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Molecular Cloning, Expression and Characterization of Hyoscyamine 6β-Hydroxylase from Hairy Roots of *Anisodus tanguticus*

Abstract

Anisodus tanguticus, one of the indigenous Chinese ethnological medicinal plants of the Solanaceae, produces anticholinergic alkaloids such as hyoscyamine, 6β-hydroxyhyoscyamine and scopolamine. Hyoscyamine 6β-hydroxylase (H6H), a key enzyme in the biosynthetic pathway of scopolamine, catalyzes the hydroxylation of hyoscyamine and epoxide formation from 6β-hydroxyhyoscyamine to generate scopolamine. A full-length cDNA of H6H has been isolated from *A. tanguticus* hairy roots. Nucleotide sequence analysis of the cloned cDNA revealed an open reading frame of 1035 bp encoding 344 amino acids with high homology to other known H6Hs. The equivalent amino acid sequence shows a typical motif of 2-oxoglutarate-dependent dioxygenase. The *A. tanguticus* H6H was expressed in *Escherichia coli* and purified for enzyme function analysis. This study characterized the recombinant *At*H6H and showed it could generate scopolamine from hyoscyamine.

Key words
Anisodus tanguticus · Solanaceae · hyoscyamine 6β-hydroxylase (H6H) · molecular cloning · expression · characterization

Introduction

Tropane alkaloids constitute a group of secondary metabolites in Solanaceae plants including four important medicinally used alkaloids: hyoscyamine, 6β-hydroxyhyoscyamine, anisodine, and scopolamine. *Anisodus, Atropa, Duboisia, Hyoscyamus,* and *Datura* are the major tropane alkaloid producing genera. Tropane alkaloids act mainly on the parasympathetic nervous system [1], [2]. 6-Hydroxyhyoscyamine may be used to improve the microcirculation [1].

Anisodus tanguticus (Maxim.) Pascher (Solanaceae) is one of the folk medicines used in Tibet and Qinghai Province in West China. It is an important medicinal plant species yet its cultivation is difficult. Tropane alkaloids are mainly present in the underground parts of the plant, and hairy roots but not suspension cells are considered capable of producing more secondary metabolites [1], [2], [3], [4]. Observations have revealed that the content of scopolamine was much higher than that of hyoscyamine in hairy roots after 4 weeks cultivation [4]. This indicated that the enzymes responsible for the conversion of hyoscyamine should be very active and might have potential use in increasing the scopolamine production rate. Hyoscyamine 6β-hydroxylase (H6H, EC 1.14.11.11) is a key enzyme in the biosynthesis of scopolamine. It belongs to the 2-oxoglutarate-dependent dioxygenase family and catalyzes two consecutive oxidation reactions: the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine and the epoxidation of 6β-hydroxyhyoscyamine to scopolamine (Fig. **1**) [5].

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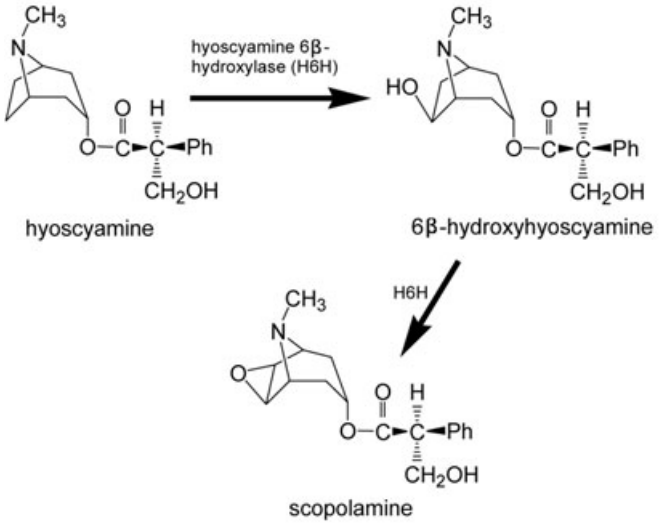

Fig. **1** Biosynthetic pathway from hyoscyamine to scopolamine. H6H catalyzes the two reactions.

The aim of this study was to clone the H6H gene of *A. tanguticus* (*AtH6H*) and analyze the function of the recombinant protein expressed by *Escherichia coli*.

Materials and Methods

Plant material

Anisodus tanguticus (Maxim.) Pascher was identified by Professor Guangcheng Xia in the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. The voucher specimen (IH #673) was deposited in the institute.

Hairy roots of *A. tanguticus*, established from aseptic seedlings by *Agrobacterium rhizogenes* (ATCC No. 15834) on Linsmayer and Skoog (LS) medium without auxins as described [4], were transferred from solid culture to 500-mL flasks containing 200 mL of liquid LS medium without auxins on a rotary shaker at 80 rpm and 25 °C in the dark [6]. The six-day hairy roots were used for RNA preparation. The voucher specimen (NP #84) was deposited in the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College.

Chemicals

l-Hyoscyamine hydrobromide was purchased from Sigma, USA. 6*β*-Hydroxyhyoscyamine and scopolamine were obtained from Minsheng Pharmaceutical Co., Ltd. Hangzhou, China.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from *A. tanguticus* hairy roots by Concert Plant RNA Reagent (Invitrogen, USA) following the manufacturer's instruction. Total RNA (5 μg) was reverse transcribed to synthesize first strand cDNA with Oligo$(dT)_{20}$ Primer (Invitrogen, USA) and SuperScript RT II Reverse Transcriptase (Invitrogen, USA).

Primers were designed from the homologous regions found in the nucleotide alignments of the hyoscyamine 6*β*-hydroxylase of *Hyoscyamus niger* (*Hn*H6H, GenBank Accession No.D26583) and *Atropa belladonna* (*Ab*H6H, GenBank Accession No. AB017153) as well as the enzymes of the 2-oxoglutarate dependent dioxygenase family. The primer pair of 5′-AACTATTACCCACCATG-3′ (sense primer) and 5′-TCCCAAGTTGACCACAAAAGC-3′ (antisense primer) was used to amplify the fragment from the first strand cDNA. PCR was performed in a total volume of 25 μL containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM each primer, 200 ng first strand cDNA, and 2.5 U Platinum *Taq* DNA polymerase (Invitrogen, USA) in 1 × reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl). After an initial 4 min denaturing step at 94 °C, 30 cycles of amplification were performed at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1.5 min. Elongation was extended to 10 min at 72 °C after the last cycle. The amplified fragment (200 bp) was ligated into pMD 18-T vector (TaKaRa, Japan) and sequenced.

3′and 5′rapid amplification of cDNA ends (RACE)

To obtain 3′and 5′-terminal sequences of the cDNA, 3′RACE and 5′RACE were performed with RACE System Version 2.0 (Invitrogen, USA) as the manufacturer described.

For 3′RACE, two gene-specific primers were designed: 5′-CGATCAACAAGACTTGCCTGGCT-3′ (3-GSP1), 5′-CGAGCCTGGCTTGCAACAACTT-3′ (3-GSP2). The 3′ RACE Adapter Primer (AP) sequence was 5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′and Abridged Universal Amplification Primer (AUAP) sequence was 5′-GGCCACGCGTCGACTAGTAC-3′.

The first strand cDNA was synthesized from the total RNA (5 μg) with primer AP. The second strand cDNA was amplified with the 3-GSP1 forward and AUAP reverse primers (94 °C for 4 min, then 30 cycles at 94 °C for 4 min, 54 °C for 1 min, and 72 °C for 1.5 min, with final extension at 72 °C for 10 min). The PCR product was diluted 100 times, and employed in a second PCR (same conditions) but substituting forward primer 3-GSP2 for 3-GSP1. The amplified fragment (~ 600 bp) was cloned into pMD 18-T vector and sequenced.

For 5′ RACE, three gene-specific primers were designed: 5′-CAGGCAAGTCTTGTTGAAGC-3′ (5-GSP1), 5′-CGATTACCATCAGAGTGTCCTCC-3′ (5-GSP2), 5′-CGACAGAGTGTCCTCCTGAACC-3′ (5-GSP3). The 5′RACE Abridged Anchor Primer (AAP) sequence was 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′ and Abridged Universal Amplification Primer (AUAP) sequence was 5′-GGCCACGCGTCGACTAGTAC-3′.

The first strand cDNA, synthesized from total RNA (5 μg) with primer 5-GSP1, was purified by S.N.A.P. column (Invitrogen, USA) and then oligo-dC tailed using terminal deoxynucleotidyl transferase (Invitrogen, USA). The second strand cDNA was amplified with the AAP forward and 5-GSP2 reverse primers (94 °C for 4 min, then 30 cycles at 94 °C for 4 min, 54 °C for 1 min, and 72 °C for 1.5 min, with final extension at 72 °C for 10 min). The PCR product was diluted 100 times, and employed in a second PCR (same conditions) with AUAP forward and 5-GSP3 reverse primers. The amplified fragment (~ 750 bp) was cloned into pMD 18-T vector and sequenced.

Heterologous expression of AtH6H in E. coli

The open reading frame (ORF) of *At*H6H was amplified using a sense primer (5′-CGCGGAATTCATGGCTACTCTTGTCTCCAAC-3′)

with *Eco*R I site (underlined) and an antisense primer (5′-CAGGGCGGCCGCGGCATTGATTTTATAAGGCT-3′) with *Not* I site (underlined). The PCR product was ligated into pMD 18-T vector and sequenced. After sequence confirmation, the ORF fragment was digested with *Eco*R I and *Not* I (TaKaRa, Japan), and directionally ligated into pET-32a (+) vector (Novagen, Germany) to generate pET*At*H6H which expressed as a histidine-tagged fusion *At*H6H protein in *E. coli* strain BL21trxB (DE3) (Novagen, Germany).

For the enzyme preparation, *E. coli* cells transformed with pET*At*H6H harboring *At*H6H gene were grown at 37 °C in 50 mL of Luria-Bertani medium, supplemented with ampicillin (20 μg/mL). The cultures were induced by the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.6 mM until the OD_{600} reached 0.6 – 0.7. After incubation for another 8 h at 20 °C, the cells were harvested by centrifugation at 8,000 × *g* for 5 min at 4 °C to give cell pellets. The strain transformed with empty vector was used as the control.

Purification of the recombinant AtH6H

All purification procedures were carried out at 0 – 4 °C. About 30 g of cell pellets were resuspended in 60 mL of buffer A (20 mM sodium phosphate, pH 7.2) with 1 mg/mL lysozyme and 10 mM DTT, and placed on ice for 30 min and then freeze-thawed for four times. This suspension was homogenized by sonication and then centrifuged at 13,000 × *g* for 10 min at 4 °C to pellet debris. After determination by SDS-PAGE, the resulting supernatant was referred to as cell-free extract and stored at –70 °C.

The cell-free extract was loaded on a HiTrap Chelating HP column (Amersham Biosciences, Sweden) previously equilibrated with the binding buffer (buffer A containing 1 M NaCl). The column was eluted stepwise at 1 mL/min with a linear gradient of imidazole from 0 to 20 mM in 6 mL of elution buffer (buffer A containing 1 M NH_4Cl). Each fraction was analyzed by SDS-PAGE. The fractions containing the recombinant *At*H6H were combined and dialyzed against buffer A.

The enzyme fraction obtained from the previous steps was loaded on a DEAE Sepharose Fast Flow column (1.0 × 20 cm, Amersham Biosciences, Sweden) previously equilibrated with buffer A. The column was washed with 100 mL of the same buffer. The enzyme was eluted with a linear gradient of NaCl from 0 to 600 mM in 150 mL of buffer A at a flow rate of 1 mL/min. Fractions containing the recombinant *At*H6H were combined and dialyzed against buffer A and stored at –70 °C.

Enzyme assay

*At*H6H was assayed by measuring the formation of products from different substrates supplemented. The complete reaction mixture contains 50 mM Tris-HCl buffer (pH 7.4), 0.4 mM $FeSO_4$, 4 mM sodium ascorbate, 1 mM 2-oxoglutarate, 0.2 mM *l*-hyoscyamine hydrobromide or 0.2 mM 6β-hydroxyhyoscyamine, 2 mg/mL catalase (Worthington Biochemical Corp., USA), and the enzyme (0.2 – 0.5 mg of the protein) [6], [7]. The volume for the enzyme assay was 40 mL in total.

The reactions were performed at 30 °C for 2 h. They were initiated by adding the enzyme and stopped by adding $NaHCO_3$ to adjust the pH value of the mixture to 9.0, followed by the extraction with an equal volume of EtOAc. The extracts were evaporated to dryness at 55 °C and the residue dissolved in 0.5 mL of MeOH. The quantitative analysis of alkaloids was performed by HPLC on an LC-6A HPLC System (Shimadzu, Japan) with a ZORBAX ODS-C_{18} column (5 μm, 4.6 × 200 mm, Agilent, USA), eluted isocratically with a mobile phase (acetonitrile/0.05 M sodium heptanesulfonate 35 : 65) at a flow rate of 1.5 mL/min and detected at 210 nm. The products yielded by the recombinant *At*H6H were purified by preparative TLC (SiO_2, toluene/acetone/EtOH/NH_4OH, 3 : 6 : 0.6 : 0.4; Dragendorff reagent; Rf 6β-hydroxyhyoscyamine = 0.23, scopolamine = 0.68) [2] and identified by ^{1}H-NMR (500 MHz, in CD_3OD) and ^{13}C-NMR (125 MHz, in CD_3OD) spectra, which were recorded on an Inova-500 (Varian, USA).

The inhibitory effects of EDTA (a metal chelating agent) and various metal ions on the enzyme activity were investigated [7]. Kinetic parameters were calculated by Lineweaver-Burk methods. K_m values were determined at the substrate concentrations between 0.005 and 0.1 mM. Each substrate concentration was measured at least three times.

The protein concentrations were estimated according to the method of Bradford using crystalline bovine serum albumin as the standard [8].

Southern blotting analysis

Genomic DNA was extracted from *A. tanguticus* hairy roots with the DNeasy Plant Maxi Kit (Qiagen, USA). Genomic DNA (20 μg) was digested with restriction enzyme *Eco*R I, *Eco*R V, *Sac* I, and *Xho* I (TaKaRa, Japan), respectively. The fragments were fractionated on 0.7% agarose gel and then transferred onto a Hybond N^+ membrane (Amersham Biosciences, Sweden). The labeling, hybridization and detection were performed following the instruction of ECL Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences, Sweden). The labeled *At*H6H full-length cDNA was used as the probe.

Results and Discussion

Cloning of AtH6H and sequence analysis

Hairy roots of *A. tanguticus* were the original materials for the cDNA preparation. RT-PCR was performed with primers that were designed on the basis of the known H6H sequences. Sequence analysis of the resulting 177-bp amplicon revealed a partial cDNA bearing 93% nucleotide-level homology to *Hn*H6H and *Ab*H6H. Based on this sequence, several gene-specific primers were designed to obtain the sequences of 3′-end and 5′-end by RACE. Fragments about 600 bp of 3′ RACE and 750 bp of 5′ RACE were amplified and sequenced, respectively. After a PCR with primers based on the sequences of 3′-end and 5′-end, the full-length sequence of *At*H6H cDNA was obtained.

The full-length cDNA was 1,305 bp in size containing 5′-untranslated region (5′UTR), 3′-untranslated region (3′UTR), poly (A^+) tail, and an ORF of 1,035 nucleotides encoding a deduced protein of 344 amino acid residues (GenBank Accession No. AY356396). The deduced amino acid sequence of *At*H6H showed 83% identity with *Hn*H6H and 84% identity with *Ab*H6H. The alignment to

*Hn*H6H, *Ab*H6H and other 2-oxoglutarate-ependent dioxygenases is shown in Fig. **2**.

Additionally, *At*H6H possesses the HXD…H motif (residues His217, Asp219, and His274, respectively) that has been found in other 2-oxoglutarate dependent dioxygenases from plants, fungi and bacteria. This sequence element has been considered to be an iron-binding site [9], [10].

Recombinant expression and purification of AtH6H

The ORF fragment of *At*H6H was sub-cloned into the expression vector pET-32a (+) and expressed in *E. coli* strain BL21trxB (DE3) cells. A protein of the appropriate size (57 kDa) was expressed in the soluble form determined by SDS-PAGE. The resulting soluble enzyme fraction was treated by HiTrap affinity chromatography followed by DEAE Sepharose Fast Flow chromatography to give the recombinant *At*H6H (Fig. **3**). The purified protein was used for the enzyme assay and catalytic characterization analysis.

Biochemical characterization of the recombinant AtH6H

The catalytic functions and characters of the recombinant *At*H6H were investigated in this study. The recombinant *At*H6H generated different products depending on the substrates supplemented. The enzyme reaction mixture containing hyoscyamine as the substrate yielded two reaction products with retention times of 2.33 min and 2.47 min on HPLC, which were corresponding exactly to the standards of 6β-hydroxyhyoscyamine and scopolamine, respectively. When 6β-hydroxyhyoscyamine was used as the substrate, only one reaction product was detected and showed retention time of 2.47 min equal to that of scopolamine. No product was detected for the control when the same assay was performed.

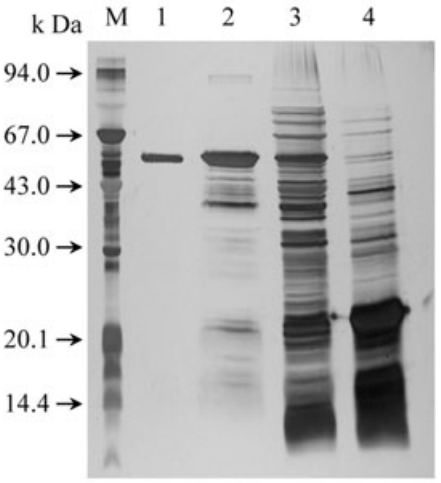

Fig. **3** Silver-stained SDS-PAGE with extracts from the bacteria expressing *At*H6H. Lanes: M, molecular mass marker; 1, pure recombinant *At*H6H (7 μg protein); 2, fractions after HiTrap column (10 μg protein); 3,cell-free extract (15 μg protein); 4, control (15 μg protein, cell-free extract of cells with empty vector).

The biosynthetic products were further prepared and purified by silica gel TLC and gave an NMR spectra identical to that of 6β-hydroxyhyoscyamine and of scopolamine [11], [12], confirming that the enzyme was *At*H6H which could convert hyoscyamine to scopolamine via 6β-hydroxyhyoscyamine [5]. The optical rotation values of the products: 6β-hydroxyhyoscyamine $[\alpha]_D^{20}$: –13.5° (*c* 1.9, MeOH) and scopolamine $[\alpha]_D^{20}$: –18° (*c* 1.5, EtOH).

The optimum temperature was 30 °C for enzymatic activity of the recombinant *At*H6H. Lower temperatures (below 20 °C) were not suitable for the catalysis, whereas higher temperatures (above 50 °C) could denature the enzyme. The pH optimum for the recombinant *At*H6H was found to be at pH of 7.4. The enzyme activity dropped to 20% at pH 5.0 and decreased rapidly when

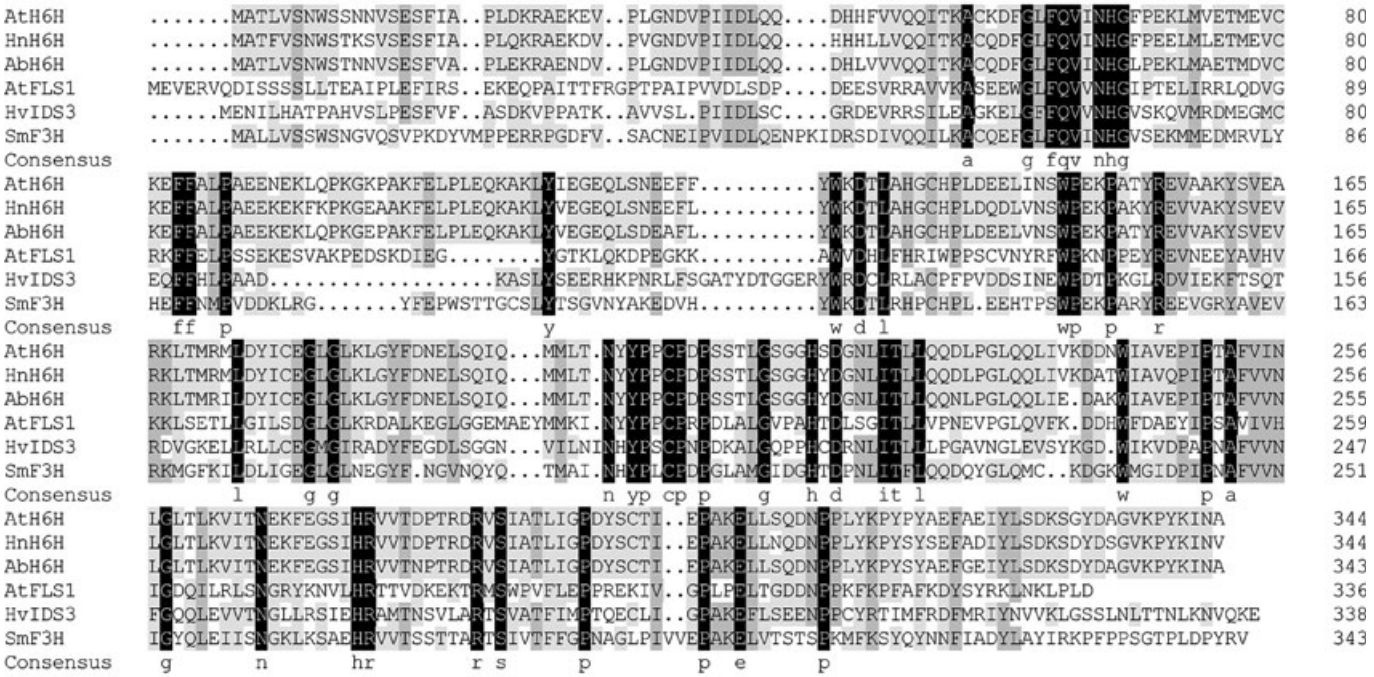

Fig. **2** Deduced amino acid sequence comparison of *At*H6H to other 2-oxoglutarate-dependent dioxygenases. *At*H6H: hyoscyamine 6β-hydroxylase from *Anisodus tanguticus* (GenBank Accession No.AY356396). *Hn*H6H: hyoscyamine 6β-hydroxylase from *Hyoscyamus niger* (D26583). *Ab*H6H: hyoscyamine 6β-hydroxylase from *Atropa belladonna* (AB017153). *At*FLS1: flavonol synthase 1 from *Arabidopsis thaliana* (NM_120951). *Hv*IDS3: iron deficiency-specific protein 3 from *Hordeum vulgare* subsp. *vulgare* (AB024007). *Sm*F3H: putative flavanone 3-hydroxylase from *Saussurea medusa* (AF509338).

the pH reached 8.8. The effect of pH on enzyme activity of *At*H6H was quite similar to that of the available *Hn*H6H except for the optimum pH value (pH 7.8 for *Hn*H6H) [7].

Enzyme activity of the recombinant *At*H6H was strongly inhibited by 5 mM Ca^{2+} (100% inhibition), Cu^{2+} (100%), Mn^{2+} (100%), Zn^{2+} (66%), Fe^{3+} (65%), Ni^{2+} (64%) and EDTA (61%), and slightly by 5 mM Mg^{2+} (14%). In contrast with the reported data [6], Ca^{2+} had a strong inhibitory effect on the *At*H6H enzyme activity, while a slight inhibitory effect was observed on the *Hn*H6H enzyme activity. The enzyme activity was decreased to 14% and 10% in the absence of catalase (2 mg/mL) and sodium ascorbate (4 mM), respectively. Without the addition of Fe^{2+}, only 1.3% of the enzyme activity was detected. It indicated that Fe^{2+} might be an essential cofactor for the enzyme activity [7].

The K_m values for hyoscyamine and 6β-hydroxyhyoscyamine at pH 7.4 were determined to be 15.1 ± 0.3 μM and 17.0 ± 0.2 μM, respectively. The *Vmax* value for 6-hydroxyhyoscyamine (4.2 ± 0.3 nKat/mg protein) was lower than that for hyoscyamine (11.1 ± 0.5 nKat/mg protein). Comparison of the K_m values between *At*H6H and *Hn*H6H indicated that *At*H6H showed a much higher affinity to the substrate than *Hn*H6H [7].

Genomic organization

Genomic DNA of *A. tanguticus* hairy roots was analyzed for the presence of H6H gene. The entire cDNA of *At*H6H was used as the hybridization probe to perform Southern blotting. Genomic DNA digested with *Eco*R I and *Sac* I yielded seven hybridizing bands, whereas *Eco*R V and *Xho* I digestion gave eight fragments (Fig. **4**). At least seven bands could be detected in Southern blotting, which gave a clue that either some *At*H6H isoenzyme genes or some restriction enzyme sites in the introns of *AtH6H* might be present in the genomic DNA of *A. tanguticus* hairy roots. Further investigation is needed to clarify whether more copies or some *At*H6H-like genes would be present in the genomic DNA of *A. tanguticus*.

In recent years, much progress has been made in transgenic plants and cell cultures. The alkaloid composition was improved in *A. belladonna* transformed with H6H gene [13]. Furthermore, by overexpressing the H6H gene in *Hyoscyamus muticus* hairy roots, the best transgenic clone had a 100-fold increase of scopolamine [14]. It also reported that *H. niger* hairy roots overexpressing putrescine *N*-methyltransferase (PMT) and H6H produced significantly higher levels of scopolamine compared with the wild-type [15]. With increased an understanding of the enzymology, molecular genetics and regulation of the tropane alkaloids pathway, metabolic engineering could be another way to provide a potential solution to produce compounds that are rare and not easily obtained.

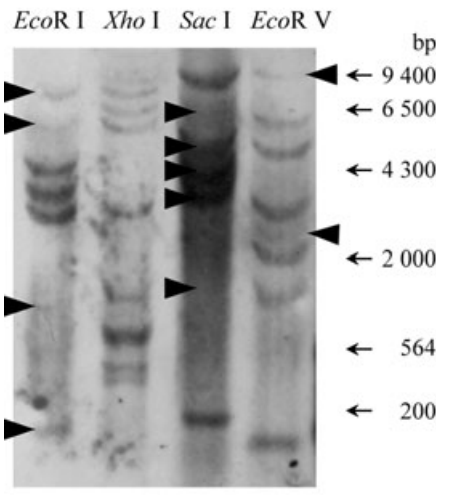

Fig. **4** Genomic Southern blotting analysis of *At*H6H gene. *Xho* I has one recognition site within the cDNA sequence, whereas *Eco*R I, *Eco*R V and *Sac* I have none. Weak signals are marked with arrows.

Acknowledgements

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