



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.

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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 Engelsdorp 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 sealed 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 N₂ 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 (DWBV), 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

Table 1

List of primers used for detection of 6 viruses.

Primers	Forward primer sequence	Reverse primer sequence	Length (bp)	Virus	Reference
ABPV	TGAGAACACCTGTAATGTGG	ACCAGAGGGTTGACTGTGTG	452	Acute bee paralysis virus	Tentcheva et al. (2004)
CBPV	AGTTGTTCATGGTTAACAGGATACGAG	TCTAATCTTAGCACGAAAGCCGAG	455	Chronic bee paralysis virus	Ribi�re et al. (2000)
DWV	TTTGCAAGATGCTGTATGTGG	GTCGTGCAGCTCGATAGGAT	395	Deformed wing virus	Stoltz et al. (1995)
KBV	GATGAACCTGCACCTATTGA	TGTGGTGGCTATGAGTCA	393	Kishmir bee virus	Shimanuki et al. (1994)
SBV	GGATGAAAGGAAATTACGAG	CCACTAGGTGATCCACACT	426	Sacbrood virus	Tentcheva et al. (2004)
IAPV	AGACACCAATCACGGACCTCAC	AGATTTGTCTCTCCAGTGCACAT	475	Israeli acute paralysis virus	Maori et al. (2007)

with Wizard SV Gel and PCR Clean-up System (Promega, USA) and then ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) followed by transformation into *E. coli* DH5  cells. Over 90% of the white colonies had variable sized inserts as determined by PCR amplifications with vector-specific primers and agarose gel analysis.

2.7. SSH library screening by dot-blot hybridizations

Transformants were randomly selected from the subtracted library to amplify the inserted sequences. Each reaction tube contained 2.5  l 10  Taq buffer, 0.5  l dNTP (10 mM each), 1  l of nested primer 1 and nested primer 2R (10  M), 18.75  l of PCR-grade water and 0.25  l Taq DNA polymerase (5 U/ l). PCR was performed according to the following parameters: 95  C for 5 min and 28 cycles at 95  C for 30 s, 68  C for 30 s and 72  C for 1.5 min. PCR products were analyzed by electrophoresis on 1.0% agarose gel. 5  l PCR product of each positive clone was mixed with 5  l 0.6N NaOH. Then 1  l of mixture was applied to a positively charged nylon membrane (Hybond-N⁺) (Amersham, NJ, USA). PCR products of subtracted cDNAs were purified separately by a PCR Purification Kit (Wizard SV Gel and PCR Clean-up System, Promega) and cleaved with RsaI. The cleaved cDNAs were probed by Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences). Two rounds of differential screening were performed to screen the subtracted library. Two copies of positively charged Hybond-N⁺ nylon membrane (Amersham, NJ, USA) were prepared for differential screening by subtracted probes. Hybridization and washing were carried out by conventional protocol. Immunological detection procedure was according to the recommendations of the manufacturer (Roche Applied Sciences). The membranes were scanned by UMAX 3.5 scanner (UMAX, USA), then analyzed by Quantity One 4.6.2 program. The positive clones up-regulated or down-regulated 1.0 folds were selected for further analysis and sequenced by using T7 or SP6 promoter primers.

Table 2Primers of the qRT-PCR analysis of differentially expressed genes of *A. cerana* and *A. mellifera*.

Product name	Gene ID	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)	Amplification efficiency ^a (R ^b)
Actin	406122	ATGCCAACACTGCCTTTCTGG	GACCCACCAATCCATACGGA	150	2.27(0.997)
Vdhl	726182	GCATCACCTTCTGACCAACC	ACCTCGTCCAACATCCTTCT	228	2.82(0.903)
Hex70c	409354	GAACGCCAGAGCTCGGAATCAC	GGTTTACCACGGGCAAGATGAG	161	2.04(1.000)
Npy-r	411614	AGGGATATTTGGTTTGATTGG	GTGGAGAAGATGCTCTGGGAT	162	2.05(0.980)
Faa	552210	GCTATCTTTGTCCGAGGA	AACCACATTTCTCCATG	183	2.05(0.980)
Mlc	409881	AATCTCTTCGCATCTCGC	CGCATCGTTGACTTCTT	165	2.13(0.998)
Headcase	724595	TATGAGTTGCCTGGAGGGTAGTGG	ATAGAAGTTGAGCCGCTGGTGTGG	198	1.87(0.959)
Dsp	725551	TTGTCTTTCGGAAATTTCTA	CTTTACTTTCTCTTTTGGTGG	153	2.25(0.998)
Pgi	551154	AATGAAGCACGAAACCGAA	CTCCAAGAATAAAAAGCGG	139	2.13(0.998)
Defensin	406143	GTCCGCCCTTCTTTCATGG	TGACCTCCAGCTTTACCCAAA	199	2.08(0.993)
PO	551550	AATCCATTACCTGAAATTTGATCTTAT	TAATCTTCCAATAATTCATACGCTCTT	200	2.04(0.995)
Pcmt	408785	CGAAAAACGTAAGCGAAGATT	ACCTTCTATAGCAACAACCTGGACA	205	2.10(0.994)
Nedd8	552822	TCTGAAAGCAAATGAATGA	AGAGAACAAGACGATTACAAGT	251	2.05(0.980)
Para	409018	TCAAGCGACCGAGGATGT	ACTTGTCCGGCTTGTGTATCTG	169	2.11(0.994)

Gene IDs refer to the cDNAs selected from the Genbank. *Actin* is the housekeeping gene; *defensin* and *PO* were selected from Yang and Cox-Foster (2005); *Pcmt*, *Nedd8* and *Para* were selected as Navajas et al. (2008); others were from these SSH libraries. Forward and reverse primer sequences and the PCR product length are indicated.

^a Amplification efficiencies were calculated from the slope of standard curves as $E = 10[-1/\text{slope}] - 1 \times 100\%$ efficiency corresponds to an amplification efficiency of 1.

^b Regression coefficient of linear standard curve.

2.8. DNA sequence analysis

cDNA sequences from the SSH libraries were sequenced. The vector was trimmed using the VecScreen (<http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html>), and the sequences were filtered for polyA⁺ tail, for size (less than 100 bp), for doubles or for sequences with low quality read. To obtain unique fragments we made an assemble of the ESTs using the CAP3 program. Unique DNA sequences were compared against non-redundant nucleotide and protein databases using BLASTn/x. Searches for potential open reading frames (ORF) were carried out using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). We considered fair putative ORFs if they covered most of the sequence and had a minimum of 100 nucleotides. Classification of sequences was performed under Gene Ontology (GO) criteria. For a general approach, we used the BLAST2GO program (<http://www.blast2go.de/>) for a similarity search. Then we used the FlyBase to have the nomenclature of *Drosophila melanogaster* as a reference.

2.9. Real-time RT-PCR transcript quantification

Clones representing candidate loci (i.e., those that appeared to be expressed differently based on the screening technique described above), immune genes (*PO*, *defensin*) (Yang and Cox-Foster, 2005) and genes (*pcmt*, *nedd8*, *para*) identified in Navajas et al. (2008) were selected further by quantitative real-time PCR (qRT-PCR). These analyses were carried out using *A. mellifera* and *A. cerana* prepupae challenged by *Varroa* mites for 8 h, and prepupae without *Varroa* mites challenge were used as the negative controls. Total RNA from these prepupae was extracted and quantified as described above. After sample purification, reverse transcription into the first strand cDNA was made with the AffinityScriptTM Multiple Temperature Reverse Transcriptase (Stratagene, USA) using Oligo (dT) primers (Clontech, USA). PCR primers used in qRT-PCR expression studies were designed using the Primer premier 5.0

Table 3
Characteristics of the dot-blot analysis of differentially expressed genes of *A. cerana* and *A. mellifera*.

No.	dbEST_ID	Length (bp)	ESTs	Gene predicted function	Accession no.	E-value	GO (molecular function)	GO (biology process)	Up/down
CF3	67920188	387	1	cg4995-isoform a	XP_001121709.1	6.02E–45	–	–	+2.7
CF4	67920189	348	9	Larval-specific very high density lipoprotein (<i>Vhdl</i>)	XP_001121939.1	2.00E–160	–	–	+2.4 to +5.3
CF7	67920190	504–510	3	Hexamerin 70c (<i>hex 70c</i>)	NP_001092187.1	5.41E–80	Oxygen transporter activity	Transport	+2.0 to +2.6
CF23	67920191	410–465	4	Aldehyde dehydrogenase (<i>Aldh</i>)	GI408559	7.60E–48	Oxidoreductase activity	Oxidation reduction	+1.2 to +2.3
CF37	67920192	554	1	Histone h3 type	GB14620	1.03E–66	DNA binding	Nucleosome assembly	+1.3
CF39	67920193	315	1	Similar to nucleoside diphosphate kinase (<i>NDK</i>)	GB17251	1.05E–44	Deoxyribonuclease activity	Negative regulation of cell proliferation	+1.6
CF63	67920194	480–532	2	Heat shock cognate 70 protein (<i>hsc 70</i>)	NP_001153522.1	9.12E–70	Nucleotide binding	Response to stress; nervous system development	+1.6 to 2.1
CF64	67920195	406	1	Stretchin-mlck cg18255-isoform a (<i>Strn-Mlck</i>)	NP_725510.1	3.32E–35	Zinc ion binding	–	+1.8
CF79	67920196	537–680	31	High glx storage protein (<i>hex 110</i>)	NP_001094493.1	4.10E–76	Oxygen transporter activity	Transport	+1.8 to +2.8
CF82	67920197	406	1	Odorant binding protein (<i>Obp14</i>)	GI677673	1.65E–26	Odorant binding	Transport	+1.7
CF83	67920198	389	1	Proteasome subunit beta type (<i>Psmb1</i>)	GB18905	3.30E–19	Threonine-type endopeptidase activity	Ubiquitin-dependent protein catabolic process	+1.8
CF128	67920199	392	1	39s ribosomal protein mitochondrial	GB10626	1.79E–49	Structural constituent of ribosome	Translation	+1.3
CF148	67920200	328	2	Similar to DAZ associated protein 2 (<i>DAZAP2</i>)	GB17466	6.00E–134	–	–	+2.2 to +9.3
CF153	67920201	413	2	ATP synthase beta subunit (<i>AtpB</i>)	GB13596	3.27E–51	Hydrogen ion transmembrane transporter activity	Ion transport	+2.2 to +3.9
CF161	67920202	320	1	Y37E3.4	XP_001123102.1	2.05E–16	–	–	+1.3
CF195	67920203	395	1	Ornithine aminotransferase (<i>Oat</i>)	GB12326	8.25E–58	Ornithine-oxo-acid transaminase activity	–	+1.4
CF205	67920204	471–534	3	Amp dependent CoA ligase	XP_001121814.1	3.26E–67	4-Coumarate-CoA ligase activity	Metabolic process	+1.2 to +2.2
CF445	67920205	374	1	Cytochrome P450 6a2 (<i>Cyp6a2</i>)	XM_001119981.1	8.00E–26	–	–	+3.5
CF447	67920206	742	1	Cytochrome oxidase ii (<i>CO II</i>)	AAG40082.1	2.35E–70	Copper ion binding	–	+3.1
CF489	67920207	310	1	Hypothetical protein	GB18183	1.25E–10	–	–	+1.3
CR4	67920219	522–546	2	rRNA promoter binding protein	XP_453843.1	1.41E–16	Receptor activity	–	–1.3 to –3.0
CR29	67920220	183–238	2	Neuropeptide y receptor-like cg5811-pa (<i>Npy-r</i>)	GB13527	2.00E–27	–	–	–2.0 to –2.2
CR71	67920221	421	1	fk506-binding protein	GB13529	1.01E–31	Catalytic activity	Protein metabolic process	–1.5
CR108	67920222	543	1	Isoform b	XM_001604577.1	4.15E–41	–	Cellular component organization and biogenesis	–1.7
CR109	67920223	363–383	3	Fumarylacetoacetate hydrolase (<i>Faa</i>)	GB13982	1.00E–59	Hydrolase activity	Amino acid and derivative metabolic process	–1.7 to –2.2
CR130	67920224	326–823	7	ac1147-like protein	GI544669	1.36E–28	–	–	–1.3 to –2.7
CR164	67920225	540	1	Protein 540	emb X89529.1	1.90E–14	–	Regulation of cellular respiration	–1.2
CR269	67920226	444	1	Ribosomal protein I27e	GB15660	8.24E–34	Structural molecule activity	Translation	–1.3
CR285	67920227	295	1	Tubulin alpha-1 chain (<i>tubα1</i>)	GI550827	4.67E–29	Hydrolase activity	Cytoskeleton organization and biogenesis	–2.6
MF49	67920237	461	1	RNA binding motif protein	XP_397567.2	3.06E–41	–	–	+1.3

MF94	67920238	537	1	Serine threonine-protein kinase (<i>STPK</i>)	GB13173	3.07E–17	Protein serine/threonine kinase activity	JNK cascade	+1.4
MF112	67920239	406	2	Protein n-terminal asparagine	GB10784	2.30E–20	–	Asparaginase activity	+1.3 to +2.3
MF139	67920240	618	1	Polypeptide c transferase 5 cg31651-pa	GI727154	1.11E–31	Polypeptide N-acetylgalactosaminyl-transferase activity	Calcium ion binding	+1.3
MF572	67920241	498	1	Myosin light chain 2 (<i>Mlc2</i>)	XP_393371.2	2.86E–42	Motor activity	–	+2.4
MF587	67920242	298	1	cg17181 cg17181-pa	GB12968	1.55E–24	–	–	+1.3
MF591	67920243	1069	1	Protein lethal (2) essential for life (<i>Efl21</i>)	GB10339	2.27E–42	–	Response to stress	+1.5
MF609	67920244	637	1	Signal peptidase complex subunit 3 (<i>Spcs3</i>)	GB14961	8.99E–48	Peptidase activity	Signal peptide processing	+1.4
MF618	67920245	723	1	DNA polymerase eta (<i>Pol eta</i>)	GB15618	5.76E–127	Damaged DNA binding	DNA repair	+1.3
MF634	67920246	1054	1	Merlin moesin ezrin radixin (<i>erm</i>)	EU179323.1	2.57E–30	Cytoskeletal protein binding	Olfactory behavior	+1.2
MF650	67920247	374	1	hect e3 ubiquitin ligase (<i>hect e3</i>)	XP_001122009.1	5.33E–49	Ubiquitin-protein ligase activity	Modification-dependent protein catabolic process	+ 2.3
MF710	67920248	543	1	Protocadherin fat 2 (<i>Fat2</i>)	XP_394631.3	1.80E–36	Cell adhesion molecule binding	Negative regulation of imaginal disc growth	+4.2
MF723	67920249	443	1	Class type 10a	XP_002121110.1	4.26E–22	Magnesium ion binding	ATP biosynthetic process	+3.5
MF814	67920250	587	2	Lissencephaly-1 CG8440-PA, isoform A (<i>Lis-1</i>)	XP_392399.1	4.60E–74	Dynein binding	Oogenesis	+3.5
MF823	67920251	738	2	Proteasome subunit alpha type-3 (<i>psma3</i>)	XP_392518.1	1.01E–73	Threonine-type endopeptidase activity	Ubiquitin-dependent protein catabolic process	+1.5 to +2.5
MF856	67920252	694	1	Headcase protein (<i>headcase</i>)	XP_001607251.1	8.32E–72	Pyridoxal phosphate binding	Negative regulation of terminal cell fate specification, open tracheal system	+3.6
MF950	67920253	456	2	Ubiquitin carboxyl-terminal hydrolase isozyme 15 (<i>uch-15</i>)	XP_397252.2	3.65E–18	Hydrolase activity	–	+3.5
MF966	67920254	411	1	Chitinase 10 (<i>chi10</i>)	XP_395734.3	1.99E–40	Chitinase activity	Chitin metabolic process	+3.2
MR11	67920275	443	1	Dorsal switch protein isoform f (<i>Dspf</i>)	XP_001121384.1	1.89E–70	Transcription regulator activity	Multicellular organismal development	–2.7
MR16	67920276	762	1	Mitotic protein phosphatase 1	XP_395550.3	1.11E–94	Protein binding	–	–1.9
MR27	67920277	362–1230	13	Hexamerin 110 (<i>hex 110</i>)	EU105212.1	1.88E–38	Transporter activity	Transport	–1.2 to –2.6
MR32	67920278	615	2	Neuropeptide y receptor-like cg5811-pa (<i>Npy-r</i>)	GB13527	9.24E–55	–	–	–1.3 to –1.5
MR35	67920279	627	2	Conserved plasmodium protein	GB11199	2.21E–43	GTPase activity	Chromatin assembly or disassembly	–1.3 to 1.5
MR58	67920280	568	1	ras-related GTP binding c (<i>rragc</i>)	GB19705	6.65E–88	Lipid binding	Signal transduction	–1.4
MR59	67920281	439	1	26s protease regulatory subunit 6b	GB19878	2.50E–35	Peptidase activity	Protein metabolic process	–1.3
MR71	67920282	1172	1	Enhancer of zeste 2 isoform a (<i>Ezh2</i>)	GB15558	8.67E–30	Transferase activity	Cellular component organization and biogenesis	–1.5
MR73	67920283	360	1	YTH domain containing 2 (<i>Ythdc2</i>)	GB15771	6.45E–23	–	–	–1.6
MR89	67920284	292	1	Saposin-related protein	GB16561	7.47E–35	–	Sphingolipid metabolic process	–1.3
MR98	67920285	738	1	Microsomal dipeptidase (<i>Mdp</i>)	GB11278	6.98E–83	Dipeptidyl-peptidase activity	Proteolysis	–1.3
MR148	67920286	337	1	Leucine zipper-ef-hand containing transmembrane protein (<i>letm1</i>)	XP_624231.1	2.59E–48	Calcium ion binding	–	–1.8
MR168	67920287	657	1	Hypothetical protein	GB19828	6.97E–62	–	–	–1.6
MR174	67920288	590	1	Kazal-type proteinase inhibitor (<i>KPI</i>)	GI408510	4.01E–25	Peptidase activity	–	–1.7
MR175	67920289	1151	1	Phosphoglucose isomerase (<i>Pgi</i>)	XP_623552.1	7.25E–123	Isomerase activity	Glucose metabolic process	–1.6
MR247	67920290	1161	1	Cytochrome c oxidase subunit 1 (<i>COX1</i>)	gb M23409.1	2.90E–110	Electron carrier activity	Generation of precursor metabolites and energy	–1.3
MR418	67920291	291	1	NADH dehydrogenase subunit 1 (<i>ND1</i>)	GI807692	4.26E–14	Catalytic activity	Metabolic process	–1.5

No., ESTs, up/down from dot-blot analysis; gene predicted function, gene ID, E-value resulting from a BLAST search of the GenBank database (BLASTx); dbEST_ID from the clone sequence submitted to dbEST bank. GO molecular function and biology process result in the Blast2GO analysis. "CF" or "CR" refers gene up-regulated or down in *A. cerana*; "MF" or "MR" refers gene up-regulated or down in *A. mellifera*.

(Table 2). Expression of *actin* gene (GI: 406122) was used as a reference (primers indicated in Table 2). The efficiency of each primer set was first validated by constructing a standard curve through five serial dilutions. PCR reactions were carried out in triplicate in an Mx3000P™ Real-Time PCR System (Stratagene, USA), using SYBR[®] Green (Brilliant II SYBR[®] Green QPCR Master Mix; Stratagene, USA). A control without template was included in all batches. The PCR program began with a single cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 55 °C for 60 s. Afterwards, the PCR products were heated to 95 °C for 15 s, cooled to 55 °C for 15 s and heated to 95 °C for 15 s, in order to measure the dissociation curves and to determine a unique PCR product for each gene. mRNA levels were calculated relative to *actin* expression using the Mx3000P™ Software (version 4.1) (Agilent, USA).

2.10. Statistical data analyses

Data were analyzed with normal one-way analysis of variance (ANOVA), using SPSS statistical software and the significance between treatments in each experiment was evaluated by Duncan's multiple range test at $P < 0.05$. The values are expressed as means \pm S.D. $P < 0.05$ was defined as statistically significant.

3. Results

3.1. Virus in the bee prepupae and mites

All the mites and prepupae used in this study were checked by qRT-PCR method for the possible presence of the following viruses, BQCV, CBPV, DWV, KBV, SBV, or IAPV. Only DWV was present in the mites. No signs of clinical American foulbrood or of viral disease were observed in these samples.

3.2. General library statistics

In order to find differentially expressed genes in the prepupae of *A. mellifera* and *A. cerana* challenged by the *Varroa* infection, we constructed two pairs of subtracted cDNA libraries for the prepupae of *A. mellifera* and *A. cerana*. The tester library was prepared with the prepupae infected by the mites, whereas the driver library was prepared with the prepupae without the infection of the mites. We screened 2640 clones from these two libraries, using the dot-blot screening protocol, generating 427 differentially expressed candidate clones (Tables 3 and 4). These candidate clones resulted in 427 sequences, 140 of which were filtered as low quality reads, polyA+ tails or vector sequences. To identify unique sequences from the resulting 287 useful ESTs, we carried out an assemble using the CAP3 program. After assembling, the 287 ESTs were clustered into 132 unique sequences distributed into 26 contigs and 106 singlets, where 49 consisted in *A. cerana* subtracted library and the rest 83 ESTs in *A. mellifera* subtracted library. These 132 sequences were deposited in the dbEST gene bank database with accession numbers from [dbEST: 67920188] to [dbEST: 67920307] and [dbEST: 67921313] to [dbEST: 67921324]. Among the 49 different regulation unique sequences of *A. cerana* subtracted library, 31 (63%) of these genes were significantly up-regulated and 18 (37%) down-regulated. There were 83 regulated genes in *A. mellifera*, 50 (60%) of which were up-regulated and 33 (40%) down-regulated. The 132 unique sequences resulting from assembling were compared against available databases of all organisms, as well as of *D. melanogaster* and *Homo species* in particular, in order to find similarities with known sequences. We carried out direct nucleotide comparison (blastn), followed by a dynamic translation comparison (blastx), and only matches with E-values lower than $1E-04$ were considered significant for labeling them as known genes. Sequences with an E-value higher than $1E-04$, were labeled as undescribed.

In summary, we found that 85% (112) sequences had counterpart genes known in other organisms (Tables 3 and 4) whereas 15% (20) were undescribed. The ORF finder program was used to predict putative open reading frames (ORFs) in the assembled unique sequences. Setting a minimal length of 100 bp and considering that the ORF covers most of the sequence as premises, results indicated that 126 of 132 unique sequences contain a putative ORF (Tables 3 and 4). Interestingly, all the up-regulated and down-regulated putative genes were different in both honey bee species, except the hexamerin-related gene (*hex 110*), which was up-regulated in *A. cerana* but down-regulated in *A. mellifera* after induction of *Varroa* mites. The *Npy-r* gene was down-regulated in both of the two species.

3.3. Gene Ontology analysis

To add biological meaning to the relatively large amount of SSH-derived data, we searched the Gene Ontology database for the putative biological processes and molecular functions for the genes that showed significant differences in expression. Among these 132 genes, 21 from the *A. cerana* subtracted library and 30 from the *A. mellifera* subtracted library had information in Gene Ontology. To unravel functional differences related to bee species, we analyzed sets of genes for overrepresentation in functional GO categories according to biological process and molecular function (Table 3).

Regarding biological process (Fig. 1A), genes with expression differences in both bee species included the following categories: transport (GO: 0006810), primary metabolic process (GO: 0044238), anatomical structure morphogenesis (GO: 0009653), cellular component organization (GO: 0016043), cellular metabolic process (GO: 0044237), macromolecule metabolic process (GO: 0043170), multicellular organismal development (GO: 0007275), response to stress (GO: 0006950), cell recognition (GO: 0008037), anatomical structure development (GO: 0048856), anatomical structure organization (GO: 0009653), regulation of biological quality (GO: 0065008), regulation of cellular process (GO: 0050794), anatomical structure organization (GO: 0009653). The *A. cerana* up-regulated gene categories that were most frequent included transport (54%) and primary metabolic processes (10%) while the most frequent down-regulated genes included cellular metabolic processes (33%) and primary metabolic processes (25%). In *A. mellifera* up-regulated genes involved in anatomical structure morphogenesis (14%), cellular metabolic processes (14%) and anatomical structure development (14%) showed significant enrichment, but down-regulated genes were mainly involved in cellular metabolic processes (33%) and primary metabolic processes (25%).

The category of molecular function included 16 functions (Fig. 1B). The molecular functions of *A. cerana* up-regulated genes were nucleotide binding GO: 0000166 (30%), hydrolase activity GO: 0016787 (25%), transferase activity GO: 0016740 (20%), protein binding GO: 0005515 (15%), and nucleic acid binding GO: 0003676 (10%), while those of the down-regulated genes in the most frequent functions were nucleotide binding GO: 0000166 (29%), hydrolase activity GO: 0016787 (21%), and signal transducer GO: 0004871 (14%). *A. mellifera* up-regulated genes were represented in 15 molecular function categories, with the most frequent function being hydrolase activity GO: 0016787 (33%), and the down-regulated genes in the most frequent function were protein binding GO: 0005515 (30%).

3.4. Expression patterns

To further evaluate the efficiency and quality of the SSH results, the expression pattern of a selection of putative genes, based on

Table 4Characteristics of the dot-blot analysis of differentially expressed genes of *A. cerana* and *A. mellifera*.

No.	dbEST_ID	Length	ESTs	ORF	Accession no.	Gene description	E-value	Up/down
CF25	67920208	328	1	Yes	GI410897	Similar to CG6013-PA	4.00E–05	+1.2
CF52	67920209	384–549	56	Yes	AF250954.1	<i>A. cerana</i> 16S ribosomal RNA gene, partial sequence; mitochondrial	0	+1.3 to +2.8
CF143	67920210	248	1	No	GI100191002	Ribosomal protein L41 (Rpl41)	8.00E–93	+1.8
CF284	67920211	300	1	Yes	FJ416891.1	Yunnan 16S ribosomal RNA gene	2.00E–144	+1.5
CF314	67920212	224	5	Yes	AB052895.1	18S rRNA, 5.8S rRNA, 28S rRNA	0	–1.4 to +2.3
CR3	67920228	200–216	4	Yes	GI544668	28S ribosomal RNA	5.00E–98	–1.4 to +2.6
CR32	67920229	244	7	Yes	EU164626.1	18S ribosomal RNA gene	5.00E–98	–1.5 to +2.8
CR48	67920230	776	1	Yes	FJ475128.1	<i>Diachasmimorpha longicaudata</i> 18S ribosomal RNA gene and internal transcribed spacer 1	0	–1.6
CR83	67920231	184	1	Yes	GI725387	Similar to CG15040-PA	2.00E–78	–1.7
CR106	67920232	420	1	Yes	FJ475128.1	<i>Diachasmimorpha longicaudata</i> 18S ribosomal RNA gene and internal transcribed spacer 1	0	–1.6
CR152	67920233	487–606	4	Yes	X89529.1	28S ribosomal RNA (D1 and D2 regions)	0	–1.6 to +2.4
CR201	67920234	775–889	2	Yes	GI550651	Similar to ribosomal protein S4 CG11276-PA	0	–1.6 to +2.6
CR239	67920235	514	1	Yes	GI410280	Similar to ran CG1404-PA	2E–142	–1.5
CR270	67920236	285–601	2	Yes	AB052895.1	<i>Myrmecia croslandi</i> genes for 18S rRNA, 5.8S rRNA, 28S rRNA	1E–73	–1.1 to +2.4
MF1	67920255	382	1	Yes	GI725647	Similar to 40S ribosomal protein S6	0	+1.2
MF8	67920256	323	1	Yes	GI724378	Similar to BMP and activin membrane-bound inhibitor (BAMBI)	4.00E–168	+1.5
MF10	67920257	595	1	Yes	GI551511	Similar to Elongin B CG4204-PA	0	+1.2
MF12	67920258	525	1	Yes	AF250955.1	16S ribosomal RNA gene	0	+1.2
MF45	67920259	676	1	Yes	GI726543	Hypothetical protein LOC726543	0	+1.4
MF67	67920260	446	1	Yes	GI550695	Similar to mitochondrial carnitine palmitoyltransferase I CG12891-PA, isoform A (CPT I)	2.00E–121	+1.7
MF86	67920261	530	1	Yes	GI408976	Similar to shaggy CG2621-PA	0	+1.5
MF98	67920262	277	1	Yes	GI726924	Similar to Synapse-associated protein 47kD CG8884-PA, isoform A	3.00E–100	+1.1
MF127	67920263	1144	1	Yes	GI551071	Similar to toutatis CG10897-PA, isoform A	0	+1.6
MF470	67920264	406	1	Yes	GI55081	Intraflagellar transport 57 homolog (Chlamydomonas) (IFT57)	2.00E–07	+1.6
MF476	67920265	761	1	Yes	GI726375	Similar to maverick CG1901-PA, isoform A	0	+1.5
MF486	67920266	387	1	Yes	GI551005	Similar to hexokinase A CG3001-PA, isoform A (HKA)	3.00E–16	+1.2
MF533	67920267	207	1	Yes	GI413534	Similar to O-sialoglycoprotein endopeptidase-like 1	5.00E–58	+1.1
MF549	67920268	589	1	Yes	GI408301	Similar to CG6707-PA, isoform A	0	+1.2
MF563	67920269	481	1	Yes	XM_393300.3	Similar to CG7033-PA	2.00E–115	+1.5
MF575	67920270	332	1	Yes	XM_391860.3	Similar to B52 CG10851-PB	1.00E–28	+2.8
MF622	67920271	298	1	Yes	XM_001120596.1	Similar to CG17181-PA	3.00E–79	+6.2
MF623	67920272	467	1	Yes	GI410956	Similar to nemo CG7892-PE, isoform E	1.00E–20	+1.6
MF648	67920273	221	1	Yes	GI724682	Hypothetical protein LOC724682	4.00E–32	+1.4
MF933	67920274	297	1	Yes	EU022152.1	Unknown	4.00E–04	+4.3
MR4	67920292	594	1	Yes	GI551350	Similar to CG31715-PA	0	–1.3
MR7	67920293	707	1	Yes	GI725238	Histone H1	0	–1.4
MR8	67920294	712	1	Yes	GI544670	Elongation factor 1-alpha (EF1-alpha)	0	–1.2
MR10	67920295	107	1	Yes	GI724187	Similar to leucine rich repeat containing 40	4.00E–40	–1.1
MR24	67920296	784	1	Yes	XM_392032.3	Similar to CG32638-PA	3.00E–96	–1.5
MR45	67920297	432	1	Yes	GI544669	18S ribosomal RNA	4.00E–88	–1.6
MR50	67920298	841	1	Yes	GI551257	Similar to CG1943-PA, isoform A	0	–1.4
MR51	67920299	334–359	2	Yes	GI552830	Similar to soluble NSF attachment protein CG6625-PA	5.00E–84	–1.2 to –1.3
MR93	67920300	406	1	Yes	XM_623633.2	Similar to CG32250-PA	0	–1.3
MR134	67920301	419	1	Yes	XM_623355.2	Similar to CG1599-PA	0	–1.6
MR248	67920302	408	1	Yes	GI550749	Similar to pugilist CG4067-PA, isoform A	1.00E–95	–1.3
MR283	67920303	348	1	No	GI409207	Similar to synaptic vesicle membrane protein VAT-1 homolog	1.00E–103	–1.4
MR309	67920304	1074	1	Yes	GI551519	Similar to CG13188-PB, isoform B	0	–1.2
MR423	67920305	733	1	Yes	XM_001120252.1	Hypothetical protein LOC725074	0	–1.1
CF526	67920213	287	1	Yes		No significant similarity found		+2.5
CF106	67920214	281	1	Yes		No significant similarity found		+1.2
CF160	67920215	301	1	Yes		No significant similarity found		+1.3
CF164	67920216	343	1	Yes		No significant similarity found		+1.6
CF180	67920217	524	1	Yes		No significant similarity found		+1.2
CF158	67920218	291	1	Yes		No significant similarity found		+1.2
MF581	67921313	486	1	Yes		No significant similarity found		+1.8

Table 4 (Continued)

No.	dbEST_ID	Length	ESTs	ORF	Accession no.	Gene description	E-value	Up/down
MF675	67921314	413	1	No		No significant similarity found		+3.5
MF711	67921315	455	1	Yes		No significant similarity found		+3.3
MF728	67921316	389	1	Yes		No significant similarity found		+3.1
MF751	67921317	587	1	Yes		No significant similarity found		+3.5
MF772	67921318	251	1	Yes		No significant similarity found		+2.9
MF816	67921319	442	1	Yes		No significant similarity found		+2.3
MF883	67921320	549	1	No		No significant similarity found		+3.4
MF890	67921321	379	1	No		No significant similarity found		+3.2
MF893	67921322	606	1	Yes		No significant similarity found		+4.1
MF929	67921323	437	1	Yes		No significant similarity found		+2.8
MF953	67921324	266	1	Yes		No significant similarity found		+2.9
MR22	67920306	165	1	Yes		No significant similarity found		-1.6
MR337	67920307	345	1	Yes		No significant similarity found		-1.2

No., ESTs, up/down from dot-blot analysis; gene description, gene ID, E-value resulting from a BLAST search of the GenBank database (BLASTn); dbEST_ID from the clone sequence submitted to dbEST bank; "CF" or "CR" refers gene up-regulated or down in *A. cerana*; "MF" or "MR" refers gene up-regulated or down in *A. mellifera*.

their predicted involvement of the mite infection, was investigated by qRT-PCR.

Differently expressed levels of eight cDNAs from *A. cerana* (*Vhdl*, *hex 70c*, *Npy-r* and *Faa* genes) and *A. mellifera* (*Mlc*, *headcase*, *Dsp* and *Pgi* genes) after 8-h induction of *Varroa* mites were quantified (Fig. 2). In all cases, expression levels quantified by qRT-PCR are represented by the mean of 3 measurements. In accord with SSH analysis, cDNAs from *Varroa*-induced *A. cerana* bees were up-regulated for genes *Vhdl* and *hex 70c* (Fig. 2A and B) or down-regulated for genes *Npy-r* and *Faa* (Fig. 2C and D); From the *Varroa*-challenged *A. mellifera* bees, expression was up-regulated for *Mlc* and *headcase* genes (Fig. 2E and F) and down-regulated for *Dsp* and *Pgi* genes (Fig. 2G and H). It was noticeable that expression of *Dsp* and *Pgi* genes in *A. cerana* was much higher than that in *A. mellifera*, although it was not different from *A. cerana* following introduction of the mites.

4. Discussion

4.1. The SSH library

In the present study, the SSH approach has proven to be efficient for isolating genes specifically expressed in the prepupae of *A. cerana* and *A. mellifera* induced by the early infection of *Varroa* mites. We obtained sequences of 33 up-regulated and 19 down-regulated genes from *A. cerana*, and 51 up-regulated and 34 down-regulated genes from *A. mellifera*. The expression patterns of eight of the selected genes from the prepupae of *A. cerana* and *A. mellifera* revealed that the up-regulated or down-regulated mRNA levels generally occur after challenged by the mites (Fig. 2), thus validating the SSH approach.

Some short sequences with no significant similarity to other known genes, from the SSH libraries (Tables 3 and 4), may derive from the construction procedure of the SSH library. During establishment of the SSH library, the double stranded cDNAs are digested with the enzyme *RsaI* to ligate an adaptor for PCR amplification. Apparently, this restriction site may be very frequent in the genome, thus promoting the formation of short fragments, which makes comparisons with databases more difficult. This circumstance can account for the relatively high percentage of undescribed sequences encountered. Another reason for explaining this high percentage may be that some ESTs could correspond to 3' or 5' untranslated regions (UTRs), which make impossible the finding of homologues in protein databases (Irles et al., 2009).

4.2. *Varroa* infection and differentially expressed genes

Varroa mites usually enter the uncapped cells containing the prepupae before the cells are capped. One of the consequences

of *Varroa* parasitism is a decline in immune capacity which appears to induce the proliferation of viruses such as deformed wings virus in bees (Yang and Cox-Foster, 2005). In this study, we did not detect seven viruses (BQCV, CBPV, DWV, KBV, SBV and IAPV) from the prepupae of either bee species infected by the mites after 8 h, although DWV was present in the mites. The reason why the DWV present in the infected mites was not detected from the prepupae was unclear. It seemed likely that the titer of the virus probably introduced by the mites into the prepupae was not high enough to be detected by the present RT-PCR method or that the time (8 h) was not long enough for virus proliferation. Given the possibility that mites can transmit disease, it may be possible that the gene expression from the prepupae in the presence of both virus and mites, as reported by Gregory et al. (2005), Evans (2006) and Navajas et al. (2008), is adaptable for both pathogens, although mite stimulation was considered to be the main induction factor.

Interestingly, upon mite introduction, 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, encoding a receptor protein, which was down-regulated in both bee species. Whether two bee species use different pathways in response to mite stimulus needs further research. Although up-regulated and down-regulated genes were present from both bee species in the present SSH libraries, we did not see a significant change (at least more than 1.0-fold) on transcript abundance of immune pathway members reported from the pupae by Gregory et al. (2005) and Evans (2006). All the differentially expressed genes identified in this study, except *Strm-Mlc*, were also different from those detected by microarrays from *A. mellifera* associated with *Varroa* infection (Navajas et al., 2008). This may be due to different induction stress. The differentially expressed genes were identified from *A. mellifera* challenged by 8 h mite infection in the present study, but associated with *Varroa* infection by Navajas et al. (2008).

We also used qRT-PCR to determine transcript abundance of the immune-related genes *PO* and *defensin* (Fig. 3), and the candidate genes involved in cellular and molecular repair (*Pcmt* and *Nedd8*) and in the behavioural tolerance (*para*) to *Varroa* reported by Navajas et al. (2008). Significant differences in *PO* and *defensin* expression was detected in the prepupae of *A. cerana* by mite introduction. However, *PO* and *defensin* expression was very low in *A. mellifera* and showed no significant mite effects. Interestingly, although no DWV viral RNA was displayed by qRT-PCR from the samples of *A. cerana* and *A. mellifera*, significant transcript abundance of the identified genes for protein repair (*Pcmt*) and the labeling of proteins for degradation (*Nedd8*) (Navajas et al., 2008) was displayed in *A. cerana* challenged by *Varroa* mites, but

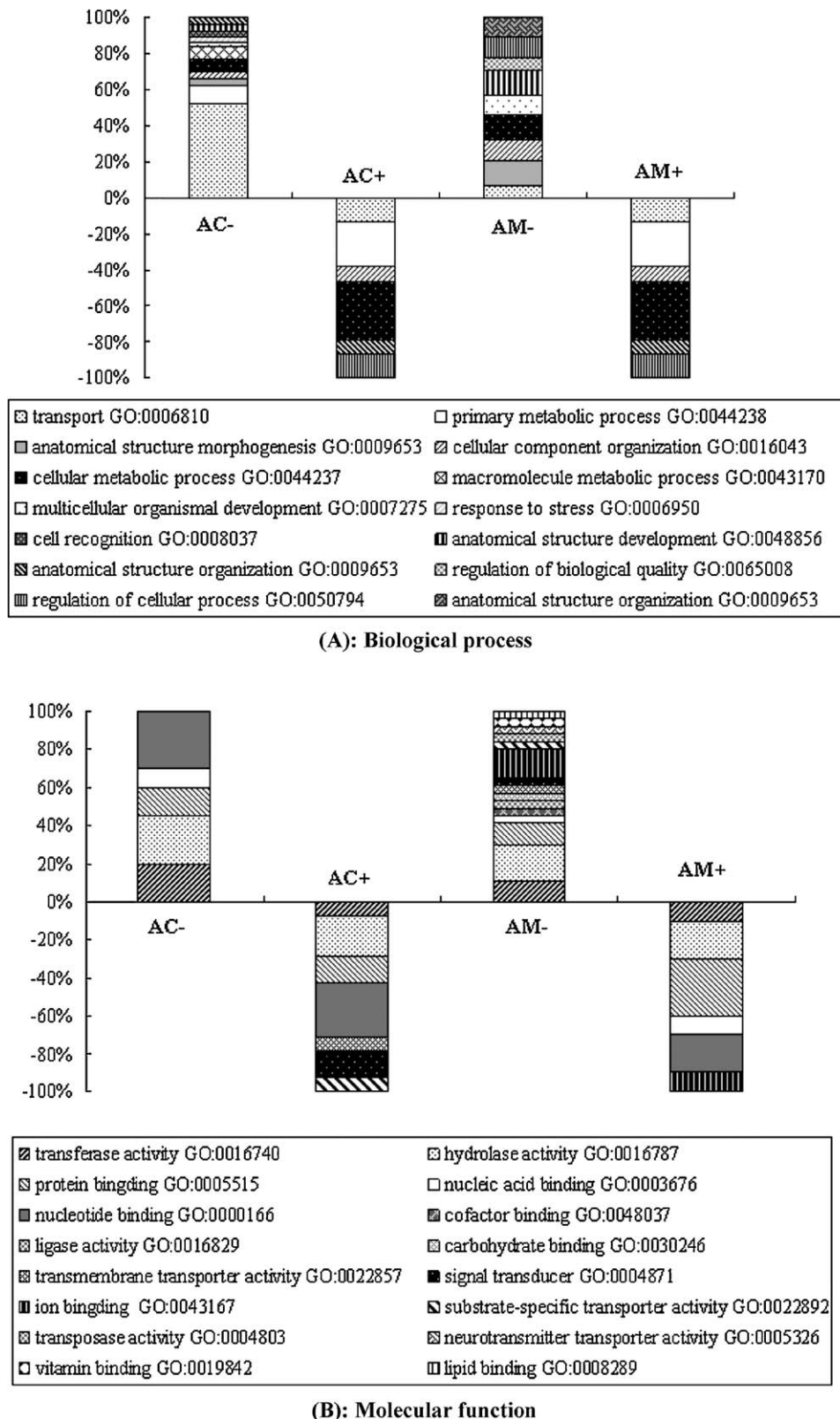


Fig. 1. Classification of ESTs according to Gene Ontology criteria. Molecular function (A) and biological process (B) clustering based on Gene Ontology of genes that show significant differences are indicated for prepupae challenged by *Varroa*. “AC+” represents genes up-regulated in *A. cerana*; “AC-” represents genes down-regulated in *A. cerana*; “AM+” represents genes up-regulated in *A. mellifera*; “AM-” represents genes down-regulated in *A. mellifera*.

not in *A. mellifera* samples. The paralytic gene (*para*), known to be important in the conducting of nerve action potentials in flies (Ganetzky, 1984), was also highly up-regulated. It should be noticed that *Pcmt* and *para* genes were expressed in *Varroa*-

tolerant *A. mellifera* population, and that *Nedd8* was affected by *Varroa* parasitism (Navajas et al., 2008). Taken together, these genes suggest a mechanism by which *Varroa*-tolerant *A. cerana* bees were more sensitive to *Varroa* stimuli than *Varroa* sensitive *A.*

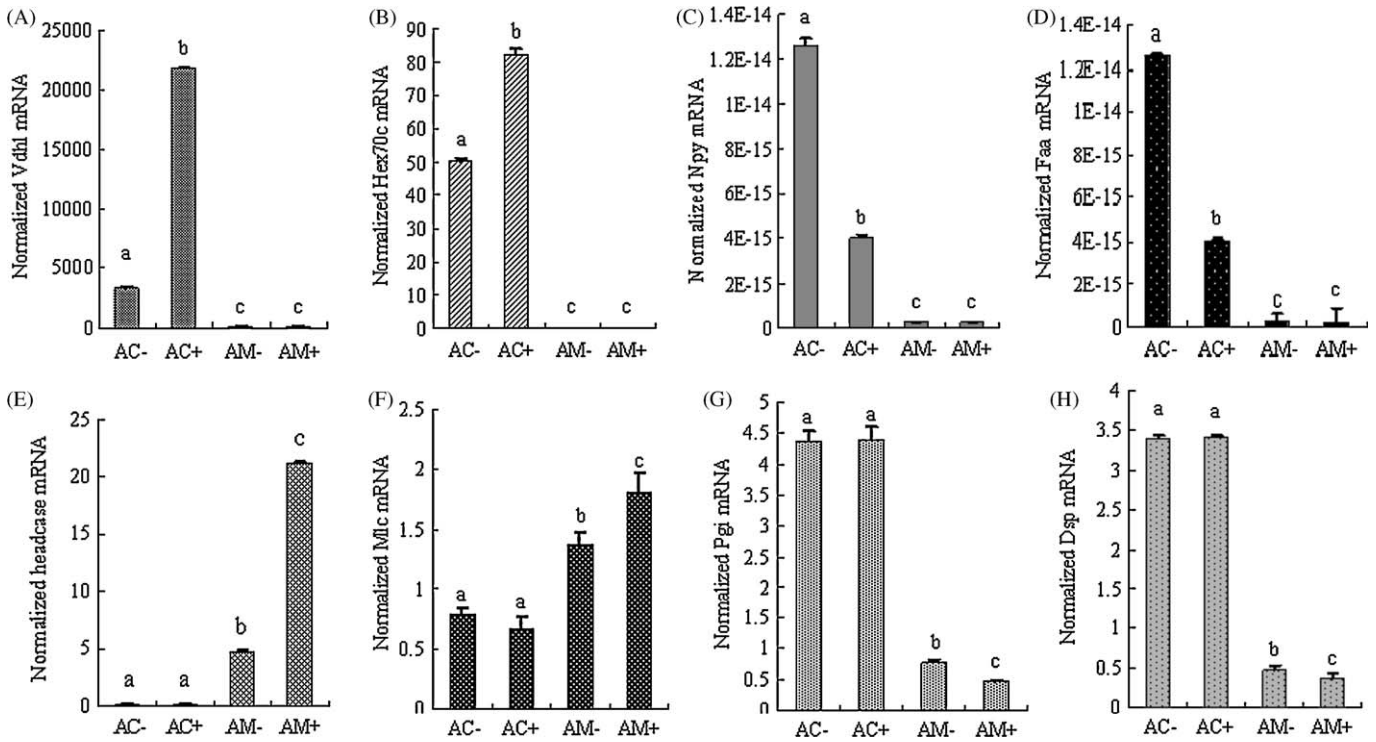


Fig. 2. cDNA levels of eight transcripts shown by SSH analysis to be significantly regulated in *A. cerana* and *A. mellifera*, respectively. Samples of *A. cerana* and *A. mellifera* bees challenged with *Varroa* (indicated as AC+ or AM+, respectively) or free of *Varroa* (indicated as AC- or AM-, respectively). All of the values shown are mean \pm S.D. The bars with different letters are significantly different (ANOVA, $P < 0.05$). (A) and (B) The expression of *Vdh1* and *hex70c* genes were up-regulated in the *Varroa*-challenged *A. cerana* bees; (C) and (D) the expression of *Npy-r* and *Faa* genes were suppressed in the *Varroa*-challenged *A. cerana* bees; (E) and (F) the expression of *headcase* and *Mlc* genes were up-regulated in the *Varroa*-challenged *A. mellifera* bees; (G) and (H) the expression of *Pgi* and *Dsp* genes were down-regulated in the *Varroa*-challenged *A. mellifera* bees.

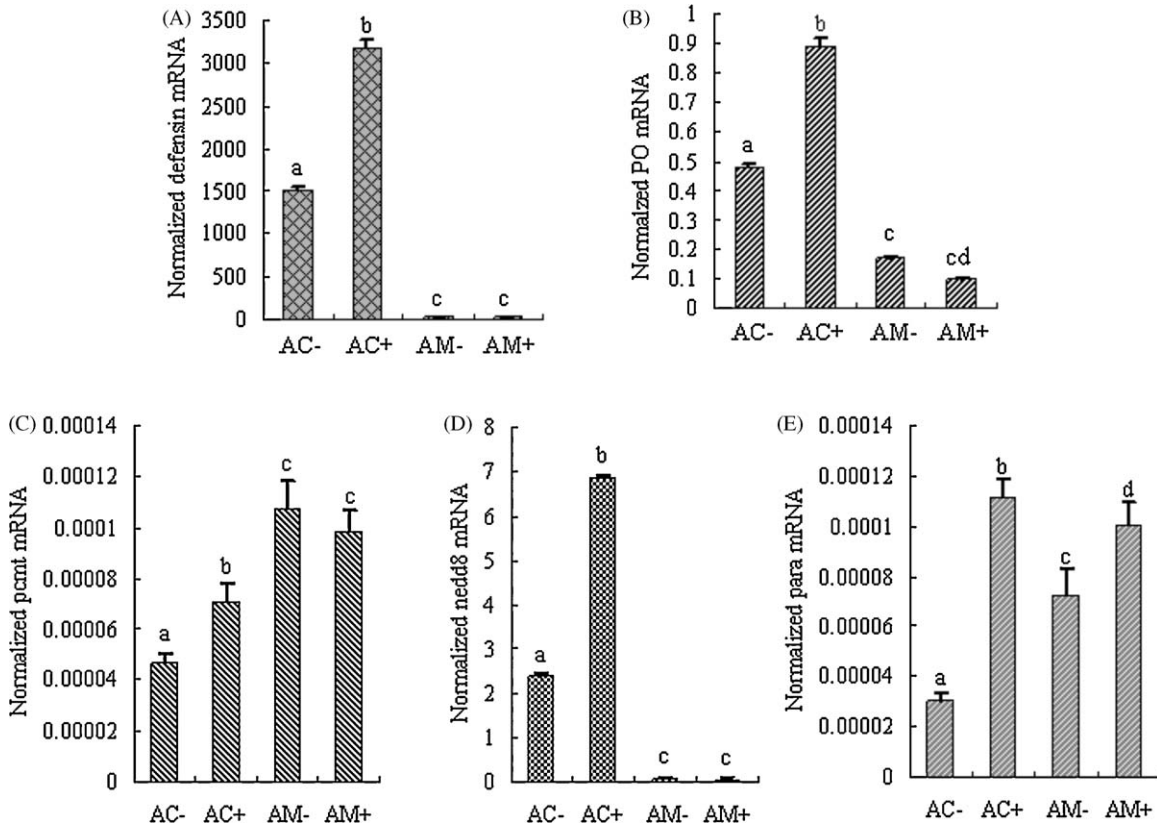


Fig. 3. cDNA levels of five regulated genes related to mite infection. Samples of *A. cerana* and *A. mellifera* bees challenged with *Varroa* (indicated as AC+ or AM+, respectively) or free of *Varroa* (indicated as AC- or AM-, respectively). All of the values shown are mean \pm S.D. The bars with different letters are significantly different (ANOVA, $P < 0.05$). (A) and (B) The expression of *defensin* and *PO* genes after 8 h mite induction; (C)–(E) the expression of *pcmt*, *para* and *nedd8* genes after 8 h mite induction.

mellifera bees. Whether these genes in *A. cerana* upon *Varroa* induction are responsible for the mite tolerance needs further investigation.

It should be pointed out that the differential expression of the above genes detected by qRT-PCR was not visualized from the SSH libraries. The reason may be related to the detection methods. We only selected the positive clones up-regulated or down-regulated 1.0-fold from SSH libraries for further sequence analysis. Although these expressed genes could be quantified by qRT-PCR, their differential expression was not high enough to be visualized by SSH libraries. It seems that qRT-PCR is more sensitive to detect differentially expressed genes than SSH method due to the limitation in sensitivity of the dot-blot.

4.3. Expressed sequences from *A. cerana*

Among twenty-six known up-regulated gene sequences from *A. cerana* prepupae (Table 3), three genes (*Aldh*, *Dazap2*, and *COII*) have been described as involved in the catalytic or oxidase activity (Santos et al., 2005; Horn and Barrientos, 2008; Guo et al., 2009), which may be important for responding invader pathogens and environmental stress. Three genes (*vdhl*, *hex 70c*, *hex 110*) encode storage proteins which are essential for construction and differentiation of adult tissues in holometabolous insects during metamorphosis (Burmester and Scheller, 1999). In particular, at least four different hexamerin genes (*hex 70a*, *hex 70b*, and *hex 70c*; *hex 80/110*) are present in honey bees (Cunha et al., 2005). In general, the abundance of Hex 70b in worker larvae and pupae is gradually decreased in honey bee hemolymph (Danty et al., 1998; Cunha et al., 2005). It seemed that infection of the *Varroa* mite increased the hexamerin Hex 70c and Hex 110, and this may reflect the possible effect of the pupal development. The cytochrome P450 6a2 gene (*cyp6a2*) is up-regulated in insecticide resistant house fly, *Musca domestica* (Zhu et al., 2008) and heat shock cognate 70 protein (*hsc 70*) is very important in activating the immune system (Javid et al., 2007). *cyp6a2* and *hsc 70* genes may play an important role in responding the infected mites by *A. cerana*. The putative odorant binding protein (*Obp14*) may act as an odor receptor. *Strn-Mlck*, which is identified as a novel member of the Titin/Myosin light chain kinase family in *Drosophila* (Champagne et al., 2000), was regulated as a function of *Varroa* infection and bee genotype and down-regulated both in *Varroa*-parasitized and tolerant bees (Navajas et al., 2008), but up-regulated in *A. cerana* in the present study.

Nineteen known down-regulated gene sequences from *A. cerana* prepupae were identified, including fumarylacetoacetate hydrolase (*Faa*) and neuropeptide y receptor-like cg5811-pa (*Npy-r*). Fumarylacetoacetate hydrolase (FAA, E.C. 3.7.1.2) is the last enzyme of the tyrosine catabolic pathway. A deficiency in FAA causes hereditary tyrosinemia type I (HTI; OMIM 276700), which is the most severe disease of the pathway in animals (Mitchell et al., 2001). The neuropeptide Y (*Npy-r*) receptor is widely distributed and well expressed in mammals, with a multitude of physiological and pathological connections (Parker and Balasubramaniam, 2008). This is a G-protein coupling receptor with seven transmembrane helices, a member of the largest group of plasma membrane receptors.

4.4. Expressed sequences from *A. mellifera*

Thirty-eight up-regulated genes from *A. mellifera* prepupae induced by the *Varroa* mite infection might represent different important putative functions (Table 3). Most of the up-regulated genes, such as t-complex protein I subunit (*tcp-1a*), myosin light chain 2 (*Mlc2*), proteasome subunit alpha type-3 (*Psm3*) and chitinase 10 (*chi10*), were involved in the hydrolase activity or

ATPase activity. Hect e3 ubiquitin ligase (*hect e3*), and protocadherin fat 2 (*Fat2*) are described in receptor down modulation and immune response (Liu, 2003), Ca²⁺ pathway (Robert, 2009) and in signaling pathway in *Drosophila* (Willecke et al., 2006), respectively. *Headcase* is described to be involved in the specification of adult ventral structures in *Drosophila* (Steneberg et al., 1998). In both mammalian and *Drosophila* neurogenesis, lissencephaly-1 (*Lis-1*), isoform A (MF814) is necessary for neuroblast proliferation and migration (Williams, 2009). Ubiquitin carboxyl-terminal hydrolase isozyme 15 (*uchl5*) is the central enzyme of nonlysosomal protein degradation in both the cytosol and nucleus (Kwon et al., 2004).

Among thirty-two down-regulated *A. mellifera* genes, *hex 110*, encoding a storage protein for the development, metamorphosis and egg production (Bitondi et al., 2006) was noticeable, because this gene was up-regulated in the *Varroa*-induced *A. cerana* prepupae. Hex 110 protein can modulate JH availability in termites (Zhou et al., 2007), while juvenile hormone JH III stimulates the proliferation of the mites (Liu and Chen, 2001). Possibly *hex 110* is involved in pheromone production and then in the control of mite development. *Npy-r* gene, encoding a receptor protein, was also down-regulated in *A. mellifera*, as we found for *A. cerana* in this study; this may indicate that this gene is involved in the control of bee development through ERK-mediated insulin signaling, as in *Drosophila* (Lee et al., 2008).

In general, most of the differentially expressed genes were involved in metabolic processes, such as *Aldh*, *NDK*, *Psmb1*, *AtpB*, *Oat*, *Faa*, *Cyp6a*, *CO II Pol eta*, *hect3*, *psma3*, *uch-15*, *Mdp*, *Pgi*, *COXI*, *CPT 1*, *HKA*, *ND I*, etc., and some were involved in nerve signaling, such as *Npy-r*, *STPK*, *Sp3c3*, *erm*, *Fat*, *hedcase*, and *Dsp*. It seemed likely that the prepupae responded to the mite infection by expressing the early signal genes, then by synthesizing various response factors. Although it is not easy to explain the functions of all these genes induced by the mite infection from both bee species, the results provide information on the molecular response of these two bee species to *Varroa* infection.

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