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Molecular cloning, expression, and characterization of adenylate isopentenyltransferase from hop (*Humulus lupulus* L.) ☆

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Abstract

A cDNA encoding adenylate isopentenyltransferase (AIPT) was cloned and sequenced from cones of hop (*Humulus lupulus* L.) by RT-PCR using oligonucleotide primers based on the conserved sequences of *Arabidopsis thaliana* AIPT isozymes (AtIPT1, AtIPT3, AtIPT4, AtIPT5, AtIPT6, AtIPT7 and AtIPT8). A full-length cDNA contained a 990-bp open reading frame encoding a molecular mass of 36,603 Da protein with 329 amino acids. Further, DNA sequencing of genomic DNA revealed absence of introns in the frame. On Southern blot analysis, a single AIPT gene was detected in *H. lupulus*, while RT-PCR analyses demonstrated that the gene was equally expressed in almost all tissues in the plant including roots, stems, leaves and cones. The deduced amino acid sequence shares 38–51% identity to those of *A. thaliana* AtIPTs. A recombinant enzyme expressed in *Escherichia coli* catalyzed isopentenyl transfer reaction from dimethylallyldiphosphate (DMAPP) to the N^6 amino group of adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP), respectively. In contrast, other nucleotides; guanosine monophosphate (GMP), inosine monophosphate (IMP), cytosine monophosphate (CMP), uridine monophosphate (UMP), were not accepted as a substrate. Interestingly, steady-state kinetic analyses revealed that the isopentenylation of ADP and ATP were more efficient than that of AMP as previously reported for *A. thaliana* AtIPT4. Finally, *H. lupulus* AIPT contains the putative ATP/GTP binding motif at the N-terminal as in the case of other known isopentenyltransferases. Site-directed mutagenesis of a conserved Asp62, located right after the ATP/GTP binding motif, with Ala resulted in complete loss of enzyme activity.

Keywords: *Humulus lupulus*; Cannabidaceae; Adenylate isopentenyltransferase; Prenylation; Cytokinin

1. Introduction

Adenylate isopentenyltransferase (AIPT) (EC 2.5.1.27) is a pivotal enzyme in the biosynthesis of cytokinins (Fig. 1) (Takei et al., 2001; Kakimoto, 2001), a group of phytohormones that play a critical role in plant growth and development (Mok and Mok, 2001). Most natural cytokinins are derivatives of N^6-prenylated adenine, and at least isopentenyladenine and *trans*-zeatin are regarded as active forms in plants. Only recently, the Arabidopsis genome project revealed the sequences of AIPTs in *Arabidopsis thaliana*. The plant contains nine isozymes (AtIPT1, AtIPT2, AtIPT3, AtIPT4, AtIPT5, AtIPT6, AtIPT7, AtIPT8 and AtIPT9) sharing sequence similarity with tRNA isopentenyltransferases (IPTs) from bacteria, prokaryotes

☆ The nucleotide sequence reported in this paper is available in the DDBJ/EMBL/GenBank™ data bases under the Accession No. AY533024.

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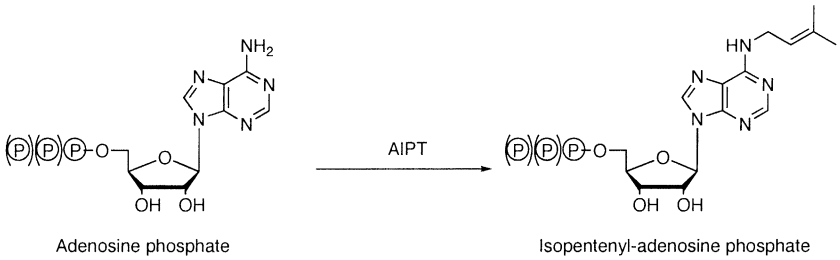

Fig. 1. Prenyl transfer reaction of adenosine phosphate (AMP, ADP or ATP) to isopentenyl adenosine phosphate by AIPT in *H. lupulus*.

and eukaryotes (Takei et al., 2001; Kakimoto, 2001). So far, AtIPT1, AtIPT3, AtIPT4, AtIPT5, AtIPT6, AtIPT7 and AtIPT8 have been shown to be involved in the de novo biosynthesis of cytokinins. Thus, recombinant enzymes heterologously expressed in *Escherichia coli* catalyzed transfer of an isopentenyl group from dimethylallyldiphosphate (DMAPP) to the N^6 amino group of adenosine phosphates (Takei et al., 2001; Kakimoto, 2001; Sakakibara and Takei, 2002; Sun et al., 2003). Although it has been pointed out that AIPT enzymes contain the putative ATP/GTP binding motif, structural details of the enzyme reaction still remain to be elucidated.

The presence of multiple AIPTs in *A. thaliana* suggested the differentiation of the physiological function of each isoform. Since some plant enzymes exhibit fairly broad substrate specificities, it would be possible that some of the AIPT isozymes are involved in the biosynthesis of secondary metabolites such as prenylated chalcones and prenylated phloroglucinols. Although these metabolites are considered to derive from prenylation of precursor compounds, the responsible enzymes involved in the isopentenyl transfer reactions have not been identified yet.

The cones of the hop plant (*Humulus lupulus* L., Cannabinaceae) have been used for centuries in the beer-brewing process as a source of lupulin components. The plant produces significant amount of bitter acids, highly prenylated phloroglucinols (e.g., humulone and lupulone), as well as prenylated chalcones (e.g. xantohumol), for which anti-microbial, anti-fungal and anti-cancer activities have been reported (Stevens et al., 2000; Zuurbier et al., 1998; Paniego et al., 1999; Okada and Ito, 2001; Okada et al., 2001). To study the biosynthesis of cytokinins as well as to shed light on the biogenesis of the prenylated secondary metabolites in *H. lupulus*, we carried out PCR cloning of AIPT cDNA using oligonucleotide primers based on the sequences of *A. thaliana* AIPTs. This is the first report of molecular cloning and characterization of a plant AIPT except those for *A. thaliana*. Functional analyses of a recombinant enzyme including enzyme kinetics, substrate specificity and site-directed mutagenesis, are also presented.

2. Results and discussion

2.1. Molecular cloning of cDNA encoding AIPT

A cDNA encoding AIPT was cloned and sequenced from cones of *H. lupulus* by RT-PCR using inosine-containing degenerate oligonucleotide primers based on the highly conserved sequences of *A. thaliana* AIPT isozymes (AtIPT1, AtIPT3, AtIPT4, AtIPT5, AtIPT6, AtIPT7 and AtIPT8) (Takei et al., 2001; Kakimoto, 2001) (Fig. 2). The terminal sequences of cDNA were obtained by 3′-RACE and 5′-inverse PCR methods. A 1642-bp full-length cDNA contained a 508-bp 5′ non-coding region, a 990-bp open reading frame encoding a molecular mass of 36,603 Da protein with 329 amino acids, and a 144-bp of 3′ non-coding region. Further, genomic DNA was also sequenced by the 5′-inverse PCR and PCR using genomic DNA as template. Interestingly, the gDNA sequence revealed absence of introns in the frame. On Southern blot analysis, a single band was detected (Fig. 3), suggesting presence of a single copy AIPT gene in *H. lupulus*, which is in sharp contrast with the case of *A. thaliana* that contains the nine AIPT genes. Moreover, RT-PCR analyses revealed that the gene was equally expressed in almost all tissues in *H. lupulus* including roots, stems, leaves and cones (Fig. 4), while it has been reported that *A. thaliana* AtIPT8 transcript was only detected in root tissues (Sun et al., 2003).

The deduced amino acid sequence of *H. lupulus* AIPT showed 51%, 39%, 47%, 39%, 46%, 38% and 49% identity to those of AIPT isozymes from *A. thaliana*, AtIPT1, AtIPT3, AtIPT4, AtIPT5, AtIPT6, AtIPT7 and AtIPT8, but exhibited only 34% and 17% identity to those of *A. thaliana* tRNA IPT isozymes, AtIPT2 and AtIPT9, respectively (Fig. 2). *H. lupulus* AIPT contains GATGTGKS (amino acids number 37–44) sequence known as the ATP/GTP binding motif which is universally observed in ATP-consuming enzymes including ATP-binding cassette transporters (Takei et al., 2001; Kakimoto, 2001). On the other hand, the putative 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

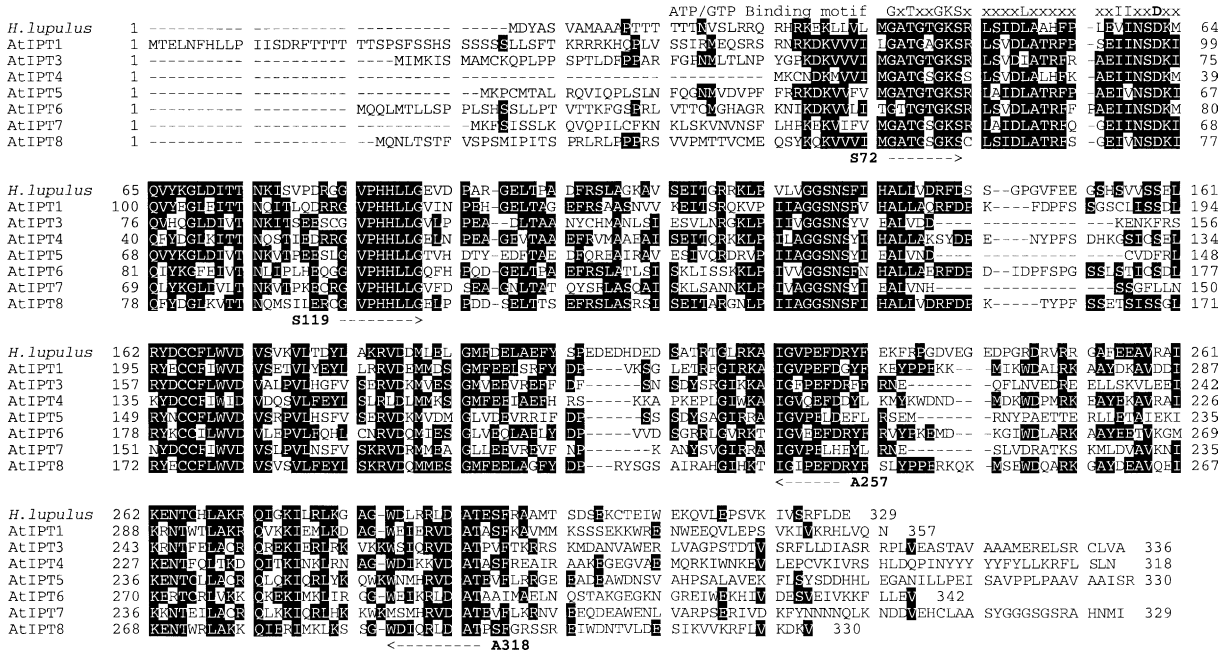

Fig. 2. Comparison of deduced amino acid sequences of *H. lupulus* AIPT and *A. thaliana* AIPT isozymes (AtIPT1, AtIPT3, AtIPT4, AtIPT5, AtIPT6, AtIPT7 and AtIPT8).

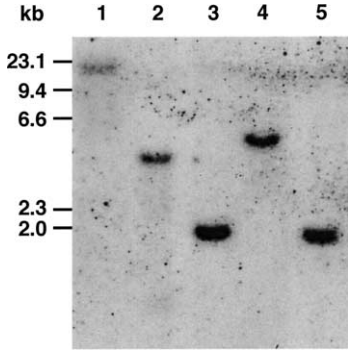

Fig. 3. Southern blot analysis of *H. lupulus* AIPT. Genomic DNA was digested with: lane 1, *Bam* HI; lane 2, *Bgl* II; lane 3, *Dra* I; lane 4, *Eco* T22I; lane 5, *Vsp* I. The positions and sizes (indicated as kilobase-pairs) of molecular size marker (λDNA; digested with *Hind* III) were shown on the left.

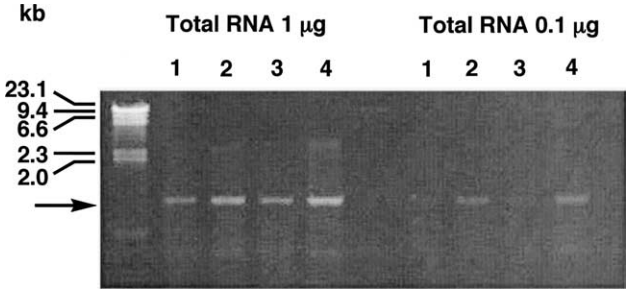

Fig. 4. RT-PCR analysis of expression pattern of *H. lupulus* AIPT: lane 1, root; lane 2, stem; lane 3, leaf; lane 4, cone. The positions and sizes (indicated as kilobase-pairs) of molecular size marker (λDNA; digested with *Hind* III), and the position of amplified ca. 0.9-kb DNA bands (indicated by arrow) were shown on the left.

in number) which is conserved in *A. thaliana* AtIPT2 and eukaryotic tRNA IPTs is not present in *H. lupulus* AIPT as in the case of *A. thaliana* AIPT isozymes (Takei et al., 2001; Kakimoto, 2001). Furthermore, like other IPTs, *H. lupulus* AIPT shares, right downstream of the ATP/GTP binding motif, a consensus pattern of X_5-(V/L/I)-X_7-(V/L/I)(V/L/I)-X_2-D-X_2-Q-X {57,60}-(V/L/I)(V/L/I)-X-GG-(S/T) (where X denotes any amino acid residue, and X{*m*, *n*} *m* to *n* amino acid residues in number) (Takei et al., 2001; Kakimoto, 2001). Within the ATP/GTP binding motif and the consensus region, *H. lupulus* AIPT exhibited 64%, 55%, 66%, 56%, 62%, 54% and 62% identity to those of AIPT isozymes in *A. thaliana*, AtIPT1, AtIPT3, AtIPT4, AtIPT5, AtIPT6, AtIPT7 and AtIPT8 and these scores paralleled well those of the overall sequence. However, the NXDDX or DDXXD putative diphosphate binding motif seen in many prenyltransferases was not found in *H. lupulus* AIPT as well as other IPTs, while Asp62, which was located 18-amino-acid downstream from the ATP/GTP binding motif, was strictly conserved among all of known IPT enzymes, suggesting possible involvement in phosphate binding via salt bridge of magnesium divalent cation. Indeed, our site-directed mutagenesis experiment demonstrated that replacement of Asp62 with Ala resulted in complete loss of enzyme activity.

In the phylogenetic tree (Fig. 5) (Thompson et al., 1994), the AIPT from *H. lupulus* (Cannabinaceae) forms a cluster with *A. thaliana* AIPTs (AtIPT1, AtIPT3, AtIPT4, AtIPT5, AtIPT6, AtIPT7 and AtIPT8) and *Lotus japonicus* AW720363 (EST clone), but a separate

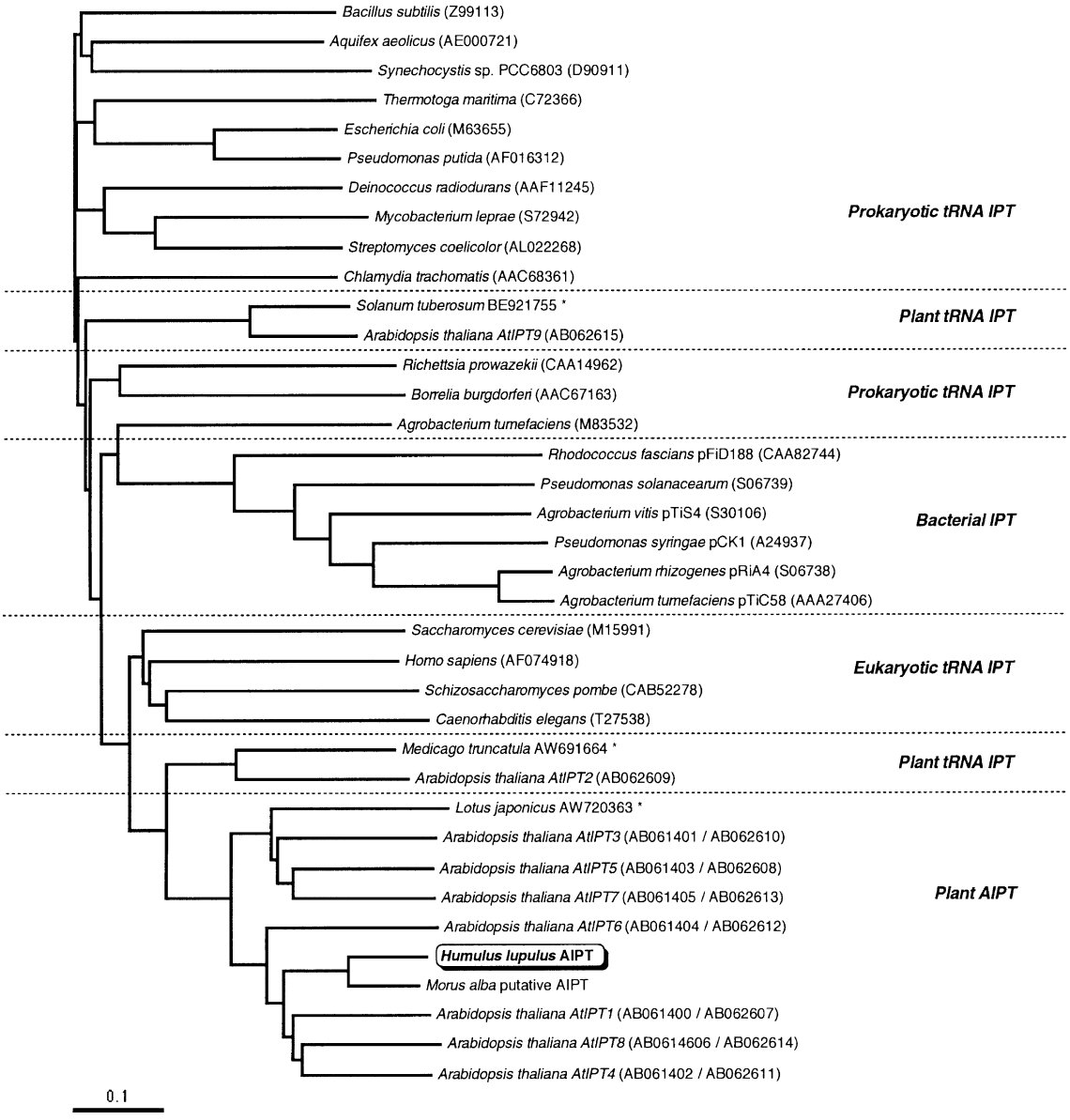

Fig. 5. Phylogenetic tree of the IPT enzymes. Multiple sequence alignment performed by CLUSTAL W (1.8). The three EST clones (*L. japonicus* AW720363, *M. truncatula* AW691664 and *S. tuberosum* BE921755) are indicated by asterisk.

cluster with other IPT enzymes including *A. thaliana* tRNA IPT (AtIPT2 and AtIPT9) and IPTs from plant-pathogenic bacteria. Further, a putative AIPT clone obtained from *Morus alba* (Moracerae) in our laboratories (Abe, I. and co-workers, unpublished data) is also included in the tree. The *H. lupulus* and *M. alba* AIPTs appear to be evolutionary more related to AtIPT1, AtIPT4, AtIPT6 and AtIPT8 than to AtIPT3, AtIPT5 and AtIPT7 isoforms.

2.2. Functional expression of AIPT

The 990-bp *H. lupulus* AIPT cDNA was cloned into pQE-80L (Qiagen) and functionally expressed in *E. coli* M15[pREP4] with an additional hexahistidine-tag at the N-terminal. Purification by Ni-chelate affinity column chromatography afforded ca. 1 mg of homogeneous recombinant AIPT from 1 g of *E. coli* cell pellet. The purified enzyme was first incubated with AMP and DMAPP as substrates under the assay condition as previously reported for *A. thaliana* AIPT isozymes (Takei et al., 2001; Kakimoto, 2001). The reaction products were directly analyzed by reverse phase HPLC, which clearly demonstrated the formation of isopentenylated product in comparison with the control reaction using boiled enzyme. Thus, *H. lupulus* AIPT catalyzed the transfer of an isopentenyl group from DMAPP to the N^6 amino group of AMP (Fig. 1). The enzyme activity of

Table 1
Steady-state kinetic parameters for enzyme reactions[a]

Substrate	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})
AMP	0.497 ± 0.009	759 ± 40	10.90
ADP	1.43 ± 0.06	19.3 ± 2.8	1238
ATP	1.31 ± 0.09	16.2 ± 3.2	1340
DMAPP	1.63 ± 0.10	19.5 ± 3.2	1397

[a] Michaelis–Menten plots of data were employed to derive the K_M and k_{cat} values (average of duplicates ± standard deviation) using EnzFitter software (BIOSOFT).

H. lupulus AIPT showed dependence on magnesium divalent cation and a broad pH optimum between 6 and 9, either in Tris–HCl or potassium–phosphate buffer systems. On the other hand, other ribonucleotides; guanosine monophosphate (GMP), inosine monophosphate (IMP), cytosine monophosphate (CMP) and uridine monophosphate (UMP), were not accepted as a substrate, suggesting that at least the purine core structure and the N^6 amino functional group is essential for the isopentenyl transfer activity.

It has been reported that some *A. thaliana* AtIPT enzymes such as AtIPT4 preferred adenosine diphosphate (ADP) and adenosine triphosphate (ATP), rather than adenosine monophosphate (AMP), as a prenyl acceptor substrate (Takei et al., 2001; Kakimoto, 2001). Steady-state kinetic analyses of *H. lupulus* AIPT revealed that the isopentenylation of ADP and ATP were more efficient than that of AMP as in the case of *A. thaliana* AtIPT4, but contrast with *Agrobacterium tumefaciens* TZS which only recognizes AMP as acceptor (Morris et al., 1993). The observed K_M and k_{cat} parameters were as follows (Table 1); 759 μM and 0.497 min^{-1} for AMP, 19.3 μM and 1.43 min^{-1} for ADP, 16.2 μM and 1.31 min^{-1} for ATP, and 19.5 μM and 1.63 min^{-1} for DMAPP, respectively. The K_M values for ATP and DMAPP in this enzyme were comparable to or a little higher than those of AtIPT4. The k_{cat}/K_M values for AMP, ADP, ATP and DMAPP were 10.90, 1238, 1340 and 1397 s^{-1} M^{-1}, respectively. The k_{cat}/K_M value for ATP was thus 123-fold higher than that of AMP. This suggested that cytokinins are also generated from ATP and ADP in hop plant as in the case of *A. thaliana*.

3. Conclusions

In summary, this is the first report of molecular cloning of AIPT enzyme from *H. lupulus*, except those for *A. thaliana* whose sequences were identified by the genome project. Functional analyses of a recombinant enzyme including enzyme kinetics, substrate specificity and site-directed mutagenesis, provided important information on the catalytic function of the enzyme. In order to elucidate the structural details of the isopentenyl transfer reaction from DMAPP to the N^6 amino group of adenosine phosphate, and to manipulate the cytokinin biosynthesis in the plant, further characterization of the enzyme is now in progress in our laboratories.

4. Experimental

4.1. Chemicals

Dimethylallyldiphosphate, adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, guanosine monophosphate, inosine monophosphate, cytosine monophosphate, and uridine monophosphate, were purchased from Sigma. [1-^{3}H] DMAPP (triammonium salt; 20 Ci/mmol) was from American Radiolabeled Chemical, Inc.

4.2. Plant materials

All plant tissues were prepared from *H. lupulus* L. (9418R). Cones were harvested 15 days after flowering from plants grown in the field of Sapporo Breweries, Ltd., Japan. All plant materials were frozen in liquid nitrogen and stored at −80 °C.

4.3. PCR and DNA sequencing

The polymerase chain reaction (PCR) was carried out with Ex-*Taq* DNA polymerase (Takara) using Robocycler Gradient 40 (Stratagene). The nucleotide sequences were determined from both strands by DNA sequencer ABI 373A (Perkin–Elmer Applied Biosystems) using Thermo Sequenase™ II Dye Terminator Cycle Sequencing Kit (Amersham Biosciences).

4.4. Preparation of genomic DNA

Genomic DNA (gDNA) was prepared from the leaves by a modified CTAB method (Murray and Thompson, 1980). The 2% CTAB solution (2% cetyltrimethylammonium bromide, 0.1 M Tris, 20 mM EDTA, 1.4 M NaCl, and 5% 2-mercaptoethanol, pH 9.5) was added to the ground leaves, mixed, and incubated at 65 °C for 30 min. The solution was extracted with $CHCl_3$ twice, and DNA was precipitated by adding an equal volume of 2-propanol to the aqueous phase. The precipitated DNA was dissolved in High Salt TE (10 mM Tris, 1 mM EDTA, and 1 M NaCl, pH 8.0) and treated with RNase at 65 °C for 30 min. The DNA was precipitated by adding an equal volume of 2-propanol to the solution and rinsed with 70% aqueous ethanol. The DNA was dried and dissolved in distilled water.

4.5. Preparation of total RNA and cDNA

The total RNA of the cone was prepared by the modified CTAB procedure (Mukai and Yamamoto, 1995). The 2% CTAB solution (2% cetyltrimethylammonium bromide, 0.1 M Tris, 20 mM EDTA, 1.4 M NaCl, and 5% 2-mercaptoethanol, pH 9.5) was added to each ground tissue, mixed, and incubated at 65 °C for 30 min. The solution was extracted with $CHCl_3$ twice, and RNA was precipitated by adding one-fourth volume of 10 M LiCl to the aqueous phase. The precipitated RNA was dissolved in DNase buffer (100 mM Na-Acetate, 5 mM $MgCl_2$, pH 5.2) and treated with DNase (RNase free) at 30 °C for 60 min. After phenol-$CHCl_3$ extraction, the RNA was precipitated by adding one-fourth volume of 10 M LiCl to the solution, then rinsed with 70% aqueous ethanol. The RNA was dried and dissolved in distilled water, then reverse transcribed using SuperScript™ (Invitrogen) and oligo dT primer (RACE 32 = 5′-GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TT-3′) according to manufacturer's protocol. The obtained cDNA mixture was diluted with TE (10 mM Tris, 1 mM EDTA, pH 8.0) and used as a template for the following PCR amplification.

4.6. PCR amplification of cDNA

Inosine-containing degenerate oligonucleotide primers (72S, 119S, 257A, and 318A, the number of primer indicates the amino acid number of corresponding *A. thaliana* AtIPT1) based on the highly conserved sequences of *A. thaliana* AIPTs were used for amplification of a core fragment of the cDNA. The sequences of the primers are as follows: 72S = 5′-GG(A/T) GC(A/C/T) AC(A/C) GGI (A/T)C IGG CAA (A/G)TC-3′, 119S = 5′-GT(A/T/G/C) CC(G/T) CA(C/A) CA(C/A) CT(C/A) CT(C/A) GG-3′, 257A = 5′-TC(A/G) AAC TC(C/G/T) GG(A/G/T) AC(A/T) CC(G/T) AT-3′, and 318A = 5′-GC(A/G) TC(A/C) ACI C(G/T)C T(G/T)(A/C/T) ATI T(A/C/T) CCA-3′. Nested PCR was carried out with the primer sets of 72S and 318A, and then with 119S and 257A, to amplify a 431-bp DNA fragment. For the PCR, 30 cycles of reactions (94 °C for 1 min, 42 °C for 1 min and 72 °C for 1.5 min) were performed each time with a 10 min final extension. The gel-purified PCR product was ligated into pT7Blue T-Vector (Novagen) and sequenced.

For the 3′-end amplification, a specific primer; 108S = 5′-GCT GGA AAA GCC GTG CTG AAA-3′, was designed based on the obtained core sequence. Then, 3′-rapid amplification of cDNA ends (3′-RACE) was carried out with the primer set of 108S and RACE32 with 30 cycles of reactions (94 °C for 1 min, 49 °C for 1 min and 72 °C for 1.5 min), to amplify an 810-bp DNA fragment. On the other hand, the 5′-end sequence was finally obtained by the 5′ inverse PCR method. Thus, the genomic DNA prepared from leaves was first digested with *Psh* BI (Takara), and circularized using the DNA Ligation Kit (Takara) according to the manufacturer's instruction, which was then used as a template for the PCR reactions using four specific primers based on the obtained core sequence. The sequences of the primers are as follows: 142A = 5′-TC AAA CCG GTC CAC CAA CAG-3′, 170A = 5′-AC CCA GAG AAA GCA ACA GTC-3′, 178S = 5′-ACC GAT TAC TTG GCG AAG GC-3′, and 196S = 5′-AG TTG GCC GAG TTC TAT AGC-3′. Nested PCR was carried out with the primer sets of 170A and 178S, and then with 142A and 196S, to amplify a 1,450-bp DNA fragment. For the PCR, 30 cycles of reactions (94 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 6 min) were performed each time with a 7 min final extension.

4.7. Southern blot analysis

Genomic DNA (10 μg) was digested with *Bam* HI, *Bgl* II, *Dra* I, *Eco* T22I, and *Vsp* I separated on a 1% agarose gel and blotted onto a nylon membrane. The recognition sites of all of these restriction enzymes were absent in the structural gene of *H. lupulus* AIPT. Hybridization with *H. lupulus* AIPT cDNA was performed using the DIG system (Roche Diagnostics) according to the manufacturer's instructions. Hybridization was done at 68 °C overnight with the digoxinlabeled probe in 5× saline sodium citrate (SSC), 50% formamide, 0.02% SDS, 0.1% *N*-lauroylsarcosine, 2% Blocking Reagent. Membrane was washed twice with 2× SSC, 1% SDS at 60 °C for 15 min.

4.8. RT-PCR analysis

Total cellular RNA prepared from *H. lupulus* was treated with RNase-free DNase I at 37 °C for 15 min. RT-PCR was performed with total RNA (1 or 0.1 μg). The first Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnosis) was used for the RT reaction according to the manufacturer's instructions. After incubation with RNase H for 30 min at 37 °C, 1 μl of the reaction mixture was used for subsequent PCR using the N-terminal and C-terminal primers as described above. For the PCR, 30 cycles of reactions (94 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 2 min) were performed with a 10 min final extension to amplify the 990-bp DNA fragment.

4.9. Phylogenetic tree

A total of 37 amino acid sequences of isopentenyltransferases were aligned and phylogenetic tree was developed with the CLUSTAL W (1.8) program (DNA Data

Bank of Japan, URL: http://www.ddbj.nig.ac.jp) (Thompson et al., 1994). Bootstrap resampling (1000 bootstrap, 111 seed) of the original data was used as a pseudo-empirical test of the reliability of the tree topology (Felsenstein, 1985; Wu, 1986). The tree was constructed by use of the majority rule and strict consensus algorithm implanted in PHYLIP (Felsenstein, 1989). The accession numbers of GenBank sequence data bank used in the analysis are as follows; *Aquifex aeolicus* (AE000721), *Agrobacterium rhizogenes* pRiA4 (S06738), *A. tumefaciens* (M83532), *A. tumefaciens* pTiC58 (AAA27406), *A. vitis* pTiS4 (S30106), *A. thaliana* AtIPT1 (AB061400/AB062607), *A. thaliana* AtIPT2 (AB062609), *A. thaliana* AtIPT3 (AB061401/AB062610), *A. thaliana* AtIPT4 (AB061402/AB062611), *A. thaliana* AtIPT5 (AB061403/AB062608), *A. thaliana* AtIPT6 (AB061404/AB062612), *A. thaliana* AtIPT7 (AB061405/AB062613), *A. thaliana* AtIPT8 (AB061406/AB062614), *A. thaliana* AtIPT9 (AB062615), *Borrelia burgdorferi* (AAC67163), *Bacillus subtilis* (Z99113), *Caenorhabditis elegans* (T27538), *Chlamydia trachomatis* (AAC68361), *Deinococcus radiodurans* (AAF11245), *E. coli* (M63655), *Homo sapiens* (AF074918), *Lotus japonicus* AW720363*, *Medicago truncatula* AW691664*, *Mycobacterium leprae* (S72942), *Pseudomonas putida* (AF016312), *P. solanacearum* (S06739), *P. syringae* pCK1 (A24937), *Rhodococcus fascians* pFiD188 (CAA82744), *Richettsia prowazekii* (CAA14962), *Solanum tuberosum* BE921755*, *Saccharomyces cerevisiae* (M15991), *Streptomyces coelicolor* (AL022268), *Schizosaccharomyces pombe* (CAB52278), *Synechocystis* sp. PCC6803 (D90911), *Thermotoga maritima* (C72366). The three EST clones are indicated by asterisk.

4.10. Cloning and expression of cDNA

A full length cDNA was obtained by RT-PCR using N-terminal and C-terminal PCR primers; 5′-TCT ATC GGA TCC ATG GAC TAC GCA TCC GTT GC-3′ and 5′-TGA CCG GGA TCC CTA CTC GTC CAA GAA GCG AC-3′ (the *Bam* HI site is underlined), with 30 cycles of reactions (94 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 3 min). The amplified DNA was digested with *Bam* HI, and cloned into the *Bam* HI site of pQE-80L (Qiagen). Thus, the recombinant enzyme contains an additional hexahistidine tag at the N-terminal. After confirmation of the sequence, the plasmid was transformed into *E. coli* M15[pREP4]. The cells harboring the plasmid were cultured in Luria–Bertani medium containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin at 37 °C until the A_{600} reached 0.7. Then, isopropyl-β-D-(−)-thiogalactopyranoside was added to a final concentration of 1 mM to induce protein expression, and the culture was incubated further at 18 °C for 24 h.

4.11. Enzyme purification

The *E. coli* cells were harvested by centrifugation, resuspended in 50 mM potassium phosphate buffer (KPB), pH 8.0, containing 0.3 M NaCl, and disrupted on ice by sonication. The cell lysate was centrifuged at 15,000*g* for 60 min. The supernatant was loaded onto a column of Ni-NTA resin (Qiagen) which contained Ni^{2+} as an affinity ligand. After washing with 50 mM KPB, pH 6.8, containing 0.3 M NaCl, the recombinant AIPT was eluted with a gradient of 0–0.5 M imidazole in the wash buffer. The fraction containing the recombinant IPT was dialyzed with 0.1 M KPB, pH 6.8, containing 1 mM DTT, and stored at −80 °C. The purified enzyme preparation gave a single band with molecular mass of 37 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentration was determined by the Bradford method (Protein Assay, Bio-Rad) with bovine serum albumin as standard.

4.12. Enzyme reaction

The standard reaction mixture contained 1 mM acceptor substrate, 100 μ MDMAPP, and 2.0 μg of the purified recombinant AIPT in a final volume of 100 μl of 100 mM Tris–HCl buffer, pH 8.0, containing 50 mM KCl, 10 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 1 mM DTT. Incubations were carried out at 25 °C for 120 min, and stopped by adding 25 μl of 10% AcOH. The products were analyzed by reverse phase high-performance liquid chromatography (HPLC) on a TSK-gel ODS-80T_S column (4.6 × 150 mm, TOSOH) with a flow rate of 1.0 ml/min. Gradient elution was performed with 0.85% H_3PO_4 and CH_3CN: 0–15 min, 100% H_3PO_4; 15–45 min, linear gradient from 0% to 80% CH_3CN. Eluants was monitored at UV 280 nm.

Steady-state kinetic parameters were determined for acceptor and donor substrate. The experiments for kinetic analyses for acceptor substrate were carried out in duplicate using five concentrations of AMP (2500, 2000, 1500, 1000 and 500 μM), ADP (100, 80, 60, 40 and 20 μM) or ATP (50, 40, 30, 20 and 10 μM) in the assay mixture, containing 100 μM DMAPP, 50 mM KCl, 10 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 1 mM DTT, and the purified recombinant AIPT in a final volume of 100 μl of 100 mM Tris–HCl buffer, pH 8.0. The experiments for kinetic analyses for donor substrate were carried out in duplicate using five concentrations of DMAPP (50, 40, 30, 20, and 10 μM) in the assay mixture, containing 100 μM ATP, 50 mM KCl, 10 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 1 mM DTT, and the purified recombinant AIPT in a final volume of 100 μl of 100 mM Tris–HCl buffer, pH 8.0. The content of the purified recombinant AIPT was 4.0 μg for

kinetic analysis for AMP, and 2.0 μg for kinetic analysis for ADP, ATP and DMAPP, respectively. Incubations were carried out at 25 °C for 30 min, and stopped by adding 25 μl of 10% AcOH. The reaction products were analyzed by HPLC, and the molar content of the reaction product was calculated from calibration curve obtained as follows. The same standard reaction as described above was carried out except that 100 μM DMAPP was replaced for 0.5 μM [1-^{3}H] DMAPP (1 μCi, 50 pmol) as a donor substrate, and quenched by adding 25 μl of AcOH–H_2O (1:9, v/v). The reaction products were separated by HPLC monitored at UV 280 nm with five different injection volumes of reaction solution (5, 10, 15, 20, and 25 μl), the radioactivity in each fraction was determined by liquid scintillation counter (Aloka LSC-3100), and the data sets of molar content and HPLC peak area were plotted to obtain the calibration curve. Michaelis–Menten plots of data were employed to derive the apparent K_M and k_{cat} values (average of duplicates ± standard deviation) using EnzFitter software (BIOSOFT).

4.13. Site-directed mutagenesis

Asp62 of *H. lupulus* AIPT was replaced by Ala using the QuikChange™ XL Site-Directed Mutagenesis Kit (Stratagene) and a pair of complementary mutagenic primers as follows: sense 5′-C CCT CTC GAA GTC ATA AAC TCG GCT AAA ATG CAG G-3′, anti sense 5′-C CTG CAT TTT AGC CGA GTT TAT GAC TTC GAG AGG G-3′ (mutated base is underlined). The plasmid containing D62A mutant was used to transform competent XL10-Gold® ultracompetent cells. Mutagenic plasmid DNA was isolated from selected transformants and analyzed by restriction mapping and DNA sequencing. The verified mutant clones were transformed into competent cells for expression and isolation as described above.

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