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Two chitinase 5 genes from *Locusta migratoria*: Molecular characteristics and functional differentiation

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ABSTRACT

The duplication of chitinase 5 (*Cht5*) into two to five different genes has been reported only in mosquito species to date. Here, we report the duplication of *Cht5* genes (*LmCht5-1* and *LmCht5-2*) in the migratory locust (*Locusta migratoria*). Both LmCht5-1 (505 aa) and LmCht5-2 (492 aa) possess a signal peptide and a catalytic domain with four conserved motifs, but only LmCht5-1 contains a chitin-binding domain. Structural and phylogenetic analyses suggest that *LmCht5-1* is orthologous to other insect *Cht5* genes, whereas *LmCht5-2* might be newly duplicated. Both *LmCht5* genes were expressed in all tested tissues with *LmCht5-1* highly expressed in hindgut and *LmCht5-2* highly expressed in integument, foregut, hindgut and fat bodies. From the fourth-instar nymphs to the adults, *LmCht5-1* and *LmCht5-2* showed similar developmental expression patterns with transcript peaks prior to each nymphal molting, suggesting that their expression levels are similarly regulated. Treatment with 20-hydroxyecdysone (20E; the most active molting hormone) and reducing expression of *EcR* (ecdysone receptor gene) by RNAi increased and decreased expression of both *LmCht5* genes, respectively, indicating that both genes are responsive to 20E. Although transcript level of *LmCht5-2* is generally 10-fold higher than that of *LmCht5-1*, RNAi-mediated suppression of *LmCht5-1* transcript led to severe molting defects and lethality, but such effects were not seen with RNAi of *LmCht5-2*, suggesting that the newly duplicated *LmCht5-2* is not essential for development and survivorship of the locust.

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1. Introduction

Chitin is a β- (1,4)-linked polymer of N-aceytylglucosamine moieties, and found in a vast variety of taxonomic groups including algae, fungi, protists, sponges, rotifers, nematodes, arthropods, cuttlefish, brachiopods, and mollusks (Merzendorfer, 2013). In insects, chitin is extensively distributed in ectodermal epithelial tissues, including cuticles, trachea, foregut and hindgut; and also serves as an important constituent of intestinal peritrophic matrices (PMs) (Moussian, 2010). During the development, insects must undergo periodically molting to allow for continued growth with increased body size, wherein the old cuticle is degraded and replaced by new synthesized one (Kramer et al., 1993). Digestion of the old cuticle is necessary prior to ecdysis and is mainly accomplished by several hydrolytic enzymes called chitinase, proteinase and lipase.

Insect chitinases belong to family 18 of glycoside hydrolase (GH18) and hydrolyze chitin by an endo-type of cleavage that retain the anomeric β-(1,4) configuration of products (Kramer and Muthukrishnan, 2005). They are highly diverse enzymes encoded by many different genes as implicated in all sequenced insect transcriptoms or genomes (Zhu et al., 2004, 2008a; Nakabachi et al., 2010; Pan et al., 2012). In *Drosophila melanogaster*, *Tribolium castaneum* and *Anopheles gambiae*, 16, 22 and 20 chitinase and chitinase-like genes have been reported, respectively (Zhang et al., 2011a). These genes differ significantly in size, and developmental and tissue expression patterns, and their deduced proteins also differ in their primary structures and domain architectures

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(Arakane and Muthukrishnan, 2009; Zhang et al., 2011a). Insect chitinases appear to play roles in cuticle turnover, regulating abdominal contraction and wing expansion, digestion, immunity and natural defense (Zhu et al., 2008b; Arakane and Muthukrishnan, 2009).

Currently, insect chitinases and chitinase-like proteins are classified into eight groups based on phylogenetic analyses of their catalytic domains. Among the eight groups, Group I has relatively been well characterized. The first cDNA of *MsCht5* belonging to Group I was isolated from *Manduca sexta* in 1993 (Kramer et al., 1993). Since then, the orthologous cDNAs of *MsCht5* have been identified or sequenced from at least 15 different insect species including *Bombyx mori*, *Hyphantria cunea* (Kim et al., 1998), *Chironomus tentans* (Feix et al., 2000), *Spodoptera litura* (Shinoda et al., 2001), *Choristoneura fumiferana* (Zheng et al., 2002), *Helicoverpa armigera* (Ahmad et al., 2003), *D. melanogaster* (Zhu et al., 2004), *An. gambiae*, *Aedes aegypti*, *Culex quinquefasciatus*, *T. castaneum* (Zhu et al., 2008a; Zhang et al., 2011a, 2011b), *Lacanobia oleracea* (Fitches et al., 2004), *Spodoptera exigua* (Zhang et al., 2012), *Mamestra brassicae* (Paek et al., 2012) and *Ostrinia furnacalis* (Wu et al., 2013).

The transcripts of Cht5 are mainly detected in the epidermis and the guts (Kramer et al., 1993; Ahmad et al., 2003), which suggest the Cht5 may be involved in chitin turnover associated tissues such as cuticular exoskeleton and peritrophic membrane. The supposed function of Cht5 was confirmed in *T. castaneum* and *S. exigua* by using RNAi technology (Zhu et al., 2008b; Zhang et al., 2012). Heterologous expression of insect Cht5 has been successfully performed in the Hi5 and Sf9 cell lines and the yeast *Pichia pastoris*, and the recombinant protein showed high levels of chitinolytic activity (Gopalakrishnan et al., 1995; Shinoda et al., 2001; Zhu et al., 2008c; Wu et al., 2013). The crystal structure of OfCht5 from *O. furnacalis* has been determined (Chen et al., 2014a), and a series of fully deacetylated chitooligosaccharides (GlcN)2–7 has been demonstrated as inhibitors of OfCht5 (Chen et al., 2014b).

Only single Cht5 gene had been reported in various insect species until 2010 with five different Cht5 genes, presumably evolved by gene duplication events, were identified in *An. gambiae* (Zhang et al., 2011b). Multiple Cht5 genes were also found in other two mosquito species including *Ae. aegypti* with four Cht5 genes and *C. quinquefasciatus* with three Cht5 genes (Zhang et al., 2011b). Although these duplicated Cht5s in *An. gambiae* were distinct in genome structures, domain architectures and expression profiles (Zhang et al., 2011b), their roles in chitin metabolism remains unknown.

In this paper, we reported two Cht5 genes possibly originated for a duplication event in *Locusta migratoria*, a serious agricultural pest in many regions of the world. Specifically, we sequenced their cDNAs, profiled their developmental and tissue expression patterns, and examined their transcription responses to 20-hydroecdysone (20E) treatment and RNAi-mediated suppression of *EcR* (the 20E receptor gene). We further examined their biological functions by using RNAi. Our results suggested that *LmCht5-1* was essential for molting, whereas *LmCht5-2* was not.

2. Materials and methods

2.1. Insect

The migratory locusts (*L. migratoria*) were maintained in the laboratory at the Research Institute of Applied Biology, Shanxi University, Taiyuan. They were reared with fresh wheat seedlings and wheat bran at 30 ± 2 °C and 40% humidity with 14:10 h light: dark cycle. The nymphs were reared in cages (25 × 25 × 25 cm) with density of approximately 150–200 insects per cage. Newly molted individuals were synchronized and transferred into glass beakers covered with net screen for dsRNA injection.

2.2. Search of chitinase 5 genes in locust transcriptome and genome databases

The candidate cDNA sequences of *L. migratoria* Cht5 were first searched against the locust transcriptome database, which was generated from the mixed mRNA samples prepared from eggs, nymphs and adults. BLASTx was performed for Cht5 homology search. We identified two distinct cDNA fragments putatively encoding two different LmCht 5 proteins based on an E-value cut-off of $8e^{-121}$. To confirm that the locust possessed only two *LmCht5* genes, we extensively searched the *L. migratoria*'s genome database (Wang et al., 2014).

2.3. Sequencing of two chitinase 5 cDNAs

Primers were designed with the software of Primer Premier 5, and used for amplification of 5'-end, 3'-end or full-length cDNA sequences. The sequences of primers and the expected sizes of PCR products were shown in Table S1. Total RNA was isolated from integument of the sixth day of the 5th-instar nymphs using Trizol Plus reagent (TaKaRa, Dalian, China). The mRNA was isolated using PolyATtract mRNA isolation systems (Promega, Madison, WI, USA). The first-strand cDNA was synthesized from 1 μg mRNA using the Smart RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA). All PCR amplifications were performed using Advantage® 2 Polymerase (Clontech). For RACE-PCR, the program was used as follows: initial 94 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 1 min; 35 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 1 min; and 72 °C for 10 min for final extension. For amplification of full-length cDNA sequences, the program was used as follows: 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 2 min; and 72 °C for 10 min. The PCR products were purified using E.Z.N.A® Gel Extraction kit (Omega Bio-Tek, Norcross, GA, USA), subcloned into pEASY-Blunt Zero plasmid (TransGen, Beijing, China), and sequenced from both directions by Life Technologies Company (Beijing, China).

2.4. Deduced amino acid sequence analysis of two chitinase 5 genes

The amino acid sequences were deduced using Translate tool on the ExPASy Proteomics website (http://web.expasy.org/translate/). The molecular mass (MM) and isoelectric point (pI) for each Cht5 were predicted by using Compute pI/Mw tool (http://web.expasy.org/compute_pi/). SMART domain analysis (http://smart.embl-heidelberg.de/) and SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) were used to predict the domain architecture and signal peptide.

The known insect Cht5 genes deposited in GenBank were used for constructing phylogenetic trees. These insects include hymenopteran (*Apis mellifera*), dipteran (*Ae. aegypti*, *An. gambiae*, *C. quinquefasciatus* and *D. melanogaster*), lepidopteran (*Bombyx mandarina*, *B. mori*, *C. fumiferana*, *H. armigera*, *H. cunea*, *L. oleracea*, *M. sexta*, *O. furnacalis*, *S. exigua*, *Spodoptera frugiperda* and *S. litura*), coleopteran (*T. castaneum*), and orthopteran (*L. migratoria*) species. Their catalytic domains were aligned using ClustalW software (http://www.ebi.ac.uk/clustalw/), and used for constructing trees by the neighbor-joining algorithm using Mega 6.06 software.

To compare the amino acid sequences and catalytic domains of two *LmCht5* genes along with those from five different insect orders, Genedoc software (http://www.psc.edu/biomen/genedoc) was used for multiple sequence alignments. Four conserved motifs were identified based on the references previously

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