Korean J. Medicinal Crop Sci. 12(2) : 135 - 140 (2004)

Production of Monoclonal Antibody about Specific Key Enzyme of Hyoscyamine 6β-Hydroxylase (H6H) in *Scopolia parviflora*

Young Min Kang*, Hee Young Jung*, Seung Mi Kang*, Byung Rae Jin**, Sang Chul Lee**, Byung Hyun Lee***, and Myung Suk Choi*,****†

*Division of Forest Science, Gyeongsang National University, Jinju 660-701, Korea.
**College of Natural Resources and Life Science, Dong-A University, Pusan 604-714, Korea.
***Division of Applied Life Science, Gyeongsang National University, Jinju 660-701, Korea.
****Environmental Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Korea.

ABSTRACT : Total RNAs were isolated from cultured roots of *Scopolia parviflora*, poly(A)$^+$ RNA was obtained through the mRNA purification, cDNA library of Hn*h6h* was constructed. Recombinant baculoviruses in *Spodoptera frugiperda* (Sf) cells were constructed by use of the transfer vector pBacPAK, which has the AcNPV sequence under the polyhedrin promoter. The expression vector carrying Hn*h6h* gene was transferred to *S. parviflora* and obtained transgenic hairy root lines. Our results confirmed the over expression of the H6H protein was used by anti-pBacPAK about cDNAs of *S. parviflora*. This study will served for production of tropane alkaloids by metabolic engineering.

Key words : *Scopolia parviflora*, Sf cell, pBacPAK, AcNPV, polyhedrin promoter, H6H

INTRODUCTION

The solanaceae genera produce a range of biologically active alkaloids that include nicotine and tropane alkaloids. Hyoscyamine and scopolamine, which are found mainly in *Hyoscyamus*, *Duboisia* and *Atropa* species, are used medicinally as anticholingergic agents that act on the parasympathetic nerve system (Hashimoto *et al.*, 1993).

Generally, secondary metabolites are produced by the plant cell culture, elicitation, metabolic engineering, feeding of precursors and bioreactor system (Verpoorte & Memelink, 2002). Regulation of secondary metabolic pathways aims to either increase or decrease the quantity of a specified compounds or group of compounds (Hamill *et al.*, 1987). The biosynthetic pathway of tropane alkaloids is initiated from ornithine and arginine, and further hyoscyamine 6β-hydroxylase (H6H), a bifunctional enzyme, is known to catalyze the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine, and the epoxidation to scopolamine (Matsuda *et al.*, 1998). Hence, H6H is a promising enzyme for metabolic and genetic engineering to enhance the yields of scopolamine in plants. In production of metabolites by metabolic engineering, it is very important to detect the expression of foreign genes. For elucidation of expression of key enzyme, preparation of antibody was very important. In order to prepare the antibody, large amounts of purified key enzymes are needed. However, these demerits could probably be due to lengthy purification steps with low recovery, lack of a paid and easy immunological assay system for detecting gene. Shoji *et al.* (1997) reported the

† Corresponding author : (Phone) +82-55-751-5493 (E-mail) mschoi@nongae.gnsu.ac.kr
Received March 20, 2004 / Accepted April 19, 2004.

efficient gene transfer method into various mammalian cells including non-hepatic cells, by baculovirus vectors

Recombinant baculoviruses were constructed by use of the transfer vectors pBacPAK, which has the AcNPV sequence under the polyhedrin promoter (Boublik *et al.*, 1995). Many viral vector have been developed for gene transfer *in vitro, ex vivo* and *in vivo*. Retrovirus, adenoviruses, and adeno-associated viruses are currently used for gene delivery into mammalian for gene therapy (Mulligan, 1993). We have constructed a recombinant baculovirus and this recombinant baculovirus was capable of efficient transcription in many cells. This method could be effective. It was specific on the post-translational regulation of the secondary metabolites related key enzyme in respect of the foreign one. Thus, we are reported here characterization of monoclonal antibody for Hn*h6h* gene by recombinant baculovirus system and conformed the overexpression in transgenic *S. parviflora* hairy root lines.

MATERIALS AND METHODS

Plant materials and *E. coli* strains

S. parviflora plants were obtained from Korea National Arboretum in Korea. Plant culture was performed according to the method of Jung *et al.* (2002). *Escherichia coli* strain used for DNA cloning was XL1-Blue MRF (Stratagens, USA). The expression of fusion protein was performed in *E. coli* BL21 (DE3) pLysS.

Construction and screening of cDNA library

Total RNAs were isolated from cultured roots of *S. parviflora*, and cDNA library constructed *Hyoscyamus niger* hyoscyamine 6β-hydroxylase (Hn*h6h*). Poly(A)+ RNAs were obtained by using an mRNA purification kit. The cDNA library was constructed by using a ZAP-cDNA Synthesis Kit (Stratagens, USA) and ZAP-cDNA GigapackIII Gold Cloning Kit. Screening of the cDNA library was performed according to Sambrook *et al.* (1989). Duplicate plaque filters (Amersham, UK) were hybridized with Hn*h6h* cDNA probe labeled by using Prime a Gene Labeling system (Promega, USA) in 50% formamide, 5×Denhardt's solution, 6×SSC buffer, 0.5% SDS and 0.1 ㎎/㎖ salmon sperm DNA at 42℃ for 16 h. Washing was performed once in 2× SSC and 0.05% SDS at room temperature and twice in 0.2×SSC and 0.1% SDS at 65℃. The washed blots were dried, and then autoradiograph at −70℃ for overnight using the two intensifying screens. After the second round of screening, cDNA inserts were in vivo excised to pBluescript SK(+/−) with an ExAssist helper phage with SOLR strain.

Preparation of antibodies

Recombinant baculoviruses were constructed by use of the transfer vector pBacPAK, which has the AcNPV sequence under the polyhedrin promoter (Boublik *et al.*, 1995). pBacPAK-Hn*h6h* were constructed by excision of the cDNA of pBluescript vector construct by digestion with XhoI, were filled in with Klenow enzyme, and were cloned into the *Xho*I site of pBacPAK after ligation of the *Xho*I site. To express the genes of *S. parviflora*, it was introduced into the baculovirus under the control of the polyhedrin promoter.

AcNPV sequence and recombinant baculoviruses were grown and assayed in *S. frugiperda* (Sf) cells in TC100 medium (Gibco Laboratories, Grand Island. NY) supplemented with 0.26% Bactotryptose broth (Difco, Detroit, MI), 100 ㎍/㎖ kanamycin and 10% fetal bovine serum (FBS). The recombinant baculovirus pBacPAK-Hn*h6h* was generated by homologous recombination of BacPak viral DNA with pBacPAK-Hn*h6h*. These recombinant were purified as follows; culture supernatants were harvested at 3 days after infection, and cell debris was removed by centrifugation at 6,000 rpm for 15 min. at 4℃. Virus was pelleted down by ultracentrifugation at 25,000 rpm in the SW28 rotor (Beckman, Palo Alto, CA) for 60 min. After it was resuspended in phosphate-buffered saline (PBS), it was loaded on 10~60% (w/v) sucrose gradients, and ultracentrifuged at 25,000 rpm in the SW41E rotor (Beckman) for 60 min. The virus band was collected, resuspended in PBS and ultracentrifuged at 25,000 rpm in the SW41E rotor for 60 min. The virus band was resuspended in

PBS, and infectious titers were determined by plaque assay (Shoji *et al.*, 1997).

Mice were injected subcutaneously with 150 μg of recombinant virus emulsified in complete adjuvant. Booster injections of recombinant virus were given with 10-day intervals. After the third immunization, blood was collected and the antiserum was isolated. The IgG fraction of anti-pBacPAK was purified by protein A-Sepharose chromatography.

Genetic transformation of *h6h* gene

The roots of *S. parviflora* were sterilized in 1% (v/v) NaOCl solution with several drops of Triton X-100, vigorously shaken for 25 min., and then rinsed several times in sterile distilled water. Shoots were induced from the roots of *S. parviflora* and then cultured on B5 (Gamborg *et al.*, 1968) basal medium supplemented with 3% (w/v) sucrose and 0.75% (w/v) agar. The pH of the medium was adjusted to 5.8 prior to autoclaving. Subculturing was conducted at 4 weeks intervals. All the cultures were maintained at 25±1℃ in 16/8 h light/dark cycle under cool fluorescent white light. The pEB expression vector was transferred to an *Agrobacterium rhizogenes* strain, KCTC (Korean Collection for Type Cultures) 2703 by the direct transfer method. Stem tissues were incubated with *A. rhizogenes* carrying Hn*h6h* gene.

Hairy roots which appeared 4 weeks after the incubation were cultured separately on B5 basal medium supplemented with 500 mg/ℓ carbenicillin to eliminate the bacteria. Rapidly growing clones were used to establish the cultures of hairy roots. After several subcultures, transformed hairy roots were transferred to half-strength B5 solid medium with 0.75% (w/v) agar and then were also cultured in B5 liquid medium without growth regulators. All the cultures were maintained as described above conditions.

Determination of H6H monoclonal antibody

Roots of native growing plant, and transgenic roots were collected, frozen with liquid nitrogen, and then homogenized. The cell homogenate was suspended in a protein extraction buffer (300 mM Tris-HCl and 1 mM EDTA) and centrifuged at 12,000 rpm for 15 min. The supernatant was measured using the BioRad protein assay kit. The crude root extracts (20 μg /lane) were separated by SDS-PAGE on a 12.5% separating gel according to the method of Laemmli (1970). After electrophoresis, electro-blotting transferred the proteins to a nitrocellulose membrane. Electro-transfer was performed at 13 mA for 1 h in SDS electro transfer buffer (2.9 g glycine, 5.8 g Tris base, 3.7 mℓ 10% SDS and 200 mℓ methanol). Immunoreactions were carried out using anti-pBacPAK as the primary monoclonal antibodies. Unbound antibodies were removed following three washes of TTBS (500 mM Tris-HCl, 1.5 M NaCl and 1% Tween 20) and the antibodies bound to the membranes were detected using peroxide-conjugated anti-mouse IgG as the secondary antibody (Sigma Co.). The antigen protein was detected by chemiluminescence's using an ECL-detecting reagent (Amersham, UK) according to the manufacturer's protocol (Sambrook *et al.*, 1989).

RESULTS AND DISCUSSION

Isolation and sequence analysis of h6h cDNAs

Screening of approximately 4.0×10^4 independent clones from a cultured root cDNA library of *S.*

```
   1 atggctactt ttgtgtcgaa ctggtctact aagagtgttt ccgaaagctt tatagcacca
  61 ttacagaaaa gagcagaaaa agatgttccc gtaggaaatg atgtccctat tattgatctc
 121 caacaacatc atcatcttct tgttcaacaa atcaccaaag cttgtcaaga ttttggtctc
 181 tttcaggtga tcaatcatgg atttccagaa gaactaatgt tagagacaat ggaagtgtgc
 241 aaagagttct ttgcactgcc agctgaggaa aaggaaaagt ttaagccaaa aggagaggca
 301 gctaaatttg aacttcctct tgagcagaaa gcaaagctat atgttgaagg agaacaactc
 361 tctaacgagg agttcttata ctggaaagac actttggctc atggttgtca tcctcttgat
 421 caagacttag tcaattcctg gcctgaaaaa ccagcaaaat atagagaggt ggttgctaaa
 481 tattcagtag aagtgaggaa gttgaccatg aggatgctgg actacatctg tgaaggactt
 541 gggcttaaat tgggctactt tgataatgaa cttagccaaa ttcagatgat gctgactaac
 601 tattacccac catgcccaga cccaagttca acattgggat caggaggaca ctatgatggt
 661 aaccttataa ctttgcttca acaagacttg cctggtttgc aacaactcat tgttaaggat
 721 gctacctgga ttgctgttca acctattcct actgcttttg tcgtcaactt gggattgact
 781 ctaaaggtta ttaccaatga aaagtttgaa ggttctatcc atagggtagt gacagatcca
 841 acaagagaca gggtttcaat tgctactttg attggtcctg attattcatg taccattgaa
 901 cctgctaaag aactactcaa ccaagacaac ccaccactct acaaacctta ttcatattct
 961 gagtttgctg acatttatct aagtgataaa tcagactatg attctggtgt taagccatat
1021 aaaatcaatg tctaa
```

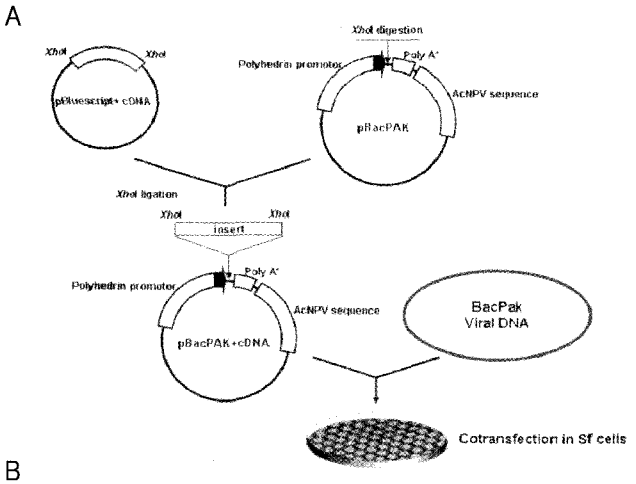
Fig. 1. Nucleotide sequence of a Hn*h6h* cDNA. Total RNAs were isolated from cultured roots of *S. parviflora*, and poly(A)+ RNAs were obtained by using mRNA purification kit. cDNA library was constructed by using a ZAP-cDNA Synthesis kit (Stratagens, USA) and ZAP-cDNA Gigapack III Gold Cloning kit. Screening of the cDNA library was performed according to Sambrook *et al.* (1989). The nucleotide sequences are numbered from the first nucleotide of insert. Hn*h6h* cDNA size is 1.035 bp.

parviflora with a Hn*h6h* cDNA probe resulted in six Hn*h6h* clones. All positive clones were confirmed that they had identical cDNAs by DNA sequencing. A full-length cDNA was referred to as Hnh6h. The cDNA sequence showed an open reading frame of 1,035 bps with a molecular mass of 36 kDa (Fig. 1).

Preparation of H6H monoclonal antibody

To generate recombinant baculoviruses, the baculovirus transfer vector pBacPAK-Hn*h6h* was constructed by inserting cDNA encoding the AcNPV sequence under the polyhedrin promoter, respectively (Fig. 2A). The recombinant proteins should be expressed at very high levels in insert cells under the polyhedrin promoter.

We determined the incorporation of the pBacPAK-Hnh6h proteins into virions and compared to wild type and infected recombinant virus (Fig. 2B). Although



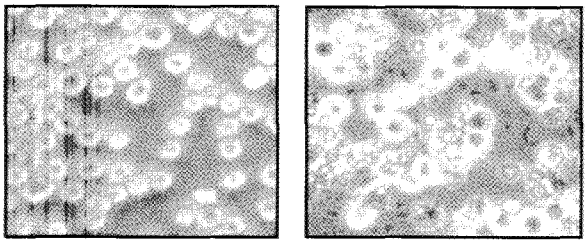


Fig. 2. Construction of pBacPAK recombinant vector and infection of recombinant Sf cells. A: Recombinant baculoviruses were constructed by use of the transfer vectors pBacPAK, which has the AcNPV sequence under the polyhedrin promoter. B: Comparison of wild type and infected with recombinant virus Sf cells.

the expression of AcNPV sequence, the cell infected with the recombinant viruses, the highest level of expression was observed in cells infected with pBacPAK (data not shown).

The purified protein was determinated by Coomassie brilliant blue staining and western blotting (Fig. 3). Recombinant protein was not readily evident in the Coomassie brilliant blue staining gels but was clearly present as demonstrated by western blot analysis. Furthermore, the purified protein possessing 5×10^6 pfu to insect cells were shown to have similar amounts of capsid proteins detected by Coomassie brilliant blue staining gels.

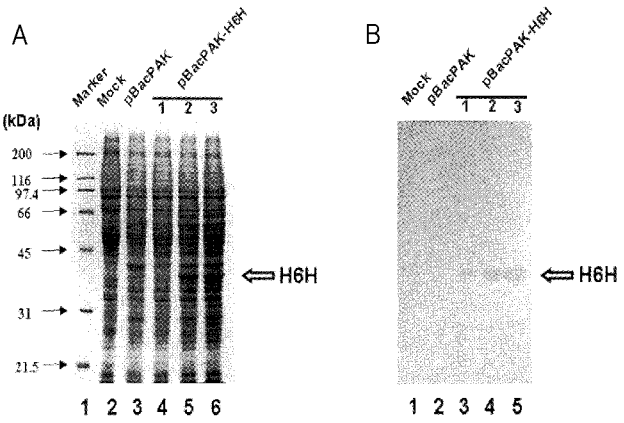


Fig. 3. Purification of pBacPAK recombinant proteins transformed with Sf cells and Western blotting of purified pBacPAK recombinant protein using pBacPAK-Hn*h6h* IgG. A: SDS-PAGE stained with Coomassie blue. It was electrophoresed on 10% polyacrylamide gels containing SDS followed by transfer to nitrocellulose sheets. Lane 1: Molecular weight marker, lane 2: Wild type of Sf mock cell, lane 3: Vector control of pBacPAK, lane 4~6: Recombinant proteins of pBacPAK with Hn*h6h*. B: The sheets were reacted with goat anti-pBacPAK IgG, followed by treatment with ECL substrate (Amersham) was detected for 5 sec. Lane 1: Wild type of Sf mock cell, lane 2: Vector control of pBacPAK, lane 3~5: Recombinant proteins of pBacPAK with Hn*h6h*.

Plant transformation and transgenic hairy roots of *S. parviflora*

The plasmid containing cDNA encoding Hn*h6h* driven by a CaMV 35S promoter with an omega

sequence and the plasmid containing a selectable marker (*npt*II) gene were simultaneously introduced into *S. parviflora* shoot tissue using *A. rhizogenes* KCTC 2703 harboring pEB expression vector (data not shown). Hairy roots were induced from shoot basal tissue after infection of *S. parviflora* shoots with *A. rhizogenes*. The induced hairy root lines were subcultured in B5 medium containing 0.1 ㎎/ℓ IBA for at least 1 month. Hairy root lines were first screened for the kanamycin-resistant phenotype and cultured in half-strength B5 basal medium without antibiotics.

The presence of Hn*h6h* gene in the genome of hairy roots was determined by PCR (data not shown). The DNA of the wild type root did not show any amplified material. However, putative engineered hairy root lines tested a band of 1.3 kb size corresponding to the Hn*h6h* gene fragments, which consequently confirmed the presence of the transgenes in the hairy root lines considered.

Determination of H6H proteins

The protein extracts prepared from transgenic hairy root lines were subjected to SDS-PAGE for Western blot analysis. A 36 kDa protein band that cross reacts with specific pBacPAK antibodies was found in protein extracts derived from transgenic hairy root lines (data not shown). Western blotting using a pBacPAK specific monoclonal antibodies showed that the Hn*h6h* polypeptide of 36 kDa was abundant in several transgenic hairy root lines.

The levels of Hn*h6h* expression in transgenic hairy root lines varied considerably among the 20 lines, respectively. In addition, the expression levels of the key enzyme in the transgenic hairy root lines were two or more times greater than in the wild type (Fig. 4).

Our results confirmed the overexpression of the Hn*h6h* protein in S. parviflora using monoclonal anti-pBacPAK. This could be a different, and it was specific post-translational regulation of the endogenous enzyme in respect of the secondary metabolites biosynthetic gene.

In further studies, to seek the controlling factors for enzymatic activities of Hn*h6h*, we will characterize the clones obtained using differential display, based on root-specific expression pattern of scopolamine biosynthesis enzymes. Thus, this study is contributed for production of tropane alkaloids by genetic and metabolic engineering of the secondary metabolic pathway and confirmation of overexpression key enzyme in *S. parviflora* using the monoclonal antibody.

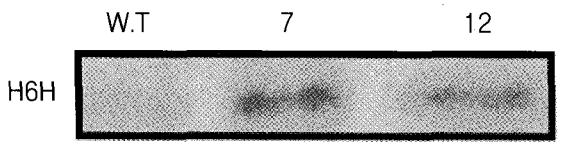


Fig. 4. Western blotting of transgenic hairy root lines. It indicates the specific proteins in Western blots obtained with SDS-PAGE separated proteins from the wild type root and transgenic hairy root lines. It was electrophoresed on 10% polyacrylamide gels containing SDS followed by transfer to nitrocellulose sheets and reacted with goat pBacPAK-Hn*h6h* IgG, followed by treatment with ECL substrate (Amersham) was detected for 5 sec. Lane 1.: Wild type root, Lane 2 and 3: Transgenic hairy root line #7 and #12.

ACKNOWLEDGEMENT

This work was supported by a grant from the Korea Science and Engineering Foundation (KOSEF) to the Environmental Biotechnology Research Center (grant #: R15-2003-002-02001-0).

LITERATURE CITED

Boublik Y, Dibonito P, Jones IM (1995) Engineering the major surface glycoprotein of the Autographa californica nuclear polyhedrosis virus (AcNPV) for the presentation of foreign proteins on the virus surface. Biotechnol. 12:1079-1084.

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of cultures of soybean root cells. Exp. Cell Res. 50:148-151.

Hamill JD, Parr AJ , Rhodes MJC, Robins RJ, Walton NJ (1987) New routes to plant secondary products. Biotechnol. 5:800-804.

Hashimoto T, Yun DJ, Yamada Y (1993) Production of tropane alkaloid in genetically engineered root culture. Phytochemistry 32:713-718.

Jung HY, Kang MJ, Kang YM, Yun DJ, Bahk JD, Chung YG, Choi MS (2002) Production of tropane alkaloids by optimum culture conditions and XAD resin on hairy root cultures from *Scopolia parviflora* Nak. Korean J. Biotechnol. Bioeng. 6:525-530.

Laemmli UK (1970) Cleavage of structural protein during the assembly of the lead of bacteriophage T4. Nature 227:680-685.

Matsuda J, Okabe S, Hashimoto T, Yamada Y (1998) Molecular cloning of hyoscyamine 6βhydroxylase,a 2-oxoglutarate dependent dioxygenase from cultured roots of *Hyoscyamus niger*. J. Biol. Chem. 266:9460-9464.

Mulligan RC (1993) The basic science of gene therapy. Science 260:926-932.

Sambrook J, Fritsch EF, Maniatis T (1989) A laboratory manual, Cold Spring Harbor Lab. Press., Cold Spring Harbor, New York.

Shoji I, Aizaki H, Tani H, Ishii K, Chiba T, Saito I, Miyamura T, Matsuura Y (1997) Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J. Gen. Virol. 78:2657-2664.

Verpoorte R, Memelink J (2002) Engineering secondary metabolite production in plants. Curr. Opin. Biotech. 13:181-187.