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sHsp22.6, an intronless small heat shock protein gene, is involved in stress defence and development in *Apis cerana cerana*

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ABSTRACT

Small heat shock proteins (sHSPs) play an important role in protecting against stress-induced cell damage and fundamental physiological processes. In this study, we identified an intronless *sHsp* gene from *Apis cerana cerana* (*AccsHsp22.6*). The open reading frame of *AccsHsp22.6* was 585 bp and encoded a 194 amino acid protein. Furthermore, a 2064 bp 5'-flanking region was isolated, and potential transcription factor binding sites associated with development and stress response were identified. Quantitative PCR and western blot analyses demonstrated that *AccsHsp22.6* was detected at higher levels in the midgut than in other tissues tested, and it is highly expressed during the shift to different development stages. Moreover, *AccsHsp22.6* was significantly up-regulated by abiotic and biotic stresses, such as 4 °C, 16 °C, 42 °C, cyhalothrin, pyridaben, H_2O_2, UV, $CdCl_2$, 20-hydroxyecdysone and *Ascosphaera apis* treatments. However, *AccsHsp22.6* was slightly repressed by other stresses, including 25 °C, phoxim, paraquat and $HgCl_2$ treatments. The recombinant AccsHSP22.6 also exhibited significant temperature tolerance, antioxidation and molecular chaperone activity. In addition, we found that knockdown of *AccsHsp22.6* by RNA interference remarkably reduced temperature tolerance in *A. cerana cerana*. Taken together, these results suggest that *AccsHsp22.6* plays an essential role in the development stages and defence against cellular stress.

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1. Introduction

Living cells are inevitably challenged by adverse conditions such as extreme temperature, oxidative stress, toxic substances or infectious agents. Protein aggregations resulting from these stresses pose a dominating threat to the cells (Basha et al., 2012). To maintain cellular protein homeostasis, cells have evolved a "protein quality control" network, which is mainly mediated by autophagy, the proteasome, and heat shock proteins (HSPs) (Liberek et al., 2008).

HSPs belong to a large protein family which is classified into several superfamilies, including HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (sHSPs), based on their molecular weight and sequence homology (Garrido et al., 2012). The sHSPs are ATP-independent molecular chaperones that comprise a less conserved, more diverse and small molecular weight (15–30 kDa) group of proteins (Basha et al., 2012). sHSPs share common structural characteristics: a conserved α-crystallin domain containing 80–100 amino acids, an extremely flexible, variable C-terminus and a disorganised N-terminus (Kostenko and Moens, 2009; Bagnéris et al., 2009). It has been suggested that the conserved α-crystallin domain is important for maintaining molecular chaperone activity and other functions of sHSP in cells whereas the diverse C-terminal and N-terminal sequences may be involved with the diverse expressions, functions, and evolutionary patterns of sHSPs (Hayes et al., 2009; Li et al., 2009). Thus, the sequences and number of sHSPs vary between and within species.

Similar to other chaperones, sHSPs can bind partially denatured proteins and facilitate correct refolding, which protects cells against protein aggregation after exposure to various stresses (Garrido et al., 2012). Thus, sHSPs are ubiquitous and crucial components

of protein quality control networks. In addition to their central role as chaperones, sHSPs participate in diverse fundamental cellular and physiological processes, such as cell proliferation and differentiation, cytoskeletal organisation, redox homeostasis, apoptosis and the immune response (Garrido et al., 2012; Kostenko and Moens, 2009). The varied expression pattern of sHsps is also involved in many human diseases, such as cataracts, neurodegenerative disorders, cardiovascular disease, and cancer (Sun and MacRae, 2005a,b).

Although the heat shock response was first observed in *Drosophila*, the understanding of sHSPs in insects is not as comprehensive and profound as in bacteria, plants, and mammals. Apart from the fundamental chaperone function, recent studies found that sHSPs might be involved in insect development, particularly in larval-pupal metamorphosis. Huang et al. (2009) found that in *Liriomyza sativa* the highest expression levels of three sHSPs were observed in the pupal stage, but the expression of three HSPs was up-regulated gradually with development. Sonoda et al. (2006) also reported similar results in the diamondback moth, *Plutella xylostella*. The increased levels of sHSPs were speculated to facilitate cell proliferation and the regeneration of new tissues and organs during metamorphosis (Huang et al., 2009).

The Chinese honeybee (*Apis cerana cerana*) is an important pollinator that plays a critical role in maintaining the balance of regional ecologies in China. Compared with the Western honeybee (*Apis mellifera*), it has some advantages, such as higher cold tolerance and disease resistance, and the capability of long-distance flight (Li et al., 2012a,b). However, the population of the Chinese honeybee has declined severely in recent decades, which is attributed to an epidemic of honeybee diseases and the deterioration of its environment, apart from invasion of *A. mellifera* (Li et al., 2012a,b; Gegear et al., 2006). To date, sHSPs have been widely considered as the first line of defence against adverse conditions. Therefore, the study of sHSPs in the Chinese honeybee is necessary. Here, we isolated and characterised an intronless *sHsp* gene from *A. cerana cerana* (*AccsHsp22.6*) and evaluated its expression profiles in different developmental stages and tissues. We also investigated the response of *AccsHsp22.6* to various stresses. Moreover, the recombinant AccsHSP22.6 protein displayed significant temperature tolerance, antioxidation and molecular chaperone activity. In addition, we knocked down *AccsHsp22.6* in *A. cerana cerana* and evaluated its protective role in cold or heat stresses. Our results provide new insights into the molecular characterisation and functions of insect sHSPs.

2. Materials and methods

2.1. Insects and various treatments

The Chinese honeybees (*A. cerana cerana*) were maintained in the experimental apiary of Technology Park of Shandong Agricultural University (Taian, China). The egg, the first to fifth day instar (L1−L5) larvae, pupae (prepupae (PP), white-eyed (Pw), pink-eyed (Pp), brown-eyed (Pb) and dark-eyed (Pd) pupae) and newly emerged adults (A1) were identified according to the criteria of Michelette and Soares (1993) and collected from the hive. The 15 day post-emergence (A15) and 30 day post-emergence adults (A30) were collected at the entrance of the hive after marking newly emerged bees with paint 15 and 30 days earlier. The 15 day post-emergence adults were divided into groups ($n = 40$/group), fed an adult diet of water, 70% powdered sugar and 30% honey and maintained in an incubator at a constant humidity (70%) and temperature (34 °C) under a 24 h dark regimen (Alaux et al., 2010). Groups 1−4 were placed at different temperatures (4 °C, 16 °C, 25 °C and 42 °C). Groups 5−12 were treated with the following

conditions: ultraviolet (UV)-light (30 mj/cm^2); H$_2$O$_2$ (0.5 µl of a 2 mM dilution was injected between their first and second abdominal segments using a sterile microscale needle); HgCl$_2$ (3 mg/ml added to food); CdCl$_2$ (0.5 mg/L added to food); or pesticide (0.5 µl of cyhalothrin, phoxim, pyridaben, and paraquat, which were applied to the thoracic notum of worker bees and the final concentrations were 20 µg/L, 1 µg/ml, 10 µM, and 10 µM, respectively). The untreated adults, as the control group, were fed normal food. Bees in group 6 injected with phosphate buffered saline (PBS) (0.5 µl/worker) were the injection controls. The larvae were collectively reared as previously described (Sun et al., 2013; Yu et al., 2012). For 20-hydroxyecdysone (20E) treatment, once the larvae reached their third instar they were transferred to an artificial diet containing different doses of 20E: 1, 0.1, 0.01 and 0.001 µg/ml. The larvae were reared in 24-well plate (Costar, Corning Incorporated, USA) which were filled with 48 larvae/plate (2 larvae/well) and three plates were treated with one concentration of 20E. Ethanol was the 20E solvent and was used in the 20E controls. Its final concentration was 0.1 mg/ml. The living larvae were collected after 96 h of feeding on the treatment diets. For microbe treatment, once the larvae reached their second instar they were inoculated with *Ascosphaera apis*. One 24-well plate was filled with 48 larvae/plate (2 larvae/well) and three plates were treated. The control larvae were fed only artificial diets. The living larvae were collected after treatment at 96 h. All of the bee specimens were immediately frozen in liquid nitrogen at the indicated time points and stored at −80 °C. For the tissue-specific expression analysis, the brain, epidermis, midgut and muscle of adults (15 d) were dissected on ice, flash-frozen in liquid nitrogen and stored at −80 °C. These experiments were performed in triplicate.

2.2. Cloning AccsHsp22.6 cDNA

The extraction of total RNA and the isolation of the full-length cDNA sequence were performed as previously described (Yan et al., 2013).

2.3. Amplification of the genomic sequence and promoter of AccsHsp22.6

The genomic DNA was extracted according to Yan et al. (2013). The *AccsHsp22.6* promoter region was obtained using inverse PCR (I-PCR) as previously described (Zhang et al., 2013). All of the PCR primers used are listed in Table S1. The MatInspector database (http://www.genomatix.de/matinspector.html) was used to predict transcription factor binding sites in the *AccsHsp22.6* promoter region. All of the PCR amplification conditions are listed in Table S2.

2.4. Bioinformatic analyses

The bioinformatics tools available at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to detect the *AccsHsp22.6* conserved domains. The DNAMAN software program version 5.2.2 (Lynnon Biosoft, Quebec, Canada) was used to identify the *AccsHsp22.6* ORF and align its homologues. The theoretical isoelectric point (pI) and molecular mass were predicted by the Expasy-Sib Bioinformatics Resource Portal (http://web.expasy.org/compute_pi/). The phylogenetic tree was constructed by the neighbour-joining method using the Molecular Evolution Genetics Analysis (MEGA version 5.2) software program. I-TASSER, an online protein structure prediction tool server (http://zhanglab.ccmb.med.umich.edu/I-TASSER), was used to predict the AccsHSP22.6 tertiary structure (Roy et al., 2010).