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The gene, expression pattern and subcellular localization of chitin synthase B from the insect *Ostrinia furnacalis*

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ABSTRACT

Insect midgut peritrophic membrane (PM) is a functional structure that protects insects against chemical damage and microorganism infection. The essential component in PM is chitin and its synthesis is catalyzed by Class B chitin synthase (CHSB), which plays a unique role in chitin-containing organisms and thus represents a potential target for eco-friendly pesticides. cDNA and gDNA of *CHSB* from a widely spread pest *Ostrinia furnacalis* (*OfCHSB*) were obtained and their sequences and transcription patterns were characterized. Results indicated that *OfCHSB* may be indirectly stimulated by ecdysone because the binding sites of only early ecdysone-inducible elements (BR-C and E74A) rather than ecdysone response elements (EcR and USP) were found within the core promoter of *OfCHSB*. In addition, the transcripts of *OfCHSB* increased *in vivo* at the feeding stage of the 4th and 5th instar larvae. The subcellular localization of OfCHSB was studied using an insect midgut cell line. Puncta structures of the recombinant OfCHSB were observed co-localized with Golgi marker Man II-GFP, suggesting a possible localization of chitin synthases under physiological conditions.

1. Introduction

The insect midgut contains a functional structure, the peritrophic membrane (PM), which protects insects against the abrasion of food and invasion of microorganisms [1]. Contained within the PM, chitin is a linear homopolymer of β-1, 4 N-acetylglucosamine and forms a fibrous component structure. Insect chitin synthase B (CHSB) is a crucial enzyme involved in the biosynthesis of chitin in PM. It has been studied as an ideal target for the design of environmental friendly agent to control pests [2].

CHSB is one of the two chitin synthases (*CHSA* and *CHSB*) in insects. These two classes of chitin synthases are closely related and share some basic properties in common. They both have a larger molecular weight of about 170 kDa and contain more transmembrane helices (about 16–17) compared to fungal enzymes. According to the amino sequences, insect chitin synthases contain three domains: an N-terminal transmembrane domain, a central catalytic domain and a C-terminal transmembrane domain [3]. In the catalytic center, all the chitin synthases share some conserved motifs such as "DXD", "EDR", and "QRRRW", which contribute to divalent cation binding, catalysis, and substrate binding, respectively, [3].

CHSA and *CHSB* can also be clearly differentiated from each other according to their properties and functions. *CHSB* is shorter than *CHSA* and encodes a smaller protein with a lower pI. The genome of *CHSB* contains no alternative splicing site while that of *CHSA* does. They can also be distinguished by expression patterns due to their different functions. *CHSA* is responsible for the synthesis of cuticular chitin [4], and is mainly expressed at larval–larval and larval–pupal molting time [5–7]. However, *CHSB* is mainly expressed during the formation of peritrophic membrane at the feeding stage [8–10]. For example, the transcripts of *MsCHSB* from *Manduca sexta* were detected at the 5th instar [11] and *TcCHSB* from *Tribolium castaneum* was shown to be mainly expressed in late larvae stage as well as adults [12], at which time the insects were actively feeding. Inhibition of the class B chitin synthase will result in insect death because of starvation [10].

The expression levels of insect chitin synthase B change with the molting cycle of the insect and are normally regulated under the control of ecdysterone. A previous study has shown that within the promoter region *of Drosophila metamorphosis CHSB* (*DmeCHSB*), early ecdysone-inducible elements (BR-C and E74A) were present, while ecdysone receptor (EcR) and ultra-spiracle (USP) binding elements were absent [13]. The expression of *DmeCHSB* was up-regulated hours after the ecdysone pulse [14]. These results demonstrated that *DmeCHSB* acted as a late gene in the ecdysone

Abbreviations: EcR, ecdysone receptor; USP, ultra-spiracle; PM, peritrophic membrane; CHS, chitin synthase; Man II, Mannosidase II.

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dependent regulatory hierarchy. However, the mechanism by which *CHSB* is regulated in insects from other orders needs to be investigated further.

Currently, the understanding about the post transcriptional molecular properties of CHSB, such as its subcellular localization is still very limited. The study of *M. sexta* CHSB (MsCHSB) showed that it is localized in the brush border of column cells. Vesicular structures immune-reacted with chitin synthase antibody were also observed in the columnar cell and may be involved in the trafficking of MsCHSB to the plasma membrane [15].

Here we focused on a new class B chitin synthase (*OfCHSB*) from *Ostrinia furnacalis* (Asian corn borer), a lepidoptera pest, which damages crops and cotton in the Asian Pacific region. The cDNA and gDNA of *OfCHSB* were isolated and characterized. The transcription elements within the core promoter of *OfCHSB* were analyzed and the expression patterns of *OfCHSB* during the insect development and in different tissues were studied. The subcellular localization of OfCHSB protein was investigated using an insect midgut cell line.

2. Materials and methods

2.1. Insect cultures

Asian corn borer (*O. furnacalis*) was kindly gifted by Prof. Kang-lai He from the Institute of Plant Protection (IPP), Chinese Academy of Agricultural Sciences (CAAS). *O. furnacalis* larvae were reared using an artificial diet at 26–28 °C [16] under a relative humidity of 70–90% and a photoperiod of 16 h light and 8 h darkness. Insects at different developmental stages were collected for further experiments.

2.2. Preparation of gDNA, total RNA and cDNA synthesis

Genomic DNA was extracted from the fifth instar larvae using a Universal Genome DNA Extraction Kit Ver 3.0 (TaKaRa, China). Total RNAs were collected from different periods of insects using RNAiso Reagent (TaKaRa, China). cDNAs were synthesized from 2 μg total RNA in a 20 μL reaction system using reverse transcriptase M-MLV (TaKaRa, China). All these procedures were carried out according to the manufacturer's instructions.

2.3. cDNA and gDNA cloning and sequencing of OfCHSB

To obtain the sequence of chitin synthase in *O. furnacalis*, degenerated primers were designed according to multiple alignment results of the other insect chitin synthases. The first 246 bp fragment of *OfCHSB* was isolated through PCR using the cDNA from the fifth instar larvae as the template. The degenerated forward primer OfCHSB-F1 (5′-GCNTGYGGNMGNATHCAYCC-3′) was designed according to the conserved amino acid sequence 'ACGRIHP' and the reverse primer OfCHSB-R1 (5′-CCANCKRTCYTCNCCYTGRTCRTAYTG-3′) was based on the conserved amino acid sequence 'YDQGEDRW'. The overlapping fragments of *OfCHSB* cDNA were obtained through PCR and RACE (Supplemental Table S1, Fig. 1A).

The overlapping PCR fragments of the genomic DNA were obtained using pairs of specific primers designed according to the corresponding cDNA sequence of *OfCHSB* (Supplemental Table S2, Fig. 1B). The 5′ and 3′ genomic DNA sequence were determined using a Genomic Walking Kit (TaKaRa, China).

2.4. DNA and protein sequence analysis

The promoter was predicted using the NNPP program at the Berkeley Drosophila Genome Project [17]. The transcription elements were predicted by TESS [18], matInspector [19], and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) programs.

The cDNA sequence of *OfCHSB* was translated into the amino acid sequence by DNAMAN software. Transmembrane helices were predicted using TMHMM v.2.0 and coiled-coil domains were predicted using the Paircoil program [20]. The MW and pI of the protein were predicted by Compute pI/Mw. All these tools for protein analysis were obtained from the ExPASy proteomics website (http://cn.expasy.org/). A multiple sequences alignment was performed by ClustalW and the phylogenic relationship of the insect chitin synthases was generated using MEGA software [21].

2.5. The expression pattern of OfCHSB during the development of O. furnacalis

The expression levels of *OfCHSB* from the 4th instar larvae to adult were detected using real-time PCR. Different larval tissues including epidermis and midgut were also collected and the expression levels of *OfCHSB* were measured.

Gene specific primers were designed according to the specific sequences of *OfCHSB* (F: 5′-CCCGTATCAAAACCGACCTAAA-3′, R: 5′-CAGGAAGATGACCAGCACGA-3′). Housekeeping gene *OfRpS3* (GenBank ID: EU275206) was chosen as an endogenous control (F: 5′-TGCAACGACTACGTCAACACC-3′, R: 5′-TCGGGCTGCGGTTTCTT-3′). cDNAs were synthesized by PrimeScript™ RT enzyme (TaKaRa, China) in a 20 μL reaction system. The real-time PCRs

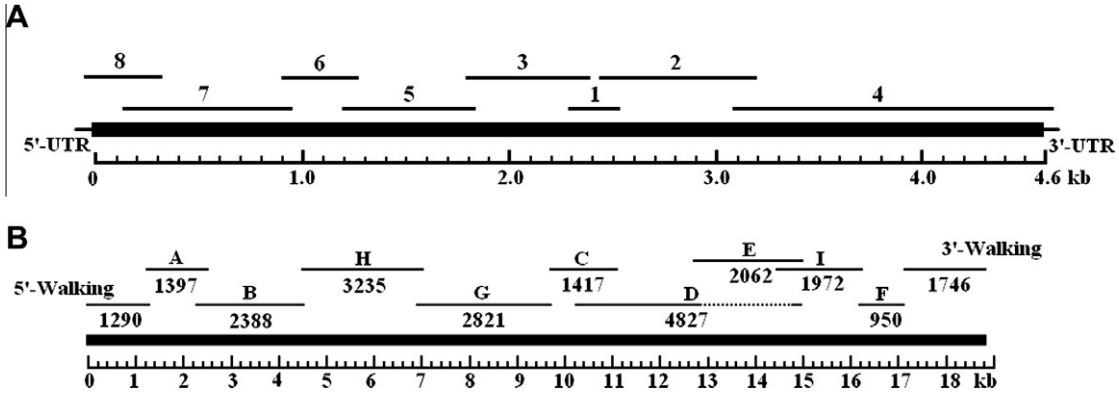

Fig. 1. Cloning strategy of cDNA and gDNA of *OfCHSB*. (A) The full length cDNA of *OfCHSB* was determined by 8 fragments. Fragments 1, 2, 3 and 7 were the PCR products. Fragment 4 was obtained by 3′-RACE and 5, 6 and 8 were the products of 5′-RACE. (B) The *OfCHSB* genomic DNA was obtained through 11 fragments. (A–G) were PCR products amplified using specific primers and the 5′ upstream and 3′ downstream were DNA-Walking products. Each fragment length is given.

were performed using a SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, China) on a real-time PCR detection system (Rotor-Gene 3000). The copy numbers of gene transcripts were normalized through the copy number of the detected gene divided by the copy number of the housekeeping gene (*OfRpS3*).

2.6. Cloning of OfCHSB into insect cells

Insect expression vector pIB-V5/His (Invitrogen) was used to express *OfCHSB* in an insect cell line CF203/2.5 (kindly provided by Prof. Guido F. Caputo from Natural Resource Canada), which originates from the midgut of *Choristoneura fumiferana* and is widely used to study insect proteins [22]. Different constructs, including the full length gene as well as some truncations (NBH5: 512-1143; BH7: 512-1521; BH5: 512-1143) were inserted between the BamHI and SacII sites of pIB-V5/His. The C-terminal V5 tag and 6× His tag were in frame with the chitin synthase gene.

The recombinant expression vectors (pIB-CHSB, pIB-NBH5, pIB-BH7, pIB-BH5) were transfected into the insect midgut cell line CF203/2.5 using cellfectin II (Invitrogen). All the methods were performed according to manufacturers' protocols.

2.7. Western blot and subcellular localization of chitin synthases

For Western blot, each construct was transfected into the insect cell CF203/2.5 in a 6-well format. After 72 h, cells were collected and lysed. The total proteins were separated by 8% SDS PAGE gel and then transferred to PVDF membrane. The membrane was blocked in 3% fat free milk for 30 min and incubated in mouse anti-V5 antibody (1:5000, Invitrogen) overnight. After 3 washes, the membrane was incubated in HRP conjugated goat anti-mouse antibody (1:10,000) for 1 h.

For immunofluorescence, cells were grown on coverslips in a 24-well format. Golgi marker Man II-GFP was co-transfected with chitin synthase. Seventy-two hours after transfection, cells were washed with PBS once and fixed by 4% paraformaldehyde for 20 min. After permeation with 0.1% triton for 5 min, 3% BSA was added and incubated for 30 min to block the unspecific binding. Cells were then incubated with 1:500 mouse anti-V5 antibody plus 3% BSA for 2 h. After washing with PBS 3 times, Cy3 conjugated goat anti-mouse antibody was added for 1 h. Cells were then washed 3 times with PBS, mounted and visualized under a fluorescence microscope.

3. Results and discussion

3.1. OfCHSB cDNA and deduced amino acid sequence

The *CHSB* from *O. furnacalis* (GenBank ID: DQ294306) was obtained based on the DNA fragments amplified by PCR using degenerate primers as well as 5′ and 3′ RACE. *OfCHSB* contains an open reading frame of 4566 nucleotides encoding 1522 amino acids and an upstream region (5′ UTR) of 141 nucleotides. The sequence alignment indicated it was a class B chitin synthase with 74% identity with MsCHSB and named OfCHSB (Supplemental Fig. S1). Like other insect chitin synthases, the OfCHSB was predicted to be a 174 kDa transmembrane protein that contains 16–17 transmembrane helical spans. Its predicted isoelectric point is pH 5.79 showing it is an acidic enzyme similar to SeCHSB (Mw: 174.6; pI: 5.64) and SfCHSB (Mw: 174.3; pI: 5.84). The acidic isoelectric point is conducive to its function in the peritrophic matrix.

OfCHSB was predicted to contain three domains, the putative catalytic domain (residues 512–904) in the middle, the N-terminal domain (residues 1–512) with 9–10 transmembrane helices and the C-terminal domain (residues 904–1512) with 7 transmembrane helices (Fig. 4A). Like other class B insect chitin synthases, OfCHSB has no coiled-coil domains which distinguish class B from class A enzymes [23]. OfCHSB contains all the critical motifs of insect chitin synthases [3], including "DXD", "EDR", "QRRRW" and "SWGTRE" (Fig. S2).

3.2. Genomic DNA structure and promoter region of OfCHSB

In order to further understand the transcriptional regulation of *OfCHSB*, the genomic sequence of *OfCHSB* (GenBank ID: EU258740) was obtained. It is 16,893 bp long, including the 5′UTR and 24 exons (Fig. 2B). Details of the genomic organization are summarized in Supplemental Table S3. As a feature of insect class B chitin synthase, no alternative splicing site was found in the genomic DNA of *OfCHSB*. The genomic structure of *OfCHSB* shares high similarity with other lepidoptera insect enzymes, such as *MsCHSB* [23], *SeCHSB* [9] and the class B chitin synthase from *Bombyx mori* (Fig. 2B). They show high complexity compared to the genomic structures of insects from other orders, such as *T. castaneum* from coleopteran, *Drosophila melanogaster* and *Anopheles gambiae* from dipteran.

The obtained 5′ upstream sequence of *OfCHSB* genomic DNA prior to the translation start site ATG is 1174 bp long. The potential core promoter region (257–304 in Fig. 2A) was predicted by NNPP [17]. The predicted transcriptional start site was further confirmed by 5′ RACE (TaKaRa, China). Since the insect chitin synthases are regulated by ecdysone, potential transcriptional regulatory elements binding sites were predicted within this core promoter region, including ecdysterone response elements EcR (ecdysone receptor), USP (ultra-spiracle) and early ecdysone-inducible elements BR-C, E74A. One consensus sequence for BR-C element at 152–160 and one consensus sequence for E74A at 412–424 were identified. No EcR and USP binding sites [24] were found. The consensus sequences for BR-C and E74A were also predicted to be within the promoter region of *DmeCHSB* [13]. These ecdysone-inducible elements indicate that *OfCHSB* might get involved in an ecdysone dependent regulatory pathway, but not directly stimulated by ecdysone.

3.3. Expression pattern of OfCHSB in O. furnacalis

To understand how the *OfCHSB* is regulated and functions, the tissue- and development- dependent expression pattern of *OfCHSB* were investigated using real-time PCR. Since the expression level of *OfCHSB* changes during the larval–larval, larval–pupal and pupal–adult molting time, the periods from the 4th instar to adult were chosen for study. As demonstrated in Fig. 3A, the transcription level of *OfCHSB* was relatively constant at the 4th instar stage but decreased between the 4th and the 5th instar molting time. Then it gradually increased throughout the whole feeding stage of the 5th instar. It dropped dramatically when the feeding ceased at the 6th day of the 5th instar. At the larval–pupal molting time, only minor amounts of *OfCHSB* transcripts could be detected. During the pupal stages, the transcription level of *OfCHSB* increased at the 3rd day and then dropped at the 4th day, suggesting that the *OfCHSB* might be involved in tissue reconstruction during the pupa–adult metamorphosis.

The tissue specificity expressions of *OfCHSB* in the epidermis and midgut were also determined. The results showed that the *OfCHSB* was mainly expressed in the midgut, whereas the transcripts in the epidermis could hardly be detected (Fig. 3B). Similar to other class B chitin synthases from *M. sexta* [23], *T. castaneum* [12] and *S. frugiperda* [8], *OfCHSB* was expressed in the midgut at the intermolt stage when the peritrophic matrix formed. This

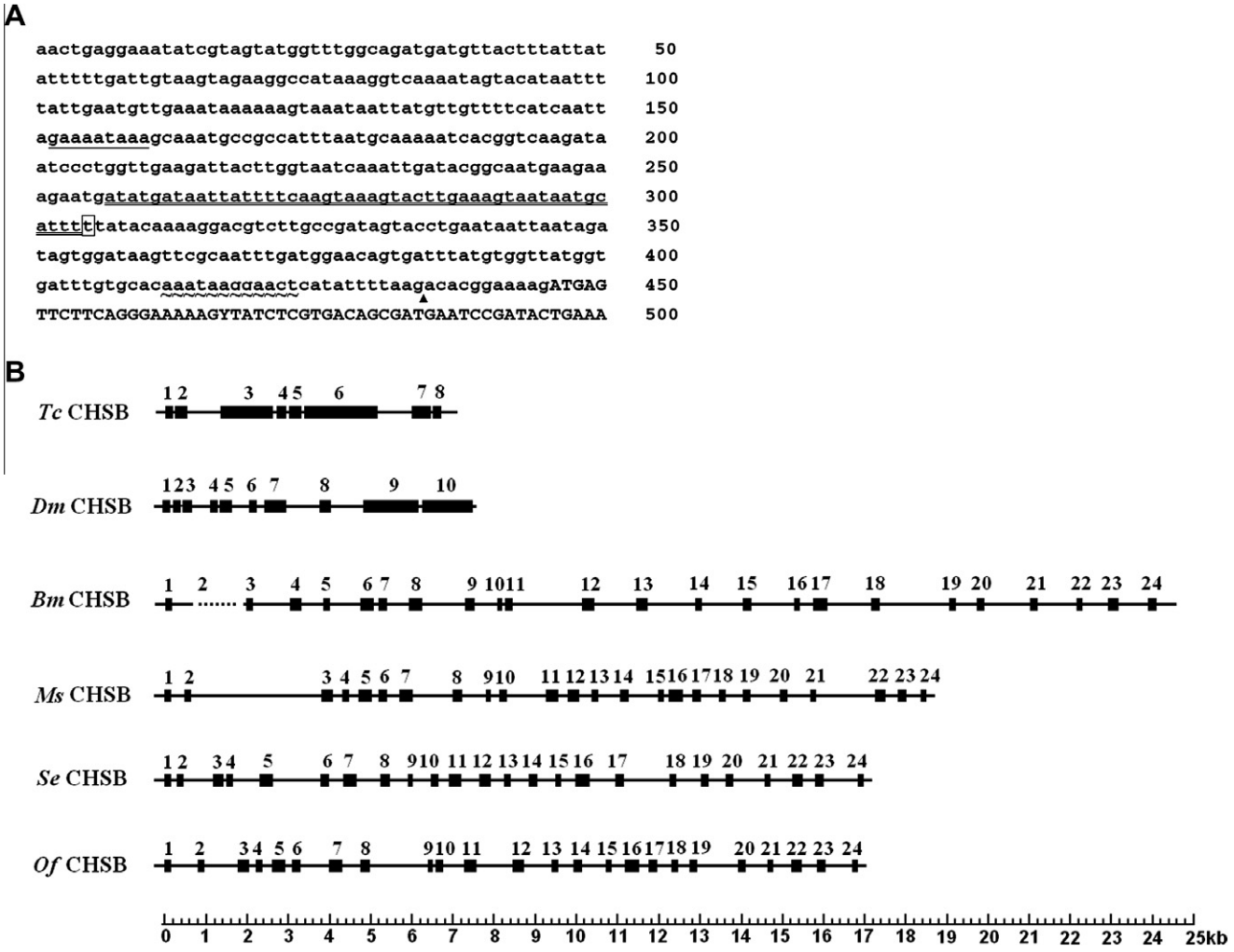

Fig. 2. Promoter region of *OfCHSB* and the genomic structure of insect *CHSB*. (A) The 5′ region of *OfCHSB* genomic DNA. The translated sequence of *OfCHSB* is in upper case letters, the predicted transcriptional start site is open boxed and the promoter sequence is underlined twice. The shadowed arrow head demonstrates where the intron 1 (729 bp long) is inserted. The transcriptional elements BR-C and E74a are highlighted by an underline and a wavy line, respectively. (B) Genomic structures of insect class B chitin synthases. The shaded boxes indicate exons and lines indicate introns as well as the 5′ upstream and 3′ downstream region. The genomic structures of class B chitin synthases were analyzed based on the sequences available in Genbank. *BmCHSB*: *Bombyx mori* BABH01010575; *DmCHSB: Drosophila melanogaster* NT_037436; *MsCHSB: Manduca sexta* AY821561; *OfCHSB*: *Ostrinia furnacalis* EU258740; *SeCHSB*: *Spodoptera exigua* EU622827; *TcCHSB*: *Tribolium castaneum* AY295879.

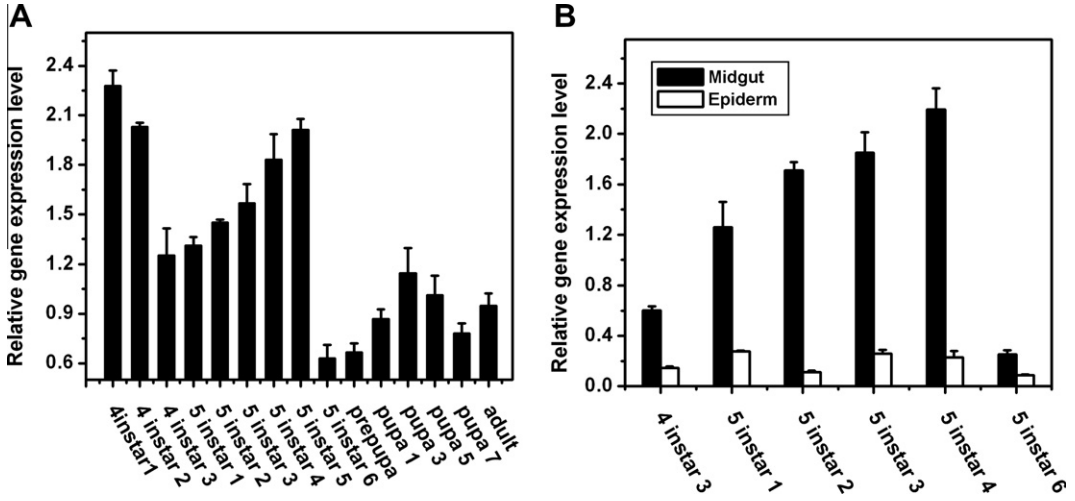

Fig. 3. Expression pattern of *OfCHSB* in *O. furnacalis*. (A) The temporal-specific expression of *OfCHSB*. (B) The tissue-specific expression of *OfCHSB*. The transcription levels of each gene were normalized by the housekeeping gene *OfRpS3*. Each sample had three repeats.

demonstrated that *OfCHSB* was the gene that contributed to the synthesis of chitin in the *O. furnacalis* peritrophic matrix.

The expression pattern of *OfCHSB* also implied that it acted differently from *CHSA*. Although *OfCHSB* changes with the molting cycle of the insect, it does not alter with the ecdysterone level like other ecdysterone directly stimulated genes [25]. The up-regulation of *OfCHSB* appeared very late after the ecdysone pulse, which further indicates that the transcription of *OfCHSB* is not directly stimulated by ecdysterone.

3.4. Subcellular localization of OfCHSB

Besides the transcription pattern, the molecular properties of insect chitin synthase are still unclear. *OfCHSB* was introduced into insect cell line CF203/2.5 to study its subcellular localization. Considering that the N-terminal and C-terminal helices of OfCHSB do not exist in other glycosyltransferases, such as rabbit N-acetylglucosaminyltransferase I [26], β-1, 3-N-acetylgalactosaminyltransferase [27] and yeast chitin synthases Chs1 and Chs2 [28], they might not be essential for the enzymatic activity. Consequently, besides the full length OfCHSB, three truncations with deletion of the N- or C-terminal transmembrane helices (NBH5, BH7, BH5, Fig. 4A) were constructed and expressed in CF203/2.5. NBH5 has the C-terminal with the last two helices deleted; BH7 has none of the N-terminal helices; BH5 has neither the N-terminal helices nor the last two helices from the C-terminal, but contains all the conserved motifs.

The full length OfCHSB was expressed successfully in the insect cell line CF203/2.5 according to Western blot analysis which gave a single band of about 170 kDa corresponding to its theoretical molecular weight (Fig. 4B, lane CHSB). Three other truncations were also expressed well and gave the expected molecular weight bands by Western blot analysis (Fig. 4B, lanes NBH5, BH7 and BH5). Furthermore, the subcellular localizations of the expressed

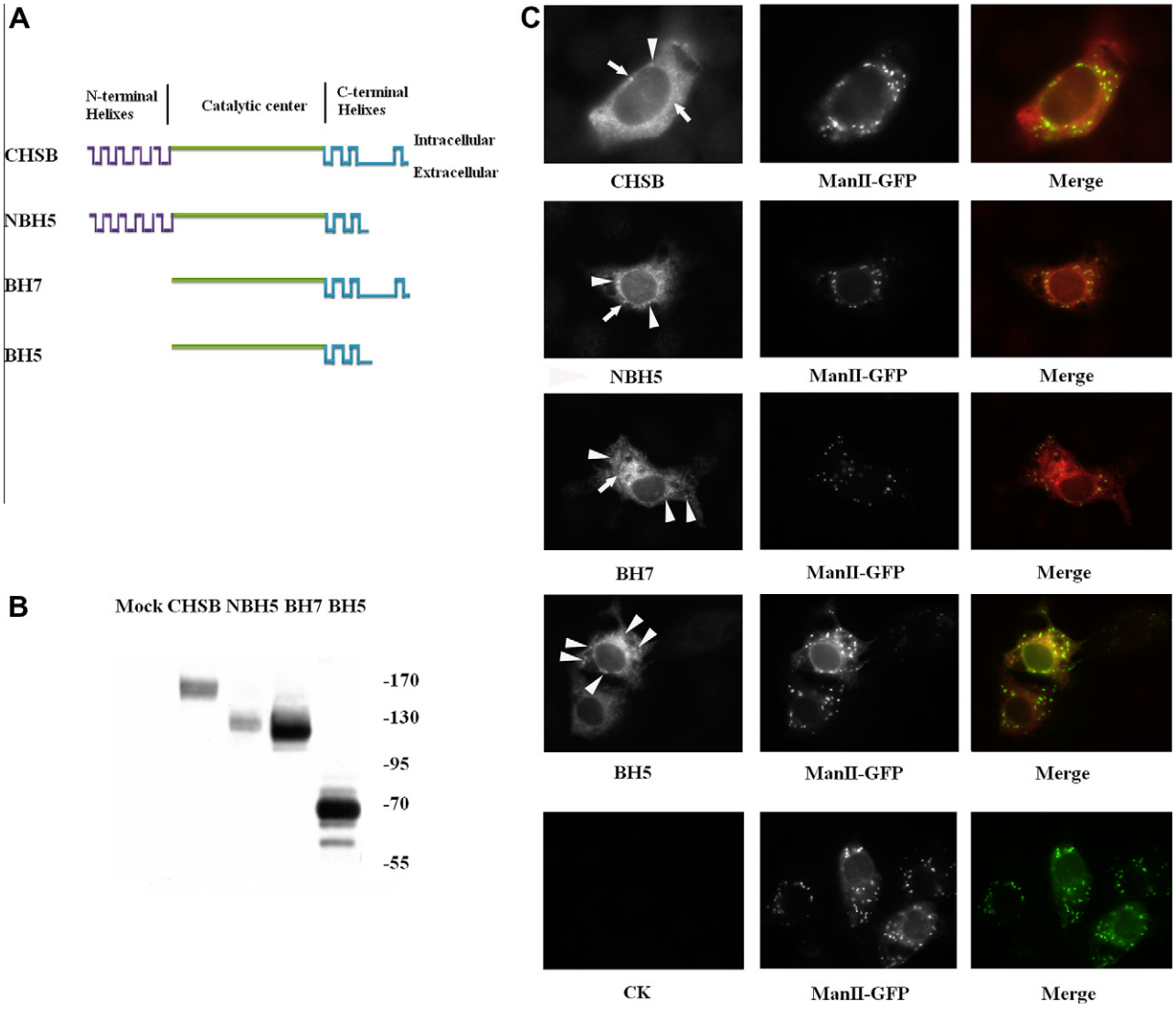

Fig. 4. Subcellular localization of OfCHSB in the insect cell line. (A) Protein structure and cloning strategy of OfCHSB in the insect cell. (B) Western blot of the recombinant proteins. Anti-V5 antibody (1:5000) was used to detect the protein. The molecular weight of the marker was indicated. (C) Immunofluorescence of different OfCHSB truncations in the insect cell line. The red signal indicates recombinant protein and the green indicates Man II-GFP in the merge column. The puncta structures not co-localized with Golgi marker are shown with an arrow while the puncta structures co-localized with Golgi marker are shown with an arrow head. (A) Protein structure and cloning strategy of OfCHSB in the insect cell. (B) Western blot of the recombinant proteins. Anti-V5 antibody (1:5000) was used to detect the protein. The molecular weight of the marker was indicated. (C) Immunofluorescence of different OfCHSB truncations in the insect cell line. The red signal indicates recombinant protein and the green indicates Man II-GFP in the merge column. The puncta structures not co-localized with Golgi marker are shown with an arrow while the puncta structures co-localized with Golgi marker are shown with an arrow head. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proteins were investigated by immunofluorescence. Puncta structures of full length OfCHSB protein were observed, most of which were not co-localized with the insect Golgi marker Man II-GFP (Fig. 4C lane CHSB, arrow), but located around the nucleus of the insect cell. The C-terminal truncation NBH5 showed more puncta structures and a few of them were co-localized with the Golgi marker (Fig. 4C lane NBH5, arrow head). For the BH7 and BH5 truncations, abundant puncta structures were observed and most were co-localized with the Golgi marker Man II-GFP (Fig. 4C lane BH7, BH5, arrow head). Several puncta structures were visible in the cytoplasm close to plasma membrane.

Chitin synthase could either integrate into the apical plasma membrane or reside in the vesicle structures near the cell surface [3]. Puncta structures which immune-reacted with chitin synthase antibody were also observed in the columnar cell of *M. sexta* [15]. This implied that vesicles may get involved in the transport of chitin synthase. Here for the first time, the subcellular localization of recombinant chitin synthase was studied in the insect cell line CF203/2.5. No obvious signals of the recombinant proteins were observed on the plasma membrane. Most of the puncta structures found were co-localized with the Golgi marker in the insect cell and might be involved in the trafficking of chitin synthases to the plasma membrane. The other puncta structures localized close to plasma membrane could be chitosome like structures found in yeast cells [29] or vesicles on their way to the plasma membrane [15]. It is assumed that under physiological conditions, OfCHSB might reside in the Golgi after translation and it will be transported to the plasma membrane or secreted to the cell surface when new chitin is synthesized.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.111.

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