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Full Length Article

Characterization of the small hive beetle transcriptome focused on the insecticide target site and RNA interference genes

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ABSTRACT

The small hive beetle (SHB), *Aethina tumida*, is an invasive pest species in most Northern Hemisphere countries, including Korea. SHB causes serious damage to apiaries by destroying overwintering honey bee colonies. To obtain basic information for efficient management of SHB, genes encoding conventional insecticide targets, specifically the voltage-sensitive sodium channel α-subunit (VSSC) and acetylcholinesterase (AChE), and RNA interference (RNAi)-related components were annotated and characterized following analysis of transcriptomes of adults and larvae. A single VSSC gene was identified but no apparent mutations associated with pyrethroid resistance were detected. Genes encoding two AChEs (AtAChE1 and AtAChE2) were identified from the SHB transcriptome. No apparent mutations associated with resistance to organophosphorus and carbamate insecticides were identified in the AtAChE1 gene, whereas the S238G mutation, originally identified from the Colorado potato beetle, was detected in the AtAChE2 gene. Native polyacrylamide electrophoresis in conjunction with western blotting revealed that AtAChE1 was the main catalytic enzyme and therefore a toxicologically more relevant target. AtAChE1 was determined to exist in both membrane-anchored and soluble forms. The main components of RNA interference (RNAi) were identified, suggesting that RNAi is likely functional in SHB and an RNAi-based approach is a feasible alternative control measure.

Introduction

The small hive beetle (SHB), *Aethina tumida*, is an invasive pest in the Northern Hemisphere that causes serious damage to apiaries by destroying overwintering honey bee colonies by feeding on honey, pollen and bee broods (Neumann and Elzen, 2004). SHB is considered a factor in the colony collapse disorder (CCD) in the United States (Vanengelsdorp et al., 2007). This destructive pest was first discovered in the United States in 1966 and is believed to have spread from South Africa through the Eastern Seaboard (Sheridan et al., 2012). While SHB causes serious damage to honey bee colonies in the United States and Australia, it is a minor pest of the honey bee in sub-Saharan Africa (Annand, 2011; Spiewok and Neumann, 2006), affecting only the weakened or diseased colonies (Ellis and Hepburn, 2006). SHB has invaded many countries including the United States, Canada and Mexico, Australia, and Portugal, Italy and England (Cervancia et al., 2016; Ellis and Ellis, 2010; Goodman and Kaczynski, 2009; Lounsberry et al., 2010). Populations of SHB that invade cold regions such as Northern Europe, Russia and Northern Asia have not caused severe damage to honey bee colonies as they have in the United States and Australia.

In Korea, severe damage by SHB was first reported by several apiaries in late fall 2016 in the southeastern province (Muan-myeon, Miryang-si, Gyeongsangnam-do, Korea), which is a typical region for bee hive overwintering. SHB populations had been informally reported in the same region for past several years without noticeable damage. Therefore, these SHB populations appeared to have invaded several years previously and adapted to their new environment. Because some apiaries in Northern Korea migrate south for overwintering and return north during spring blooming season, SHB infestation in overwintering sites would accelerate the spread of SHB in Korea. Therefore, an efficient SHB management system must be established prior to the spread of SHB.

Several insecticides or acaricides have been used to control SHB. Fluvalinate and coumaphos, which are representative acaricides used for Varroa mite control, are also recommended for treating bee hives for

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SHB (Mostafa and Williams, 2002). In addition, permethrin is used to treat soil surrounding bee hives (Hood, 2000). Since pyrethroid (fluvalinate and permethrin) and organophosphorus (coumaphos) insecticides are currently the only option for SHB control, understanding the properties of their targets in SHB, which are voltage-sensitive sodium channel (VSSC) and acetylcholinesterase (AChE), respectively, is essential for effective control. Since SHB is an invasive species, determining whether invading SHB populations carry predisposed target site mutations associated with resistance is of practical importance.

Despite the pending threat SHB creates, little molecular information on SHB is available so far. In this study, the transcriptomes of adult and larval SHB were analyzed and annotated. Genes encoding VSSC and AChE were characterized to obtain basic information on the targets of SHB-control insecticides. Genes encoding the RNA interference (RNAi) machinery were identified and characterized to determine the potential of RNAi as an alternative control option against SHB.

Materials and methods

Insects

Small hive beetles were originally collected from the first report site (Muan-myeon, Miryang-si, Gyeongsangnam-do, Korea) and have been maintained in the Animal and Plant Quarantine Agency, Korea.

RNA extraction, library construction and sequencing

Five SHB adults were used for RNA extraction. Insects were homogenized in a mortar and pestle with liquid nitrogen and immediately placed in 500 µl TRIzol reagent (MRC, Cincinnati, OH, USA). Total RNA was extracted according to the manufacturer's protocol and treated with RNase-free DNase (Qiagen, Hilden, Germany) to remove genomic DNA. RNA concentration was assessed with a NanoDrop8000 spectrophotometer (Thermo, Waltham, MA, USA) and integrity with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA with $OD_{260/280} \geq 1.8$ and integrity ≥ 7.0 was used in experiments. cDNA sequencing libraries were constructed with 2.5 µg total RNA using Illumina TruSeq RNA Sample Preparation Kits (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Libraries were amplified, yielding ~400 ng cDNA with an average fragment size of ~350 bp. Libraries were paired-end sequenced (2 × 100 bp) with Illumina Hiseq 2500.

Sequence processing, de novo assembly and annotation

Files in Fastq format containing results of paired-end sequencing were analyzed with Cutadapt (Martin, 2011) for processing with default parameters to obtain quality trimmed (phred quality > 30), adaptor-removed and length-trimmed (< 100 bp) sequences (Martin, 2011). Total preprocessed sequences were pooled and assembled using default values of Trinity assembler v.2.0.6, which was developed to recover more full-length transcripts than other de novo assemblers (Grabherr et al., 2011). Transcripts > 200 bp were selected for inclusion in the reference transcriptome. Assembled unigenes were mapped by BlastX (e-value cut-off $1e^{-5}$) against the National Center for Biotechnology Information (NCBI) non-redundant protein and Swiss-prot databases using Blast2GO.

Transcripts of VSSC (*Atvssc*) and AChE (*Atace*) genes were identified from the *A. tumida* transcriptome. Transcripts related to RNAi pathways were identified from the transcriptome data and manually rechecked with data in NCBI using BlastX.

Presence or absence of 25 VSSC mutations associated with pyrethroid resistance were determined for *Atvssc*. Likewise, the deduced amino acid residues in *Atace1* and *Atace2* were checked at the 15 AChE mutation sites associated with resistance to the organophosphorus (OP) and carbamate (CB) insecticides in various arthropod pests.

Cloning of full-length Atvssc transcript

Deduced amino acid sequences of seven contigs identified as *Atvssc* were aligned with reference sequences (XP_019866360.1) using MEGA6 (Tamura et al., 2013) with the option of MUSCLE alignment. A set of sequence-specific primers (5′-GATGACATTCCTCTGTGTGG-3′, 5′-TTCCTGAGCATCGAGATAGG-3′) were designed from contig sequences annotated as *Atvssc*. A cDNA fragment covering the missing gap sequences (1231–1662 bp) was obtained by PCR. Reaction mixture (50 µl) contained 10× buffer, 5 pM each primer, 200 µM dNTPs, and 25 ng cDNA template. Thermal PCR conditions were 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min and 72 °C for 2 min for final extension. PCR products were separated by agarose gel electrophoresis and target DNA bands were extracted from agarose gels using Qiaquick gel extraction kits (Qiagen, Hilden, Germany). Extracted DNA was cloned into p-GEM-T Easy vector (Promega, Madison, Wisconsin, USA) and sequenced (Macrogen sequencing company, Seoul, Korea).

Phylogenetic analysis of Atvssc transcripts

The deduced amino acid sequence of *Atvssc* was used for alignment by CLC (QIAGEN Bioinfomatics, Hilden, Germany) and phylogenic analysis with representative *vssc* genes of *Tribolium castaneum* (tr|C9D7C7|), *Apis mellifera* (gi|259906449|), *Blatella germanica* (AAC47484.1) and *Drosophila melanogaster* (gb|AAB59195.1| and gi|386768616|) to determine their orthologous nature based on sequence identity and the presence of *vssc*-specific motifs.

Identification of GPI-anchored and soluble transcripts of Atace1

The amino acid sequences of two *Atace1* transcripts (TBIM036458 and TBIM036459) were used for GPI-anchor prediction by PredGPI (Pierleoni et al., 2008). To confirm the presence of two different transcripts (GPI-anchored and soluble), sequence-specific primers were designed for the exon with or without GPI-anchoring (Supplementary Table 1). Primers were used to amplify the transcripts encoding either GPI-anchored or soluble form of *Atace1*. PCR reactions (50 µl) contained 10× buffer, 5× HQ buffer, 5 pM each primer, 200 µM dNTPs and 25 ng cDNA template. Thermal PCR condition were 95 °C for 2 min and 35 cycles of 98 °C for 10 s, 60 °C for 30 s and 72 °C for 1 min. Methods for gel extraction and cloning for sequencing were the same as in the previous section.

Protein extraction, electrophoresis, activity staining and western blotting

Heads and thoraxes were isolated from five SHBs, chopped with dissecting scissors and homogenized in 0.1 M Tris-HCl (pH 7.8) containing 0.5% Triton X-100 (*v*/v) and 1% protease inhibitor cocktail (v/v) using a bullet blender with 0.5–2 mm beads (Nextadvance, NY, USA) at maximum speed for 5 min. Homogenized samples were centrifuged at 12,000 ×*g* for 10 min at 4 °C. Supernatants were transferred to EzClear & mini columns type D (GeneAll, Seoul, Korea) and centrifuged at 12,000 ×*g* for 1 min. Polyacrylamide electrophoresis (PAGE), activity staining and western blotting were as previously described (Kim et al., 2010). Primary and secondary antibodies against AChE1 and AChE2 were as previously described (Kim and Lee, 2013).

Phylogenetic analysis of Atace transcripts

The deduced amino acid sequence of *Atace* was aligned with *ace* genes from other insects (*Leptinotarsa dicemlineata*, *A. mellifera*, *Anopheles gambiae*, *Culex pipiens*, *Aphis gossypii*, *Tetranychus urticae*, *D. melanogaster* and *Musca domestica*) and phylogenetically analyzed with bootstrap value 1000 to identify how each transcript, annotated as AtAChE, was distinguished by phylogenetic clade.