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Differential gene expression of the honey bees *Apis mellifera* and *A. cerana* induced by *Varroa destructor* infection

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ABSTRACT

Varroa destructor mite is currently the most serious threat to the world bee industry. Differences in mite tolerance are reported between two honey bee species *Apis mellifera* and *Apis cerana*. Differential gene expression of two honey bee species induced by *V. destructor* infection was investigated by constructing two suppression subtractive hybridization (SSH) libraries, as first steps toward elucidating molecular mechanisms of *Varroa* tolerance. From the SSH libraries, we obtained 289 high quality sequences which clustered into 132 unique sequences grouped in 26 contigs and 106 singlets where 49 consisted in *A. cerana* subtracted library and 83 in *A. mellifera*. Using BLAST, we found that 85% sequences had counterpart known genes whereas 15% were undescribed. A Gene Ontology analysis classified 51 unique sequences into different functional categories. Eight of these differentially expressed genes, representative of different regulation patterns, were confirmed by qRT-PCR. Upon the mite induction, the differentially expressed genes from both bee species were different, except *hex 110* gene, which was up-regulated in *A. cerana* but down-regulated in *A. mellifera*, and *Npy-r* gene, which was down-regulated in both species. In general, most of the differential expression genes were involved in metabolic processes and nerve signaling. The results provide information on the molecular response of these two bee species to *Varroa* infection.

1. Introduction

The honey bees *Apis mellifera* and *Apis cerana* are important economic insects, not only for honey production, but also for crop pollination. From an economic standpoint, the value of crops created by honey bee pollination is 100 times higher than that by honey production (Morse and Calderone, 2000).

The external parasitic mite *Varroa destructor* (Anderson and Trueman, 2000) is currently the most serious threat to beekeeping around the world (De Jong et al., 1982). This haemolymph-feeding mite not only weakens adult, pupal and larval bees but also serves as a vector and inducer of viral infections, causing severe damage to bee populations world wide (Ball and Allen, 1988; Ball, 1994). Furthermore, the *Varroa* mite has been attributed, in part, to the recent widespread Colony Collapse Disorder (CCD) as a disease vector (van Englesdorp et al., 2007; Anderson et al., 2008).

Recently, there are several methods developed to control the mites, including physical, genetic, and chemical controls. Physical control measures, such as natural products, smoke, thermal treatments, and cell size modifications, mite trapping devices, etc., provide various degrees of success, but are labor-intensive (Fries and Hansen, 1993; Schmidt-Bailey et al., 1996; Sammataro et al., 2000; Maggi et al., 2008). Although a longer-term solution is the development of genetically-resistant honey bee populations to limit the build-up of mite populations or reduce the effect of the secondary pathogens associated with the mite infection, more research and practice on this genetic method is needed (Wilkinson et al., 2001).

Genetic differences exist in the ability of honey bees to tolerate *Varroa* parasitism. Microarray analyses of differences in gene expression of *A. mellifera* due to both mite parasitization and genotypic differences in bee tolerance were reported (Navajas et al., 2008). Colonies of the Asian honey bee *A. cerana* (the original host of *V. destructor*) suffer less damage from this parasite than *A. mellifera* in spite of the presence of the mite in the hives, and several factors have been implicated, including grooming and hygienic behavior, and differences in developmental timing (Peng et al., 1987).

As mites can transmit disease, it may be adaptive for bees to respond to mite presence by up-regulating their immune

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responses. It was observed that in a humoral immune response of bees parasitized by *V. destructor*, antibacterial peptides, such as abaecin and defensin, known to be immune-responsive, changed non-linearly with respect to the number of mites parasitizing honey bee pupae (Gregory et al., 2005). Honey bees appear also to mount a cellular immune response at wound sites caused by *V. destructor* (Kanbar and Engels, 2003). Bees also possess a humoral immune response leading to an up-regulation of several antimicrobial peptides in response to both wound infections (Casteels-Josson et al., 1994) and oral bacterial infections (Evans, 2006).

The mechanisms underlying genetic differences for honey bee tolerance to *Varroa* mites are unknown. It would be interesting to see the genes involved in the mite-tolerant mechanisms. Insights into these mechanisms may lead to new molecular tools for both *Varroa* diagnosis and selective breeding of mite-tolerant honey bees for the bee industry. These issues are now amenable to study thanks to new genomic resources available for honey bees.

Several methods were applied to identify gene expression of honey bees in response to pathogens. Gene expression concerning honey bee immunity in responses to microbial pathogens was investigated by a quantitative-PCR array (Evans, 2006). The expression of seven immune-related genes in the honey bee head after a bacterial challenge with *Escherichia coli* was determined by qRT-PCR (Scharlakena et al., 2008). The expression of genes encoding three antimicrobial peptides (abaecin, defensin, and hymenoptaecin) and four immunity-related enzymes (phenol oxidase, glucose dehydrogenase, glucose oxidase, and lysozyme) were measured by qRT-PCR, as markers for the difference in the immune response of *A. mellifera* with the *Varroa* infestation (Yang and Cox-Foster, 2005). Genes of *A. mellifera* bees linked to the presence of *Varroa* or to differences in bee tolerance were identified by microarrays (Navajas et al., 2008). Of these methods, suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) has been successfully used for studying genes specifically involved in particular processes of insect development, like morphogenesis (Gonzalez-Aguero et al., 2005) and metamorphosis (Dong et al., 2005), or to obtain genes specifically expressed under certain physiological conditions (Ursic-Bedoya and Lowenberger, 2007). These favorable results led us to choose the SSH approach for our purposes.

The objective of this study was to investigate and compare the differential gene expression of the honey bees *A. mellifera* and *A. cerana* prepupae challenged by *Varroa* infection, in order to identify the genes probably involved in the mite parasitism, and ultimately to provide cues for developing environmentally friendly agent for the control of this serious pathogen of honey bees.

2. Materials and methods

2.1. Insects

Workers and prepupae of *A. mellifera* or *A. cerana* from a single mated honey bee queen in a healthy apiary at Conghua, Guangdong Province, were collected according to the method of Kanbar and Engels (2003), for bioassay in the laboratory. Chinese honey bee *A. cerana* was reared also in a healthy apiary at Conghua, Guangdong Province. The honey bee stages were classified according to their developmental state as described in Bitondi et al. (1998).

2.2. Mite collection

Mites were collected from an infested brood of *A. mellifera* colonies. Worker brood cells were opened and mature female mites were collected from pupae, using a camel hair brush. The mites were placed in sterile Petri dishes (diameter = 9 cm; 20 mites per dish) and used for the bioassays within an hour after they were collected.

2.3. Varroa mite challenge and sample collection

20 prepupae of *A. cerana* or *A. mellifera* were chosen randomly from different hives and challenged by *V. destructor* mites. A small hole was pricked on the honeycomb ceiling of the prepupae by a sterile needle, and two *Varroa* mites were introduced, then ceiled by beeswax artificially (Garrido and Rosenkranz, 2003). The control prepupae was pricked in the same way but with no mites. These prepupae were put in an identical laboratory cage and incubated in the same incubator in the dark at 32 °C, 80% RH in a growth cabinet (SANYO, Japan, Tokyo). After 8 h mite challenge, the prepupae were collected from the capped cells, and snap frozen using liquid N_2 and stored at −80 °C until RNA extraction. 8 h challenge was considered enough for the mite challenge because the prepupae are usually stimulated repeatedly by the mites after administered together and their haemolymph is fed during this period (Garrido and Rosenkranz, 2003; Kanbar and Engels, 2003). More importantly, the possible proliferation of the virus transmitted by the mites may cause significant interaction effects on the gene expression by long challenge, apart from the mite induction.

2.4. RNA isolation

Total RNA was isolated of the prepupae, using the RNAqueous Kits (Ambion, USA), following the manufacturer's protocols. The isolated RNA was quantified by spectrophotometry. DNA was removed using 45 min DNaseI incubation at 37 °C (5 U DNaseI in appropriate buffer with the RNAse inhibitor RNAsin; Invitrogen, USA). The quality of the final RNA (integrity and size distribution of total RNA) was verified by 1.2% agarose gel electrophoresis and quantified by spectrophotometric analysis.

2.5. Virus check in the bee prepupae and mites

All the mites and prepupae used in this study were checked by RT-PCR method (Chen et al., 2004; Yan et al., 2009) for the absence of the viruses before or after mite challenged. RNA was extracted as described above. Primers for detection analysis of 6 viruses were shown in Table 1. These viruses were Acute bee paralysis virus (ABPV), Chronic bee paralysis virus (CBPV), Deformed wing virus (DWV), Kishmir bee virus (KBV), Sacbrood virus (SBV) and Israel acute paralysis virus (IAPV). cDNA synthesis was performed using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa). Amplification profile of PCR consisted of an initial 5-min denaturation at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C and finally 7-min cycle at 72 °C. PCR products were diagnosed by 1% agarose electrophoresis. Positive samples were sent to Invitrogen Company for sequencing using ABI Prism 337 DNA sequencer with corresponding specific primers.

2.6. SSH library construction

SSH was performed using the Clontech PCR-Select cDNA Subtraction Kit (BD Bioscience Clontech, No. 637401). Two sets of SSH libraries were constructed. The first set of SSH libraries included *A. cerana* prepupae challenged (used as tester) or not challenged (used as driver) by *V. destructor* mites. The second set of SSH was done using cDNA from *A. mellifera* prepupae challenged by *V. destructor* mites and those not challenged prepupae for forward and reverse selections. Total RNA was isolated from these prepupae and cDNA was synthesized according to the method (SMART™ PCR cDNA Synthesis Kit, Clontech, Mountain View, USA, No. 634902). cDNA was then processed by restriction digestion of RsaI, and adaptors were added, hybridized in two rounds, followed by two rounds of PCR amplification at the condition of 94 °C 10 s, 68 °C 30 s, 72 °C 1.5 min, 14 cycles. PCR products were purified