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Cloning and characterization of a novel CRAL_TRIO domain gene *BPHsec1* from brown planthopper

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Abstract

SEC14 superfamily members are essential catalytic components of lipid metabolism and they play critical roles in secretory protein transportation. A putative CRALBPL (cellular retinaldehyde-binding protein-like) gene (Genebank number, KU296276) from the brown planthopper, *Nilaparvata lugens* Stal, named as *BPHsec1*, was cloned and characterized, using rapid amplification of cDNA ends (RACE) method. The full length of this gene is 1,589 bp. Its open reading frame is 939 bp. The gene encodes a 312 amino acids protein, and the estimated molecular weight is around 35.3 kDa. Except the highly conserved SEC14 domain, *BPHsec1* also includes a phospholipid binding pocket and some salt bridge motifs. Reverse transcription-qPCR (RT-qPCR) results showed that *BPHsec1* existed at all different developmental stages of brown planthopper, with the highest mRNA expression in the 1st instar nymph. RNA interference (RNAi) could lead to a significant decrease in *BPHsec1* mRNA expression in brown planthopper, which indicated that RNAi technique could silence *BPHsec1* expression without causing observable morphological abnormalities because these nymphs eventually died. This study will pave the foundation for further functional analysis of *BPHsec1* and its role during the interaction between insects and plant immune responses.

Key words: SEC14, CRAL_TRIO, RNAi, brown planthopper, *Nilaparvata lugens*.

Introduction

Brown planthopper (BPH) *Nilaparvata lugens* Stal (Hemiptera Delphacidae) is a rice insect that causes significant crop losses in the world (Wang *et al*., 2005; Cheng *et al*., 2013). Chemical pesticides and BPH-resistant rice varieties are two main methods in the long history of BPH controlling. However, apart from the serious environmental contamination caused by pesticides, BPH has also evolved and developed strong adaptability to BPH-resistant rice varieties (Du *et al*., 2009; Hao *et al*., 2015). In order to control BPH, it is important to understand the mechanism of interactions between BPH and its host rice. At least 24 BPH-resistant genes have been identified and mapped on rice chromosomes and one of them, *bph14*, has even been cloned (Du *et al*., 2009; Huang *et al*., 2013). In addition, a lot of expressed sequence tags (ESTs), transcripts and proteins, all with different expressions, have been identified in both BPH and rice plants (Yuan *et al*., 2004; Wang *et al*., 2005; 2015a). Recently, some important genes, such as *COOH*, *Mp42*, and *Mp10* from piercing insect saliva, were reported to act as effector to suppress or as elicitor to trigger the plant resistance response (Caplan *et al*., 2008; Mutti *et al*., 2008; Hogenhout *et al*., 2009; Bos *et al*., 2010; Bonaventure, 2012). Although many salivary genes and ESTs have been isolated from BPH, the molecular characteristics are still poorly studied (Noda *et al*., 2008; Ji *et al*., 2013). Hence, a detailed analysis of genes or proteins from saliva at the molecular, cellular, and functional levels is necessary for a deep understanding of the interactions between sucking insect and its host plants.

Yeast Sec14-protein (Sec14p) belongs to the SEC14 domain (Smart entry: smart00516) protein family. SEC14 domain is also named as CRAL_TRIO domain in many mammalian proteins (Pfam: PF00650, SMART: SM00516), because it was first identified in cellular retinaldehyde binding protein (CRALBP) and Trio, a guanine nucleotide exchange factor (GEF) (Panagabko *et al*., 2003). The SEC14 superfamily protein exists in most eukaryotes, including yeast, plants, and animals (Saito *et al*., 2007) and it plays an important role in the transportation and exchange of secretory proteins from Golgi complex to lipid membrane bilayers (Howe and McMaster, 2006). CRALBP (Intres *et al*., 1994; Deeg *et al*., 2007), protein tyrosine phosphatase (PTP) (Huynh *et al*., 2003), α-tocopherol transfer protein (α-TTP) (Kruger *et al*., 2002), supernatant protein factor (SPF) and tocopherol-associated protein (TAP) (Zimmer *et al*., 2000) share a characteristic lipid-binding domain similar to the three-dimensional structure of CRAL_TRIO domain. SEC14 proteins also play a role in the lipid metabolism because they are involved in binding or transporting several small hydrophobic molecules, such as tocopherol and retinaldehyde (Kong *et al*., 2006).

Previous studies of SEC14 superfamily were mainly focused on model organisms such as yeast and human (Tripathi *et al*., 2014). However, with the rapid development of genome sequencing, an increasing number of CRALBP proteins has been identified from different insect species. Smith and Briscoe (2015) identified 43 proteins with CRAL_TRIO domain by screening against GenBank database, and they characterized CRAL_TRIO proteins from insects for the first time. However, no SEC14 protein from BPH has ever been reported. In our previous research on candidate effector isolation from BPH salivary gland, we discovered an EST with CRAL_TRIO domain, whose expression increased significantly after BPH fed on the hopper-resistant rice. Because it was the first SEC14 superfamily gene from

BPH, we named it as *BPHsec1*. To better understand this novel gene, we further investigated the characteristic and expression of this novel CRALBPL gene with CRAL-TRIO domain from BPH. The results would be of great importance for future study on its role in the adaption for the rice defense response.

Materials and methods

Nilaparvata lugens

The rice BPHs (*N. lugens*) were provided by Wuhan University (Wuhan, China) and were reared on 1~2 month-old rice plants (susceptible variety Taichung Native1, TN1) under controlled conditions (25 ± 2 °C, 70-80% relative humidity, 16 h light/8 h dark) in School of Life Sciences, Guangzhou University.

Total RNA extraction

RNA was extracted using RNeasy Mini Kit (QIAGEN, Germany). For gene cloning, a mixed sample of 30~40 BPHs were collected for RNA extraction, including both 3~5 instar nymphs and male/female adults. The whole body of BPH sample was grinded into powder by liquid nitrogen, and then the powder was collected into extraction buffer (Buffer RTL) offered by the kit. After homogenizing, same volume of 70% ethanol was added to the lysate. Then the sample (~700 µl) was transferred to the RNeasy Mini spin column. After centrifugation, washed the membrane with 350 µl Buffer RW1, and then 80 µl DNase I incubation mix was added to the membrane of the column and incubated for 15 min at 20~30 °C. Washed the column firstly with 700 µl Buffer RW by centrifuge, then washed twice with 500 µl Buffer PRE. Finally, 25~50 µl RNase-Free water was added to the column to collect RNA by centrifuge. RNA concentration and quality were determined using Nanodrop spectrophotometer (Thermo Fisher, USA).

Cloning of full-length CDS by RACE

The 5' and 3' ends of *BPHsec1* cDNA were cloned with SMARTer rapid amplification of cDNA ends (RACE) cDNA Amplification Kit (Clontech, CA, USA) according to the procedure reported by Li *et al*. (2012). Approximately, 2 µg of total RNA extracted from BPH were used as template to synthesize the first-strand cDNA template for 3' RACE or 5' RACE. PCR reactions were performed in a Peltier Thermal Cycler (MJ Research, Canada). All primers used were listed in table 1. All final PCR products were sequenced on both directions (Sangon, Shanghai, China). The gene-specific primers were designed using Primer premier 5.0 (http://www.PremierBiosoft.com) and Oligo 6.0 (http://www.oligo.net).

Sequence analysis and structure prediction

Using the above sequence as the query, a Blastp searching was conducted and 25 sequences from several different species (23 from insects, one from yeast and one from human) were identified for phylogenetic analysis. The sequences were aligned using the ClustalX software (http://clustalx.software.informer.com) (Larkin *et al*., 2007). The assessment of transmembrane regions was carried out with TMHMM Server v. 2.0 (http://genome.cbs.dtu.dk/services/TMHMM/). Protein secondary structure was predicted using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). N-glycosylation site and O-glycosylation site were identified using NetNGlyc 1.0 and NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/), respectively.

Table 1. Primers used in this study

Primers	Sequences
RACE	
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT(long) CTAATACGACTCACTATAGGGC(short)
NUP	AAGCAGTGGTATCAACGCAGAGT
3'GSP1	TCCGCCGTTGTCAACTGAATTGGA
3'GSP2	ATGGCAAAACAACCTCACATTCCCC
5'GSP1	TTAATCGACGCTTAGTTG
5'GSP2	TCAGAGGCCAGCTGCTGTTGTGA
ORF	
ScF	CGGAATTCATGGTTGTAAGCGAGCTG
ScR	CGGAATTCTTAATCGACGCTTAGTTG
q-PCR	
β-actin-F	TGCGTGACATCAAGGAGAAGC
β-actin-R	CCATACCCAAGAAGGAAGGCT
qSecF1	GCTGTTCTTCCTACCGGGTC
qSecR1	ATGTGGAGGCTGCTGAAGTC
dsRNA synthesis	
dssec-F	AATTAACCCTCACTAAAGGGTCCGCCGTTGTCAACTGAATTGGA*
dssec-R	AATTAACCCTCACTAAAGGGTCAGAGGCCAGCTGCTGTTGTGA*
dsGFP-F	AATTAACCCTCACTAAAGGGTCATACGTGCAGGAGAGGAC*
dsGFP-R	AATTAACCCTCACTAAAGGGTCCAGATTGTGTGGACAGG*

*Underlined sequences are T3 promoter.

MEGA 5.0 (http://www.megasoftware.net) was used to construct the phylogenetic tree (Kumar *et al*., 2008). The sequence logo was aligned with WebLogo (http://weblogo.berkeley.edu/). The protein structure was predicted using I-TASSER (Iterative threading assembly refinement) (Yang *et al*., 2015).

Relative expression analysis of BPH at different developmental stages

Several rice plants with newly hatched BPH were selected and their moulting times as their instars were documented. Two days after each moulting, BHP samples at different developmental stages (1st, 2nd, 3rd, 4th and 5th instar nymphs and adult BPH with long wings and short wings) were collected for RNA extraction. RNA extraction procedure was the same as mentioned before. For nymphs, 30~40 nymphs were used for RNA extraction, while 20~30 adults were used. The genomic DNA was also eliminated by on-column DNase digestion as mentioned before. First-strand cDNA was synthesized as described above.

QRT-PCR was performed using GoTaq® qPCR Master Mix (Promega, USA). Every 20 µl of qPCR reaction consists of 10.0 µl 2x GoTaq® Green Master Mix, 0.4 µl forward primer, 0.4 µl revers primer, 0.5 µl cDNA and 8.7 µl dH_2O. In the negative control, the template was replaced by dH_2O, and *tublin* and *RPs* were chosen as internal reference genes. The qPCR primers for *BPHsec1* were qSecF1 and qSecR1 (table 1). Real-time qPCR was monitored by ABI 7300 system according to procedures previously reported by Zhang *et al*. (2012).

RNA interference by double-stranded RNA injection or feeding

To synthesize double-stranded RNA (dsRNA), T3 promoter (AATTAACCCTCACTAAAGGGTC) was conjugated to both the forward and reverse primers at the 5'-end (dssec-F and dssec-R, table 1) for the partial coding sequence of BPHsec1 gene. The PCR product (883 bp) was ligated into the pUCm-T vector. After sequencing, the correct plasmid was amplified by PCR with above primers, and then the purified PCR products were served as template for dsRNA synthesis. We used 30 U T3 Polymerase (Thermo Fisher) to transcribe 0.5 µg template at 37 °C for 2 h, then, stop reaction at 75 °C for 5 min. RNAs transcribed from two directions were reversely complementary and they could bind with each other to form dsRNA. After agarose gel detection, 2 U DNase I (Takara, Dalian, China) and 0.5 µl RNase were added to remove the template DNA and ssRNA by incubation at 37 °C for 15 min. Phenol/chloroform extraction was performed to inactivate DNase I and RNase. Then, the dsRNA was isopropanol precipitated, resuspended in ultra-pure water, quantified using a microvolume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA), and its purity and integrity were determined by agarose gel electrophoresis. As control, 688 bp fragment of GFP (ACY56286) dsRNA was also produced as described above (Hao *et al.*, 2015). Another control, sterile water was also used as control. The resulted dsRNA was diluted in DEPC water to 1,000 ng/µl for use.

Two methods of RNA interference (RNAi) experiments were performed as follows.

(1) The dsRNA injection was carried out according to the previously reported procedures (Wang *et al*., 2015b). 40-50 healthy 3rd instar BPHs were chosen and anesthetized on ice, before they were fixed on a 2% agar plate for injection using FemtoJet® injector (Eppendorf). 0.2 µl of dsRNA was injected into the second thorax surface of BPH exoskeleton. Three repetitions were per-

Figure 1. Nucleotide and deduced amino acid sequence of BPHsec1. The sequence was analyzed by DNAMAN. The start codon ATG is indicated bold italic and the stop codon TAA is bold italic and noted with an asterisk. Polydenylation signal AATAAA is bold underlined. The amino acid of the position 293 is an O-glycosylation site with red large case. Shadowed characters denote the CRAL-TRIO domain by searching the prosite database.

formed and the injected nymphs were reared on rice plants of TN1 with 4-5 leaves at 28 °C, 85% RH and 16:8 h (L:D) in three cages for parallel repetition. 3~5 nymphs were taken out from each cage on days 1, 3 and 5 for RNA extraction.

(2) The effects of dsRNA feeding were also tested (Chen *et al*., 2010; Liu *et al*., 2013). A two-way glass tube was used, with one end covered with double-layer parafilm. Between the two layers, 180 µl of 2.5% sucrose and 20 µl of *dsBPHsec1* were injected at super clean bench. 180 µl of 2.5% sucrose with sterile water or *dsGFP* were used as control, respectively. After 12 h starvation, 35-40 BPHs were transferred into the tube and then the other end of tube was covered with gauze to keep BPHs from escaping. Several live BPHs were randomly selected for RNA extraction and qPCR on days 1, 3 and 5 after treatment. Three repetitions were conducted.

Results

Nucleotide and deduced Amino Acid Sequences of *BPHsec1*

The full-length *BPHsec1* cDNA was cloned using the RACE method. The cDNA is 1,589 bp long and contains an open reading frame coding from 366 to 1,304 nt, encoding a putative 312 amino acids protein. At the 3' end of the sequence, there is a putative typical poly(A) signal (AATAAA) (figure 1). The estimated pI of the encoded protein is 7.67, with the estimated molecular weight at 35.3 kDa. There are one 365-bp 5' untranslated region (UTR) upstream from the start codon and another 276-bp 3' UTR downstream from the stop codon. The cDNA sequence was deposited at GenBank and the accession number was KU296276.

Through sequence database search, a conserved CRAL-TRIO domain (103 to 254 amino acids in length) was identified in this protein (figure 1), suggesting it belonging to the SEC14-like protein family with CRAL-TRIO lipid binding domain. Analyzed by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), BPHsec1 showed no signal peptide which is necessary for a protein to be secreted outside the cells. Neither transmembrane domain existed in this protein therefor the protein was likely located inside the cells. No potential N-glycosylation site except an O-glycosylation site was detected at the position of 293 amino acids (figure 1).

Phylogenetic relationship of BPHsec1 with other SEC14 members

A blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of GenBank revealed that there are many SEC14 superfamily members homology to BPHsec1. Our search showed that BPHsec1 shared the greatest similarity (37%) with *Halyomorpha halys* clavesin-1-like isoform X2 (GenBank XP_014289947) and another 34% amino acid identity with a *Cimex lectularius* uncharacterized protein LOC106663953 (XP_014244701). To analyze the sequence homology and phylogenetic rela-

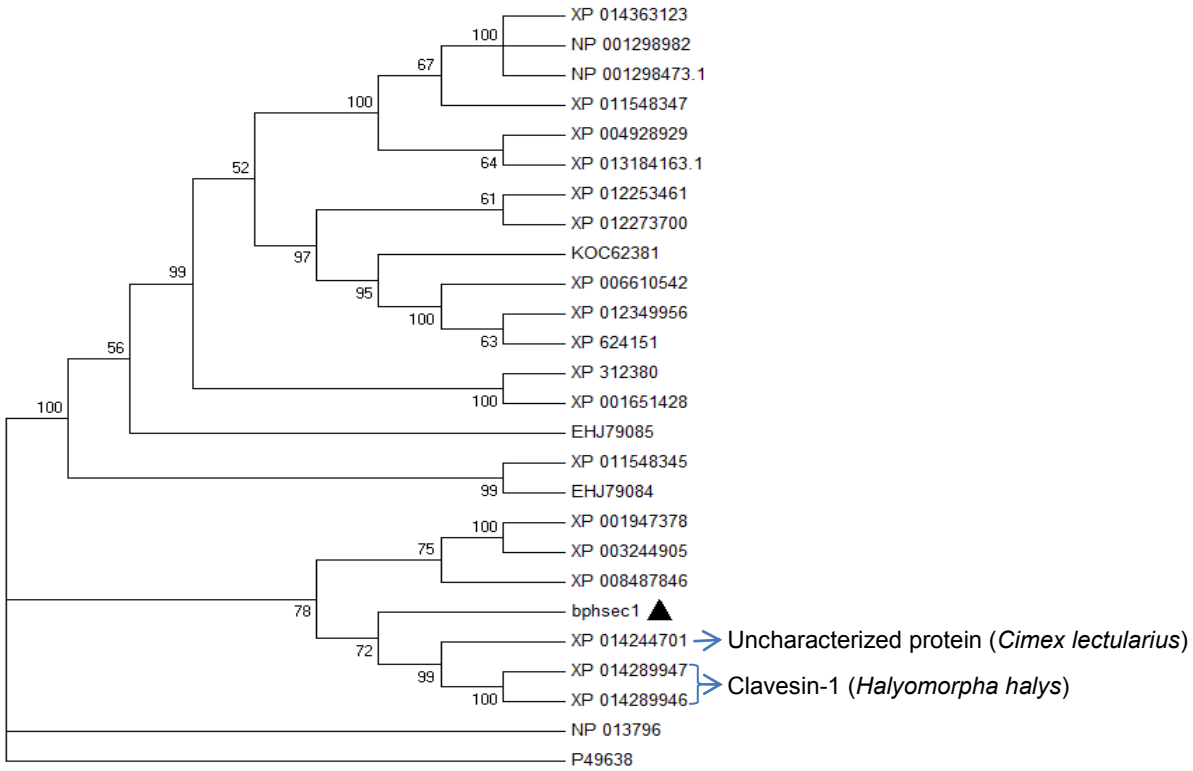

Figure 2. Phylogenetic relationship of bphsec1 and other insect SEC14 genes shown by GenBank accession numbers. The phylogenetic tree was inferred using the NJ method. The consensus tree was inferred from 1,000 bootstrap replicates. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1,000 replicates), is shown next to the branches. Phylogenetic analyses were conducted in MEGA 5.05.

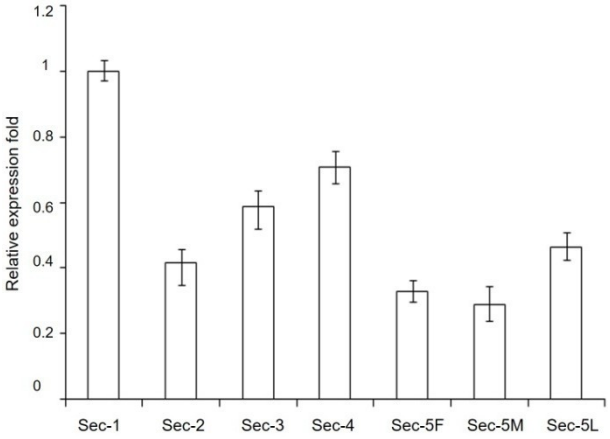

Figure 3. Expression levels of Bphsec1 cDNA at different developmental stages. 1-5: first to fifth-instar nymph, Sec: BPHsec1 gene, 5F: the fifth female instar nymph with short wing, 5M: the fifth male instar nymph with short wing 5L: the fifth instar with long wing.

tionships, 23 insect SEC14 genes, one yeast *Sec14p* and one human *CARLBP* gene were downloaded from GenBank and aligned with *BPHsec1* by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A NJ phylogenetic tree was constructed using MEGA 5.05 (figure 2). The resulted phylogenetic tree showed that *BPHsec1* was close to the SEC14 domain genes of insect, most closely related to that of *H. halys*, but far from those of human or yeast.

The mRNA expression of *BPHsec1* in different developmental stages

The expression patterns of *BPHsec1* were investigated using samples collected from male/female adult BPHs at 1st to 5th developmental stages (figure 3). The results showed that *BPHsec1* was expressed in all stages, with the highest expression in 1st instar nymphs and relatively lower expressions at 2nd and 5th stages. Expression patterns for adult female and male were almost the same, indicating that *BPHsec1* expression is not related to the gender. Figure 4A also showed that the expression level was lower in short wing BPHs than in long wing ones.

Knockdown effects of BPHsec1

Qualitative two-step RT-PCR expression analysis was used to confirm if microinjections or feeding of dsRNA could trigger the disruption of *BPHsec1* mRNA expression. 3rd instar BPHs were used because their size was suitable for dsRNA injection. Samples were taken on days 1, 3 and 5 after BPHs were treated with dsRNA. RT-qPCR results showed that the mRNA abundance of *BPHsec1* decreased from day 1 to day 5 in all groups (figure 4A). Injection of ds*BPHsec1* inhibited the expression of *BPHsec1*, resulting in a significant reduction of mRNA abundance to more than 50% of that of untreated groups on day 1. The mRNA expression remained at low level till the insects died. However, the disruption of target BPHsec1 mRNA expression was not detected until

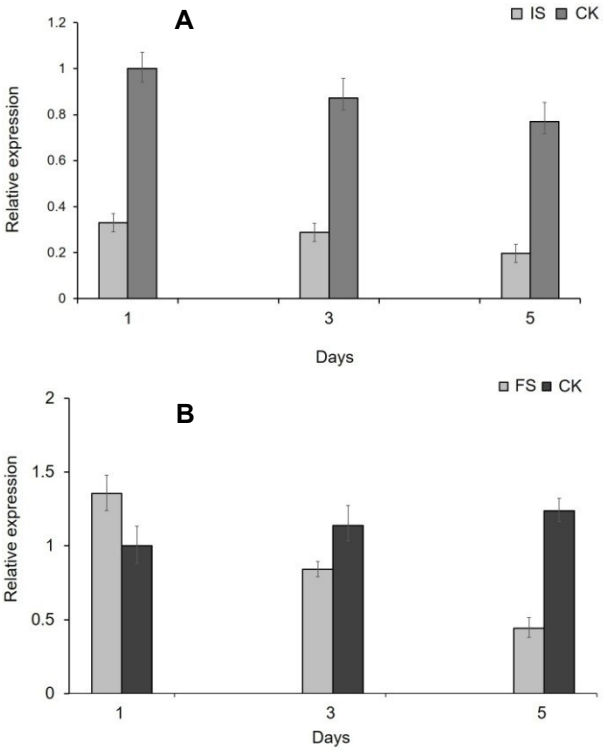

Figure 4. Measurement of RNAi efficiency and its impact on bphsec1 mRNA expression by RT-qPCR. (**A**) Injection-based RNAi. (**B**) Feeding-based RNAi. IS: Injected-based bphsec1- dsRNA; FS: Feeding-based *BPHsec1*- dsRNA. CK: control group with *dsGFP*. The data represent the mean value of three replicates. 1, 3, 5 indicate the mRNA expression on days 1, 3 and 5 after dsRNA injection or feeding.

day 3 for the dsRNA-feeding group. Results showed that from day 3, the expression dropped below that of the control group (figure 4B). The above results showed that the knockdown effect of feeding method was significantly postponed compared with that of injection method. This also can be deduced by reduced survival rate of BPH (figure 5). At first day, comparing to the survival rate of 84.76% by feeding diet, RNAi by injection lead to a sharp fall survival to 62.86%. The survival rate is obviously different by the two methods, though the BPHs are almost died at day 7th.

Structure prediction

Predicted secondary structure of BPHsec1 showed that BPHsec1 was composed of more than 10 helices, 4 strands, and several coils (figure 6). Weblogo identified 4 highly conserved amino acid sites compared with yeast Sec14p and human alpha-tocopherol transfer protein. When compared with insects' CRAL_TRIO domain, there are 8 identical amino acid residues identified (figure 7). In addition, 3D structure was predicted using I-TASSER (Roy *et al*., 2010; Yang *et al*., 2015), which applies both multiple threading alignments and *ab initio* modeling with further refinement to obtain the most likely 3D structure and potential active binding site residues. The final 3D structure was predicted from consensus sequences of 159 insect SEC14 domains, resulting in

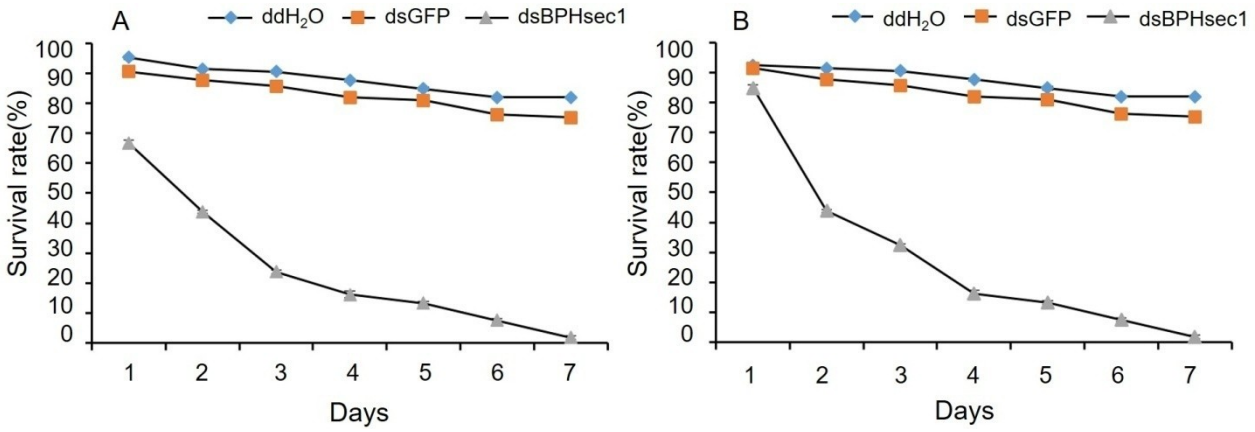

Figure 5. Survival rate of the dsBPHsec1 injection and feeding. A: ddH_2O: fed on 2.5% sucrose (untreated diet); dsGFP: fed on 0.1 μg/μl dsGFP control in 2.5% sucrose; dsBPHsec1: fed on 0.1μg/μl dsBPHsec1 in 2.5% sucrose; B: ddH_2O: sterile water; dsGFP: 0.1μg/μl dsGFP control; dsBPHsec1: 0.1μg/μl dsBPHsec1.

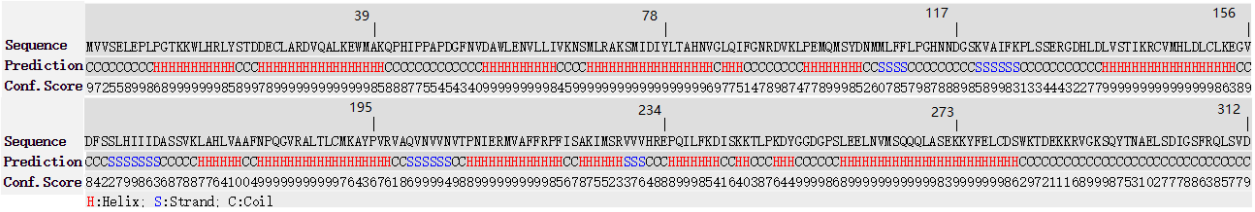

Figure 6. Secondary structure predicted. Analyzed by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). H: Helix; S: Strand; C: Coil.

a topological model (figure 8) with a confidence score (C-score) of −0.67. Furthermore, top10 highly similar structures in PDB (as identified by TM-align) were also identified (table 2). The predicted 3D structure showed great homology to that of Retinaldehyde-binding protein 1(RLBP1, 3hy5A) with a TM-score of 0.798. The top enzyme homologue of Beta-N- acetylhexosaminidase with C-score of 0.232 and the 15 prediced specific ligand binding sites (124, 137, 144, 165, 167, 186, 187, 190, 201, 203, 210, 213, 217, 221, 229) of BPHsec1 with RET ligands of C-score of 0.32 were inferred. Gene ontology predicts the biological, molecular and cellular functions of BPHsec1 protein by COACH based on the I-TASSER structure prediction, which showed that it would be involved in vitamin E binding (GO:0008431), phospholipid transporter activity (GO:0005548) and enzyme regulator activity (GO:0030234).

Discussion

Up to date, over 1,550 proteins from *Saccharomyces*, *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, etc, were annotated with SEC14 domains in the NCBI database and more are still being discovered (Katoh *et al*., 2009). Although SEC14 proteins from diverse insect species have been well researched (Smith and Briscoe, 2015), little is known about the SEC14 protein in *N. lugens*. The present study investigated the cloning and the preliminary function of BPH gene with SEC14 domain. The SEC14 domain is an important lipid-binding domain responsible for PtdIns/PtdCho exchange in plants and animals and for the secretory proteins transporting in yeast. It carries out complicate lipid-mediated regulatory functions between membranes and is essential for protein viability and secretion (Simon *et al*., 1998). Amino acid alignment and phylogenetic tree analysis showed that BPHsec1 was more similar to an uncharacterized protein from *C. lectularius* and clavesin-1 like

Table 2. Top 10 identified structural analogs in PDB.

Rank	PDB Hit	TM-score	RMSD[a]	IDEN[a]	Cov
1	3hy5A	0.798	1.76	0.14	0.849
2	1oizA	0.778	2.22	0.149	0.833
3	3hx3A	0.709	1.8	0.148	0.756
4	3b7zA	0.616	4.45	0.102	0.808
5	4fmmA	0.579	5.05	0.133	0.798
6	1auaA	0.567	4.97	0.109	0.788
7	4tlgA	0.564	4.24	0.095	0.737
8	1olmE	0.555	4.44	0.108	0.74
9	1o6uA3	0.46	3.17	0.133	0.538
10	1qbaA	0.439	5.64	0.037	0.651

RMSD[a] is the RMSD between residues that are structurally aligned by TM-align. IDEN[a] is the percentage sequence identity in the structurally aligned region. Cov represents the coverage of the alignment by TM-align and is calculated by dividing the number of structurally aligned residues with the length of the query protein.

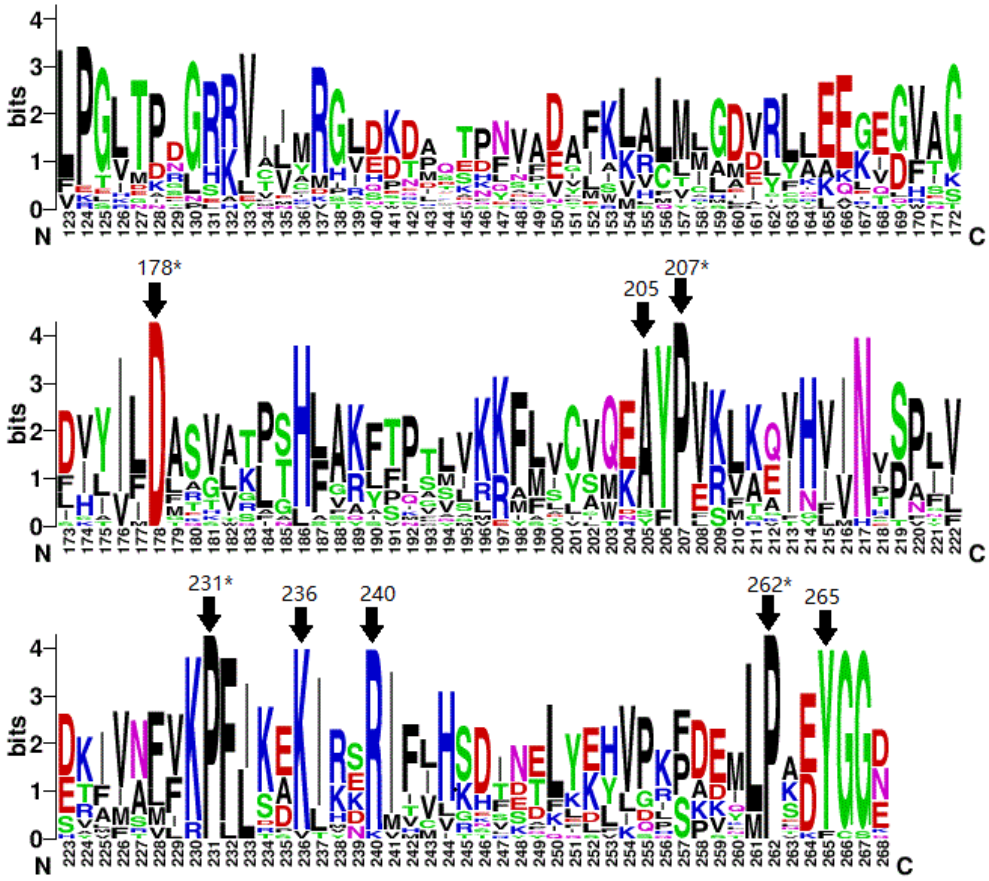

Figure 7. The sequence logo of the CRAL_TRIO domain created by WebLogo. Analyzed by WebLogo (http://weblogo.berkeley.edu/). The height of each stack is proportional to the sequence conservation, measured in bits, and the height of each letter is proportional to the frequency of residues at that position. Black arrows denote highly conserved amino acid positions in sucking insects. Black arrows combined with asterisks denote conserved sites among insect, human and yeast SEC14p sequences. Numbers above arrows show the position of that amino acid within the yeast SEC14p protein (NCBI Black arrows accession: NP_013796).

Figure 8. Protein structure and conservation of the SEC14 domain in insects. The three-dimensional protein structure was predicted using I-TASSER and visualized in Jmol using the consensus of 159 insect sequences (α-helices shown in pink, β-strands in yellow).

isoform from *H. halys* than to SEC14 domain proteins of other insects, yeast or human. Clavesins, predominantly associated with clathrin coated vesicles (CCVs), originate from the trans-Golgi network (TGN), a major transport pathway from the secretory system to endosomes/lysosomes. Clavesin 1 and 2 (clathrin vesicle-associated Sec14 protein) were found to be neuron-specific proteins that play an important role in the regulation of endosome/lysosome morphology (Katoh *et al*., 2009). Since functional characterizations of proteins usually depend on their 3D structures, proteins sharing evolutionally conserved three-dimensional structures usually have similar function. In I-TASSER searching, TM-score has a value in the range of 0 to 1 and it is a good indicator of similarity between two structures. Higher TM-score signifies a fold with more confidence in SCOP/CATH (He *et al*., 2009). In this research, the highest TM score of BPH CRAL_TRIO domain sequence against the templates currently available in Protein Data Bank (PDB) is 0.798, which means that the predicted fold model is highly similar to the crystal structure 3hy5A of human RLBP1 protein (accession No. P12271). The human RLBP1 protein is involved in the visual cycle and carries 11-cis-retinaldehyde or 11-cis-retinal as physiologic ligands (He *et al*., 2009).

These alignment and structure prediction results suggested that BPHsec1 might be a specific protein of BPH and play a role in protein transport.

Gupta *et al*. (2012) surveyed CRAL_TRIO domains across a broad range of species, including yeast, plants, fish and human, with only one conserved residue located at the position D178 in yeast Sec14p. After comparing BPHsec1 with other insect SEC14 proteins from *D. melanogaster*, *Anopheles gambiae*, *Apis mellifera*, *Tribolium castaneum*, *Bombyx mori*, *Manduca sexta* and *Danaus plexippus*, we identified eight highly conserved amino acid residues in the CRAL_TRIO domain, providing further evidence that these eight conserved residues could be insect specific. When compared with yeast Sec14p and human alpha-tocopherol transfer protein, four similar amino acids were identified. In addition to the position D178 in yeast Sec14p that is conserved according to Smith and Briscoe's (2015) study and ours, three more amino acids (position 207, 231 and 262 in yeast Sec14p) were identified to be conserved. Interestingly, these three amino acids are proline, a kind of imino acid. Whether the proline embedded in CRAL_TRIO domain is specific for the secondary structures and it is worthy of further study.

Several transcripts were isolated from saliva of aphid and planthopper. Among them, certain genes named effectors would suppress the plant defense response. Contrastingly, the biological functions of *C002* that are knockdown are thought to be associated with foraging and feeding behaviour of aphids (Mutti, 2008). Our RT-PCR expression data showed that *BPHsec1* expression was the highest in 1st instar nymphs while the lowest expression was detected in the male adult with short wings. In figure 4A, the expression levels in female and male are almost the same, which means *BPHsec1* is not related to the gender. *BPHsec1* expression is lower in short-wing BPHs than in long wing ones, which give us a clue that *BPHsec1* might be involved in regulating the development of wings. To further understand the biological significance of *BPHsec1*, RNAi were performed, with only partial *BPHsec1* silencing observed (figure 4). Though all insects died 7 days after RNAi, results in our study showed that the efficacy of RNAi varied along with dsRNA administration methods (injection or mouth feeding). After dsRNA injection, *BPHsec1*expression significantly declined on day one, with the low level lasting till the fifth day. *BPHsec1* dsRNA feeding didn't lower *BPHsec1* mRNA expression till day 3. Our results showed that dsRNA feeding or injection did not initiate RNAi uniformly. DsRNA injection is more effective in silencing genes at BPH. It's possible that the presence of dsRNase in BPH might cause feeding method less effective (Liu *et al*., 2012).

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