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Differential responses of *Apis mellifera* heat shock protein genes to heat shock, flower-thinning formulations, and imidacloprid

Jinmo Koo [a], Tae-Gwon Son [b], Soo-Yeon Kim [c], Kyeong-Yeoll Lee [a,d,*]

[a] School of Applied Biosciences, Kyungpook National University, Daegu, Republic of Korea
[b] Apple, Kyungpook National University, Daegu, Republic of Korea
[c] Gyeongsanbuk-Do Agricultural Research & Extension Services, Daegu, Republic of Korea
[d] Institute of Plant Medicine, Kyungpook National University, Daegu, Republic of Korea

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ABSTRACT

The honey bee, *Apis mellifera*, is a cosmopolitan pollination insect. Recently, global populations of honey bees have rapidly declined owing to colony collapse disorder (CCD), the mechanism of which is still unknown. Here, we used mRNA levels of heat shock protein (HSP) genes as molecular markers of response to three types of external stress: thermal shock, flower-thinning agents, and pesticides. When worker bees were exposed to temperatures of 4, 27, 40, 45 and 50 °C for 1 h, decreased survival occurred only at 50 °C. Further, increased levels of *hsp70*, *grp78*, and *hsp90*, but not *hsp40*, were detected, and reached a maximum at 45 °C, particularly in the hypopharyngeal glands and fat bodies. Artificial ingestion of two flower-thinning agents containing either 0.1% boron and zinc, or 1% sulfur increased *hsp70* and *grp78* levels at different rates without affecting *hsp40* and *hsp90* levels, and had no effect on workers' mortality. However, ingestion of imidacloprid solution (0.5–50 ppm) increased mortality in workers and decreased the levels of *hsp70*, *grp78*, and *hsp90* in a dose-dependent manner. Our results showed that the responses of honey bees to each *hsp* are differential and highly specific to different stresses. This study suggests that the unique expression profiles of *hsps* can be used as valuable tools for monitoring the susceptibility of honey bees to various environmental impacts.

Introduction

The honey bee, *Apis mellifera*, is a cosmopolitan pollination insect that contributes to the pollination of wild and cultivated plants globally. In the last two decades, the population of honey bees has been declining as a result of colony collapse disorder (CCD), which is a phenomenon of the abrupt disappearance of European honey bee colonies (vanEngelsdorp et al., 2009). CCD has caused a significant economic impact owing to the decline of pollination of agricultural crops as well as wild plants (Klein et al., 2007; Schacker, 2008). The mechanisms of CCD remain unknown, although many possible causes have been proposed, such as pesticides (primarily neonicotinoids), infectious pathogens (e.g., *Varroa* mites and viruses), malnutrition, genetic factors, immunodeficiency, loss of habitat, changed beekeeping practices, or a combination of these factors (Becher et al., 2013; Smith et al., 2013). However, the effects of these impacts have been evaluated mostly at the whole organism level by determining mortality or infertility in bee colonies. It is equally important to study responses to stress at the cellular and physiological levels to understand why honey bees fail to adapt adequately to various environmental impacts capable of triggering CCD (Johnson et al., 2009).

Heat shock proteins (HSPs) are a group of evolutionarily highly conserved molecules (Lindquist and Craig, 1988; Boorstein et al., 1994; Richter et al., 2010). HSPs are divided into several sub-groups according to their size, structure, and function, namely small HSPs (sHSPs), HSP60, HSP70, HSP90, and HSP100 (Lindquist and Craig, 1988; Boorstein et al., 1994). Most HSPs are constitutively expressed and perform essential roles in normal cells. The HSP70 family is the largest group and contains many different isoforms that are specifically distributed in cellular organelles. For example, GRP78 is the endoplasmic reticulum (ER) HSP70 and is also called binding immunoglobulin protein (Bip) in mammals or heat shock 70-kDa protein 5 (HSPA5) in humans (Lee, 2001). GRP78 acts as an ER chaperone responsive to ER stress in mammals. Further, its transcription and protein levels are influenced by nutritional as well as thermal stresses (Lee, 2001). Our previous study showed that the level of *grp78* in the Indian mealmoth *Plodia interpunctella* was highly associated with nutrition uptake during the fifth instar larval stage (Shim et al., 2014). The *grp78* level increased in feeding larvae but decreased in wandering larvae, which do not feed, as well as in nutritionally deprived larvae. Expression levels of *grp78* were also upregulated in

* Corresponding author at: School of Applied Biosciences, Kyungpook National University, Daegu, Republic of Korea. Tel.: +82 53 950 5759; fax: +82 53 950 6758.
E-mail address: leeky@knu.ac.kr (K.-Y. Lee).

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P. interpunctella larvae envenomated by the ectoparasitoid *Bracon hebetor* (Shim et al., 2008).

The role of HSPs in the stress responses of many organisms has been well-documented (Feder and Hofmann, 1999; Denlinger et al., 2001). At the cellular level, HSPs act as chaperones to protect proteins from damage during synthesis, folding, assembly, and localization of proteins in the cell (Feder and Hofmann, 1999; Hartl and Hayar-Hartl, 2002). At the ecological level, HSPs act to protect organisms from various environmental stressors such as heat, cold, desiccation, toxins, pathogens, and others (reviewed by Feder and Hofmann, 1999; Kregel, 2002). In this regard, levels of transcripts and translated proteins encoded by HSP genes can be used as biomarkers to monitor cellular and physiological responses to various environmental stimuli (Gibney et al., 2001; Nazir et al., 2003).

Honey bees are highly thermotolerant and the roles played by some HSPs against thermal stress have been studied previously (Severson et al., 1990; Gregorc and Bowen, 1999; Elekonich, 2009; Xu et al., 2010). The honey bee genome has been deciphered and it is known to contain at least 36 *hsps* (Honey Bee Genome Sequencing Consortium, 2006; Elsik et al., 2014). However, changes in the expression of different *hsps* in response to various environmental stresses have not been studied in detail in honey bees. The patterns of transcripts and proteins of various *hsps* may provide important information for the understanding of the cellular and physiological mechanisms of susceptibility of honey bees to different environmental challenges. Here, using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) we determined cellular stress response profiles by measuring the expression levels of various *hsps* (*hsp40*, *hsp70*, *grp78*, *hsp90*) in worker honey bees in response to several stress conditions, namely temperature changes, flower-thinning agents, and imidacloprid pesticide.

Materials and methods

Exposure of honey bees to heat and cold shocks, flower-thinning agents, and imidacloprid

Adult workers (10–20 days old) were collected from beehives in Yeongcheon, Korea. The workers were kept in a plastic cage ($19 \times 12 \times 11$ cm^3) containing a piece of cotton moistened with a 20% sucrose solution at 26–27 °C and 50–60% relative humidity for 1 day before the experiments commenced.

The worker bees were exposed to cold (4 °C) and heat (40, 45, and 50 °C) shocks for 1 h and maintained at 27 °C for 1 h before RNA extraction. The commercial flower-thinning agent Koduri containing 0.1% water-soluble boron and zinc (Apple Co. Ltd., Daegu, Korea) was diluted with a 20% sugar solution to generate 0.1% and 1% dilutions. The resulting solutions contained boron and zinc at final concentrations of 1 and 10 ppm, respectively. The commercial formulation Eco-sulfur containing 1% lime sulfur (Saeil Co. Ltd.; Chilgok, Korea) was also diluted with a 20% sugar solution to prepare 0.1%, 1%, and 10% dilutions. The final concentrations of lime sulfur in the resulting solutions comprised 10, 100, and 1000 ppm, respectively. The neonicotinoid pesticide Konido containing 10% imidacloprid (Dongbu Farm; Hannong, Seoul, Korea) was diluted with a 20% sugar solution to prepare 0.0005%, 0.005%, and 0.05% dilutions that contained imidacloprid at final concentrations of 0.5, 5, and 50 ppm, respectively. Cotton balls were soaked in each diluted solution. Worker honey bees ($n = 20$) were placed in the plastic box and allowed to ingest the sugar solutions for 4 days at 27 °C.

Quantitative real-time RT-PCR

Total RNA was extracted from the abdomen ($n = 1$) or dissected tissues ($n = 5$) of workers using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Concentrations of purified RNA samples were determined using a Nanophotometer (Implen, Schatzbogen, Germany). Reverse transcription (RT) reactions were performed with 2 μg of total RNA as a template for cDNA synthesis using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR primers for each gene were designed using sequences identified from the National Center for Biotechnology Information (NCBI) database (Table 1). PCR amplifications were performed in a 25 μL reaction volume containing 0.2 μL cDNAs as a template, 0.2 μL of gene-specific primers at a concentration of 10 pM, 11.9 μL of distilled water, and 12.5 μL of Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The amplified signals were monitored continuously with the 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The amplification protocol was as follows: 1 cycle (95 °C for 10 min), 40 cycles (95 °C for 15 s, 55 °C for 20 s, 72 °C for 35 s), and 1 cycle for dissociation (95 °C for 15 s, 60 °C for 30 s, 95 °C for 15 s). Threshold cycle (Ct) values were used to calculate the relative quantities of HSPs and actin. Data were analyzed using the formula: $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ \text{treatment} - \Delta Ct\ \text{reference}]}$ (Livak and Schmittgen, 2001). The levels of HSPs mRNA were normalized by those of actin mRNA in the same samples.

Statistical analysis

Statistical analysis of the data was conducted using the SPSS v. 12.0 software package for Windows (SPSS Inc.; Chicago, IL, USA). The data were analyzed by one-way analysis of variance or the Student's *t*-test. Data that were not normally distributed were analyzed with Duncan's multiple range test and the same letters are not significantly different ($P < 0.05$).

Results

When worker bees (10–20-day-old adults) were exposed to cold or heat shocks at 4, 27, 40, 45, and 50 °C for 1 h, decreased survival rate occurred only at 50 °C heat shock into 77.0 ± 10.4% (data not shown). Workers upregulated *hsps* 1 h after treatment but levels of expression were dependent on temperature. Both *hsp70* and *hsp90* levels were highest after the 45 °C shock, with their expression being potentiated by 3- to 10-fold more than at any other temperature. However, the *hsp40* level was not changed by cold or heat shocks, but the *grp78* level was significantly increased by both cold and heat shocks at any temperature in comparison with the control temperature (27 °C) conditions (Fig. 1).

In a comparison of different tissues, such as brain, hypopharyngeal glands, fat bodies, midgut, and flight muscle, *hsp* levels were tissue-specific at both normal and stress temperatures. For example, at 27 °C *hsp40* was highly expressed in hypopharyngeal glands, but *hsp70* was expressed in flight muscles (Fig. 2). After heat shock at 45 °C, *hsp70* was highly upregulated in fat bodies but both *grp78* and *hsp90* were found in the hypopharyngeal glands (Fig. 2). A moderate increase of both *grp78* and *hsp90* was also detected in the brain, fat bodies, and midgut, but not in the flight muscles.

Artificial feeding with two kinds of flower-thinning formulations, Koduri (0.1% and 1% dilutions) and Eco-sulfur (0.1%, 1%, and 10% dilutions), did not increase mortality when Koduri and Eco-sulfur were used at approximately the commercially recommended concentrations of 0.1% and 1%, respectively (data not shown). In addition, we observed an increase in the expression levels of *hsp70* and *grp78* as a result of these treatments, whereas the expression levels of both *hsp40* and *hsp90* were not altered. Furthermore, both *hsp70* and *grp78* levels were augmented to a similar extent, regardless of the different concentrations of Koduri, but reduced by the higher (1%) dose of Eco-sulfur (Fig. 3).

Exposure to 0.5–50 ppm imidacloprid solutions significantly increased the mortality of workers in a dose-dependent manner (Fig. 4). Complete mortality was detected 2 days after ingestion of 5 ppm and higher doses. We observed a dose-dependent downregulation in the levels of all tested *hsps* (Fig. 5). However, the rates of decease in *hsp70*, *grp78*, and *hsp90* were greater than in *hsp40*.