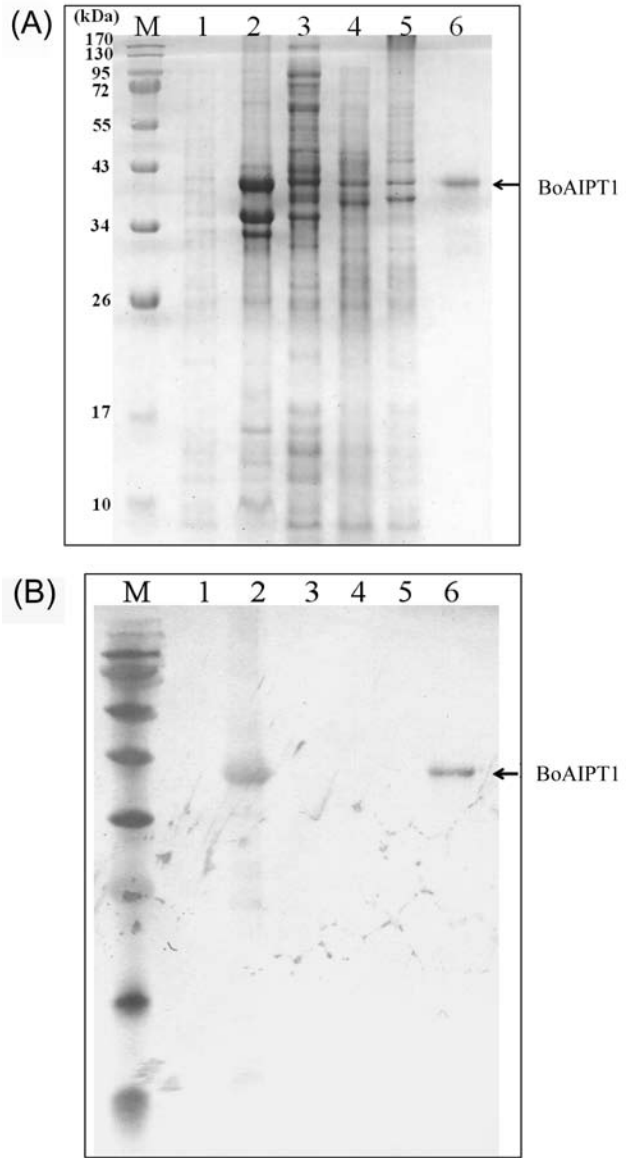


**Figure 2.** SDS-polyacrylamide gel electrophoresis of recombinant BoIPT1 in *Escherichia coli* by affinity chromatography. Samples from different purification steps were monitored by (A) 15% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue R-250, or was preceded to (B) Western blot analysis and detected by using anti-His antibody followed by DAB color development. Lane 1: bacteria lysate without IPTG; lane 2: IPTG induced soluble faction of bacteria lysate; lane 3: eluted sample from buffer containing 10 mM imidazole; lane 4: 50 mM imidazole elution; lane 5: 100 mM imidazole elution; and, lane 6: 250 mM imidazole elution. M, pre-stained protein molecular size marker.

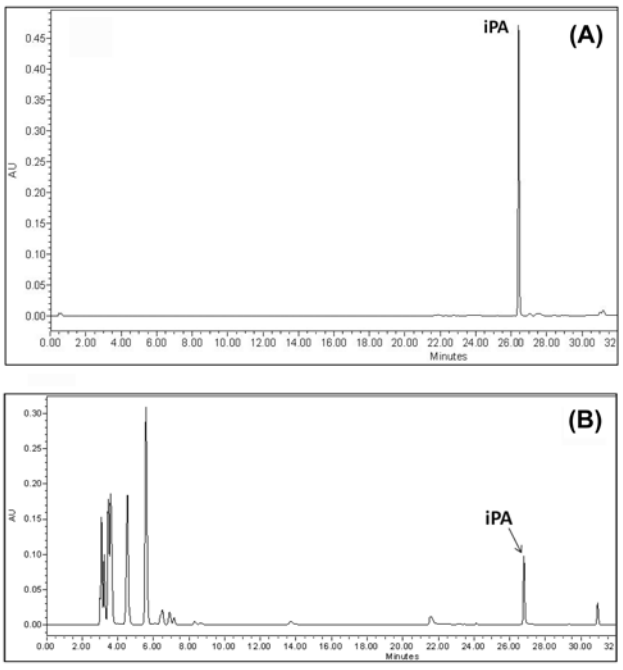


**Figure 3.** HPLC analysis of the BoIPT1-catalyzed reaction product. (A) HPLC chromatograms of the iPA standard (Sigma-Aldrich). (B) Elution profile of the calf intestine alkaline-phosphatase-treated isopentenylation reaction product. The arrow indicates iPA retention time.

Optimum protein expression was obtained by incubating the IPTG-induced culture at 18°C for 18 hr.

## Activity assay of recombinant BoIPT1

The enzyme activity of purified recombinant BoIPT1 was analyzed by incubation with DMAPP and ATP. CIAP-treated reaction products were then analyzed by reverse phase HPLC (Figure 3), which clearly demonstrated the formation of an isopentenylated product.

## Real-Time RT-PCR

In order to analyze the RNA expression level of *BOAIPT1* at different developmental stages, the total RNA of etiolated shoots and green shoots and sheath of etiolated shoot base were isolated and analyzed with Real-time RT-PCR (Figure 4). Results revealed a significant difference in the expression levels of both etiolated shoots and green shoots. *BOAIPT1* mRNA expression level in the green shoots showed an incremental increase from base to top, with the expression level of the top section 3 times greater than the middle section and 5 times greater than the base section. In etiolated shoots, the RNA expression level of all three sections (top, middle, and base) showed no significant differences. Compared to the top section of the green shoot, the expression level is approximately 5 times less. The results clearly demonstrate that, as a bamboo develops, *BOAIPT1* gradually increases, and the green shoots have a higher expression level than the etiolated shoots. The top sections of the green shoots display a higher expression level of BoAIPT1 than the base section.

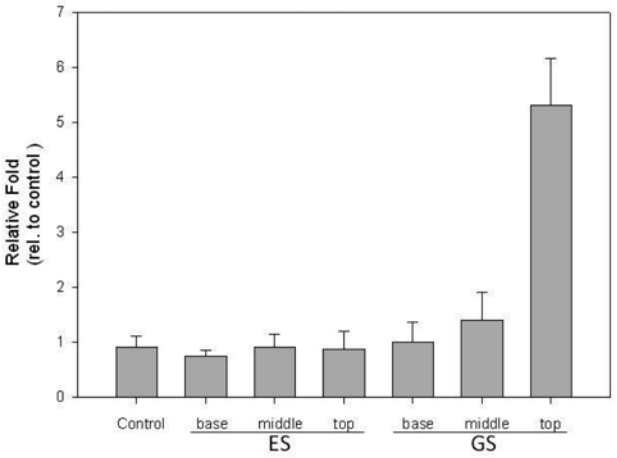


**Figure 4.** Expression profile of *BoAIPT1* analyzed by real-time RT-PCR. Relative expression level of *BoAIPT1* at different growth stages, ES (base, middle and top) and GS (base, middle and top). Sheath of ES base (control). The data shown are means ±SE (*n*=3).

## DISCUSSION

BoAIPT1 is an intronless gene that shares a similar gene structure with other plant AIPTs (Takei et al., 2001; Sakano et al., 2004; Abe et al., 2007). In rice and *Arabidopsis*, approximately 20% of genes are intronless. One proposition is that intronless gene families develop from gene duplication or reverse transcription. The conservation of the lack of introns shows that evolution of AIPT is alike regardless of gene structure or biochemical characteristics.

From the alignment results, we observed that BoAIPT possesses the conserved ATP/GTP-binding sites (P-loop) motif and positively charged amino acid cluster ($Lys^{83}$, $Arg^{218}$, $Arg^{261,}$ and $Lys^{265}$) (Chu et al., 2009). We also noticed the second amino acid of the cluster is always either Arg or Lys in all the different plant AIPTs. The zinc finger-like motif, designated as C-$X_2$-C-X{12,18}-H-$X_5$-H, (where X denotes any amino acid residue, and X{m, n} m to n amino acid residues in number) (Kakimoto, 2001; Sakamoto et al., 2006), which is conserved in eukaryotic tRNA-IPT is absent in the BoAIPT1 C-terminal. From activity analysis, we determined that BoAIPT1 uses DMAPP and ATP as its substrate to synthesize iPTP, an iP precursor.

Results from real-time RT-PCR analysis showed that, when bamboo emerges from underground, the expression of *BoAIPT1* increases as well, predominantly in the green shoot top section. Expression level in the green shoot tip can be 5-times greater than the expression level in etiolated shoot.

The promoter sequence of BoAIPT1derived from chromosome sequencing underwent Plant *Cis*-acting Regulatory DNA Elements (PLACE) promoter bioinformatics analysis. Results revealed that the *BoAIPT1* promoter has three light-induced regions, suggesting that the *BoAIPT1* expression level may be induced by the presence of light. As the shoots emerge from the ground and are exposed to light, *BoAIPT1* expression level increases. This agrees with real-time RT PCR analysis where expression level is lower in etiolated shoots than green shoots, and the base section of the green shoot has a lower expression level than the top section. Cytokinins have been demonstrated to induce shoot growth. When a bamboo emerges from the ground, it grows rapidly. In our study, we found that the green shoot top section has high level of BoAIPT1 expression. This could indicate that the fast growth phenomenon may be related to the high expression level of BoAIPT1.

We will continue to analyze *BoAIPT1* to gain a further understanding of its biochemical properties and application for agricultural uses. In other monocot plants, such as *Zea mays* and *Oryza sativa*, more than one *AIPT* gene has been identified (Sakamoto et al., 2006; Brugière et al., 2008). However, based on the bamboo genomic library screening, only one *AIPT* has been successfully screened. Presently, we are continuing the screening process.

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# 以大腸桿菌系統分子鑑定綠竹異戊烯基轉移酶

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細胞分裂素 Cytokinins (CKs) 爲植物賀爾蒙，具有調控植物發育的功能，例如細胞分裂與植物生長過程的調節，CKs 生合成路徑關鍵酵素爲腺嘌呤核苷酸異戊烯基轉移酶 Adenylate isopentenyltransferase (AIPT)。本研究中，根據蛋白質保守性序列設計出 DNA 引子，進行 PCR 得到部分綠竹 *AIPT* DNA 序列。由染色體基因庫篩選出基因全長核酸序列。利用專一性引子進行 5'-RACE 與 3'-RACE 得到全長 cDNA 序列名爲 *BoAIPT1*，*BoAIPT1* 讀碼框內序列含 1,035 bp，可表現出具有 344 個胺基酸，分子量 37.7 kDa 的蛋白質。*BoAIPT1* 基因不含插入序列 (intron)。分析 BoAIPT1 與其他物種 AIPT 相似在 N 端具有 ATP/GTP 結合區。大腸桿菌表現 BoAIPT1 重組蛋白具有活性可將 DMAPP 中異戊烯側鏈 (isopentenyl side chain) 轉移至腺嘌呤核苷酸中 $N^6$ 位置。Real-time PCR 分析結果顯示 *BoAIPT1* 表現情況因綠竹生長而增加，且莖頂具有最高表現量。

**關鍵詞：**綠竹；腺嘌呤核苷酸異戊烯基轉移酶；分子選殖；大腸桿菌表現系統。