



Proteomic Analysis of *Apis cerana* and *Apis mellifera* Larvae Fed with Heterospecific Royal Jelly and by CSBV Challenge

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Abstract

Chinese honeybee *Apis cerana* (*Ac*) is one of the major Asian honeybee species for local apiculture. However, *Ac* is frequently damaged by Chinese sacbrood virus (CSBV), whereas *Apis mellifera* (*Am*) is usually resistant to it. Heterospecific royal jelly (RJ) breeding in two honeybee species may result in morphological and genetic modification. Nevertheless, knowledge on the resistant mechanism of *Am* to this deadly disease is still unknown. In the present study, heterospecific RJ breeding was conducted to determine the effects of food change on the larval mortality after CSBV infection at early larval stage. 2-DE and MALDI-TOF/TOF MS proteomic technology was employed to unravel the molecular event of the bees under heterospecific RJ breeding and CSBV challenge. The change of *Ac* larval food from RJC to RJM could enhance the bee resistance to CSBV. The mortality rate of *Ac* larvae after CSBV infection was much higher when the larvae were fed with RJC compared with the larvae fed with RJM. There were 101 proteins with altered expressions after heterospecific RJ breeding and viral infection. In *Ac* larvae, 6 differential expression proteins were identified from heterospecific RJ breeding only, 21 differential expression proteins from CSBV challenge only and 7 differential expression proteins from heterospecific RJ breeding plus CSBV challenge. In *Am* larvae, 17 differential expression proteins were identified from heterospecific RJ breeding only, 26 differential expression proteins from CSBV challenge only and 24 differential expression proteins from heterospecific RJ breeding plus CSBV challenge. The RJM may protect *Ac* larvae from CSBV infection, probably by activating the genes in energy metabolism pathways, antioxidation and ubiquitin-proteasome system. The present results, for the first time, comprehensively describe the molecular events of the viral infection of *Ac* and *Am* after heterospecific RJ breeding and are potentially useful for establishing CSBV resistant populations of *Ac* for apiculture.

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Introduction

Apis mellifera (*Am*) and *A. cerana* (*Ac*) are major honey bee species in the global beekeeping industry [1,2]. They are heavily infected by different vital viruses [3,4]. Chinese sacbrood virus (CSBV) is the most stricken pathogen of *Ac*, which results in severe and deadly infections of the colony and eventually losses of the entire colony [5]. As a small RNA virus (picorna-like virus) that has an icosahedral virion with a diameter of 26–30 nm, CSBV genome consists of a single positive-strand RNA molecule with 8.8 kilo bases (kb) [7]. Although CSBV shows a close genetic relationship to its western counterpart sacbrood virus (SBV), there is no cross infection [8]. Since this viral disease broke out in 1972 in southern China [8,9], some efforts have been made to study this virus, such as diagnostic methods (electron microscopy, enzyme-linked immunosorbent assay and reverse transcription-polymerase chain reaction (RT-PCR)) [10], and the control of this disease by RNA interference [11]. It is interesting that *A. mellifera* is not sensitive to CSBV in general beekeeping practice [12]. Similar to all other insects, the honeybees lack a classically adaptive immune

system as in the case of mammalian [6]. To survive, they have evolved cellular and humoral immune responses to cope with microbial infections. *A. mellifera* may develop cellular and humoral immune responses to various pathogens such as bacteria [13,14], viruses [15], microsporidian [16–18] and *Varroa* mites [19,20]. Usually, the viral infection causes cell apoptosis, tissue damage and even functional disorder of the whole organism and all these changes can be further reflected in proteome alteration [21]. Recent study revealed the pathological mechanism of Chinese sacbrood disease to *Ac* [6]. However, no information on the difference in the immune responses of two bee species to CSBV is reported.

In the honey bees, queen and workers have different behavior and reproductive capacity despite possessing the same genome. The primary substance that leads to this differentiation is royal jelly (RJ), which contains a range of proteins, carbohydrates, lipids, minerals, vitamins, and a large number of bioactive substances, especially immunological peptides and antibacterial proteins [22–24]. Major Royal Jelly Proteins (MRJPs) are the prime RJ ingredients, which are crucial in regulating reproductive maturation

tion [22]. There are quantitative differences in nucleic acids and protein composition in fresh RJ between *Am* and *Ac* [24]. The most recent discovery is that RJ contains microRNAs which may play a role in caste differentiation [25–27]. Whether the RJ also influences the resistant behavior of *Am* to CSBV is unknown. The molecular event for CSBV resistance of *Am* needs to be also determined.

Two-dimensional gel electrophoresis (2-DE) based proteomics technology and matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) are powerful molecular tools. In recent years, proteomic techniques have been successfully used to profile the proteome change in insect growth and development [6,28–30]. The present study employed gel-based (2-DE) and shotgun proteomic (label-free LC-MS based) strategies, which have complementary natures, to gain an in-depth understanding of the resistant mechanism of *Am* to the fatal CSBV disease by comparison of the proteome-wide change of the healthy and sick worker larvae of both *Am* and *Ac* fed with RJ either from *Am* (RJM) or *Ac* (RJC).

In the present study, we performed comparison analyses of the proteome in *Ac* and *Am* after heterospecific RJ breeding (i.e. *Am* larvae fed with RJC or *Ac* larvae fed with RJM), and then CSBV challenge, by two-dimensional electrophoresis and MALDI-TOF MS analysis, to systematically search for the molecular basis of the crossbreeding and CSBV challenge. A total of 101 differentially expressed nonredundant proteins (≥ 3 folds change) were identified. Furthermore, gene expression patterns in two bee species were investigated at mRNA levels. The results show that differential expression proteins are involved in the regulation of metabolism and response to CSBV challenge.

Materials and Methods

Ethics Statement

Honey bee colonies (*Am* and *Ac*) were raised at Conghua, Guangdong Province, China (113°17' E, 23°8' N), by Guangdong Entomological Institute, by standard beekeeping techniques. According to the present regulation from Ministry of Agriculture *Am* and *Ac* used in this studies are not endangered or protected species. No specific permissions were required for performing these experiments.

Honey Bees

To obtain age controlled second instar larvae, the queen was caged on a comb and left to lay eggs for 6 h. Twenty hours after larval eclosion, or 92 h after oviposition, the comb containing second instar larvae was retrieved from the colony, and placed in the laboratory for treatments at 32–34°C. All the larvae used in this study were detected by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) method for the absence of the following viruses, black queen cell virus (BQCV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), kashmir bee virus (KBV), Chinese sacbrood virus (CSBV) and Israeli acute paralysis virus (IAPV), with the primers from Ai *et al.* [4]. RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, USA) and cDNA synthesis was performed using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Kyoto, Japan). Amplification profile of PCR consisted of an initial 2-min denaturation at 94°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. No signs of clinical American foulbrood or other viral disease were observed in these larvae.

Virus

CSBV-infected larvae with typical symptom were collected from an apiary at Xinhua Village from Hunan Province, and kept at –80°C for less than 10 days before use. The presence of CSBV virus was confirmed by observation of the morphological symptom under electronic microscopy and RT-PCR according to Chen *et al.* and Yan *et al.* [3,10].

To obtain CSBV, the larvae (approximately 0.2 g) infected by CSBV were ground in 6 mL sterile phosphate-buffered solution (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) with a sterile grinder. The resulting solution was centrifuged at 14 000 g at 4°C for 10 min, and the supernatant was further passed through a 0.45 μ m cell filter first, then through a 0.22 μ m cell filter [31]. The CSBV concentration was quantitated by absolute quantification assay [11]. CSBV polyprotein gene (SBV1) was amplified to detect copy number of CSBV according to Liu *et al.* [11]. PCR reactions were carried out in triplicate in an Mx3000P Real-Time PCR System (Stratagene, California, USA), using SYBR_Green (Brilliant II SYBR-Green QPCR Master Mix; Stratagene, California, USA). The CSBV concentration for the experiments was approximately 7.9×10^5 copies/mL.

Harvest of RJM and RJC

RJM and RJC were produced according to standard practices in China [32]. Briefly, the queen was confined inside a queen excluding cage. Queen cups with young larvae (one-day old) were introduced into the colony and allowed to be fed by workers for 2 days. 3-day old larvae were first carefully removed by using either a grafting tool or a pair of forceps, then the royal jelly was removed by using a spatula.

Larvae Fed with RJM or RJC and Challenged with CSBV

To determine the effects of the changing food (RJs) on the mortality and protein expression of the third instar larvae, which are the most sensitive stage to CSBV infection, the second instar larvae of *Ac* and *Am* were collected carefully from the combs, respectively transferred to 96-cell culture plates containing 30 μ L RJC or RJM, in each cell, then placed in a cabinet (Sanyo, Tokyo, Japan) at 80% relative humidity at 32–34°C. After 24 h, the third instar larvae of both bee species fed with RJM or RJC were transferred to new plates, and challenged with 30 μ L CSBV solution per cell, prepared as described above. After 8 h, the larvae were washed with sterile distilled water for 3 times, and kept at –80°C for use. 8 treatments (Table 1) were established with 3 replicates containing 20 larvae in each replicate. Each 20 larvae were pooled as one biological replicate and three independent biological replicates were produced.

The same treatments were also established to examine the mortality of bee larvae fed with RJM or RJC and challenged by CSBV after 24 h. The death of the larvae was confirmed when they had no response after stimulating with a soft tip, and by the color change of their bodies (from white to yellow or even dark).

Protein Extraction and 2-DE

Larval protein extractions were carried out according to the previously described method with some modifications [33,34].

Bee larvae were manually homogenized for 30 min and sonicated for 2 min on ice in lysis buffer (8 M urea, 0.2% w/v Bio-Lyte 3/10 Ampholyte (Bio-Rad, USA), 4% CHAPS, 65 mM DTT) containing Protease Inhibitor Cocktail (Calbiochem, Germany)(w/1 = 1 mg/4 μ L). The mixture was centrifuged 15 000 g at 4°C for 30 min, and the supernatant was collected. The

Table 1. Treatments of the two honey bee species by RJs and CSBV.

No.	Honey bees	Treats	Abbreviation
A	<i>A. cerana</i>	fed on <i>A. cerana</i> royal jelly	AC-RJC
B		fed on <i>A. cerana</i> royal jelly, and challenged with CSBV	AC-RJC+CSBV
C		fed on <i>A. mellifera</i> royal jelly	AC-RJM
D		fed on <i>A. mellifera</i> royal jelly, and challenged with CSBV	AC-RJM+CSBV
E	<i>A. mellifera</i>	fed on <i>A. cerana</i> royal jelly	AM-RJC
F		fed on <i>A. cerana</i> royal jelly, and challenged with CSBV	AM-RJC+CSBV
G		fed on <i>A. mellifera</i> royal jelly	AM-RJM
H		fed on <i>A. mellifera</i> royal jelly, and challenged with CSBV	AM-RJM+CSBV

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resulting precipitates were treated three times with the same procedures. All the supernatants were centrifuged 15 000 g at 4°C for 60 min. The final supernatant was used or stored at -80°C for 2-DE. Protein concentrations were determined by the Bradford method using Modified Bradford Protein Assay Kit (Sangon, Shanghai, China).

The 2-DE was performed according to the methods described previously and the manufacturer's instruction [35]. The first dimension (isoelectric focusing) was conducted using the IPGphor IEF system (Bio-Rad, California, USA) at 20°C. For analytical gels, 350 µg protein was solubilized in 400 µL rehydration solution (8 M urea, 0.2% w/v Bio-Lyte 3/10 Ampholyte, 4% CHAPS, 65 mM DTT, 0.001% w/v bromophenol blue), and loaded onto a 17 cm pH 3–10 NL IPG strip (Bio-Rad). Focusing was performed for 13 h at 50 V, 1 h at 500 V, 1 h at 1000 V, and 30 min and 6 h at 8000 V (total = 48 kVh). The IPG strips were equilibrated as previously described. The second dimension was performed with 13% (w/v) SDS-polyacrylamide gels using the Protean II xi 2D Multicell system (Bio-Rad, California, USA). Proteins were stained with silver nitrate, and gels were digitized using Image ScannerII (Amersham Biosciences, USA). Digitized 2-DE gel patterns were edited and matched using the PDQUEST V8.0.1 software package (PDI, Humington Station). Triplicate experiments were run to confirm the reproducibility of results.

Protein Identification by MALDI-TOF/TOF MS

Protein digestion and peptide extraction were done according to our previously established protocol [36]. Spots of interest in gels staining with silver nitrate were cut out, washed, reduced, S-alkylated with iodoacetamide and in-gel digested at 37°C overnight with sequencing grade porcine trypsin (Promega, USA). After extraction in extractant of 50% ACN (Fisher, Waltham, MA, USA) and 2.5% TFA (Sigma, St Louis, MO, USA), peptide mixtures were analyzed using a saturated solution of 5 mg/mL α -cyano-4-hydroxycinnamic acid (CHCA, Sigma, St Louis, MO, USA) in ACN containing 0.1% TFA (Sigma, St Louis, MO, USA) (50/50 v/v) using a 4800 Proteomics Analyzer equipped with matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Applied Biosystems, Framingham, MA, USA). For MS calibration, the trypsin autolysis peptides were used as internal calibrants. Monoisotopic peak masses were automatically determined within the mass range of 800–4000 Da, with a minimum S/N of 50. Five of the most intense ion signals were selected as precursors for MS/MS acquisition. Combined MS and MS/MS queries were performed with the MASCOT search engine (V2.1, Matrix Science, UK) embedded in GPS-Explorer Software (V3.6, Applied Biosystems,

Framingham, MA, USA), using the *A. mellifera* database (Gene DB). MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 61 were considered statistically significant ($P \leq 0.05$). The individual MS/MS spectrum with statistically significant (confidence interval >95%) best ion score (based on MS/MS spectra) were also accepted.

Based on the UniProt Knowledgebase (<http://www.uniprot.org/>), identified proteins were submitted to KEGG (Kyoto Encyclopedia of Genes and Genomes) and classified according to KEGG pathway maps (<http://www.genome.jp/kegg/pathway.html>) and Gene Ontology (<http://www.geneontology.org/>), grouped on the basis of their biological process of GO terms (<http://www.blast2go.com>) [19]. Protein interaction networks of the differentially regulated proteins were analyzed using the online database resource Search Tool for the Retrieval of Interacting Genes (STRING: <http://string-db.org>) [37,38].

Quantitative Real-Time PCR (qRT-PCR)

To verify the gene expression of differentially expressed proteins, 11 proteins (FBA, LOC408516, MDH, SOD, Jafrac1, LOC725646, AGO1, Che-3, Ald, Pxd and Tpi) were randomly selected for qRT-PCR analysis of their encoding genes by quantitative real time PCR, according to our previously method with some modifications [19]. Briefly, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA). The RNA was dissolved in diethylpyrocarbonate (DEPC) treated water and quantified by measuring ultraviolet absorbance at 260 and 280 nm. Reverse transcription was performed on 1 µg of total RNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Kyoto, Japan). The gene-specific primers for the analysis are listed in Table 2. Expression of *actin* gene (GI: 406122) was used as an internal control. 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, California, USA), using SYBRGreen (SYBR Premix Ex Taq II, Takara, Kyoto, Japan). 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 48°C for 60 s. Afterwards, the PCR products were heated to 95°C for 15 s, cooled to 48°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, California, USA). The fold change was calculated using the $2^{-\Delta\Delta C_t}$ method. Each sample was analyzed independently and processed in triplicate.

Table 2. Primers for qRT-CR analysis of 12 differentially expressed genes.

Product name	Gene ID	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
Actin	gij406122	ATGCCAACACTGTCCTTTCTGG	GACCCACCAATCCATACGGA	150
FBA	gij66526635	TTTCTAGTATCTAAAGCATG	ACTGCTATTGCTACTGCT	228
LOC408516	gij66524882	GATCCAGATGCACCAAG	GGACAGAATCGTGGAAA	235
MDH	gij66513092	AAGGCTGGCACAGGTTT	TAAATGCAGCGATCCCA	239
SOD	gij33089104	ACTTGTCTGTTCCGTGTA	CACATTTCAACCCATTA	229
Jafrac1	gij66548188	AAACTCATTGCAGCATC	GGGAGGTCTGTTGATGA	250
LOC725646	gij110755974	ACCCGAATTGTACTTTA	TAGATGAAGGTGCTCAA	221
AGO1	gij110777044	TGCCCCAGATCAAGTAGAGC	AATTTGATAGCGTTTGTTGAT	200
Che-3	gij110757336	TCCTGTCCGAATTTTACCTGT	GAAGCTGCGTTTGCGTCTA	250
Ald	gij110748949	TGCGTACTGTTCCACCT	GAGGGCTAAGGCTAACA	233
Pxd	gij110757934	CGCTGTTCCAAGAGGCT	ACGACAAACGCCAGATC	250
Tpi	gij148224276	TCAGTAACAGCAGGAAAT	TTTATGTCATGTTCTGCAA	269

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Western Blot

To further verify the variation tendency of differentially expressed proteins identified by the proteomic approaches, glyceraldehyde-3-phosphate dehydrogenase 2 isoform 1 (GAPDH), fructose-1,6-bisphosphate phosphatase (FAH), heat shock protein (Hsp)60, phosphoglycerate kinase (PGK), triosephosphate isomerase 1 (Tpi), CuZn superoxide dismutase (SOD) and fructose-bisphosphate aldolase (FBA) were randomly selected for Western Blot analysis by the method of Han *et al.* [6] with minor modifications. Briefly, equal amount of protein sample (30 µg/lane) were separated by stacking (5%) and separating (12%) SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels and then transferred to a nitrocellulose transfer membrane (0.2 µm pore size) (Invitrogen, Eugene, OR) using the iBlot apparatus (Invitrogen, Eugene, OR). After blocking, the membranes were incubated over night at 4°C with primary rabbit polyclonal antibodies of anti-GAPDH, FAH, Hsp60, PGK, Tpi, SOD, and FBA antibodies (Abcam, Cambridge, MA) at a dilution of 1:1000. Following three washes, the membranes were further incubated with horseradish peroxidase-conjugated rabbit anti-goat secondary antibody (BOSTER, Wuhan, China) at a dilution of 1:2000 for 1 h. Immunoreactive protein bands were detected using the DAB Western Blotting Substrate (BOSTER, Wuhan, China) and quantified by densitometry using Quantity-one image analysis system (Bio-Rad, Hercules, CA). Actin was detected simultaneously as loading control of the analysis, which immunoreacted by BCIP/NBT Western Blotting Substrate (BOSTER, Wuhan, China). Quantification of the protein bands was conducted by scanning the films and importing the images into the Quantity One 1-D analysis software (Bio-Rad, Hercules, CA). Scanning densitometry was used for semiquantitative analysis of the data.

Data Analysis

The statistical analysis of gene expression was performed by one-way ANOVA test followed by Tukey's test for pair comparisons (SPSS version 16.0, SPSS, Inc.). Comparison of larval mortality differences between different groups was performed by independent samples t test. An error probability $p < 0.05$ was considered statistically significant.

Results

Effects of RJM and RJC on the Mortality of Two Bee Species after CSBV Challenge

Experiments were carried out to examine whether RJM would enhance the resistant ability of *Ac* larvae to CSBV infection. The larval mortalities of the treatments after 56 h were indicated in Figure 1. The dead larvae showed the symptom of CSBV infection. Without CSBV infection, the mortalities of *Ac* larvae fed with RJC or RJM were not significantly different ($t = 2.040$, $df = 4$, $p = 0.111$; Figure 1 A); meanwhile, the mortalities of *Am* larvae fed with RJM or RJC were also not significantly different ($t = 1.572$, $df = 4$, $p = 0.191$; Figure 1 E). This indicated that the RJs did not significantly influence the mortalities of the larvae of two bee species, at least at key early larval stage.

However, the mortality (72.2%) of *Ac* larvae fed with RJC and subsequent CSBV infection was significantly higher than that of the larvae fed with RJC (26.7%) but without CSBV ($t = -9.642$, $df = 3.801$, $p = 0.001$; Figure 1 B); When *Ac* larvae were fed with RJM and subsequently challenged by CSBV, their mortality was not significantly different than that of those larvae fed with RJM and without CSBV ($t = -2.447$, $df = 4$, $p = 0.071$; Figure 1 C); Interestingly, when *Ac* larvae were fed with RJC or RJM and subsequently challenged with CSBV, the mortality (72.2%) of *Ac* larvae fed with RJC was significantly higher than that of those larvae fed with RJM (27.8%) ($t = 10.674$, $df = 4$, $p = 0.000$; Figure 1 D). This indicated that RJM conferred antiviral activity for *Ac* larvae.

When *Am* larvae were fed with RJC or RJM, and subsequently challenged with CSBV, no significant differences in mortalities of *Am* larvae were observed ($t = 1.572$, $df = 4$, $p = 0.191$, Figure 1 E; $t = -0.941$, $df = 4$, $p = 0.400$, Figure 1 F; $t = -0.512$, $df = 4$, $p = 0.636$, Figure 1 G; $t = 2.000$, $df = 4$, $p = 0.116$, Figure 1 H). So the RJs did not influence the mortality of *Am* larvae after CSBV challenge. As usual, *Am* is not sensitive to CSBV infection.

Qualitative Comparisons and Identification of Differentially Expressed Proteins

To determine the effects of the RJ on the protein expression of two bee species after CSBV infection, the protein spots were visualized by 2-DE from the bee larvae of *Ac* and *Am* fed with RJM or RJC, and with RJM or RJC for 24 h and then challenged

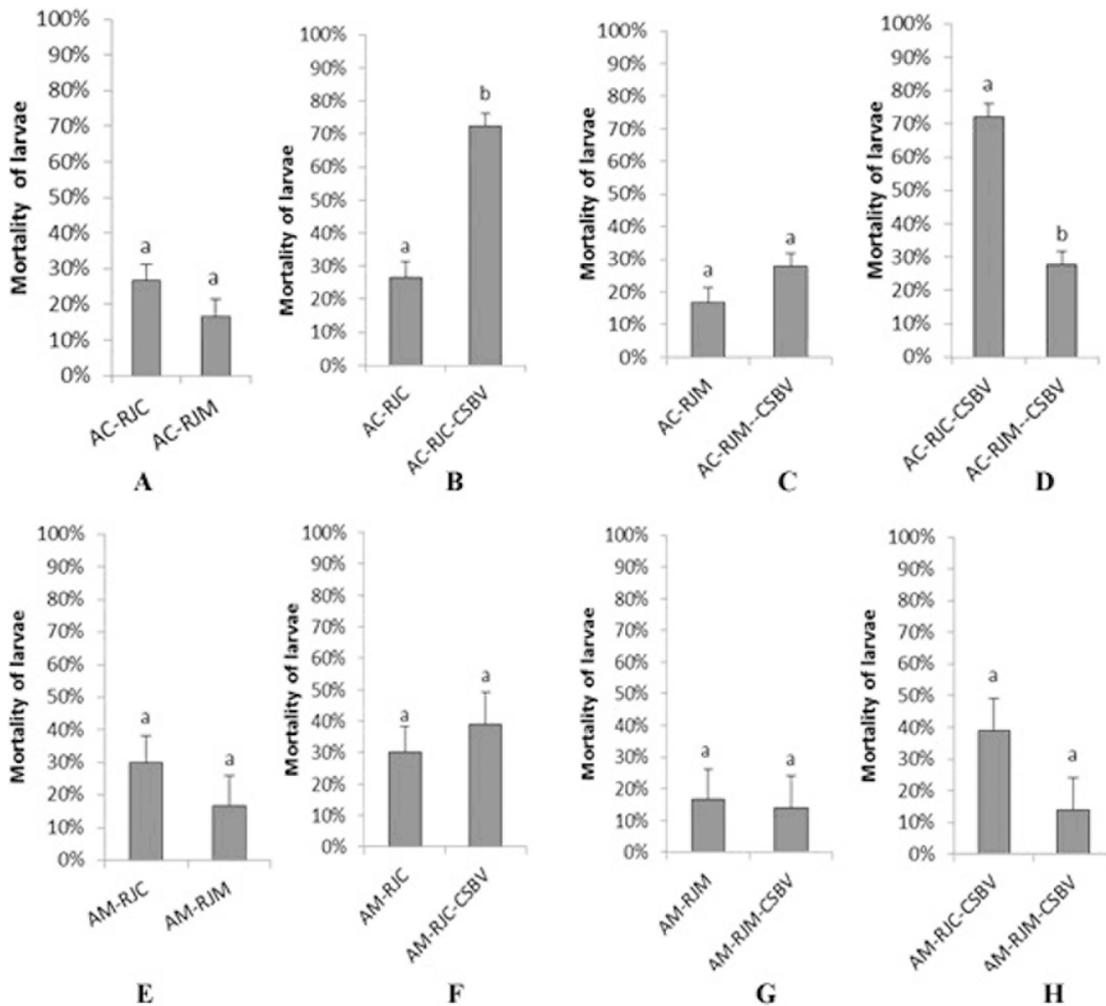


Figure 1. The larval mortalities of *A. cerana* and *A. mellifera* under different treatments after 56 hours. RJC: royal jelly from *A. cerana*; RJM: royal jelly from *A. mellifera*; AC-RJC: 3-day *Ac* larvae fed with RJC. AC-RJM: 3-day *Ac* larvae fed with RJM. AC-RJC+CSBV: 3-day *Ac* larvae fed with RJC, and infected with CSBV. AC-RJM+CSBV: 3-day *Ac* larvae fed with RJM, and infected with CSBV. AM-RJC: 3-day *Am* larvae fed with RJC. AM-RJM: 3-day *Am* larvae fed with RJM. AM-RJC+CSBV: 3-day *Am* larvae fed with RJC, and infected with CSBV. AM-RJM+CSBV: 3-day *Am* larvae fed with RJM, and infected with CSBV. The data are averages from three replicates. The error bars indicate standard deviations. Bars with different letters mean significantly different ($P < 0.05$, independent samples t test). doi:10.1371/journal.pone.0102663.g001

by CSBV for 8 h. 8 h after CSBV challenged, there was no dead larvae, although the virus was detected in the challenged larvae.

The total proteins were separated on 2-DE gels spanning pH 3–10, silver stained, and analyzed by MS. Protein levels were expressed as percentage volume, which corresponds to the percentage ratio between the volume of a single spot and the total volume of all spots present in a gel. The mean values of spot intensity were calculated using at least three gels. Spots showing more than 15% variation were not considered (Student's test, with 7 degrees of freedom, $P < 0.05$). Little deviation was observed in the patterns on replica gels.

530–670 spots were revealed on the silver-stained 2-DE patterns of the total proteins from different larvae, depending on bee species and the treatments (Figure 2). Protein spots were distributed over the 3–10 pH range.

Quantitatively, 296 total spots showed significant changes of expression (by a factor of ≥ 3 -folds and $p < 0.05$). All differentially expressed spots were identified by a MALDI-TOF/TOF mass spectrometer, and submitted to the NCBI nr database or the

NCBI EST_ database. 163 differentially expressed proteins were successfully identified (Figure 2), among which 44 were identified as MRJPs, and 119 were clustered to 101 non-redundant proteins. The up-regulated and down-regulated proteins from the 3-day old *Ac* and *Am* larvae fed with RJC or RJM, and with CSBV infection were listed (Table 3).

In *Ac* larvae, 6 differential expression proteins (6 spots) were identified from heterospecific RJ breeding only, 21 differential expression proteins (22 spots) from CSBV challenge only and 7 differential expression proteins (7 spots) from heterospecific RJ breeding plus CSBV challenge. In *Am* larvae, 17 differential expression proteins (17 spots) were identified from heterospecific RJ breeding only, 26 differential expression proteins (36 spots) from CSBV challenge only and 24 differential expression proteins (31 spots) from heterospecific RJ breeding plus CSBV challenge (Figure 3).

Some proteins were not identified either because their abundance was too low to produce enough spectra or because

Table 3. Identification of differentially expressed proteins.

No.	Protein Name	Gene	Accession No.	Protein MW (kDa)/PI	Protein Score C.I. %	GO	EC No.	Fold change
AC-RJM-up								
22	SF2 CG6987-PA	SF2	gil66548276	28.4/9.88	100	F:nucleotide binding	-	↑∞
98	vasa intronic gene CG4170-PA, isoform A	Vig	gil66565092	46.9/8.85	99.69	P:RNA interference	-	3.22
104	glyceraldehyde-3-phosphate dehydrogenase 2 isoform 1	Gapdh	gil48142692	35.8/8.11	100	F:NAD/NADP binding; P: glucose metabolic process	EC:1.2.1.12	3.21
AC-RJM-down								
15	Gamma-actin	Act5C	gil178045	25.8/5.65	100	P:adherens junction organization; F:protein kinase binding;	-	↓∞
21	ubiquitin conjugating enzyme E2	ben	gil156551253	17.3/5.71	100	P:response to anesthetic; F: ubiquitin-protein ligase activity;	EC:6.3.2.19	↓∞
77	abnormal CHEmotaxis family member (che-3) isoform 1	LOC411639	gil110757336	451.7/6.14	97.72	P:regulation of transcription, DNA-dependent; F:microtubule motor activity;	EC:3.6.1.3	↓∞
AM-RJC-up								
54	pORF2	pORF2	gil16508047	149.5/9.7	99.97	P:RNA-dependent DNA replication; F:RNA-directed DNA polymerase activity;	-	↑∞
56	yellow-e2 CG17044-PA	yellow-e2	gil110776421	39.6/8.29	99.39	P:defense response to fungus/G+/G-; caste determination, influence by environmental factors;	-	↑∞
63	Hypothetical protein Bd0095	Bd0095	gil42521740	41.4/6.17	95	-	-	↑∞
64	mitochondrial malate dehydrogenase precursor isoform 1	LOC408950	gil66513092	35.8/9.33	100	P:tricarboxylic acid cycle; F:L-malate dehydrogenase activity;	EC:1.1.1.37	↑∞
85	Ornithine aminotransferase precursor CG8782-PA	Oat	gil66524972	47.3/8.5	99.99	P:metabolic process; F:ornithine-oxo-acid transaminase activity;	EC:2.6.1.13	↑∞
158	40S ribosomal protein S12	Rps12	gil66548338	15.3/5.48	100	P:translation; F:structural constituent of ribosome;	-	5.14
AM-RJC-down								
67	Heat shock protein cognate 5 CG8542-PA	Hsc70-5	gil66501507	75.4/6.38	99.9	P:protein folding; F:2-alkenal reductase [NAD(P)] activity;	EC: 1.3	↓∞
68	thioredoxin peroxidase 1 CG1633-PA, isoform 1	Tpx-1	gil66548188	21.7/5.65	100	P:oxidation-reduction process; F:glutathione/thioredoxin peroxidase activity;	EC: 1.1	↓∞
69	Elongation factor 2 (EF-2) isoform 1	EF2b	gil66508439	94.5/6.11	100	P: translational elongation; F:translation elongation factor activity;	-	↓∞
70	Fumarylacetoacetase (Fumarylacetoacetate-hydrolase)(Beta-diketonase)	Faa	gil66522657	41.6/6.09	100	P:aromatic amino acid family metabolic process; F:fumarylacetoacetase activity;	EC: 3.7	↓∞
71	Putative invasion protein	invA	gil16266003	7.4/9.64	97.68	P:nucleobase-containing compound metabolic process; F:hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	EC: 3.6	↓∞
86	T-complex Chaperonin 5 CG8439-PA, isoform A	Cct5	gil66522349	59.4/5.7	97.50	-	-	↓∞

Table 3. Cont.

No.	Protein Name	Gene	Accession No.	Protein MW (kDa)/PI	Protein Score C.I. %	GO	EC No.	Fold change
100	hypothetical protein LOC726980	CG13321	gi 110755727	14.4/5.52	99.99	F:transferase activity	-	3.21
101	40S ribosomal protein S12	Rps12	gi 66548338	15.3/5.48	100	P:translation; F:structural constituent of ribosome;	-	3.19
102	60 kDa heat shock protein, mitochondrial-like	Hsp60	gi 66547450	60.4/5.64	100	P:response to stress; F:solute:hydrogen antiporter activity;	-	6.64
103	Glyceraldehyde 3 phosphate dehydrogenase 1	Gapdh1	gi 66517066	31.6/7.6	100	P:glucose metabolic process; F:glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity;	EC: 1.2	3.27
136	hexamerin 110	Hex110	gi 155369750	112.1/6.43	99.98	-	-	3.21
AC-RJC-CSBV-up								
8	Vacuolar h	Vha55	gi 66531434	55.1/5.41	99.57	P:ATP metabolic process; F:hydrogen-exporting ATPase activity, phosphorylative mechanism;	EC: 3.6	↑ ∞
9	HSP70	hsp70	gi 92430370	70.8/5.42	99.97	P:response to stress; F:ATP binding;	-	↑ ∞
10	40S ribosomal protein S3a (C3 protein)	Rps3A	gi 66547340	30.0/9.67	100	F:structural constituent of ribosome; P:translation	-	↑ ∞
72	MTA1-like CG2244-PB, isoform B	MTA1-like	gi 110756803	94.4/9.68	95.86	F:zinc ion binding; P:regulation of transcription, DNA-dependent	-	↑ ∞
73	peroxidase-like isoform 2	pxd	gi 110757934	87.6/7.11	99.053	P:response to oxidative stress; F:peroxidase activity;	EC: 1.1	↑ ∞
74	Argonaute 1 CG6671-PB, isoform B	AGO1	gi 110777044	103.6/9.35	98.599	P:targeting of mRNA for destruction involved in RNA interference; F:miRNA binding;	-	↑ ∞
93	phosphatidylethanolamine-binding protein homolog F40A3.3-like isoform 1	LOC408516	gi 66524882	20.6/8.84	99.793	-	-	3.55
133	60S ribosomal protein L9	Rpl9	gi 66565444	21507.7/9.88	99.962	-	-	3.17
132	10 kDa heat shock protein, mitochondrial-like	LOC552531	gi 66547447	11.4/8.01	100	P:response to stress; F:ATP binding;	-	5.25
AC-RJC-CSBV-down								
7	Hypothetical protein V12801_09746	V12801_09746	gi 84388955	20.5/5.61	99.616	-	-	↓ ∞
89	tyrosyl-trna cytoplasmic	Aats-tyr	gi 110762892	73.7/8.91	99.548	P:tyrosyl-tRNA aminoacylation; F:tyrosine-tRNA ligase activity;	EC: 6.1	3.64
91,128	fructose-bisphosphate aldolase	FBA	gi 110748949	39.6/7.57	99.959	P:glycolysis; F:fructose-bisphosphate aldolase activity;	EC: 4.1	3.79 3.50
92	aldose reductase-like isoform 1	AKR-1	gi 66525576	36.1/6.26	99.997	P:oxidation-reduction process; F:oxidoreductase activity	-	3.97
120	ribosomal protein s9	Rps9	gi 48101950	22.5/10.74	97.835	P:translation; F:rRNA binding	-	4.47
122	peroxiredoxin 1	Jafra1	gi 66548188	21.7/5.65	95.152	P:hydrogen peroxide catabolic process; F:thioredoxin/glutathione peroxidase activity;	EC: 1.1	8.45
124	phosphoglycerate kinase	Pgk	gi 110763826	44.9/8.15	100	P:phosphorylation; F:phosphoglycerate kinase activity;	EC: 2.7	9.74
125	60 kDa heat shock protein, mitochondrial-like	Hsp60	gi 66547450	60.4/5.64	100	P:response to stress; F:solute:hydrogen antiporter activity;	-	3.45

Table 3. Cont.

No.	Protein Name	Gene	Accession No.	Protein MW (kDa)/PI	Protein Score C.I. %	GO	EC No.	Fold change
126	t-box protein h15	<i>mid</i>	gi 110757297	9.7/9.36	99.914	F:sequence-specific DNA binding transcription factor activity; P:regulation of transcription, DNA-dependent;	-	14.10
127	prohibitin protein wph	<i>wph</i>	gi 48097857	29.9/6.54	100	Required for larval metabolism or for the progression of the larva into a pupa	-	3.72
129	pyruvate kinase	<i>LOC552007</i>	gi 66548684	55.8/6.92	100	P-phosphorylation; F:potassium ion binding;	EC: 2.7	3.93
131	similar to CG31531-PA, isoform A isoform 1	<i>LOC409705</i>	gi 66500352	113.1/6.62	98.889	Primaginal disc-derived wing morphogenesis; F:zinc ion binding	-	3.09
AC-RJM-CSBV-up								
13	eukaryotic translation initiation factor 5a	<i>eIF-5A</i>	gi 110767655	17.6/5.19	97.017	F:translation initiation factor activity; P:translational initiation;	-	↑ ∞
14	Triosephosphate isomerase1	<i>Tpi</i>	gi 148224276	26.9/7.77	99.999	P:gluconeogenesis; F:triose-phosphate isomerase activity	EC: 5.3	↑ ∞
87	neurochondrin homolog	<i>CG2330RA</i>	gi 66509434	84.2/5.89	99.177	P:metabolic process; F:phosphoglycolate phosphatase activity	EC: 3.1	3.24
135	actin, indirect flight muscle-like	<i>Act88F</i>	gi 110775533	37.3/5.37	99.948	P:phagocytosis, engulfment; F:ATP binding;	-	3.04
AC-RJM-CSBV-down								
75	grip and coiled-coil domain-containing protein 1	<i>CG10703</i>	gi 110750733	58.3/8.69	95.142.	P:cellular process	-	↓ ∞
76	60 kDa heat shock protein, mitochondrial precursor (Hsp60)	<i>HSP60</i>	gi 66547450	60.4/5.64	100	P:response to stress; F:transporter activity;	-	↓ ∞
134	yellow-e2, CG17044-PA	<i>CG15040</i>	gi 110776421	39.6/8.29	99.773	P:killing of cells of other organism;	-	3.36
AM-RJC-CSBV-up								
31	lambda-crystallin homolog	<i>Cry1</i>	gi 66521282	34.4/6.3	100	F:3-hydroxyacyl-CoA dehydrogenase activity; P:oxidation-reduction process	EC:1.1.1.35	↑ ∞
32	GL19229	<i>Dper\GL19229</i>	gi 194106483	17.3/6.05	99.505	-	-	↑ ∞
34	Catalase	<i>Cat</i>	gi 25990773	57.9/8.39	100	P:hydrogen peroxide catabolic process; F:protein kinase activity;	EC:1.11.1.6	↑ ∞
82	metallo-beta-lactamase domain-containing protein 1-like	<i>CG9117</i>	gi 110775625	16.1/5.86	95.029	F:hydrolase activity	-	↑ ∞
83	transposase	<i>LOC725919</i>	gi 110766128	22.6/9.6	98.985	P:transposition, DNA-mediated; F:transposase activity;	-	↑ ∞
84	Translation elongation factor eEF-1 alpha chain	<i>Eif</i>	gi 58585198	50.5/9.16	99.948	F:translation elongation factor activity; P:translational elongation;	-	↑ ∞
108	40S ribosomal protein S20	<i>RpS20</i>	gi 110766823	13.7/9.95	100	P:translation; F:structural constituent of ribosome;	-	6.08
109	serine protease easter	<i>Sp7</i>	gi 110757145	41.0/7.83	98.199	P:proteolysis; F:serine-type endopeptidase activity;	EC:3.4.21.0	5.08
110	atp synthase subunit mitochondrial	<i>LOC551766</i>	gi 110762902	55.1/5.25	100	P:ATP synthesis coupled proton transport; F:ATP binding;	EC:3.6.3.6	3.80
111	CuZn superoxide dismutase	<i>Sod</i>	gi 33089104	15.6/6.21	100	P:superoxide metabolic process; F:superoxide dismutase activity;	EC:1.15.1.1; EC:1.11.1.7	4.13

Table 3. Cont.

No.	Protein Name	Gene	Accession No.	Protein MW (kDa)/PI	Protein Score C.I. %	GO	EC No.	Fold change
112	Nucleoside diphosphate kinase (NDK) (NDP kinase) (Abnormal wing disks protein) (killer of prune protein)	<i>Awd</i>	gi 66520497	17.6/6.75	100	P:nucleoside diphosphate phosphorylation; F: nucleoside diphosphate kinase activity;	EC:2.7.4.6	4.15
113	fructose-bisphosphate aldolase-like	<i>FBA</i>	gi 110748949	39.6/7.57	100	P:glycolysis; F:fructose-bisphosphate aldolase activity	EC:4.1.2.13	3.49
114	ornithine aminotransferase, mitochondrial	<i>Oat</i>	gi 66524972	47.3/8.5	100	P:metabolic process; F:ornithine-oxo-acid transaminase activity;	EC:2.6.1.13	4.46
33, 115,116,118	glyceraldehyde-3-phosphate dehydrogenase	<i>LOC410122</i>	gi 66517066	31.6/7.6	100	F:NAD/NADP binding; P:glucose metabolic process;	EC:1.2.1.12	↑∞ 4.29 3.16 3.89
117	larval cuticle protein a3a-like	<i>CPB31A</i>	gi 110764443	34.3/5.54	100	F:structural constituent of cuticle	-	3.25
119,137,138	glyceraldehyde-3-phosphate dehydrogenase 2 isoform 1	<i>Gapdh</i>	gi 48142692	35.8/8.11	100	F:NAD/NADP binding; P:glucose metabolic process;	EC:1.2.1.12	8.54 3.08 3.14
AM-RJC-CSBV-down								
24, 25, 26	60 kDa heat shock protein, mitochondrial precursor	<i>Hsp60</i>	gi 66547450	60.4/5.64	100	P:response to stress; F:transporter activity	-	↓∞ ↓∞ ↓∞
27	60 kDa heat shock protein, mitochondrial	<i>Hsp60</i>	gi 170045840	60.4/5.39	100	P: protein metabolic process; response to abiotic stimulus; F: nucleotide/protein binding; catalytic activity;	EC: 4.2	↓∞
30	G116304	<i>Hsp60</i>	gi 193906980	60.7/5.65	99.812	P: protein metabolic process; response to abiotic stimulus; F: nucleotide/protein binding; catalytic activity;	EC: 4.2	↓∞
78	Spectrin beta chain	<i>Beta-Spec</i>	gi 110759783	268.6/5.37	97.139	F:phosphoprotein phosphatase activity;	EC: 3.1	↓∞
80	Stretchin-Mlck CG18255-PA, isoform A	<i>Strn-Mlck</i>	gi 110757372	38.1/5.45	99.212	-	-	↓∞
81	yellow-h CG1629-PA	<i>Yellow-h</i>	gi 110761428	33.7/5.71	100	-	-	↓∞
105	fatty acid binding protein	<i>FABP</i>	gi 27531027	15.5/5.46	100	F:lipid binding; P:transport;	-	8.52
107	protein NPC2 homolog	<i>NPC2</i>	gi 110756609	16.1/7.55	100	-	-	3.22
AM-RJM-CSBV-up								
50	c6 transcription	<i>SS1G_11931</i>	gi 156039589	50.2/8.99	97.085	F:sequence-specific DNA binding RNA polymerase II transcription factor activity; P:regulation of transcription from RNA polymerase II promoter;	-	↑∞
51,161	protein disulfide-isomerase	<i>Pdi</i>	gi 110768510	24.3/4.74	100	F:electron carrier activity; P:cell redox homeostasis;	EC: 5.3; EC: 2.4	↑∞ 18.95
52	aldehyde dehydrogenase mitochondrial-like	<i>Aldh</i>	gi 66526635	45.4/5.25	100	F:retinal dehydrogenase activity; P:oxidation-reduction process	EC: 1.2	↑∞
53	hydroxysteroid dehydrogenase-like protein 2-like	<i>CG5590</i>	gi 66530010	45.2/8.69	100	P:oxidation-reduction process; F:glucose 1-dehydrogenase [NAD(P)] activity;	EC: 1.1	↑∞
153	heat shock protein cognate 3	<i>Hsc70-3</i>	gi 229892214	72.8/5.29	100	F:metal ion binding; P:porphyrin-containing compound biosynthetic process;	EC: 4.2	4.15

Table 3. Cont.

No.	Protein Name	Gene	Accession No.	Protein MW (kDa)/PI	Protein Score C.I. %	GO	EC No.	Fold change
154	phosphoglycerate kinase isoform 1	PGK	gi 110763826	44.9/8.15	100	P:glycolysis; phosphorylation; F:phosphoglycerate kinase activity	EC: 2.7	6.13
155	a-kinase anchor protein 9	Cp309	gi 110758909	81.4/8.39	98.602	P:microtubule cytoskeleton organization; F:protein binding;	-	7.52
156	heat shock protein cognate 4	Hsc70-4	gi 229892210	71.0/5.43	100	F:ATP binding; P:response to stress;	-	4.99
157	glyceraldehyde-3-phosphate dehydrogenase 2 isoform 1	Gapdh	gi 48142692	35.9/8.11	100	F:NAD/NADP binding; glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity; P:oxidation-reduction process;	EC: 1.2	3.68
161	26S proteasome non-ATPase regulatory subunit 13	Rpn9	gi 66547365	43.6/6.07	95.252	-	-	18.95
163	myosin heavy muscle isoform 1	Mhc	gi 110759191	224.7/5.78	99.86	P:ecdysone-mediated induction of salivary gland cell autophagic cell death; F:2-alkenal reductase [NAD(P)] activity;	EC: 1.3	3.14
AM-RJM-CSBV-down								
35	30S ribosomal protein S3	RpS3	gi 73661978	24.0/9.8	99.657	P:translation; F:RNA binding;	-	↓ ∞
37, 142,143	thioredoxin peroxidase 1 CG1633-PA	Jafrac1	gi 66548188	21.8/5.65	100	P:response to stress; F:antioxidant activity;	EC: 1.1	↓ ∞, 3.36, 4.50
38	aldo-keto reductase	CG6084	gi 66525576	36.1/6.26	100	P:metabolic process; F:catalytic activity	-	↓ ∞
36,39,42 141, 144,145,148	60 kDa heat shock protein, mitochondrial precursor	Hsp60	gi 66547450	60.4/5.64	100	P:response to stress; F:transporter activity;	-	↓ ∞ ↓ ∞ ↓ ∞ 7.55 3.95 29.95 3.24
40	tyrosyl-tRNA cytoplasmic	Aats-tyr	gi 110762892	73.7/8.91	100	P:translation; F:catalytic activity; RNA binding;	EC: 6.1	↓ ∞
43, 46	heat shock protein 60	Hsp60	gi 156541962	60.5/5.5	100	P:response to stress; F:transporter activity;	-	↓ ∞ ↓ ∞
47	fructose-bisphosphate aldolase	FBA	gi 110748949	39.6/7.57	100	P:carbohydrate metabolic process; F:catalytic activity	EC: 4.1	↓ ∞
49	heat shock cognate 70	Hsp70	gi 66537940	71.7/5.43	100	P:response to stress; F:nucleotide binding;	-	↓ ∞
139	arylphorin subunit alpha	Lsp2	gi 149939401	79.5/6.72	100	-	-	4.00
140	atp synthase subunit mitochondrial	Vha68	gi 110762902	55.1/5.25	100	P:generation of precursor metabolites and energy; F:hydrolase activity;	EC: 3.6	3.07
147	similar to Pyruvate kinase CG7070-PB, isoform B	LOC552007	gi 66548684	55.8/6.92	100	-	-	3.99
149	hexamerin 110	Hex110	gi 155369750	112.1/6.43	100	-	-	4.27
150	protein NPC2 homolog	NPC2	gi 110756609	16.1/7.55	100	-	-	6.33
151	grpe protein mitochondrial-like	Roe1	gi 66525522	26.4/7.82	100	P:protein metabolic process; F:protein binding;	-	3.66
152	n-acetylneuraminase lyase-like	LOC725646	gi 110755974	34.0/8.3	100	P:metabolic process; F:catalytic activity	-	4.19

Spot numbers and folds change correspond to the number of the protein spots in Figure 2. The theoretical molecular weight (Mr) and isoelectric point (pI) of the identified proteins were retrieved from the protein database of NCBItr limited to *Apis mellifera*. The Mascot score was searched against the *A. mellifera* database. Protein name is given when proteins were identified by MALDI-TOF/MS. The taxonomy is *A. mellifera*. Accession number is the unique number given to mark the entry of a protein in the NCBItr database. GO molecular function and biology process result in the Blast2GO analysis, P, Biological process; F, Molecular function. doi:10.1371/journal.pone.0102663.t003

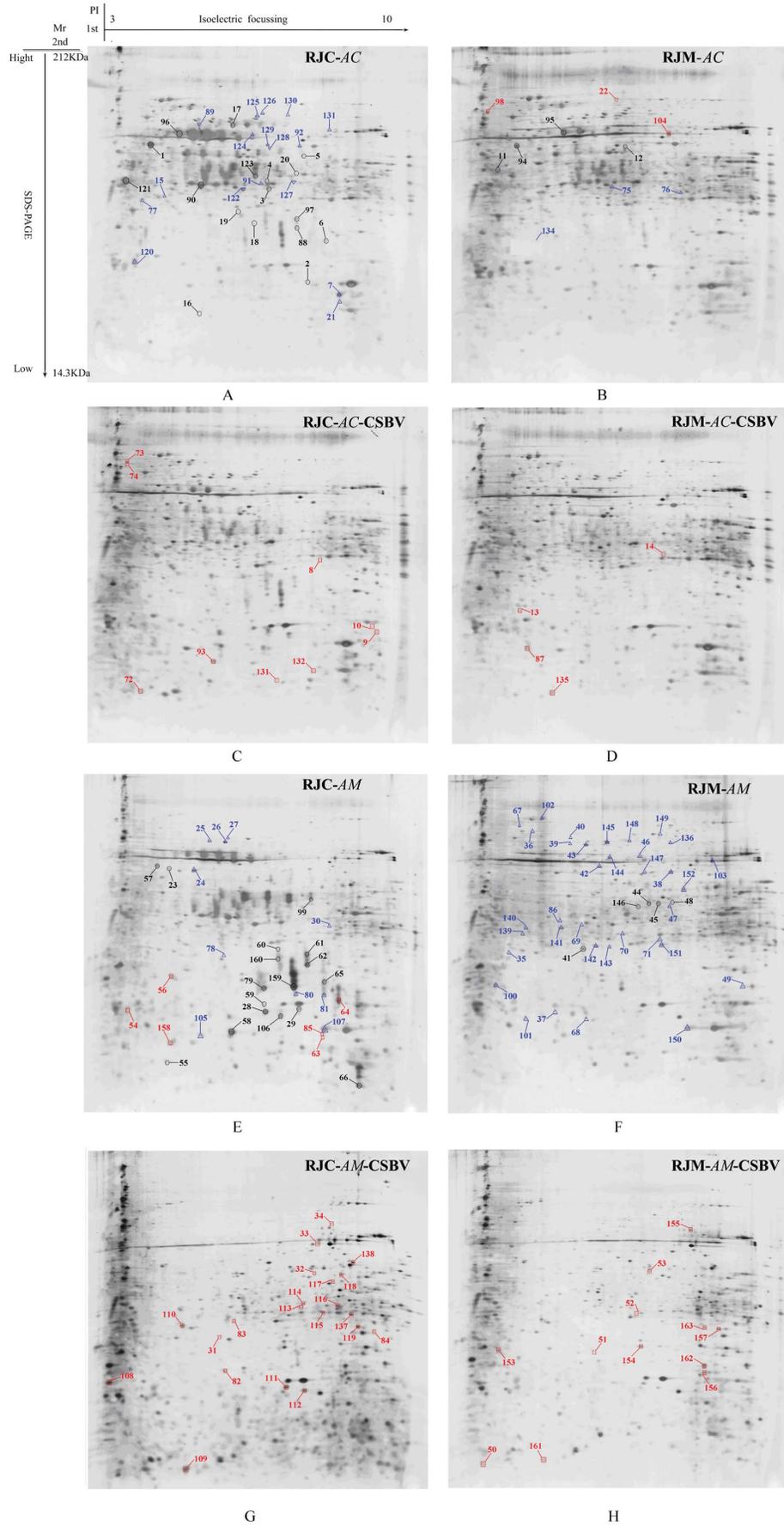


Figure 2. 2-DE pictures of *A. cerana* and *A. mellifera* 3-day old larvae fed with RJs and by CSBV infection. RJC: royal jelly from *A. cerana*; RJM: royal jelly from *A. mellifera*; A: 3-day old *Ac* larvae fed with RJC. B: 3-day old *Ac* larvae fed with RJM. C: 3-day old *Ac* larvae fed with RJC and by CSBV infection. D: 3-day old *Ac* larvae fed with RJM and CSBV infection. E: 3-day old *Am* larvae fed with RJC. F: 3-day old *Am* larvae fed with RJM. G: 3-day old *Am* larvae fed with RJC and CSBV infection. H: 3-day old *Am* larvae fed with RJM and by CSBV infection.
doi:10.1371/journal.pone.0102663.g002

the database search scores can not yield unambiguous results (> 95%).

GO (Gene Ontology) Functional Term Enrichment

To understand the functions of differentially expressed proteins from the bee larvae, which resulted from heterospecific RJ breeding or CSBV infection, 101 identified proteins were analysed by gene ontology (GO) mapping respectively. At least one GO term could be assigned to 88 of 101 detected differentially expressed proteins (Table 3). Thirteen (12.8%) proteins were deemed of unknown function after blast searches against the nr databases and annotation augmentation. The GO terms assigned to individual proteins were shown in Table 3. Based on the GO annotations, proteins were grouped by their broad biological processes (BP) (Figure 4, 5).

Three major BPs of the up-regulated proteins from the *Ac* larvae fed with RJM were metabolic processes (40.0%), cellular processes (20%) and biological regulation (20%), whereas those of down-regulated proteins were mainly metabolic processes (17.6%), cellular processes (17.6%) and developmental process (11.8%). The major BPs of the increasing proteins from *Ac* fed with RJC, and then by CSBV challenge were metabolic process (18.2%), cellular processes (18.2%) and response to stimulus (18.2%), whereas the major BPs of the decreasing proteins were metabolic processes (37.5%), cellular processes (33.3%) and biological regulation (12.5%). The major BPs of the increasing proteins

from *Ac* fed with RJM, and then by CSBV challenge were metabolic processes (25.0%), cellular processes (25.0%) and cellular component organization or biogenesis (16.7%), whereas the major BPs of the decreasing proteins were metabolic processes (25.0%), cellular processes (25.0%) and biological regulation (12.5%).

Three major BPs of the increasing proteins from the *Am* larvae fed with RJM were metabolic processes (22.2%), cellular processes (11.1%) and cell killing (11.1%), whereas those of decreasing proteins were metabolic processes (32.0%), cellular processes (28.0%) and response to stimulus (16%). The major BPs of the increasing proteins from *Am* fed with RJM, and then by CSBV challenge were cellular processes (28.6%), biological regulation (19.0%) and cellular component organization (9.6%), whereas the major BPs of the decreasing proteins were metabolic processes (37.5%), cellular processes (29.2%) and response to stimulus (16.7%). The major BPs of the increasing proteins from *Am* fed with RJC, and then by CSBV challenge were metabolic processes (60.9%), cellular processes (30.4%) and response to stimulus (4.3%), whereas the major BPs of the decreasing proteins were localization (28.5%), response to stimulus (21.4%) and metabolic processes (21.4%).

Network Analysis of Differentially Expressed Proteins

Proteins function jointly in a living cell through networks by forming protein-protein interactions (PPI), modifications and

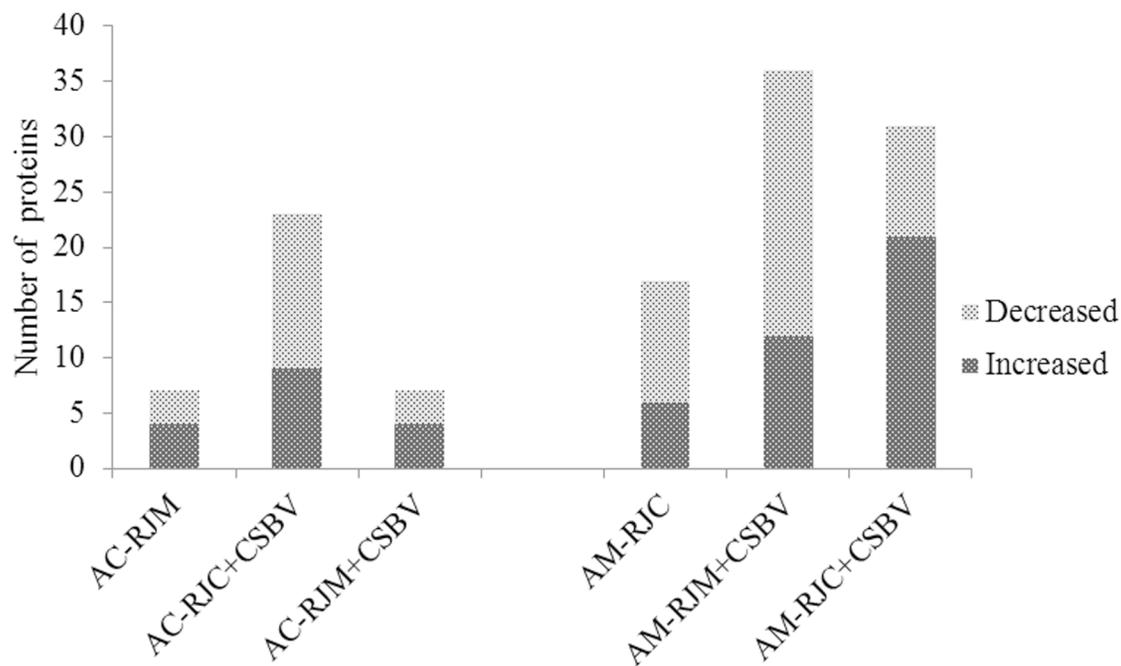


Figure 3. Numbers of up-regulated and down-regulated proteins from the bee larvae fed with RJs and challenged by CSBV. On the basis of triplicate replications analyses, only the proteins that changed ≥ 3.0 -fold in relative ratios ($p < 0.05$) were considered. RJC: royal jelly from *A. cerana*; RJM: royal jelly from *A. mellifera*; AC-RJM: 3-day old *Ac* larvae fed with RJM. AC-RJC+CSBV: 3-day old *Ac* larvae fed with RJC and infected with CSBV. AC-RJM+CSBV: 3-day old *Ac* larvae fed with RJM and infected with CSBV. AM-RJC: 3-day old *Am* larvae fed with RJC. AM-RJC+CSBV: 3-day old *Am* larvae fed with RJC and infected with CSBV. AM-RJM+CSBV: 3-day old *Am* larvae fed with RJM and infected with CSBV.
doi:10.1371/journal.pone.0102663.g003

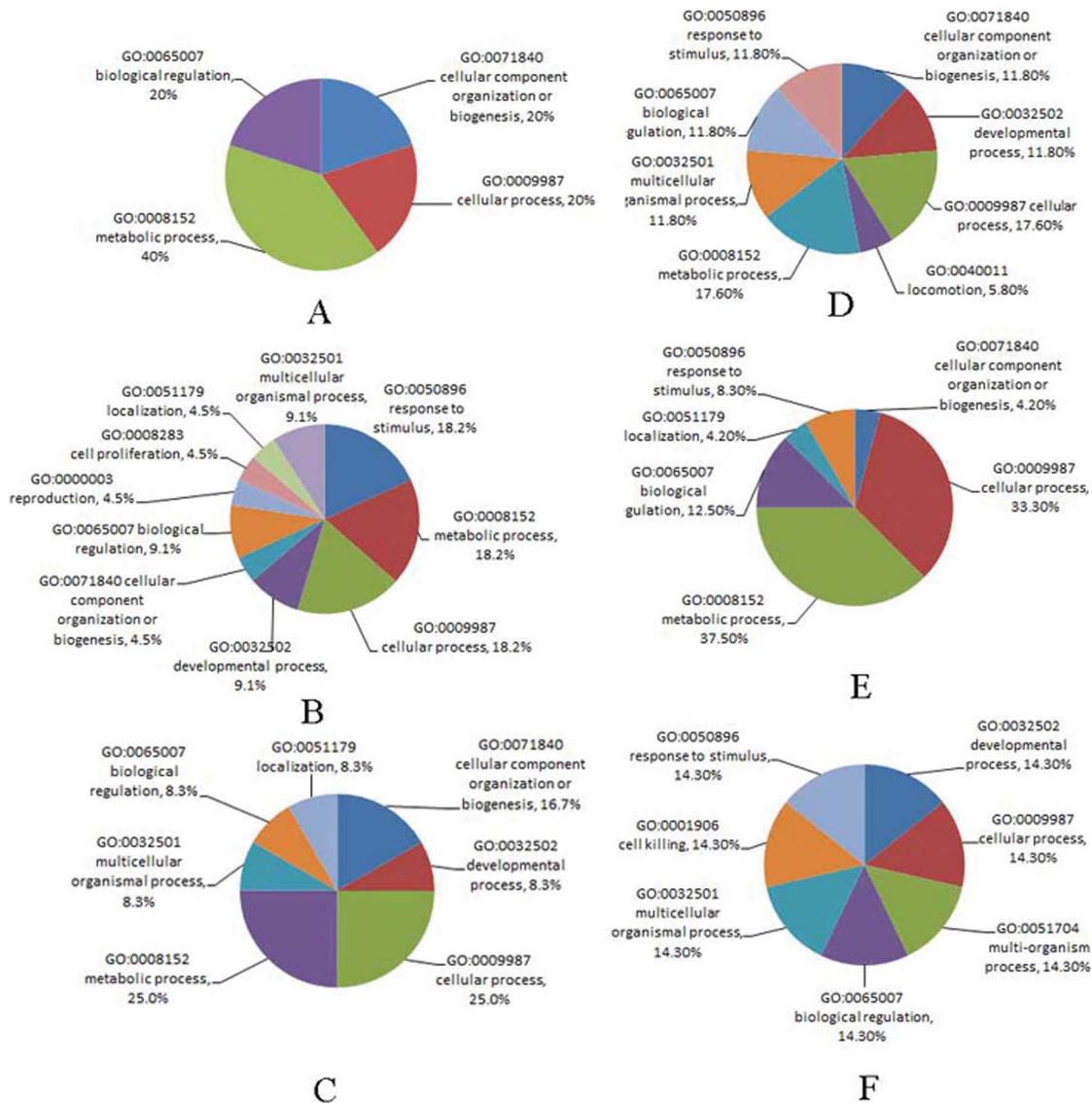


Figure 4. Based on the GO annotations, the proteins from *A. cerana* larvae fed with RJM or RJC, and by CSBV challenge, were grouped by the broad biological processes (BP). A, B, C: up-regulated proteins; D, E, F: down-regulated proteins. RJC: royal jelly from *A. cerana*; RJM: royal jelly from *A. mellifera*; A, D: 3-day *Ac* larvae fed with RJM; B, E: 3-day *Ac* larvae fed with RJC, and infected with CSBV; C, F: 3-day *Ac* larvae fed with RJM, and infected with CSBV. doi:10.1371/journal.pone.0102663.g004

regulation of expression relationships. Of all the identified proteins in this study, 107 proteins with the established functions based on *Drosophila melanogaster* were selected and their PPI mapping was constructed using STRING. The result showed that all the treats almost divided into two distinct PPI clusters with several others operating without connectivity. The first cluster primarily consisted of cytoskeletal proteins, while the second cluster mainly consisted of proteins involved in metabolism (Figure 6).

Verification of Differentially Expressed Proteins

To test the tendency of protein expression between its encoding gene at the transcript level, 11 proteins (FBA, LOC408516, MDH, SOD, Jafrac1, LOC725646, AGO1, Che-3, Ald, Pxd and Tpi) were randomly selected for qRT-PCR analysis of their encoding genes. The trend of mRNA expression of the selected genes showed consistent patterns with their protein expression (Figure 7).

Western Blot Analysis

Western blot analysis was conducted to verify the expression of the proteins which are closely related to energy metabolism of heterospecific royal jelly and CSBV resistance, such as GAPDH, PGK, Tpi, FAH, SOD, and FBA. The results are consistent well with the proteomic data (Figure 8).

Discussion

Heterospecific RJ breeding means during the growth or reproduction period of the colony or individual, changing the comb from *Ac* to *Am* or from *Am* to *Ac* or feeding RJ artificially. In recent years, heterospecific RJ breeding between *Ac* and *Am* has been concerned in the bee industry [39]. This technology was used between different species of western honeybees in 1957. *Am* fed with RJC showed some morphological characteristic of *Ac*, such as proboscis, forewings, abdomen and thorax, and body weight of

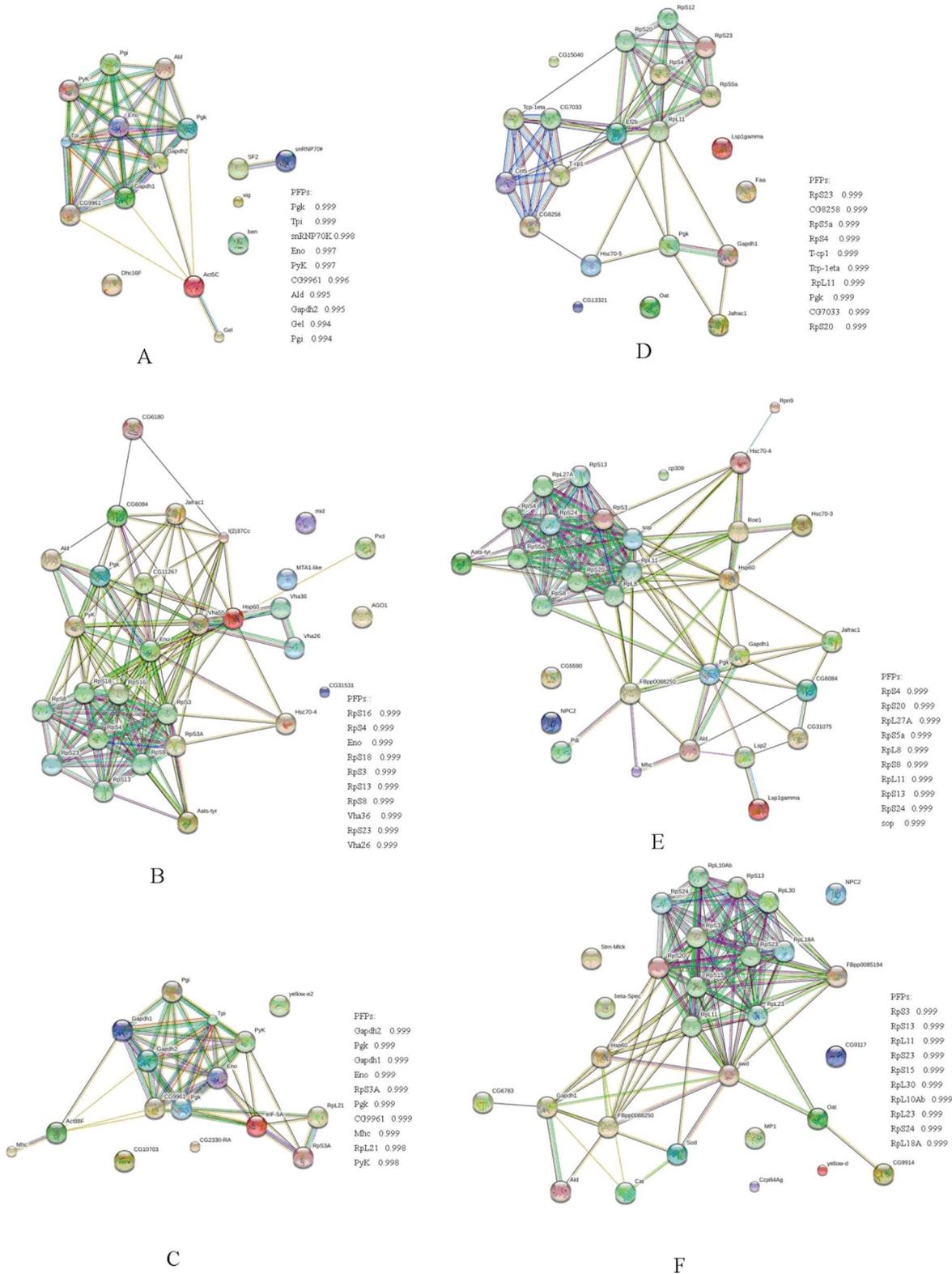


Figure 6. Protein-protein interaction networks of identified differentially expressed proteins from nutritional crossbreeding and CSBV challenge were constructed by Search Tool for the Retrieval of Interacting Genes (STRING). Lines represent the existence of the different types of evidence used in predicting the associations. A red line indicates the presence of fusion evidence; a green line, neighborhood evidence; a blue line, co-occurrence evidence; a purple line, experimental evidence; a yellow line, text mining evidence; a light blue line, database evidence; a black line, coexpression evidence. FFPs: Predicted Functional Partners. A: 3-day *Ac* larvae fed with RJM. B: 3-day *Ac* larvae fed with RJC, and infected with CSBV. C: 3-day *Ac* larvae fed with RJM, and infected with CSBV. D: 3-day *Am* larvae fed with RJC, and infected with CSBV; F: 3-day *Am* larvae fed with RJM, and infected with CSBV. doi:10.1371/journal.pone.0102663.g006

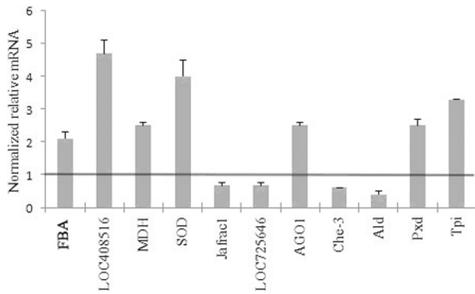


Figure 7. Verification of 11 differentially expressed proteins at mRNA level by qRT-PCR analysis. The normalized relative mRNA levels (>1) of the genes of the corresponding differentially expressed proteins indicate up-regulation and those (<1) indicate down-regulation. Error bar is standard deviation. The names of the proteins are referred in Table 2. doi:10.1371/journal.pone.0102663.g007

unknown protein), and 12 proteins down-regulated (V12B01_09746, Aats-tyr, Ald, AKR-1, RpS9, Jafracl, PGK, Hsp60, mid, wph, LOC552007, LOC409705).

The up-regulated proteins were mainly involved in the stress response and in the defense from viral infection. Such as, vacuolar h (Vha55) couples ATP hydrolysis to the build up of a H⁺ gradient. Metastasis associated gene 1 (MTA1) is part of the NURD (nucleosome remodeling and deacetylating) complex and plays a role in cellular transformation and metastasis [61]. Peroxidase-like (Pxd) is response to oxidative stress. Argonaute 1 (AGO1) is part of the RNA-induced silencing complex (RISC) in

the RNA interference pathway which can defense insects from virus infection [62,63].

The down-regulated proteins were mainly involved in the energy process and in the development regulation. For example, tyrosyl-trna cytoplasmic (Aats-tyr) are involved in amino acid synthesis, cell cycle control, RNA shear modification and transport of tRNA [64,65]. Fructose-bisphosphate aldolase (FBA) and aldose reductase-like isoform 1 (AKR-1) are involved in glycolysis. PGK and pyruvate kinase are involved in energy metabolism. PGK is the enzyme responsible for the first ATP generating step of glycolysis and the *in vivo* activation of l-nucleoside pro-drugs effective against retroviruses such as HIV and hepatitis [66]. Pyruvate kinase (PK) regulates the final rate-limiting step of glycolysis. In tumor cells, the glycolytic pyruvate isoenzyme M2 (PKM2, M2-PK) determines whether glucose is converted to lactate for regeneration of energy or used for the synthesis of cell building blocks [67]. Cell proliferation only proceeds when metabolism is capable of providing a budget of metabolic intermediates that is adequate to ensure both energy regeneration and the synthesis of cell building blocks in sufficient amounts. In this study, the three important enzymes of glycolysis were significantly down regulated after *Ac* larvae fed with RJC and by CSBV challenge, indicating that the energy metabolism pathway may be important in antiviral activity of the bees. Prohibitin protein wph (Wph) is required for larval metabolism or for the progression of the larva into a pupa. LOC409705 is responsible for imaginal disc-derived wing morphogenesis. These proteins were significantly down-regulated, probably directly impacting the larval development.

As indicated in Figure 1, the *Ac* larvae fed with RJC were not resistant to CSBV infection. Although the up-and down-regulated protein expressions were detected from those bee larvae, it seemed

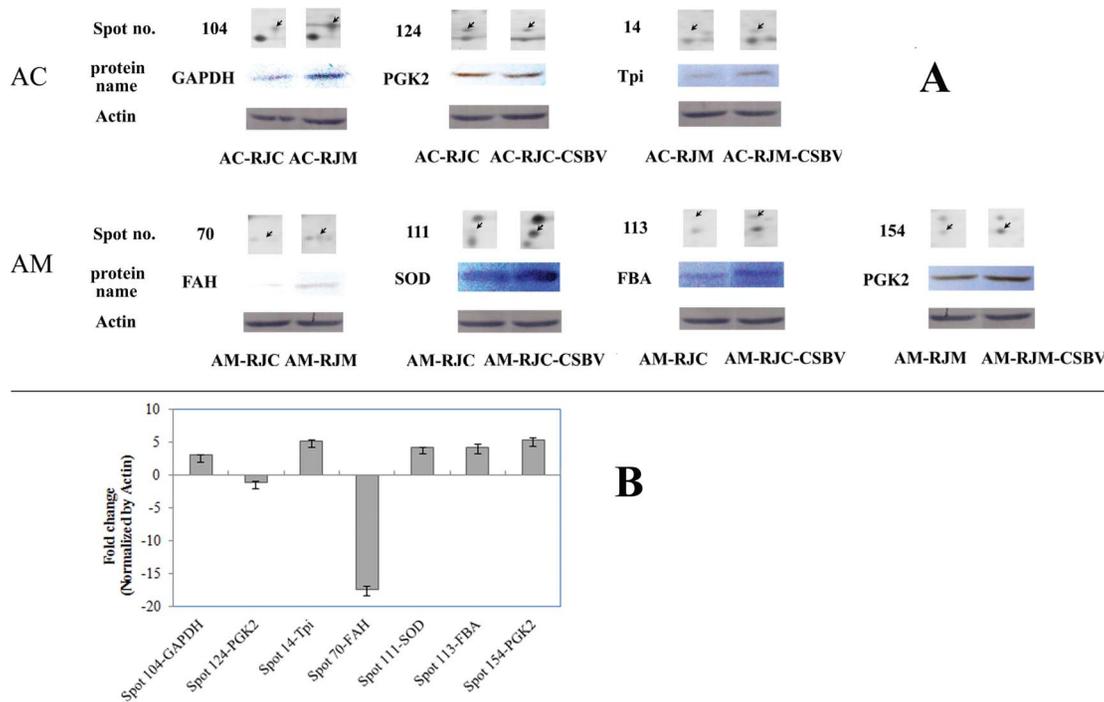


Figure 8. Western blot Analysis of differentially expressed proteins. Six proteins were selected. Proteins were separated on a 12% SDS-PAGE gel and immunoblotted with anti-GAPDH, anti-PKG2, anti-Tpi, anti-FAH, anti-SOD, and anti-FBA. β -actin was used as an internal control. (A) The Western blot images of GAPDH, PKG2, TPI, FAH, SOD, FBA and Actin. The protein spots from Figure 2. (B) Quantification of target protein expression. The relative fold change of GAPDH, PKG2, Tpi, FAH, SOD, FBA (normalized by Actin) was visualized by using Quantity One Software. The expression of GAPDH, SOD, FBA and Tpi were up-regulated. On the other hand, FAH and PKG were down-regulated. Error bar is standard deviation. doi:10.1371/journal.pone.0102663.g008

that these proteins were not actively involved in effectively protecting the larvae from CSBV infection. Whether some *Ac* expressed proteins were active for helping viral infection needs further study.

Differentially Expressed Proteins from *Ac* Larvae Fed with RJM and by CSBV Challenge

Comparing to the 3-day old *Ac* larvae fed with RJM but without CSBV infection, 4 highly expressed protein spots (eIF-5A, Tpi, CG2330RA, Act88F) and 3 down-regulated proteins (CG10703, HSP60, yellow-e2) were found from the *Ac* larvae fed with RJM and by CSBV infection.

Eukaryotic translation initiation factor 5a (eIF-5A) plays an important role in protein translation extending, and in stress tolerance [68]. Triosephosphate isomerase (Tpi) is a glycolytic enzyme [69]. Act88F (actin, indirect flight muscle-like) is involved in phagocytosis [51]. HSP 60 and yellow-e2 are both activated by environmental factors as discussed above. They were down-regulated may indicated that the influence of CSBV were weakened when the *Ac* larvae fed with RJM.

Interestingly, CSBV infection did not significantly increase the mortality of *Ac* larvae fed with RJM comparing to those fed with RJM and without viral challenge (Figure. 1). So RJM may protect *Ac* larvae from viral infection probably by promoting energy metabolism and activating phagocytosis. Another possibility is that the specific components from RJM, such as Peroxiredoxin 2540, glutathione S-transferase S1, and MRJP5, which are detected only in the RJ of *A. mellifera ligustica* [24], may inhibit the viral replication.

Differentially Expressed Proteins from *Am* Larvae Fed with RJC and by CSBV Challenge

In comparison to 3-day old *Am* larvae fed with RJC and without CSBV infection, 24 proteins were detected from 3-day old *Am* larvae fed with RJC and CSBV infection, in which 17 proteins were up-regulated (Cry11 (lambda-crystallin homolog), Dper\GL19229, Cat, CG9117 (metallo-beta-lactamase), LOC725919 (transposase), Eif, RpS20, serine protease easter (Sp7), LOC551766 (atp synthase subunit mitochondrial), SOD, Abnormal wing disks protein (Awd), FBA, ornithine aminotransferase (Oat), GADPH, larval cuticle protein a3a-like (CPR31A)) and 7 proteins down-regulated (Hsp60, Beta-Spec, Strn-Mlck, Yellow-h, FABP, NPC2, an unknown protein).

Most of the up-regulated proteins were involved in energy metabolism, such as Cry11, CG9117, LOC725919, Sp7, LOC551766, FBA, Oat, LOC410122, and GAPDH. The regulation pattern of FBA and GADPH was opposite to that in the *Ac* larvae fed with RJC and challenged with CSBV, maybe due to different species of honey bees. But in response to viral invasion, two bee species showed the same way in regulation of antioxidative enzymes, such as SOD, CuZn superoxide dismutase and Catalase, which were biomarkers to evaluate the toxic effects of organisms [70]. The proteins (Awd and CPR31A) related to development were up-regulated, to some degree, but the

cytoskeletal proteins (Beta-Spec and Strn-Mlck) were significantly down-regulated. These differentially expressed proteins might be involved in protecting *Am* larvae from CSBV infection, although these larvae were fed with RJC (Figure 1). *Am* larvae are not sensitive to CSBV infection [6]. The food change from RJM to RJC did not lower the resistant ability of *Am* to CSBV.

Differentially Expressed Proteins from *Am* Larvae Fed with RJM and by CSBV Challenge

In response to CSBV challenge, 11 up-regulated proteins (SS1G_11931, Pdi, FBA, CG5590, Hsc70-3, PGK, Cp309, Hsc70-4, GAPDH, Rpn9, Mhc), and 15 down-regulated proteins (RpS3, Jafrac1, CG6084, Hsp60, Aats-tyr, ALD, Hsp70, Lsp2, Vha68, LOC552007, Hex110, NPC2, Roe1, LOC725646) were found from the larvae fed with RJM and with CSBV infection.

Among the up-regulated proteins, those involved in energy metabolism were the majority, including PDI, FBA, ALD, PGK and GAPDH. This was in according with the expression pattern of *Ac* larvae fed with RJM. 26S proteasome (Rpn9) plays a fundamental role in eukaryotic homeostasis [71,72]. The ubiquitin-proteasome system mediated viral protein degradation constitutes a host defense process against some RNA viral infections [73]. In the present study Rpn9 and HSPs were both up-regulated, indicating the possible involvement of these proteins in antiviral activity. Correspondingly, chaperonins or store proteins were down-regulated. This may guarantee the normal growth of *Am* larvae. As a source of amino acids for tissue reconstruction during pupal development, hexamerins are first synthesized by a larval fat body and released into the hemolymph where they accumulate to extraordinarily high concentrations to build adult structures [74].

Conclusions

Although *Ac* is frequently damaged by CSBV, *Am* is usually resistant to this virus. The present study showed that the change of *Ac* larval food from RJC to RJM could enhance the bee resistance to CSBV, at least at early larval stage. Proteomic technology was employed to unravel the molecular events of the bees under heterospecific RJ breeding and CSBV challenge. There were 101 proteins with altered expressions after heterospecific RJ breeding and viral infection in two bee species. The RJM may protect *Ac* larvae from CSBV infection, probably by activating the genes in energy metabolism pathways, antioxidation and ubiquitin-proteasome system. The results, for the first time, comprehensively describe the molecular events of the viral infection of *Ac* and *Am*, and are potentially useful for establishing CSBV resistant populations of *Ac* for apiculture.

Author Contributions

Conceived and designed the experiments: RCH YZ GZZ. Performed the experiments: YZ GZZ XH. Analyzed the data: RCH YZ GZZ. Contributed reagents/materials/analysis tools: GZZ YZ XH. Wrote the paper: RCH YZ.

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