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Full Length Research Paper

Cloning and expression pattern of chitin synthase (CHS) gene in epidermis of *Ectropis obliqua* Prout

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***Ectropis obliqua* Prout is a major pest in tea fields. Chitin synthase (CHS) plays an important role in biosynthesis of chitin and growth of the pest. A cDNA sequence encoding the CHS and its expression pattern during development of *E.obliqua* was investigated. The CHS cDNA sequence was 5496 bp nucleotides, with an open reading frame of 4692 bp encoding a protein of 1563 amino acids. It belonged to CHS-A member of CHS gene family. Alternative splice was found in the CHS-A cDNA and the alternatively spliced fragments were 177 bp and shared 65% identity with each other at the nucleotide level. The CHS-A expression was the strongest in the third and fourth instar larvae, during which the growth rate of *E. obliqua* larvae was the rapidest. Catalysis model of CHS-A enzyme in *E. obliqua* was also hypothesized according to the specific motifs and topological structure prediction of the protein. This study provided an important information for further research on development of RNA interference (RNAi) technology to control *E. obliqua*.**

Key words: *Ectropis oblique*, tea pest, chitin synthase, gene cloning, RNAi, biological control.

INTRODUCTION

The insect cuticle protects an insect from external attack and plays an important role in insect wriggling movement. Chitin, a polymer of N-acetyl-β-D-glucosamine, is a major component of insect cuticle. It functions as scaffold material and is always associated with cuticle proteins, which determines the mechanical properties of the cuticle (Merzendorfer and Zimoch, 2003; Merzendorfer, 2006).

Chitin synthase (CHS) catalyzes the chitin biosynthesis and it is indispensable to insect growth and development. Dysfunction of insect CHS at the level of DNA, mRNA and protein is proved to result in abnormal phenotype and high lethality by the experiments of gene mutation (Ostrowski et al., 2002; Devine et al., 2005; Moussian et al., 2005), RNA interference (Arakane et al., 2005; Arakane et al., 2008) and enzyme inhibitor application (Gangishetti et al., 2009). The CHS genes were cloned from many insects, such as *Lucilia cuprina* (Tellam et al., 2000), *Aedes aegypti* (Ibrahim et al., 2000), *Drosophila melanogaster* (Gagou et al., 2002), *Tribolium castaneum* (Arakane et al., 2004), *Manduca sexta* (Hogenkamp et al., 2005), *Spodoptera frugiperda* (Bolognesi et al., 2005), *Plutella xylostella* (Ashfaq et al., 2007) and *Spodoptera exigua* (Chen et al., 2007; Kumar et al., 2008). CHS genes are a gene family and can be classified into CHS-A and CHS-B. CHS-A gene is used to encode the isoform of the enzyme for the synthesis of chitin in the cuticle and tracheae, whereas CHS-B gene is utilized exclusively for the synthesis of peritrophic membrane- associated chitin in the midgut (Tellam et al., 2000; Zimoch and Merzendorfer, 2002; Hogenkamp et al., 2005; Arakane et al., 2005; Zimoch et al., 2005; Kato et al., 2006). CHS-A gene is also responsible to encode the enzyme for chitin synthesis during embryogenesis of insects (Moussian et al., 2005; Rezende et al., 2008). In spite of the increasing information on insect chitin synthases, knowledge on their real structure and catalytic mechanism are rather

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Abbreviation: CHS, Chitin synthase; **PTGS,** post-transcriptional gene silencing; **RNAi,** RNA interference; **RACE,** rapid amplification of cDNA ends; **TMH,** transmembrane helices; **RT-PCR,** reverse transcription polymerase chain reaction; **NPV,** nucleopolyhedrovirus.

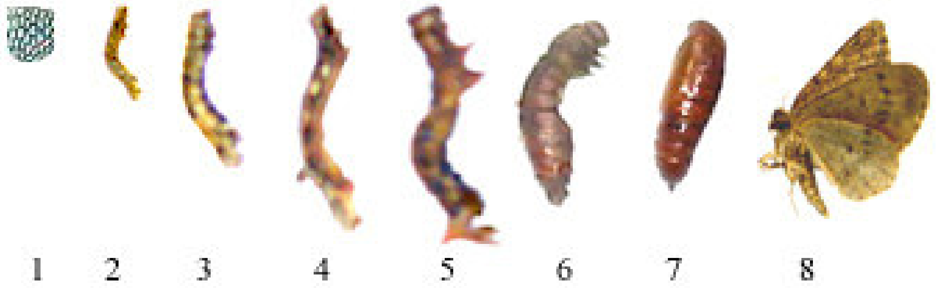

Figure 1. The lifespan of *E. oblique*. 1: egg; 2: 1st instar; 3: 2nd instar; 4: 3rd instar; 5: 4th instar; 6: prepupae; 7: pupae; and 8: adult.

limited.

Ectropis obliqua prout is one of the most serious pests in tea field. It always experiences the development stages of egg, larvae (about 4 instars), prepupae, pupae and adult (Figure 1). In around one month lifespan, the fastest growth and strongest appetite are witnessed during the stage of 3rd and 4th instars. *E. obliqua* was usually controlled using chemical pesticides, resulting in pesticide residues in tea products which were widely concerned by the consumers (Sarmah et al., 2009). Development of biological pesticides to control *E. obliqua* is urgently needed in tea industry. Suppressing the expression of CHS gene or deactivating the catalysis of the enzyme will block chitin synthesis and inhibit the growth and development of the pests, resulting in control of pest population. RNA interference (RNAi) is a specific method to knock out target gene and leads to post-transcriptional gene silencing (Kelly and Fire, 1998). It can be used as an exploring tool for gene function, but also envisaged as an insect control tool through targeting vital genes, although efficient systems of RNAi formulation and delivery must be developed (Bellés, 2010). It was reported that CHS-A specific RNAi disrupted moult of *T. castaneum*, whereas CHS-B RNAi led to cessation of feeding (Arakane et al., 2005). Furthermore, when double-stranded RNA (dsRNA) for CHS-A was injected into male or female pharate adults of *T. castaneum*, all insects died 5-7 days after the adult molt stage, and when adults were treated with dsRNA for CHS-B, insects exhibited little or no chitin in their peritrophic membrane and died about 2 weeks after injection (Arakane et al., 2008). Blocking the chitin synthesis pathway by the infection of nucleo-polyhedrovirus (NPV) recombined with dsRNA gene for CHS, seems to be a potentially effective biocontrol of the *E. oblique* (Liang et al., 2010). The knowledge of the CHS gene sequence and its expression in *E. obliqua* is the prerequisite for developing a RNAi method to control *E. obliqua.* However, there is no study on the CHS gene in *E. obliqua.*

In this study, a cDNA sequence of CHS gene was cloned from *E. obliqua* and its expression pattern during the development of *E. obliqua* larva was investigated. The study will provide useful information for developing an RNAi method to control *E. obliqua.*

MATERIALS AND METHODS

Materials

The *E. obliqua* larvae were reared in pest rearing containers under 27°C and 16 h light/8 h dark cycle conditions and fed with fresh tea leaves. Larvae samples from second instar to fourth instar were collected on the second day post molt. The epidermis of larvae from second to fourth instar, prepupae, and pupae were dissected manually and ground in a mortar with liquid nitrogen, and then stored at -80°C for further use.

TRIzol reagent was ordered from Invitrogen Life Technologies (Carlsbad, CA, USA). Taq DNA polymerase, PrimeScript™ 1st strand cDNA Synthesis kit, 5'/3' RACE kit and pMD18-T vector were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). The degenerated primers (Table 1) were designed according to the insect CHS genes of *A. aegypti* (GenBank accession No. AF223577), *D. melanogaster* (GenBank accession No. AF227729, NM_079485) and *M. sexta* (GenBank accession No. AY062175; AY821560) using the software of PrimerSelect in DNAStar package (DNAStar Inc, Madison WI, USA).

Cloning and sequencing of CHS cDNA

Total RNA was extracted using TRIzol reagent and the first strand cDNA was synthesized by using PrimeScript™ 1st strand cDNA Synthesis kit (Borthakur et al., 2008). A full-length cDNA sequence was obtained by RT-PCR, 5'-RACE and 3'-RACE using the primers in Table 1. The PCR reaction system comprised 0.5 μl 1st strand cDNA, 2.5 μl Mg^{2+} (25 mM), 2.5 μl 10 × buffer (100 mM Tris-HCl, 500 mM KCl, and 0.8% Nonidet P - 40), 0.5 μl dNTP (each 10 mM), 0.5 μl primer each (20 μM), 0.2 μl (2U) Taq DNA polymerase and 17.8 μl dd H_2O. The reactions were performed on a PTC-221 DNA Engine (MJ Research, Waltham, Massachusetts, USA) according to the procedure: preheating at 94°C for 4 min; followed by 35 cycles of 45 s melting at 94°C, 45 s annealing at 55°C and extension at 72°C for 30 s, and finally one more extension cycle at 72°C for 10 min. The PCR products were fractionated by electrophoresis on 1.5% (w/v) agarose gel. The 5'- and 3'- end amplicons were obtained by gene-specific primers and anchor primers (Table 1) using 5'- and 3'- rapid amplification of cDNA ends (RACE) with 5'/3' RACE kit, respectively. The obtained amplicons were cloned into pMD18-T vector and sequenced by the dideoxynucleotide method (Sanger et al., 1977). The full-length cDNA of CHS was obtained by overlapping the fragments using the software of SeqMan in DNAStar package (DNAStar Inc, Madison WI, USA).

Table 1. Primers for cloning CHS cDNA.

PCR fragments	Primers		
	Direction	Type[a]	Nucleotide sequence
F1	Forward	D	5’-CARGARACRAARGGRTGG
	Reverse	D	5’-TTYTCCCAMMARCC
F2	Forward	D	5’-CAARYHACBGSNTTYDTYGTNTGG
	Reverse	D	5’-GTCARARAACWNATCATYTC
F3	Forward	D	5’-AHDYNTGYGCBACWATGTGG
	Reverse	D	5’-GTCGCYTTTTGCAGCCARTG
F4	Forward	D	5’-GTCDCARGTNATGTACATG
	Reverse	D	5’-ARATSARGARCADRTACA
F5	Forward	D	5’-TTCYKATGWTGGTGGGHGC
	Reverse	D	5’-CCVAABGGCCAYTTNAHGTG
3’-RACE		G	5’-ACGAGGCGAGGCATTACGTGC
		A	5’-GTTAGACGCCCTGGCTGAA
5’-RACE		G	5’-GGAACGCGTAACTGAACCCTTG
		A	5’-GGGAATAGCGAAAGCAGCCAAGAT

[a] D: degenerate primer; G: gene-specific primer; A: anchor primer.

Sequence analysis

The CHS cDNA sequence was compared with the CHS sequences registered in the GenBank using the “BLAST-N” and “BLAST-X” tools at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov). The amino acid sequence encoding by the obtained CHS cDNA was deduced using the software EditSeq (DNAStar Inc, Madison WI, USA). The phylogenetic tree was constructed by software MEGA 4.1(Tamura et al., 2007) based on the amino acids homology of the known CHS proteins. A bootstrap analysis was carried out and the robustness of each cluster was verified in 1000 replications. Specific motifs and topology predictions of the CHS protein were carried out by the Predict Protein Server (Rost et al., 2004) through the Web http://www.predictprotein.org.

Investigation of CHS gene expression pattern in various instars of *E. obliqua*

Total RNA was extracted and first strand cDNA was synthesized as above method. The CHS gene expression pattern was analyzed by RT-PCR using the first strand cDNA as template and two primers (forward, 5’-cgtatctccaaagatctaaa; reverse, 5’-ccatttgaagtgaagggag; expected length 126 bp). The PCR reaction system and procedure were the same as above.

RESULTS AND DISCUSSION

CHS cDNA sequence

The full-length sequence of the obtained CHS cDNA from epidermis of *E. obliqua* is 5496 bp nucleotides and it was registered as accession No. EU482034 in GenBank. It has an open reading frame of 4692 bp (Figure 2). It shared 79% identity with the chitin synthase mRNA of *Mamestra brassicae* (GenBank accession No.GQ281761), 78% identity with that of *S. exigua* (GenBank accession No. DQ062153), 77% with *P. xylostella* (GenBank accession No. AB271784) and *M. sexta* (GenBank accession No. AY062175), 73% with *D. melanogaster* (GenBank accession No. NM_169052), and 71% with *T. castaneum* (GenBank accession No. AY291475 and NM_001039402). After comparing to other CHS-A genes, the obtained cDNA of *E. obliqua* belonged to ‘a’ type of the alternately spliced variant because the alternate fragment shared higher identity with the exon ‘8a’ of *T. castaneum* (Arakane et al., 2004), the exon ‘20a’ of *M. sexta* (Hogenkamp et al., 2005), the exon ‘17a’ of *S. exigua* (Chen et al., 2007) and the exon ‘6a’ of *A. aegypti* (Rezende et al., 2008). Another spliced variant (utilizing the ‘b’ type exon) of CHS-A in *E. obliqua* was also cloned by RT-PCR method with using the sequence special primer pairs (Cs1b_F:5'-aaggcggaaaaggcaagg, Cs1b_R: 5'-caccataattagggcgaag, forward primer anchoring the beginning site of alternate domain and reverse primer anchoring the non-alternate domain, the expected length being 267 bp), and it was also deposited in GenBank (GenBank accession No. EU644450). Alignment showed that the alternately spliced cDNA sequence of CHS-A in *E. obliqua* was located between 3896-4072th position (177 bp in length) of the full length cDNA, and shared 65% identity with each other (Data not shown). The length and identity between two type variants were similar to previous findings from other insects (Arakane et al., 2004; Hogenkamp et al., 2005; Chen et al.,2007; Ashfaq et al., 2007; Rezende et al., 2008).

Protein prediction

Predicted protein sequence encoded by the CHS cDNA

```
                         gagcgca cgcgcacgac atgcgcaccg acaacgaccc      37
gcgagtgcac cccaccttac attgtgaagt gacacctaat ctaaacactg attacaaacg      97
cttaacccca ttcctggcta atattcagta acaatggcga cgtcgggggg gagacggcgg     157
                                     M  A  T  S  G  G  R  R  R         9
gaggagggca gcgacaactc cgatgacgag cttacaccgc tcgcgcacga tatttatggc     217
 E  E  G  S  D  N  S  D  D  E  L  T  P  L  A  H  D  I  Y  G           29
ggcagccaac gcacagtaca ggagacaaag ggatgggacg tattccggga gttcccgccg     277
 G  S  Q  R  T  V  Q  E  T  K  G  W  D  V  F  R  E  F  P  P           49
aagcaggaca gtgggtcgat ggagagtcag aaatgcctgg agttcacagt ccgactgctg     337
 K  Q  D  S  G  S  M  E  S  Q  K  C  L  E  F  T  V  R  L  L           69
aagatcttcg cttacgcctt agtgtttatt gtggtcctca gttccggagt gctggccaaa     397
 K  I  F  A  Y  A  L  V  F  I  V  V  L  S  S  G  V  L  A  K           89
ggaaccacgc tctttatgac gtctcagctg aggaaagaca ggcggatagc ttattgtaat     457
 G  T  T  L  F  M  T  S  Q  L  R  K  D  R  R  I  A  Y  C  N          109
aggaatttag gtagagataa acaattcgta gtttcgttac cggacgagga gcgcgtggcg     517
 R  N  L  G  R  D  K  Q  F  V  V  S  L  P  D  E  E  R  V  A          129
tggatgtggg ccatcttggc tgctttcgct attcccgaga taggcgcctt catccgcgct     577
 W  M  W  A  I  L  A  A  F  A  I  P  E  I  G  A  F  I  R  A          149
ttgaggatct gcttcttcaa gtcttcgcgt cgaccgacca gcgcgcagtt cattgttgtg     637
 L  R  I  C  F  F  K  S  S  R  R  P  T  S  A  Q  F  I  V  V          169
ttcgtagccg aaacgcttca caccatctgc gtgggtctgc tgttcttcaa gatcctgcca     697
 F  V  A  E  T  L  H  T  I  C  V  G  L  L  F  F  K  I  L  P          189
gaattagacg tagtaaaagg agcaatggtg acgaactgcc tttgtgtcat tccggcgatc     757
 E  L  D  V  V  K  G  A  M  V  T  N  C  L  C  V  I  P  A  I          209
ctcagcctcc tatccagaaa taaccgcgag tcaaagcgat tcgtcaaagt gattgtggac     817
 L  S  L  L  S  R  N  N  R  E  S  K  R  F  V  K  V  I  V  D          229
atggtagcaa tagtcgctca agtcaccggt ttcatcgtct ggcccctgct ggagaataag     877
 M  V  A  I  V  A  Q  V  T  G  F  I  V  W  P  L  L  E  N  K          249
ccagttttat ggctgatccc aatatcgtgc atctgtatat ctcttggttg gtgggagaac     937
 P  V  L  W  L  I  P  I  S  C  I  C  I  S  L  G  W  W  E  N          269
tatgtcactc ggcagagtcc tataggtata atcaaaagcc ttggcagact caaagatgaa     997
 Y  V  T  R  Q  S  P  I  G  I  I  K  S  L  G  R  L  K  D  E          289
ttgaacttca ctcgctactt cacttaccgg ttcatgtcgt tatggaagat tgtggtcttt    1057
 L  N  F  T  R  Y  F  T  Y  R  F  M  S  L  W  K  I  V  V  F          309
ctcatggtta tcctgttcag catctggact gacggcgatg accccgcgat gttcttccaa    1117
 L  M  V  I  L  F  S  I  W  T  D  G  D  D  P  A  M  F  F  Q          329
ctcttcaatg cgggcttcgg accacacaac atcgttgttg aagaaattca aattcatttg    1177
 L  F  N  A  G  F  G  P  H  N  I  V  V  E  E  I  Q  I  H  L          349
ggtggaacag tgatcccaaa tttagcaaac gtgacgctaa caggcgactc tgtagaagta    1237
 G  G  T  V  I  P  N  L  A  N  V  T  L  T  G  D  S  V  E  V          369
gcagctgcgt ataaatctgc attctacgtg acgttgatcc aaatattcgc agcctacatc    1297
 A  A  A  Y  K  S  A  F  Y  V  T  L  I  Q  I  F  A  A  Y  I          389
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tgttacatct tcggaaagtt tgcgtgcaag attctgattc aagggttcag ttacgcgttc   1357
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cccataaacc tggtgatccc tctggtcgtg aatcttttga tcgctgcttg tggcattagg   1417
 P  I  N  L  V  I  P  L  V  V  N  L  L  I  A  A  C  G  I  R        429
aatggtgaca cctgtttctt ccacggatca gtgcccgact atttatactt cgaaagccct   1477
 N  G  D  T  C  F  F  H  G  S  V  P  D  Y  L  Y  F  E  S  P        449
ccagttttca cgctaagcga cttcatttcc cgacaaatgg cttgggtatg gctcctatgg   1537
 P  V  F  T  L  S  D  F  I  S  R  Q  M  A  W  V  W  L  L  W        469
ctgctgtctc agacatggat cacaatacac atatggacgc cgaaggccga gcgtcttgcc   1597
 L  L  S  Q  T  W  I  T  I  H  I  W  T  P  K  A  E  R  L  A        489
tccactgaga agttgttcgt actgccgatg tataacggac tgcttatcga ccagagcatg   1657
 S  T  E  K  L  F  V  L  P  M  Y  N  G  L  L  I  D  Q  S  M        509
gccatgaata ggaaaagaga cgatcagaag gatgtgaaga ccgaagacct agccgaaatc   1717
 A  M  N  R  K  R  D  D  Q  K  D  V  K  T  E  D  L  A  E  I        529
gagaaagaaa aaggcgacga gtactacgaa accatatcag tgcacacaga caacacgggc   1777
 E  K  E  K  G  D  E  Y  Y  E  T  I  S  V  H  T  D  N  T  G        549
tcttctccca agacggtgaa gtcgtccgac cagataacga ggatctacgc atgcgcgact   1837
 S  S  P  K  T  V  K  S  S  D  Q  I  T  R  I  Y  A  C  A  T        569
atgtggcacg agacgaaaga cgaaatgatg agtttttga cgtctgtgat acggagtgat    1897
 M  W  H  E  T  K  D  E  M  M  S  F  L  T  S  V  I  R  S  D        589
gatgatcaaa gcgcgcgacg agttgctcaa aaatatttgg gtatcgtcga cccagactac   1957
 D  D  Q  S  A  R  R  V  A  Q  K  Y  L  G  I  V  D  P  D  Y        609
tacgagtttg aagcaaacat tttcatggac gactcattcg aaatttccga tcatagctgg   2017
 Y  E  F  E  A  N  I  F  M  D  D  S  F  E  I  S  D  H  S  W        629
gaggacatgc aagtgaatcg cttcgtgaag tgcctcatcg ataccattga tgatgccgtg   2077
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 T  Y  L  L  A  L  D  G  D  I  D  F  K  P  N  A  V  T  L  L        749
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 V  D  L  M  K  K  D  K  N  L  G  A  A  C  G  R  I  H  P  V        769
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 K  A  T  E  H  M  I  G  C  V  L  C  S  P  G  C  F  S  L  F        809
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 R  V  K  A  L  M  D  D  N  V  M  K  K  Y  T  L  T  S  H  E        829
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Q R G Y R V E Y S A A S D A Y T H C P E 869

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G F N E F Y N Q R R R W V P S T I A N I 889

atggacttac tcgcagatta caaacacacg ataaagatca acgacaacat atcgtctcct 2857

M D L L A D Y K H T I K I N D N I S S P 909

tacatagcat accagatgat gttgatgggt ggtacgatcc tgggtcccgg aactatattt 2917

Y I A Y Q M M L M G G T I L G P G T I F 929

cttatgttgg tgggtgcctt cgtggctgct ttccgaatcg ataattggac ttcatttgaa 2977

L M L V G A F V A A F R I D N W T S F E 949

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Y N L Y P I L I F M F V C F T M K S D Y 969

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Q L L V A Q I L S T A Y A M I M M A V I 989

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V G T A L Q L G E D G V G S P S A I F L 1009

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V V P G V I Y L L S I P S M Y L L L I L 1049

tactctatca taaatctgaa caacgtatct tggggaaccc gcgaagtaga ggttaagaag 3337

Y S I I N L N N V S W G T R **E V E V K K** 1069

actaagaagg aaatcgaggc agagaagaag gaagcagaag aggcaaagaa gagggcgaaa 3397

T K K E I E A E K K E A E E A K K R A K 1089

cagaagtctt tgttgggctt ccttcaaggt gtaaacagta atgaagaaga aggatctata 3457

Q K S L L G F L Q G V N S N E E E G S I 1109

gagttctcgt tcgcgggtct attcaaatgt ttattgtgca cgcatccgaa aggcaacgaa 3517

E F S F A G L F K C L L C T H P K G N E 1129

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E K M Q L M H I A S T L D K L E K K L E 1149

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N V E R S I D P H G A S R S R K L S V G 1169

caccgcggga gcacaaacgg cgaccaccag ttagacgccc tggctgaagc cccagaagac 3697

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L I N P Y W I E D P D L K K G E V D F L 1229

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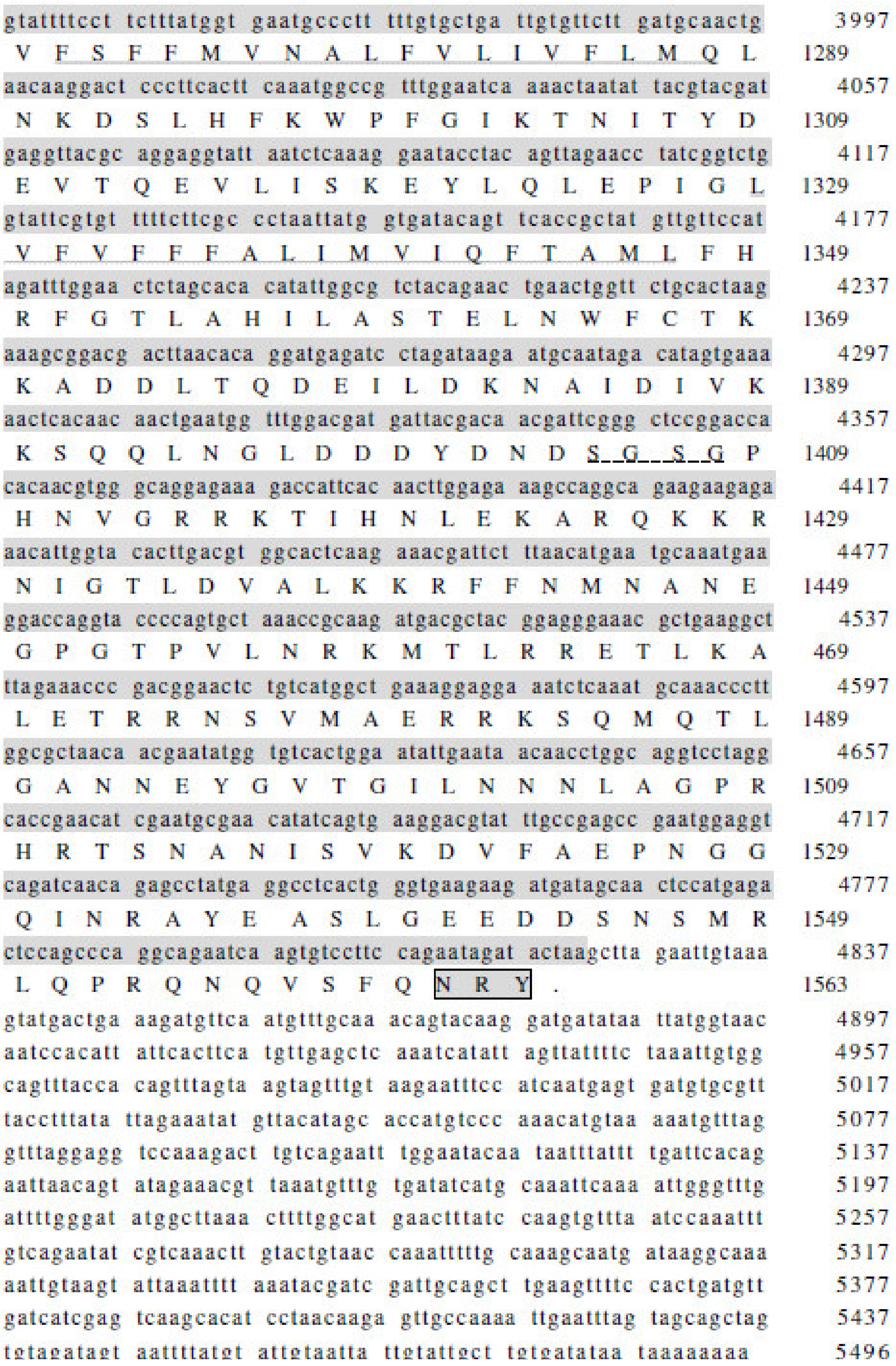

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Figure 2. CHS-A cDNA sequence and deduced amino acids sequence from *E. obliqua*.
Shade nucleotides: open reading frame; amino acids with solid underline: the conserved motif; amino acids with dash underline: glycosaminoglycan attachment site; amino acids with wave underline: transmembrane helix; amino acids with box: membrane lipoprotein lipid attachment site; amino acids with bold font: coiled-coil domain; and amino acids with shade box: the microbodies C-terminal targeting signal.

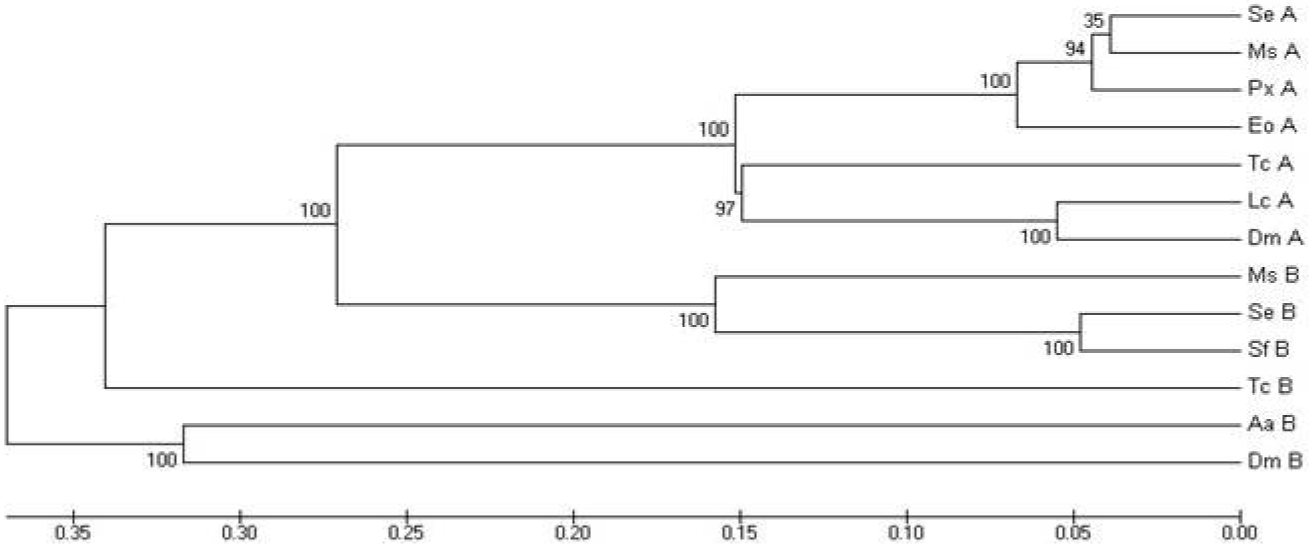

Figure 3. Phylogenetic tree diagram. Aa B: CHS-A from *Aedes aegypti* (AF223577, previously referred to as CHS-A and now as CHS-B); Dm A: CHS-A from *Drosophila melanogaster* (NM_079509); Dm B CHS-B from *Drosophila melanogaster* (NM_079485); Eo A: CHS-A from *Ectropis obliqua* (EU482034); Lc A: CHS-A from *L. cuprina* (AF221067); Ms A: CHS-A from *Manduca sexta* (AY062175); MS B: CHS-B from Manduca sexta (AY821560); Px A: CHS-A from *Plutella xylostella* (AB271784); Sp A: CHS-A from *S. exigua* (DQ062153); Sp B: CHS-B from *Spodoptera exigua* (DQ912929); Sf B: CHS-B from *S. frugiperda* (AY525599); Tc A: CHS-A from *T. castaneum* (AY291475); Tc B: CHS-B from *T. castaneum* (AAQ55061).

from *E. obliqua* has 1563 amino acid residues, with molecular weight (MW) about 178.3 kDa at a p*I* 6.7. Phylogenetic analysis of the *E, oblique* CHS protein sequence (EoA) showed 88% identity with that of *S, exigua* CHS-A (SeA), 86% with *M, sexta* CHS-A (MsA), 85% with *P, xylostella* CHS-A (PxA), 74% with *D, melanogaster* (DmA), and 69% with *T, castaneum* (TcA) (Figure 3). The identity of the EoA to CHS-A proteins was significantly higher than that to CHS-B (Figure 3), suggesting that the obtained cDNA seq-uence be belonged to CHS-A instead of CHS-B.

It is known that the conserved motifs play a key role in activity of chitin synthase and are related to the evolution of fungi and insects. There were 11 conserved motifs in the CHS-A protein of *E. obliqua* and they were showed as amino acids underlined (amino acids No.568 - 586, 631 - 648, 669 - 673, 686 - 700, 733 - 738, 762 - 766, 778 - 786, 797 - 810, 839 - 847, 865 - 877 and 1059 - 1063) in Figure 2. The conserved motifs 5-11 are usually found in insect, nematode and fungi while conserved motifs 1 - 4 coexist in insect and nematode.

The result of predicted protein showed that CHS-A enzyme of *E. obliqua* was a transmembrane protein. According to the previous studies, such as Duran and Cabib (1978) and Zhu et al. (2002), the chitin synthase was presumed to be located in plasma membrane. At the moment, there are many arguments about the enzyme location due to lack of direct evidences, such as in the plasma membrane or in the transport vesicles or chitosomes, however, many reports were apt to believe that insect chitin synthases might reside in plasma membrane together with some co-factors for helping orientation of chitin fibril (Tellam et al., 2000; Arakane et al., 2004; Hogencamp et al., 2005; Merzendorfer, 2006; Gangishetti et al., 2009) because chitin fibrils had never been observed to be present within a vesicle or free in the cytoplasm of insect cells (Reynolds, 1987; Tellam et al., 2000). Meanwhile, another polysaccharide synthesis enzyme with similar catalysis mechanism, cellulose synthase, was also predicted to be associated with plasma membrane (Doblin et al., 2002). Therefore, the CHS-A enzyme of *E. oblique* might be a plasma membrane-associated protein. The prediction also showed that the conserved motifs 1-10 were located in the catalytic center domain (domain B) which faced cytoplastic direction, and the conserved motif 11 was located ahead of the coiled-coil domain which faced extracellular direction (Figures 2 and 4). There were ten transmembrane helices (TMH) at N-terminal and seven TMH at the C-terminal (Figure 4). The TMH amount of CHS-A from *L. cuprina* (Tellam et al., 2000), *M. sexta* (Zhu et al., 2002; Hogenkamp et al., 2005), *T. castaneum* (Arakane et al., 2004) and *A. aegypti* (Rezende et al., 2008) was reported to range from 15 to 18. From this view, CHS-A enzyme of insects possesses the similar topological structure. Interactions between transmembrane helices play an important role in the regulation of diverse biological functions. Modest but specific stabilization of helix associations is realized via packing of complementary small and large groups on neighboring helices. A highly conserved dissociable motif plays a vital and widespread role as an on-off switch that can integrate with other control elements during integrin

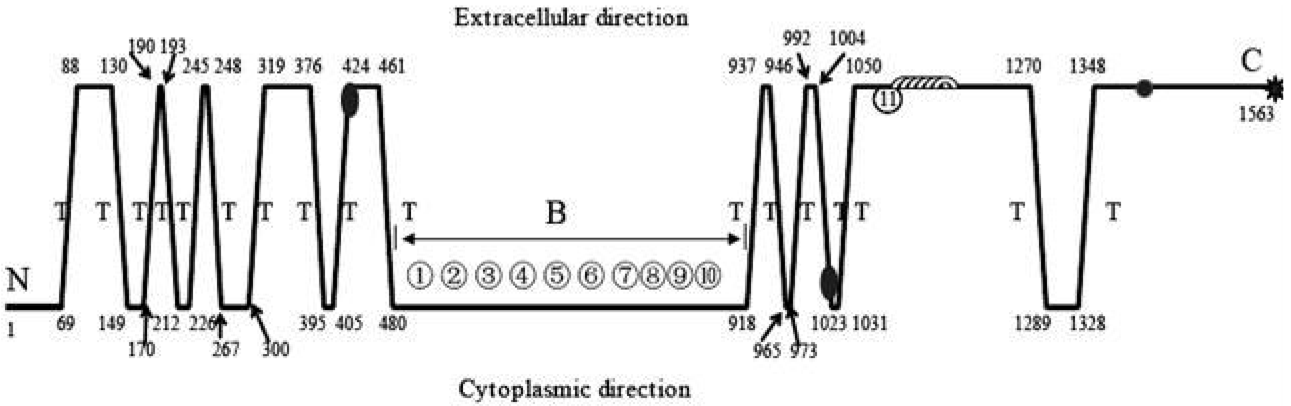

Figure 4. Predicted topology structure of CHS-A protein in *E. obliqua*. N: N-terminal; B: catalytic center domain; C: C-terminal; T: transmembrane helix; ⬮: membrane lipoprotein lipid attachment site; 〰: coiled-coil domain; ●: glycosaminoglycan attachment site; ✱: microbodies C-terminal targeting signal; numbers in circle: 11 conserved regions; number at the bottom: the amino acid number from N-terminal to C-terminal.

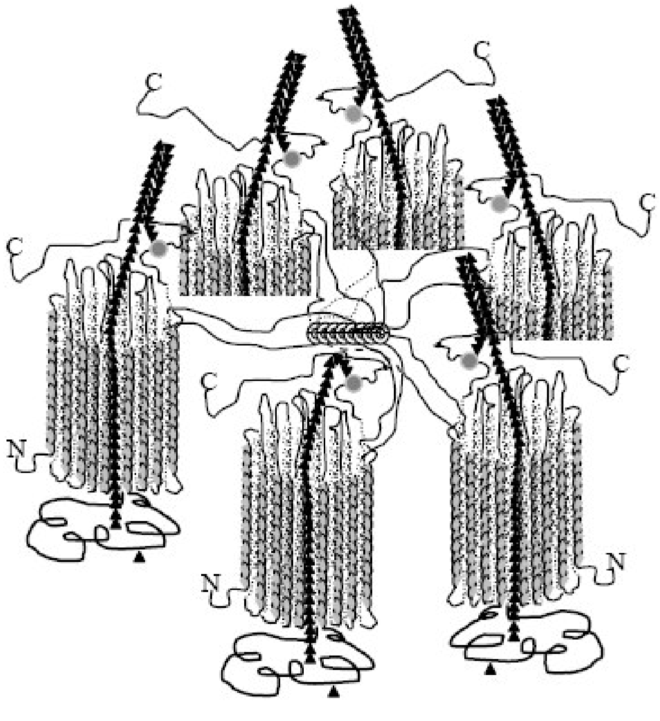

Figure 5. Hypothetical cooperative catalysis model of oligomerized CHS-A and formation of alpha-chitin in epidermis of *E. obliqua*. N: N-terminal; C: C-terminal; Shaded column: transmembrane helix; 〰: coiled-coils domain; ●: glycosaminoglycan attachment sites; Chains consisted of dark triangles: chitin chain, each triangle represented a residue of N-acetyl-glucosamine.

activation (Berger et al., 2010). The structure of multiple transmembrane helices might not only help the enzyme associate itself with membrane, but also be involved in the formation of hydrophobic pore responsible for the translocation of the nascent chitin chain. The coiled-coil domain (amino acid No. 1064 - 1094) and the glycosaminoglycan attachment site (amino acid No. 1405 - 1408) were located in the C-terminal (Figure 4). The helical coiled-coil is one of the principal subunit oligomerization domains in proteins. The architecture of a particular coiled-coil domain determines its oligomerization state, rigidity and ability to function as a molecular recognition system (Burkhard et al. 2001). Interestingly, cellulose synthases from plants, which have some similarities with chitin synthases, are organized in rosettes consisting of six oligomerized subunits (Doblin et al., 2002). It implied that the CHS-A enzyme of *E. obliqua* might be organized as oligomerized plasma membrane plaques in epidermis via coiled-coil domain in similar manner with the cellulose synthase (Figure 5). According to the analysis result of PredictProtein software, almost all of the CHS-A from other insects also possess the glycosaminoglycan attachment site whereas CHS-B lacks this domain (Data not shown). The glycosaminoglycan attachment sites might be associated with delivery and fold of the chitin chain (Merzendorfer, 2006). When the nascent chitin chain was delivered through the pore formed by multiple transmembrane helices, the head of chitin chain might be captured by the glycosaminoglycan attachment site. Along with the extension of the chain, double strands were folded and strengthened via the formation of intramolecular or intermolecular hydrogen bonds in antiparallel orientation (Figure 5). Chitin strands assembled in antiparallel orientation is called alpha-chitin (Imai et al., 2003). Many studies in insects showed that CHS-A expressed only in epidermis and trachea (Tellam et al., 2000; Hogenkamp et al., 2005; Arakane et al., 2004; 2005; Chen et al., 2007). The protein structure and its tissue expression mode of CHS-A might be explained that alpha-chitin was mainly observed in cuticle.

There was a membrane lipoprotein lipid attachment site at N-terminal (amino acid No. 416-426) and at C-terminal

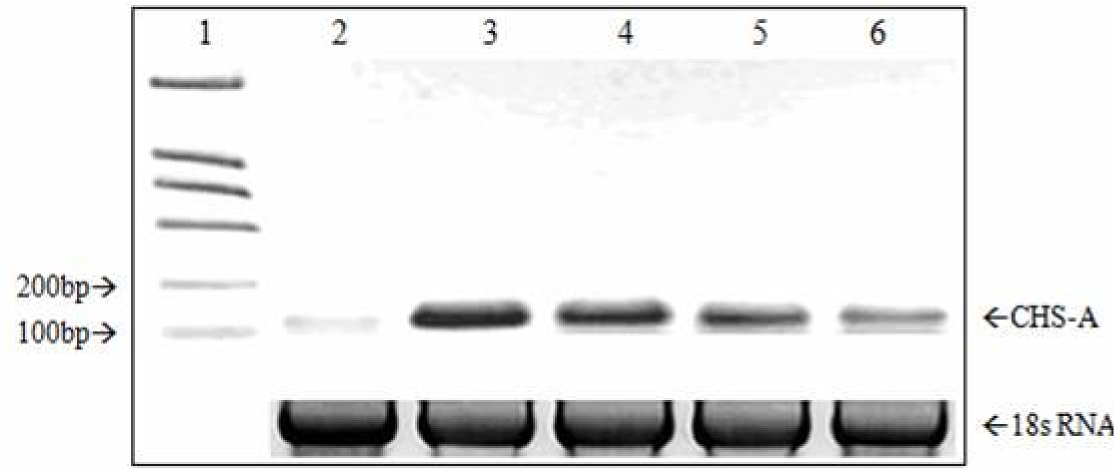

Figure 6. Expression pattern of CHS-A in epidermis **of** *E. obliqua***.** Lane 1: DNA maker DL2000 (TAKARA); lane 2: 2nd instar larvae; lane 3: 3rd instar larvae; lane 4: 4th instar larvae; lane 5: prepupae; lane 6: pupae.

(amino acid No. 1011 - 1021), respectively. The membrane lipoprotein lipid attachment site will help the chitin synthase be bound tightly on membrane lipids. There was a microbody C-terminal targeting signal (amino acid No. 1561 - 1563) at the end of C-terminal (Figure 4), which was also observed in CHS-A amino acid sequence from *D. melanogaster* (Moussian et al., 2005). This signal might be responsible for the recognition of CHS-A enzyme by the cytoplasmic transporting vesicles (Bairoch et al., 1997). These structures might be important for translocation and stabilization of the CHS-A enzyme in *E. obliqua*.

Expression pattern of CHS-A in *E. obliqua*

The expression strength of CHS-A in epidermis varied with the development of *E. obliqua.* It was the strongest in the third and fourth instar larvae, the prepupae the next, while the second instar larva and the pupae the weakest (Figure 6). Observation in insect rearing showed that growth rate of *E. obliqua* larvae was the rapidest during the third and fourth instar stages. It might be that a large amount of chitins were needed for the structure of cuticle during these stages, resulting in high level of the CHS-A expression. Although CHS-A of *T. castaneum* was expressed predominantly in the embryonic and pupal stages (Arakane et al., 2004), it was required for the chitin synthesis during all three types of moult stage (larval–larval, larval–pupal and pupal–adult), in detail, the 'a' type spliced variant was necessary for both the larval–larval and larval–pupal moults while 'b' type only for pupal–adult moult (Arakane et al., 2005). CHS-A of *M. Sexta* expressed relatively constant in the epidermis during the feeding stage of the fifth instar, and gradually increased during the larval–pupal moult stage (Zhu et al., 2002). However, the relative transcript amounts of the two spliced variants also varied substantially during development, i.e., the ratio of 'a' type spliced variant to 'b' type was high during the larval feeding and pupal stages in the epidermis, while the ratio was the lowest during the prepupal stage (Hogenkamp et al*.,* 2005). The transcript of 'a' type CHS-A variant increased highly at 9–12 h after egg laying during the *A. aegypti* chitinized serosal cuticle formation, while the transcripts of 'a' and 'b' type variants expressed simultaneously during larval cuticle synthesis (Rezende et al., 2008). CHS-A of *S. exigua* highly expressed at a certain level in the cuticle during the early and late stages of each larval instar, and consistently expressed in high level during the pupal stage (Chen et al., 2007). Relatively higher expression of CHS-A was observed in third instar and pupal of the *P. xylostella* (Ashfaq et al., 2007). Transcription of the CHS-A increased concurrently with the pupal lamellate procuticle formation of *D. melanogaster* after pupariation (Gagou et al., 2002). Furthermore, the CHS-A expression was not affected in *D. melanogaster* and *P. xylostella* (Ashfaq et al., 2007; Gangishetti et al., 2009) or was enhanced in *Anopheles quadrimaculatus* (Zhang and Zhu, 2006) by the chitin synthesis inhibitors, which implied that the effect of the inhibitors might play the role on the level of the enzyme activity or the substrates availability instead of transcription and products translocation. The expression pattern of CHS-A from *E. obliqua* was in some cases similar to that from *P. xylostella* and *S. exigua*.

Catalytic function of CHS-A enzyme is required for morphogenesis of insects and nematodes. When gene specific interference dsRNA for CHS-A was injected into *Tribolium*, the development of beetles was blocked; the beetles showed abnormal phenotype of cuticle, and then died consequently (Arakane et al., 2005; 2008). After CHS-A specific dsRNA was delivered into the *Caenorhabditis elegans* by microinjection or feeding, the eggs were fragile and permeable to small molecules, and the embryonic cell division ceased (Zhang et al., 2005). Interference of CHS delayed emergence of juveniles in *Meloidogyne artiellia* by soaking the eggs with dsRNA (Fanelli et al., 2005). Studies about gene mutation (Devine et al., 2005; Moussian et al., 2005) and enzyme inhibitor (Gangishetti et al., 2009) also showed that CHS genes and their products were essential for the development of the *D. melanogaster*. These results showed that RNAi for CHS can be used as an efficient tool to control the population of pests because of its high efficiency of post-transcriptional gene silencing (PTGS). Double-stranded RNA applied in these experiments was synthesized *in vitro* and easily broken down in environment, and therefore, not suitable for application in fields. Alternative approach should be explored by using self-reproducible system of dsRNA. NPV is a good vector for expressing foreign genes in insect cells because it can infect insects efficiently and exclusively (Luckow and Summers, 1988). Recently, Liang et al. (2010) established a novel method for producing CHS dsRNA *in vivo* in insect cells with the help of the NPV infection and expression system. In that report, an inverted repeat CHS gene fragment was inserted into NPV, which can be transcribed into a long double-stranded hairpin RNA homologous to endogenous CHS gene, and then RNA interference was triggered after the recombinant NPV infected the insect cell. Therefore, gene cloning and expression pattern of CHS-A from insects will help for accumulating the information about the catalytic behavior of CHS enzyme and for designing the novel biological control strategy targeted to CHS gene and its product.

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