Comparative Subcellular Proteomics Analysis of Susceptible and Near-isogenic Resistant *Bombyx mori* (Lepidoptera) Larval Midgut Response to BmNPV infection

Xue-yang Wang, Hai-zhong Yu, Jia-ping Xu, Shang-zhi Zhang, Dong Yu, Ming-hui Liu & Lin-ling Wang

The molecular mechanism of silkworm resistance to *Bombyx mori* nucleopolyhedrovirus (BmNPV) infection remains largely unclear. Accumulating evidence suggests that subcellular fractionation combined with proteomics is an ideal technique to analyse host antiviral mechanisms. To clarify the anti-BmNPV mechanism of the silkworm, the near-isogenic line BC9 (resistant strain) and the recurrent parent P50 (susceptible strain) were used in a comparative subcellular proteomics study. Two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry (MS) was conducted on proteins extracted from the cytosol, mitochondria, and microsomes of BmNPV-infected and control larval midguts. A total of 87 proteins were successfully identified from the three subcellular fractions. These proteins were primarily involved in energy metabolism, protein metabolism, signalling pathways, disease, and transport. In particular, disease-relevant proteins were especially changed in microsomes. After infection with BmNPV, differentially expressed proteins (DEPs) primarily appeared in the cytosolic and microsomal fractions, which indicated that these two fractions might play a more important role in the response to BmNPV infection. After removing genetic background and individual immune stress response proteins, 16 proteins were identified as potentially involved in repressing BmNPV infection. Of these proteins, the differential expression patterns of 8 proteins according to reverse transcription quantitative PCR (RT-qPCR) analyses were consistent with the 2-DE results.

The silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae) has been domesticated for more than 5000 years and still plays an important role in many developing countries. *B. mori* is also a good model organism for the study of insect genetics and immunology. *B. mori* nucleopolyhedrovirus (BmNPV) is a primary silkworm pathogen and annually causes serious economic losses. Notably, some silkworm strains exhibit high resistance to BmNPV infection, but the molecular mechanisms of the resistance to BmNPV infection have not been fully elucidated.

In recent decades, a series of studies of the silkworm response to BmNPV infection have been reported. *B. mori* serine lipase-1, protease-2, and alkaline trypsin protein extracted from the digestive juice of the larval midgut have been reported to exhibit strong antiviral activity *in vitro*. NADH-oxidoreductase-like protein inhibited the capability of BmNPV particles to infect BmN cells *in vitro*. Arginine kinase was found to be involved in silkworm resistance to BmNPV infection using 2-DE to analyse the proteomes of different resistant strains. Beta-N-acetylglucosaminidase 2 and aminocyclase were shown to be up-regulated in BmNPV-resistant strains.

1School of Life Sciences, Anhui Agricultural University, Hefei, 230036, People’s Republic of China. 2Institute of Sericulture, Anhui Academy of Agricultural Sciences, Hefei, 230061, People’s Republic of China. 3School of Life Sciences, Chongqing Normal University, Hefei, 401331, People’s Republic of China. Correspondence and requests for materials should be addressed to J.-p.X. (email: jiapingxu@163.com)
using 2-DE to compare differences in protein patterns in different resistant strains hemolymph. In our laboratory, a total of 12 proteins potentially related to viral infection were obtained using one- and two-dimensional electrophoresis, followed by virus overlay assays. The functions of these proteins can be classified into three groups: endocytosis, intracellular transportation, and host responses. However, these previous results do not clarify the molecular mechanism of silkworm resistance to BmNPV.

Recently, comparative subcellular proteomics has become a useful strategy to reduce sample complexity and protein overlapping in exploring disease-resistant mechanisms. As “energy factories,” mitochondria are related to the chemical energy metabolism of adenosine triphosphate (ATP) and thus are involved in cell cycle and growth, as well as other biological processes, such as signal transduction, cellular differentiation, and cell death. In addition to their well-appreciated roles in biological processes, mitochondria appear to function as centrally positioned hubs in viral infection; some viruses have been reported to regulate host cell apoptosis based on mitochondrial regulation, including human immunodeficiency virus (HIV), influenza A virus (IAV), and hepatitis C virus (HCV). The microsome is a vesicle-like structure that is re-formed from pieces of endoplasmic reticulum and primarily participates in endoplasmic reticulum-like protein synthesis, protein glycosylation, and lipid synthesis. The differential expression of microsomal proteomes is consistently related to extraneous pathogen infection. Microsomes from DCs armed with peptide-MHC complexes can be an important alternative to DC-based vaccines for protection against viral infection. The cytosol contains multiple levels of organization, including concentration gradients of various ions, large complexes of enzymes, the cytoskeleton, and protein compartments. Almost all biological reactions must rely on certain cytosolic components or occur in the cytosol. Thus, understanding the cytosolic proteome is also crucial. Subcellular proteomics can largely reduce the complexity of protein samples and spot overlapping. Furthermore, to our knowledge, differentially expressed proteins (DEPs) in different-resistant silkworm strains following BmNPV infection have not been analysed based on subcellular proteomics.

In this study, subcellular proteomic maps of the near-isogenic line BC9 (resistant strain) and the recurrent parent P50 (susceptible strain) midguts following BmNPV infection were constructed and analysed based on three subcellular protein fractions: cytosol, mitochondria, and microsome. Many DEPs were identified in the three subcellular fractions using 2-DE combined with matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS); some of these proteins were potentially involved in resisting BmNPV infection. Our study provides an overview of the subcellular proteomic profiles of silkworm responses to BmNPV infection and lays a foundation for clarifying the mechanism of silkworm resistance to BmNPV.

Materials and Methods

Silkworm and virus. The recurrent parent P50 (susceptible strain) and donor parent A35 (resistant strain) were maintained in the Key Laboratory of Sericulture, Anhui Agricultural University, Hefei, China. The near-isogenic line BC9 was constructed according to the protocol of Yao et al.,. In brief, the recurrent parents were crossed with the donor parents; progeny were repeatedly backcrossed with the recurrent parents for nine generations, and each progeny was screened with BmNPV. Thus, the genetic background of BC9 is very similar to the recurrent parent P50, but BC9 should have the resistant background derived from A35.

BmNPV (T3 strain) was maintained in our laboratory and purified according to the protocol reported by Rahman et al. A haemocytometer was used to calculate the concentration of BmNPV (OB/mL).

Silkworm resistance level bioassays. The level of silkworm resistance to BmNPV was assayed according to the protocol of Cheng et al.,. In brief, the first day of fourth instar larvae were infected with different concentrations of BmNPV; inoculations were conducted in triplicate. The resistance level of the silkworm was calculated using IBM SPSS Statistics 20 (IBM, USA).

Silkworm midgut sample preparation. On the first day of fifth instar, all larvae were starved for 24 h. Then, 30 silkworms from each group were treated orally with 5 μL of BmNPV suspended in sterile water (1.0 × 10^4 OB/mL) per larva. The rapid proliferation of BmNPV was detected at approximately 24 hours post-inoculation (hpi); thus, this time was considered optimal for sample collection. Silkworm larvae were dissected, and midgut tissues were removed and then washed in DEPC (Sangon, China)-treated H₂O. Thirty larval midguts were mixed together to minimize individual genetic differences. Samples were flash-frozen in liquid nitrogen and pulverized, and 100 mg of each sample was placed directly into RNase-free microcentrifuge tubes containing 1.0 mL of TRIzol Reagent (Invitrogen, USA) and stored at −80 °C for later use.

Subcellular protein extraction. Subcellular proteins were extracted according to the method described by Lu et al. with some modification. A total of 50 μg of pulverized midgut sample was homogenized in 500 μL of potassium phosphate buffer (pH 7.6) containing 0.1 M potassium phosphate and 1.0 mM PMSF. The homogenate was centrifuged at 1,000 × g for 5 min at 4 °C to remove debris. The supernatant (total protein) was transferred to a new tube for the next step. To fractionate mitochondrial, microsomal and cytosolic proteins, the supernatant was further centrifuged at 10,000 × g for 10 min at 4 °C. The pellet was collected as mitochondrial proteins and then washed three times with potassium phosphate buffer, centrifuged at 10,000 × g for 10 min at 4 °C, and re-suspended in deionized water. The supernatant containing cytosolic and microsomal proteins was transferred to a new tube, mixed with an equal volume of 16 mM CaCl₂, incubated on ice for 5 min, and then centrifuged at 10,000 × g for 10 min at 4 °C. The pellet was collected as microsomal proteins and washed three times with potassium phosphate buffer, centrifuged at 10,000 × g for 10 min at 4 °C, and then re-suspended in deionized water. The resulting supernatant (cytosolic protein source), mitochondrial proteins, and microsomal proteins were transferred into 4 × volumes of precooled acetone, and incubated overnight at −20 °C. The fractions were then centrifuged at 10,000 × g for 10 min at 4 °C and washed three times with precooled acetone. Only the cytosolic proteins...
were cleaned using the 2-D Clean-up Kit (GE Healthcare, USA) according to the manufacturer’s instructions. The final proteins in the three subcellular fractions were dissolved in a rehydration solution containing 7 M urea, 2 M thiourea, 60 mM DTT, 65 mM CHAPS, 2% Triton X-100, and 0.2% ampholytes 5–8 for later use. The extraction of subcellular protein was performed in triplicate. The protein concentration was calculated using the Bradford method.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For the SDS-PAGE procedure, 5 × loading buffer (50 mM Tris-HCl, pH 8.0, 250 mM DTT, 5% SDS, 50% glycerol, and 0.04% bromophenol blue) was added to the protein sample. Samples were boiled for 8 min and subjected to SDS-PAGE with a 12% SDS polyacrylamide gel. Electrophoresis was performed on the Mini-protean Tetra system (Bio-Rad, USA), and gels were stained with Coomassie brilliant blue R250.

Two-dimensional gel electrophoresis (2-DE). For 2-DE, 800 μg of each sample protein dissolved in 300 μL of rehydration solution with 0.3 μL of 1% bromophenol blue dye was loaded onto a 17-cm immobilized linear dry strip (pH 5–8, Bio-Rad, USA). The strip was actively rehydrated at 20°C for 13 h at 50 V. The rehydrated strip was automatically focused using the following program: 100 V, slow, 1 h; 200 V, slow, 1 h; 300 V, slow, 1 h; 500 V, slow, 2 h; 1,000 V, slow, 2 h; 5,000 V, slow, 2 h; 10,000 V, linear, 2 h; 10,000 V, rapid, 80,000 V,h; and 500 V, rapid, 24 h. The current for each strip was limited to 50 μA. After IEF separation, the strips were immediately equilibrated with gentle shaking for 14 min 30 s in equilibration buffer (6 M urea, 20% glycerol, 2% SDS and 0.375 mM Tris-HCl, pH 8.8) containing 2% (w/v) DTT, followed by an equilibration for 14 min 30 s in the above equilibration buffer but containing 2.5% (w/v) IAM instead of DTT. Equilibrated IPG strips were further separated on a 10% SDS-PAGE gel. The procedures were performed at 10 mA/gel for 30 min and then 30 mA/gel until the bromophenol blue dye ran out from the bottom of the gels.

Gel visualization and image analysis. For visualization, gels were stained with Coomassie brilliant blue G-250. The stained gels were scanned at a resolution of 600 dpi. Spot detection, matching and quantitative intensity analysis were performed using PDQuest software (version 8.0, Bio-Rad, USA). Each protein sample was analysed in triplicate. Differential analyses of images were performed by matching the spots of the control and experimental groups. The unique protein spots or significantly differentially expressed protein spots (more than 2-fold) were selected and subjected to MS identification.

MALDI-TOF MS and database searching. In-gel digestion was performed as described earlier. Differentially expressed protein spots were excised from the stained gels, washed twice with Milli-Q water, and de-stained at room temperature for 5 min. The de-staining solution was removed, and samples were washed twice and incubated in 50% acetonitrile for 5 min, followed by removal of the acetonitrile and addition of 100% acetonitrile for 3 min. The gel was rehydrated in 4.0 μL of trypsin solution (Promega, Madison, USA) (20 μg/mL in 25 mM NH₄HCO₃) for 30 min. Next, 20 μL of cover solution (25 mM NH₄HCO₃) was added for digestion at 37°C for 16 h. The supernatant was transferred into a new tube and extracted once with 50 μL of extraction buffer (67% acetonitrile and 5% TFA). The peptide extract and supernatant were combined and then completely dried. The prepared sample was re-suspended with 5.0 μL of 0.1% TFA, followed by mixing with an equal volume of a matrix consisting of a saturated solution of α-cyano-4-hydroxy-trans-cinnamic acid in 50% acetonitrile and 0.1% TFA; 1.0 μL of mixture was spotted onto a new target plate. Peptide MS and MS/MS data were obtained with an ABI 5800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, USA). The data were obtained in a positive MS reflector using a CalMix5 standard to adjust the instrument (ABI5800 Calibration Mixture). The GPS Explorer V3.6 software (Applied Biosystems, USA) with default parameters was used to integrate and process both the MS and MS/MS data.

Database searching was performed based on a 95% or higher confidence interval of the scores of proteins in the Mascot V2.3 search engine (Matrix Science Ltd., U.K.) using the following parameters: NCBI nr database; trypsin as the digestion enzyme; one missed cleavage site; fixed modifications of carbamidomethyl (C); partial modifications of acetyl (Protein N-term), deamidated (NQ), dioxidation (W), oxidation (M); 100 ppm for precursor ion tolerance and 0.5 Da for fragment ion tolerance.

Protein identification and annotation. Protein identification used the following criteria: peptide mass fingerprinting was considered sufficient for identification if several peptides specific for a given protein were found, and protein identifications were accepted if they could be determined with greater than 95.0% probability and contained at least two unique peptides. Additionally, the calculated molecular weight (MW) and isoelectric point (pI) resulting from the MASCOT analysis agreed with the observed values to the greatest extent possible. Significant annotation was also associated with the identified organism and function in relevant biological contexts. Identified proteins were annotated based on the literature and information available in various databases, including the NCBI, Swiss-Prot/TrEMBL, and Gene Ontology (GO) databases.

Bioinformatics analysis. In living cells, many proteins can interact with each other, and these interacting proteins are expected to be involved in the same biological process or to handle in the same subcellular compartment, which is supported by the evidence that proteins in the same pathway are more interconnected. STRING (http://string-db.org/) contains abundant resources on physical and functional interactions and collects information from numerous sources, including experimental repositories, computational prediction methods, and public text collections. In this study, STRING was adopted to analyse the protein–protein interactions (PPIs) of the selected proteins. Due to the lack of proteomics information on B. mori in STRING, the PPIs network was built using the database of another well-studied insect, Drosophila melanogaster (D. melanogaster).
The related KEGG pathways of the selected proteins were analysed according to the method used by Qin et al.30. Briefly, the annotations of identified proteins were obtained by searching UniprotKB (http://www.uniprot.org/) using Bombyx mori and Drosophila database; then, KEGG pathways were analysed in accordance with the KEGG database (http://www.genome.ad.jp/kegg/pathway.html).

RNA isolation and cDNA synthesis. Total RNA of silkworm midgut was extracted with TRIzol Reagent (Invitrogen, USA) according to the manufacturer’s instructions. A NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to quantify the concentrations. The purity of all RNA samples were assessed at absorbance ratios of A260/280 and A260/230 and the integrity of the RNA was confirmed by 1% agarose gel electrophoresis. The first strand cDNA was synthesized using an RT reagent kit (TaKaRa, Japan) according to the manufacturer’s instructions.

Reverse transcription Quantitative PCR (RT-qPCR). To confirm the results of selected proteins, the relative expression levels of these proteins were validated by RT-qPCR assays. Primers used in the RT-qPCR assays are shown in Supplementary Table S1. RT-qPCR reactions were prepared with a SYBR Premix Ex Taq™ Kit (TaKaRa, Japan) according to the manufacturer’s instructions. The thermal cycling profile consisted of an initial denaturation at 95 °C for 30 s and 40 cycles at 95 °C for 5 s and 60 °C for 30 s. All assays were performed in triplicate. Relative expression levels were calculated using the 2−ΔΔCt method. In this study, B. mori glyceraldehyde-3-phosphate dehydrogenase (BmGAPDH) was selected as an internal control. Statistical analyses were conducted using the SPSS software (IBM, USA).

Results

Resistance to BmNPV of different silkworm strains. The median lethal concentration (LC50) was used to evaluate the level of silkworm resistance to BmNPV. In this study, the LC50 value of A35 was approximately 26-fold greater than that of BC9 and over 500-fold greater than that of P50. The value of BC9 was 23-fold greater than that of P50 (Table 1).

Variations in protein banding patterns among the subcellular protein fractions. It is essential to note that complete purification of subcellular fractions is nearly impossible, with few exceptions. However, subcellular fractionation is still a flexible and adjustable approach to reducing sample complexity and protein overlapping31. In this study, total protein and three subcellular protein fractions (mitochondrial, microsomal, and cytosolic) of the silkworm midgut were extracted. To detect the quality of the subcellular proteins, SDS-PAGE was used. Many bands with varying levels of intensity were observed among the three subcellular fractions. The bands in the total protein sample had matching bands with the same MW in one or more of the subcellular protein fractions; for example, 55 kDa bands existed in all of the subcellular fractions, whereas the 57 and 29 kDa bands in the total protein sample had only one matching band in the mitochondrial and microsomal fractions, respectively. Notably, some matching bands in the subcellular protein fractions exhibited much higher intensity compared with the total protein (Fig. 1). Overall, the subcellular protein samples were suitable for subsequent 2-DE analysis.

Changes in the cytosolic proteome profile in different resistant strains following BmNPV infection. Initially, IPG strips that ranged from pH 3 to 10 were used to analyse DEPs in the two strains following BmNPV infection. However, the dense area of spots was primarily distributed in the regions from pH 5 to 8; therefore, pH 5–8 IPG strips were used to improve spot resolution. Three repetitions for each sample were analysed using PDQuest software. Based on the normalized volume of each spot, DEPs were analysed in two strains following BmNPV infection. A large number of DEPs were obtained from the comparative analysis, but those with a ratio > 2 were selected for further analysis. Thus, a total of 38 spots were determined to be differentially expressed in the cytosol (Fig. 2), and the results are summarized in Table 2. The number of DEPs in P50+ vs. P50−, BC9− vs. P50−, and BC9+ vs. BC9− was 20, 14, and 4, respectively. In P50+ vs. P50−, a half number of proteins were up-regulated. In BC9− vs. P50−, 8 proteins were up-regulated, whereas in BC9+ vs. BC9−, 3 proteins were down-regulated (Table 2).

Identification of differentially expressed mitochondrial proteins in different resistant strains following BmNPV infection. Fractions of mitochondrial proteins from P50 and BC9 following BmNPV infection were also analysed using pH 5–8 IPG strips. The 2-DE images of mitochondrial proteins are shown in Fig. 3. The DEPs were filtered according to the method described above. After removing unqualified spots, 14 DEPs with significant changes were selected for further analysis, and the results are summarized in Table 3. The number of proteins differentially expressed in P50+ vs. P50−, BC9− vs. P50−, and BC9+ vs. BC9− was 8, 3, and

| Strains | LC50 (OB/mL) | 95% fiducial limits |
|---------|--------------|-------------------|
|         | Lower | Upper         |
| BC9     | 2.27 × 10^6 | 4.58 × 10^6 | 1.74 × 10^7 |
| A35     | 5.90 × 10^6 | 2.14 × 10^7 | 3.22 × 10^6 |
| P50     | 1.03 × 10^6 | 3.96 × 10^6 | 2.24 × 10^6 |

Table 1. The LC50 values of different resistant silkworm strains.
Changes in the microsomal proteome profile in different resistant strains following BmNPV infection. Microsomal proteins of the two strains following BmNPV infection were also analysed using pH 5–8 IPG strips. The results for the analysis of microsomal proteins are shown in Fig. 4. After discarding unqualified spots, 35 protein spots were observed to be significantly differentially expressed, and the results are summarized in Table 4. The number of DEPs in P50+ vs. P50−, BC9− vs. P50−, and BC9+ vs. BC9− was 16, 11, and 8, respectively. In P50+ vs. P50−, 10 proteins were up-regulated, and in BC9− vs. P50−, 8 proteins were up-regulated. In BC9+ vs. BC9−, 4 proteins were down-regulated.

KEGG pathway analysis of differentially expressed proteins (DEPs) in each subcellular fraction. Among the 87 identified DEPs, 63 proteins were involved in specific KEGG pathways (Fig. 5). To obtain overview information on these proteins, each of the subcellular fractions were individually classified. The relevant pathways were classified into five main categories and 33 subcategories according to the KEGG classifications. In microsomes, proteins that were differentially expressed following BmNPV infection primarily participated in pathways related to energy metabolism (28%), disease (39%), and transport (15%). In the mitochondria, proteins that were differentially expressed mainly participated in energy metabolism (16%) and disease (66%). In the cytosol, proteins that were differentially expressed were primarily involved in energy metabolism (40%) and protein metabolism (35%).

Additionally, the number of up-regulated proteins related to energy metabolism was increased in P50 following BmNPV infection, but decreased in BC9 following virus infection. In relation to protein metabolism, the number of up-regulated proteins was decreased in P50 and increased in BC9 following BmNPV infection. All the proteins were up-regulated in the signalling pathways in P50 following virus infection, whereas only half of the proteins were up-regulated in BC9 following infection. In relation to transport, all the proteins were down-regulated in P50 following virus infection, whereas several proteins were up-regulated in BC9 following infection. After BmNPV infection, the proteins related to primary bile acid biosynthesis were all down-regulated in P50. Notably, we found that a large number of proteins related to disease were altered following BmNPV infection; far more of these proteins were observed in P50 than in BC9, and many were up-regulated in P50 following BmNPV infection (see Supplementary Table S2).

Analysis of differentially expressed proteins (DEPs) that potentially contribute to BmNPV infection. Those proteins specifically expressed in the susceptible strain were beneficial for analysing the mechanism of silkworm resistance to BmNPV. In this study, 26 proteins (region of blue colour) were uniquely differentially expressed in P50 following BmNPV infection based on a Venn diagram analysis (Fig. 6). There were 11, 5 and 10 proteins distributed in the cytosol, mitochondria, and microsomes, respectively. In the cytosol, 6 proteins were up-regulated in P50 following infection. In the mitochondria, 2 proteins were up-regulated in P50 following infection. In the microsomes, half of the proteins were up-regulated in P50 following infection.
Theoretically, these up-regulated proteins may be involved in the BmNPV-stimulated or pathologic response in host cells.

Analysis of differentially expressed proteins (DEPs) potentially participating in the resistance to BmNPV infection. The near-isogenic line BC9 was constructed based on the recurrent parent P50, and the two strains have a highly similar genetic background. After removing DEPs relevant to the genetic background and immune stress response, 13 proteins (region of yellow colour) that were uniquely differentially expressed in BC9 following infection were obtained according to the Venn diagram analysis (Fig. 6), which were potentially involved in the BmNPV-stimulated or pathologic response. However, the number of varied proteins

Figure 2. 2-DE images of cytosolic protein extracts from P50 and BC9 following BmNPV infection. P50−, treated with sterile water; P50+, infected with BmNPV; BC9−, treated with sterile water; BC9+, infected with BmNPV. Proteins were separated by pH 5–8 IPG strips, followed by SDS-PAGE on 10% gels. The gels were stained with Coomassie brilliant blue G-250. DEPs are marked by a label with a number. All samples were processed in parallel. The full-length gels were included in the Supplementary Fig. S2.
| Spot no. | BC9 - vs. P50 | Ratio | Accession no. | Protein name | Theoretical/Observed P1<sup>d</sup> | Theoretical/Observed MW (kDa)<sup>d</sup> | Matched unique peptides | Sequence coverage (%)<sup>e</sup> | Protein score<sup>e</sup> | Molecular/biological function<sup>f</sup> |
|---------|---------------|-------|---------------|-------------|-----------------------------------|----------------------------------------|------------------------|-------------------------------|--------------------------|-------------------------------------|
| c1      | down          | 49.13 | g|525652881     | Dnaj (Hsp40) homologue 3          | 5.56/6.47                            | 40/35.25                            | 6                            | 23%                       | 456                      | Protein folding                    |
| c2      | down          | 65.71 | g|512914963     | Probable methylmalonate-          | 7.59/7.53                            | 56/57                                | 9                            | 22%                       | 808                      | Aldehyde dehydrogenase (NAD) activity, fatty-acyl-CoA binding, methylmalonate semialdehyde dehydrogenase (acylating) activity, thymine metabolic process, valine metabolic process |
| c3      | down          | 149.67| g|512936895     | Acetyl-CoA hydrolase             | 7.67/7.83                            | 52/48.4                             | 8                            | 16%                       | 523                      | Hydrolase activity, acetyl-CoA metabolic process |
| c4      | down          | 6.07  | g|512902782     | Uncharacterized protein LOC101738880 isoform X1 | 5.75/5.72                            | 25/22.73                            | 7                            | 34%                       | 578                      |                                        |
| c5      | down          | 5.34  | g|17136564      | Alpha-tubulin at 84B [Protophila melanogaster] | 5.00/5.62                            | 51/55.44                            | 8                            | 27%                       | 767                      | GTPase activity, GTP binding, structural constituent of cytoskeleton, antimicrobial humoral response, mitotic spindle assembly checkpoint |
| c6      | down          | 2.4   | g|512934077     | 10kDa heat shock protein,         | 6.74/7.38                            | 11/15.22                            | 4                            | 52%                       | 396                      | ATP binding, protein folding        |
| c7      | down          | 2.86  | g|827563568     | Electron transfer flavoprotein subunit alpha, mitochondrial | 8.43/7.2                             | 35/29.68                            | 8                            | 36%                       | 827                      | Electron carrier activity, flavin adenine dinucleotide binding |
| c8      | down          | 3.72  | g|827558088     | 3-hydroxyisobutyryl-CoA hydrolase, mitochondrial | 8.08/7.43                            | 41/35.39                            | 7                            | 27%                       | 584                      | Hydrolase activity                |
| c9      | down          | 8.15  | g|512898603     | Glyoxylate reductase/             | 8.76/7.65                            | 40/36.95                            | 10                           | 33%                       | 849                      | NAD binding, oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor |
| c10     | down          | 148.54| g|112984390     | Elongation factor 1-alpha         | 9.24/6.69                            | 51/49.5                            | 6                            | 18%                       | 390                      | GTPase activity, GTP binding, translation elongation factor activity |
| c11     | up            | 3.09  | g|112983556     | 90-kDa heat shock protein         | 4.99/5.52                            | 83/86.37                            | 9                            | 16%                       | 713                      | ATP binding, response to stress, protein folding |
| c12     | up            | 5.75  | g|512901366     | Aldose reductase-like isoform X1   | 6.09/6.68                            | 36/31.36                            | 8                            | 30%                       | 491                      | Oxidoreductase activity            |
| c13     | up            | 4.58  | g|827560339     | Prolyl endopeptidase              | 7.90/6.61                            | 90/76.65                            | 4                            | 6%                        | 309                      | Serine-type endopeptidase activity, serine-type exopeptidase activity |
| c14     | up            | 3.66  | g|512888904     | Cytoplasmic aconitate hydratase-like isoform X1 | 5.84/6.73                            | 97/94.81                            | 8                            | 13%                       | 55                       | Metabolic process                 |
| c15     | up            | 5.93  | g|512939991     | Cystathionine beta-synthase-like   | 6.02/6.86                            | 54/54.31                            | 8                            | 18%                       | 481                      | Cystathionine beta-synthase activity, metal ion binding, pyridoxal phosphate binding |
| c16     | up            | 3.12  | g|357613322     | 26S protease regulatory subunit 6 A [Danausplexippus] | 5.11/5.73                            | 48/49.32                            | 7                            | 23%                       | 493                      | ATP binding, peptidase activity, protein catabolic process |
| c17     | up            | 3.14  | g|312597598     | Inorganic pyrophosphatase         | 4.96/5.4                             | 32/29.85                            | 9                            | 28%                       | 452                      | Inorganic diphosphatase activity, magnesium ion binding, phosphate-containing compound metabolic process |
| c18     | up            | 6.1   | g|512923641     | Fatty acid-binding protein-like    | 5.04/5.4                             | 16/18.89                            | 5                            | 38%                       | 296                      | Lipid binding, transporter activity |
| c19     | up            | 4     | g|512923641     | Fatty acid-binding protein-like    | 5.04/5.4                             | 16/16.44                            | 5                            | 33%                       | 200                      | Lipid binding, transporter activity |
| c20     | up            | 8.06  | g|512917297     | Fatty acid-binding protein 1-like isoform X1 | 6.59/6.67                            | 15/15.61                            | 7                            | 71%                       | 573                      | Lipid binding, transporter activity |

| Spot no. | BC9 – vs. P50 | Ratio | Accession no. | Protein name | Theoretical/Observed P1<sup>d</sup> | Theoretical/Observed MW (kDa)<sup>d</sup> | Matched unique peptides | Sequence coverage (%)<sup>e</sup> | Protein score<sup>e</sup> | Molecular/biological function<sup>f</sup> |
|---------|---------------|-------|---------------|-------------|-----------------------------------|----------------------------------------|------------------------|-------------------------------|--------------------------|-------------------------------------|
| c21     | down          | 4.29  | g|512907055     | Grpe protein homologue, mitochondrial | 6.97/6.2                             | 24/21.21                            | 6                            | 44%                       | 491                      | Adenylyl-nucleotide exchange factor activity, protein folding |
| c22     | down          | 2.13  | g|114051229     | Microtubule-associated protein RP/EB family member 1 | 5.48/6.27                            | 31/29.98                            | 9                            | 37%                       | 829                      |                                        |
| c24     | down          | 2.15  | g|291045214     | Isopentenyl-diphosphate delta isomerase | 6.37/6.57                            | 30/27.16                            | 8                            | 31%                       | 325                      | Hydrolyase isopentenyl-diphosphate delta-isomerase activity, isoprenoid biosynthetic process |
| c25     | down          | 2.53  | g|512892238     | Carbonic anhydrase 2              | 5.92/6.41                            | 31/27.45                            | 5                            | 28%                       | 420                      | Carbonate dehydratase activity, one-carbon metabolic process |
| c26     | down          | 2.32  | g|160333678     | Glutathione S-transferase sigma 2 | 5.85/6.89                            | 23/24.17                            | 9                            | 53%                       | 864                      | Transferase activity               |

Continued
was significantly lower than that of P50. The number of the DEPs in the cytosol, mitochondria, and microsomes was 4, 3, and 6, respectively. In the cytosol, 3 proteins were up-regulated, and one was down-regulated following infection. In the mitochondria, two proteins were down-regulated, and one was up-regulated following infection. In microsomes, half the number of the proteins was up-regulated following infection. In particular, certain proteins (overlap region of yellow and green colour) exhibited varied expression levels between the near-isogenic line BC9 (resistant strain) and the recurrent parent P50 (susceptible strain) and were significantly different.

### Table 2. Identified proteins from cytosolic fraction that changed significantly in different resistant strains following BmNPV infection.

| Spot no. | P50+ vs. P50− | Ratio | Accession no. | Protein name | Theoretical/ Observed PI | Theoretical/ Observed MW (kDa) | Matched unique peptides | Sequence coverage (%) | Protein score | Molecular/biological function |
|----------|---------------|-------|---------------|--------------|--------------------------|-----------------------------|------------------------|----------------------|---------------|--------------------------------|
| c27      | down          | 4.37  | gi|112982671     | Ribosomal protein S12 | 5.79/6.21                  | 15/15.97                 | 6                      | 75%              | 385              | Structural constituent of ribosome, translation |
| c28      | up            | 64.59 | gi|49868         | Beta-actin (aa 27–375) [Mus musculus] | 5.78/6.13                  | 39/32.55                  | 5                      | 19%              | 389              | ATP binding, identical protein binding, kinase binding, nitric-oxide synthase binding, RNA polymerase II core promoter proximal region sequence-specific DNA binding |
| c29      | up            | 72.24 | gi|114051866     | Isocitrate dehydrogenase | 6.24/6.91                  | 47/43.92                  | 7                      | 15%              | 329              | Isocitrate dehydrogenase (NADP+) activity, magnesium ion binding, NAD binding, iso- cyclic metabolic process, tricarboxylic acid cycle |
| c30      | up            | 2.96  | gi|153792114     | Phosphatidylethanolamine-binding protein isoform 2 | 5.96/5.81                  | 22/20.57                  | 5                      | 39%              | 230              | Defence response to Gram-negative/positive bacteria, regulation of antimicrobial humoral response |
| c31      | up            | 9.61  | gi|152902782     | Uncharacterized protein LOC101738880 isoform X1 | 5.75/5.55                  | 25/20.54                  | 8                      | 45%              | 791              | ATPase activity, ATP binding, proteasome activating ATPase activity, TBP-class protein binding |
| c32      | up            | 4.1   | gi|4574740       | Tat-binding protein-1 [Drosophila melanogaster] | 5.39/5.73                  | 48.4/49.32                | 3                      | 13%              | 277              | Glyceraldehyde-3-phosphate dehydrogenase (NAD+) activity, NAD binding, carbohydrate metabolic process, NAD+ dependent dehydrogenase activity |
| c33      | up            | 4.88  | gi|51555848      | Glyceraldehyde3-phosphate dehydrogenase-2 | 5.62/6.4                  | 39/31.69                  | 10                     | 34%              | 850              | Glyceraldehyde-3-phosphate dehydrogenase (NAD+) activity, NAD binding, carbohydrate metabolic process, NAD+ dependent dehydrogenase activity |
| c34      | up            | 2.53  | gi|114053311     | 26S protease regulatory subunit 6B | 5.09/5.61                  | 47/50.98                  | 6                      | 17%              | 337              | ATP binding, peptidase activity, protein catalytic process |
| c35      | up            | 58.24 | gi|347326520     | DNA supercoiling factor | 4.48/5.53                  | 40/40.96                  | 7                      | 27%              | 476              | Calcium ion binding |

---

**Molecular/biological function**: GO annotations were obtained from the gene ontology database (http://www.geneontology.org/).

---

**Table 2.** Identified proteins from cytosolic fraction that changed significantly in different resistant strains following BmNPV infection. aSpot no. corresponding to the numbers in the 2-DE gels of Fig. 2. bThe expression intensity ratios of P50 and BC9 following BmNPV infection or strain BC9 to that of P50. cDetailed information on the identified proteins can be viewed via their accession numbers on http://www.ncbi.nlm.nih.gov/. dObserved molecular weight (MW) and isoelectric point (PI) values were obtained from PDQuest analysis. Theoretical MW and PI values were obtained from a Mascot analysis. eThe three parameters, matched unique peptides, sequence coverage and protein score were generated from a Mascot analysis. fMolecular/biological functions were annotated using the gene ontology (GO) database (http://www.geneontology.org/).
(Tables 2, 3 and 4). Theoretically, these three proteins may be involved in the response of the resistant silkworm strain to BmNPV infection.

**Protein-protein interactions (PPIs) network analysis of anti-BmNPV-relevant differentially expressed proteins (DEPs).** To further investigate the relationship of the 16 DEPs of interest, which included the 13 proteins (region of yellow colour) uniquely differentially expressed in BC9+ vs. BC9− and the three proteins (overlap region of yellow and green colour) that exhibited varied expression in BC9− vs. P50 and BC9+ vs. BC9−, the functional association of these proteins was analysed using STRING 9.1 online software. A combined score was assigned for every protein-protein association pair in the software. This score was computed by combining the probabilities from several pieces of evidence and correcting for the probability of randomly

Figure 3. 2-DE images of mitochondrial protein extracts from P50 and BC9 following BmNPV infection. P50−, treated with sterile water; P50+, infected with BmNPV; BC9−, treated with sterile water; BC9+, infected with BmNPV. Proteins were separated with pH 5–8 IPG strips, followed by SDS-PAGE on 10% gels. The gels were stained with Coomassie brilliant blue G-250. DEPs are marked by a label with a number. All samples were processed in parallel. The full-length gels were included in the Supplementary Fig. S3.
observing an interaction. As illustrated in Fig. 7, most proteins could be constructed into one network with medium confidence (0.707), except for carbonic anhydrase 2 and selenium-binding protein 1 isoform X2 (SB1). Notably, the multifunctional chaperonin CTP1 had close interaction with its member alpha-tubulin, Tudor-SN, and other functional partners, such as the vacuolar ATP synthase catalytic subunit A (V-ATPase subunit A), $H^+$-transporting ATP synthase beta subunit isoform 2 (ATP5B), and Ndufs8, ribosomal protein S12 (RpS12), eukaryotic translation initiation factor 3 subunit I (eIF3), proteasome subunit alpha type-1 isoform X2 (PA1), and PEBP. The chaperonin also closely interacted with energy metabolism-relevant proteins, including L-lactate dehydrogenase (LDH), methylmalonate-semialdehyde dehydrogenase (MSD), pyruvate dehydrogenase (PD), and thiol peroxiredoxin (TPx), and these proteins also can interact closely with each other.

Validation of anti-BmNPV-relevant differentially expressed proteins (DEPs) using RT-qPCR. To analyse the differential expression patterns of the 16 DEPs of interest following BmNPV infection, RT-qPCR was used. As illustrated in Fig. 8, 8 proteins exhibited highly similar differential expression patterns

| Spot no. | BC9− vs. BC9+ | Ratio | Accession no. | Protein name | Theoretical/ Observed P1 | Theoretical/ Observed MW (kDa)2 | Matched unique peptides3 | Pep. Count4 | Protein Score5 | Molecular/biological function6 |
|----------|----------------|-------|---------------|---------------|--------------------------|-------------------------------|-------------------------|-------------|----------------|--------------------------------|
| mc1      | up             | 2.58  | gi|87248369 | NADH dehydrogenase (ubiquinone) Fe-S protein 8 | 6.15/5.79 | 25.5/21.83 | 13 | 100 | 4 iron, 4 sulfur cluster binding, oxidoreductase activity, acting on NAD(P)/H |
| mc10     | up             | 3.86  | gi|38260562 | Thiolo peroxiredoxin | 6.09/7 | 22.07/19.63 | 10 | 100 | Peroxiredoxin activity |
| mc11     | up             | 3.62  | gi|98990259 | Cytochrome b-c1 complex subunit Rieske | 8.59/7.25 | 29.4/21.47 | 11 | 99.982 | 2 iron, 2 sulfur cluster binding, metal ion binding, ubiquinol-cytochrome c reductase activity |

Table 3. Identified proteins from mitochondrial fraction that changed significantly in different resistant strains following BmNPV infection. a, Spot no. corresponds to the numbers in the 2-DE gels of Fig. 3.b,c,d,e,f are the same as in Table 2.
at the translational and transcriptional levels in BC9 following infection, including Tudor-SN, alpha-tubulin, V-ATPase subunit A, TPx, eIF3, ATP5B, MSD, and LDH. Additionally, nearly all of these proteins exhibited significant differences in expression in BC9 following infection. However, other proteins displayed different patterns at the translational and transcriptional levels in BC9 following infection. This phenomenon was not unexpected because numerous factors are involved in gene expression, including mRNA stability, splicing, translational regulation, protein post-translational processing, and protein turnover\(^\text{32}\). Nonetheless, proteomics data are more relevant to biological responses because proteins, not RNAs, are the functional products of genes.

Figure 4. 2-DE images of microsomal protein extracts from P50 and BC9 following BmNPV infection. P50−, treated with sterile water; P50+, infected with BmNPV; BC9−, treated with sterile water; BC9+, infected with BmNPV. Proteins were separated with pH 5–8 IPG strips, followed by SDS-PAGE on 10% gels. The gels were stained with Coomassie brilliant blue G-250. DEPs are marked by a label with a number. All samples were processed in parallel. The full-length gels were included in the Supplementary Fig. S4.
| Spot no. | P50+ vs. P50− | Ratio | Accession no. | Protein name | Theoretical/ Observed PI | Theoretical/ Observed MW (kDa) | Matched unique peptides | Sequence coverage (%) | Protein score | Molecular/biological function |
|----------|----------------|-------|---------------|---------------|--------------------------|-------------------------------|--------------------------|-----------------------|---------------|-------------------------------|
| ms1      | down           | 33.35 | gi|148298800 | Enolase         | 5.62/6.1                   | 47/47.82                  | 9                     | 30%           | 795 Magnesium ion binding, phosphopyruvate hydratase complex, glycolytic process |
| ms2      | down           | 27.28 | gi|512913423 | Uncharacterized protein LOC101745964 | 6.04/6.02                  | 51/53                    | 4                     | 11%           | 193 ATPase activity, hydrolase activity |
| ms3      | down           | 78.57 | gi|112983322 | Transitional endoplasmic reticulum ATPase TER94 | 5.3/5.94                  | 90/101.55                | 8                     | 15%           | 589 Translation initiation factor activity, formation of translation preinitiation complex, regulation of translational initiation |
| ms4      | down           | 48.18 | gi|114051800 | Eukaryotic translation initiation factor 3 subunit I | 5.71/6.39                  | 37/36.77                 | 10                    | 42%           | 707 |
| ms5      | down           | 13.14 | gi|512892238 | Carbonic anhydrase 2 | 5.92/6.39                  | 31/28.44                 | 4                     | 22%           | 265 Carbonate dehydratase activity, one-carbon metabolic process |
| ms6      | down           | 65.11 | gi|512912927 | Sorting nexin lst-4 | 5.61/6.4                   | 64/71.17                 | 9                     | 17%           | 415 Phosphatidylinositol-3,4,5-trisphosphate binding, intracellular protein transport, phagosome-lysosome fusion involved in apoptotic cell clearance |
| ms7      | down           | 15.99 | gi|114053117 | Eukaryotic translation initiation factor 3 subunit K | 5.38/6                    | 25/24.27                 | 3                     | 12%           | 170 Ribosome binding, translation initiation factor activity, formation of translation preinitiation complex, regulation of translational initiation |
| ms8      | down           | 2.83  | gi|112983906 | Eukaryotic translation initiation factor 3 subunit H | 5.68/5.92                  | 39/54.61                 | 5                     | 16%           | 343 Translation initiation factor activity, formation of translation preinitiation complex |
| ms9      | down           | 9.05  | gi|112983898 | Elongation factor 1 gamma | 5.83/6.44                  | 49/46.85                 | 8                     | 21%           | 587 Translation elongation factor activity |
| ms10     | down           | 2.68  | gi|112983010 | Translation elongation factor 2 isoform 1 | 6.23/7.16                  | 98/113.15                | 4                     | 9%            | 223 GTPase activity, GTP binding, translation elongation factor activity |
| ms11     | up             | 2.5   | gi|827548126 | Pyruvate dehydrogenase E3 component beta subunit isoform X1 | 6.03/6.04                  | 40/32.43                 | 10                    | 35%           | 594 Pyruvate dehydrogenase (acetyl-transferring) activity, acetyl-CoA biosynthetic process from pyruvate |
| ms12     | up             | 15.59 | gi|82741809 | Enoyl-CoA hydratase precursor 1 | 8.44/6.72                  | 32/26.8                  | 7                     | 31%           | 452 Catalytic activity |
| ms13     | up             | 9.14  | gi|827537214 | Probable enoyl-CoA hydratase, mitochondrial | 9.28/7.14                  | 32/26.74                 | 10                    | 42%           | 874 Catalytic activity |
| ms14     | up             | 3.08  | gi|827537214 | Probable enoyl-CoA hydratase, mitochondrial | 9.28/7.69                  | 32/26.84                 | 10                    | 39%           | 896 Catalytic activity |
| ms15     | up             | 2.09  | gi|114052278 | ATP synthase | 9.21/6.91                  | 60/51.66                 | 4                     | 5%            | 128 ATP binding, proton-transporting ATPase activity, proton-transporting ATP synthase activity |
| ms16     | up             | 3.87  | gi|153792309 | Pyruvate dehydrogenase | 8.07/7.43                  | 44/34.96                 | 8                     | 27%           | 410 Pyruvate dehydrogenase (acetyl-transferring) activity, glycolytic process |

Spot no. | BC9− vs. P50− | Ratio | Accession no. | Protein name | Theoretical/ Observed PI | Theoretical/ Observed MW (kDa) | Matched unique peptides | Sequence coverage (%) | Protein score | Molecular/biological function |
|----------|----------------|-------|---------------|---------------|--------------------------|-------------------------------|--------------------------|-----------------------|---------------|-------------------------------|

Continued
| Spot no. | PS0+ vs. PS0− | Ratio | Accession no. | Protein namea | Theoretical/ Observed PIb | Theoretical/ Observed MW (kDa)d | Matched unique peptidesc | Sequence coverage (%)c | Protein scorec | Molecular/biological functionf |
|----------|----------------|--------|--------------|---------------|--------------------------|---------------------------------|------------------------|-----------------------|----------------|--------------------------------|
| ms17     | down           | 99.96  | gi|512903088     | Mitochondrial import receptor subunit Tom70 | 5.55/6.31 | 62/62.95 | 8 | 16% | 391 | Receptor |
| ms18     | down           | 5.77   | gi|512899307     | Esterase FE4-like | 5.27/6.12 | 68/66.96 | 5 | 5% | 206 | Hydrolyase activity |
| ms19     | down           | 64.93  | gi|112983574     | Carboxylic ester hydrolase | 7.09/6.85 | 55/71.74 | 6 | 16% | 217 | Hydrolyase activity |
| ms20     | up             | 166.38 | gi|336454478     | Heat shock protein 70–3 | 5.12/5.59 | 73/71.7 | 10 | 21% | 647 | ATP binding |
| ms21     | up             | 65.6   | gi|304307739     | Tudor staphylococcus/ micrococcal nulease | 8.56/6.12 | 99/26.35 | 9 | 13% | 664 | Transcription cofactor activity, posttranscriptional gene silencing by RNA |
| ms22     | up             | 38.05  | gi|112983926     | Arginine kinase | 5.87/6.75 | 40/42.06 | 9 | 32% | 561 | ATP binding, kinase activity |
| ms23     | up             | 33.53  | gi|124245114     | Glucose-regulated protein 78 [Fennoropenaeus chinensis] | 5.00/6.74 | 72.8/48.85 | 5 | 11% | 491 | ATP binding, Nucleotide-binding |
| ms24     | up             | 34.91  | gi|112982960     | Ferritin precursor | 6.75/7.56 | 26/26.95 | 4 | 31% | 441 | Ferric iron binding, ferroxidase activity, cellular iron homeostasis, iron ion transport |
| ms25     | up             | 8      | gi|153792257     | Trypsin-like protease | 5.62/6.5 | 28.5/25.84 | 3 | 17% | 184 | Serine-type endopeptidase activity |
| ms26     | up             | 10.16  | gi|5751          | Actin a3 | 5.47/6.05 | 42/68.45 | 4 | 12% | 238 | ATP binding |
| ms27     | up             | 2.5    | gi|827537214     | Probable enoyl-CoA hydratase, mitochondrial | 9.28/7.69 | 32/26.84 | 10 | 39% | 893 | Catalytic activity |
| ms28     | down           | 2.06   | gi|827548126     | Pyruvate dehydrogenase e1 component beta subunit isofrom x1 | 6.03/6.04 | 40/32.43 | 10 | 35% | 625 | Pyruvate dehydrogenase (acytly-transferring) activity, acetyl-CoA biosynthetic process from pyruvate |
| ms29     | down           | 6.04   | gi|32000724      | Alpha-tubulin [eukolpleura dioica] | 4.94/5.64 | 51/55.62 | 9 | 31% | 805 | GTPase activity, GTP binding, structural constituent of cytoskeleton |
| ms30     | down           | 2.76   | gi|148298878     | Vacuolar ATP synthase catalytic subunit a | 5.27/6.18 | 69/58.88 | 9 | 16% | 622 | ATP binding, proton-transporting ATPase activity, ATP hydrolysis-coupled proton transport, ATP metabolic process |
| ms31     | down           | 2.3    | gi|15213812      | Ribosomal protein s12 [spodoptera frugiperda] | 5.79/6.17 | 15/15.67 | 6 | 62% | 542 | Structural constituent of ribosome, translation |
| ms32     | up             | 198.68 | gi|153092309     | Pyruvate dehydrogenase | 8.07/7.43 | 44/34.96 | 9 | 28% | 511 | Pyruvate dehydrogenase (acyetyl-transferring) activity, glycolytic process |
| ms33     | up             | 2.47   | gi|112982996     | Thiol peroxidoxin | 6.09/7.13 | 22/20.17 | 6 | 37% | 456 | Peroxidoxin activity |
| ms34     | up             | 5.79   | gi|112983898     | Elongation factor 1 gamma | 5.83/6.48 | 49/46.85 | 10 | 26% | 704 | Translation elongation factor activity |
| ms35     | up             | 3.4    | gi|114051800     | Eukaryotic translation initiation factor 3 subunit I | 5.71/6.52 | 37/36.46 | 10 | 32% | 672 | Translation initiation factor activity, formation of translation preinitiation complex, regulation of translational initiation |

**Table 4.** Identified proteins from microsomal fraction that changed significantly in different resistant strains following BmNPV infection. a, Spot no. corresponds to the numbers in the 2-DE gels of Fig. 4. b,c,d,e,f are the same as in Table 2.
Discussion

Many technologies, such as differential gel electrophoresis and shotgun proteomics, have been used to study the molecular mechanism of silkworm resistance to BmNPV. However, these strategies by nature do not provide a global proteomic profile of silkworm anti-BmNPV mechanisms. Recently, subcellular proteomics combined with MS has been widely adopted in researching disease-resistant mechanisms to reduce the sample complexity and protein coverage, as well as to provide a spatial description of proteins in the cell\(^3\). In this study, a dynamic overview of the changed host proteins in response to BmNPV infection was obtained by comparing differences in the abundance of proteins isolated from the cytosol, mitochondria, and microsome of the recurrent parent P50 (susceptible strain) and the near-isogenic line BC9 (resistant strain) following BmNPV infection. Many distinctly different protein patterns were observed in 1-DE gels (Fig. 1). In the 2-DE gels, few of the same proteins existed in two or three subcellular fractions, except carbonic anhydrase 2, which indicated that the quality of our subcellular protein extractions was good. The spots identified as the same proteins were primarily located in the vertical or horizontal levels, such as mc7 and mc6, which might be caused by protein degradation, protein modification after translation, or the different structure of proteins. This phenomenon must be further confirmed in future studies.

In this study, most of the altered proteins in microsome and mitochondria mainly participated in disease pathways. Changes in these proteins might affect other metabolic processes and lead to diseases. For example, the catalase activity of lipid peroxidation in microsome and mitochondria was reduced by increased expression and led to prostate disease\(^33\). In this study, these proteins were changed significantly following BmNPV infection,
indicating that these proteins were potentially involved in the response to BmNPV infection. Therefore, subcellular proteomics could not only enhance the resolution of the proteome but also be beneficial for finding proteins relevant to BmNPV resistance.

In the process of BmNPV infection, the virus must penetrate the cytomembrane to enter the intracellular space. Once inside the endosome, nucleocapsids must rely on host cytoskeleton to infect the nucleus and then replicate and assemble within it. In our study, 16 DEPs of interest were identified that were primarily involved in transmembrane transport, viral replication and assembly, energy metabolism, repressing viral infection, and apoptosis.

Endocytosis is an important pathway by which budded viruses (BVs) of baculoviruses enter host cells. This process requires an acidic environment to promote membrane fusion of the BV and endosome to release the nucleocapsid into the cytoplasm. V-ATPase subunit A is an important transport protein for pH regulation and promotes the infection of baculovirus by acidifying endosomes; carbonic anhydrase 2 catalyses the interconversion of carbon dioxide and water to regulate acid balance. These results indicate that resistant silkworm strains may have lower expression levels of V-ATPase subunit A and carbonic anhydrase 2 to repress virus transmembrane transport. In this study, the expression levels of V-ATPase subunit A and carbonic anhydrase 2 in BC9 following BmNPV infection were significantly down-regulated. However, the expression levels of the two proteins did not exhibit differential expression in P50. Once in the cytoplasm, virus require highly conserved host proteins, such as those in the cytoskeleton, to assist transport and reproduction. Tubulin is a major protein constituent of cytoskeletal filaments and is involved in many essential cellular processes, including mitosis, cell motility, and intracellular transport. Fang et al. reported that Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) could be facilitated by interacting with beta-tubulin. In this study, the expression level of alpha-tubulin was down-regulated more than 6-fold in BC9 following BmNPV infection but was not differentially expressed in P50. Thus, the down-expression of alpha-tubulin indicated its role in nucleocapsids transport in host cells.

After entry into cells, viruses need to rely on the host cell to complete replication. For example, a virus requires the host cell cytoskeleton to assist in transport and assembly, as well as amino acids to synthesize the nucleocapsid. In this study, several proteins including Tudor-SN, RPS12, CTP1, and PA1 potentially related to viral replication were classified according to relevant references. Tudor-SN is a novel co-activator of E2F-1 that plays essential roles in the G1/S transition during cellular division. An analysis of intracellular antigens revealed the presence of reovirus in dividing but not quiescent hepatocytes, indicating that cellular division could promote virus replication. RPS12 is a component of the ribosome 40S subunit that monitors the complementarity of tRNA and mRNA during protein translation. Inoue et al. reported that the subunit of CTP1 could ligate to Negri bodies to promote rabies virus transcription and replication. The primary function of a proteasome is to degrade unneeded or damaged proteins by proteolysis. Amaya et al. reported that this process is used by many viruses to enhance multiplication and sustain persistent infection. According to the PPIs analysis, the CTP1 protein could directly interact with its functional partners Tudor-SN, RPS12, and PA (Fig. 7). Based on their roles...
Figure 8. Enlarged spot images and RT-qPCR analysis of the expression levels of anti-BmNPV-relevant DEPs. The data were normalized using BmGAPDH and are represented as the means ± standard errors of the means from three independent experiments. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical analysis was conducted using the SPSS software. Significant differences are indicated by different letter, e.g. a, b, c (P < 0.05).
in viral replication, the resistant silkworm strain must decrease the expression of these proteins to prevent the replication of a virus, thus explaining the notable down-regulation of the four proteins in BC9 following BmNPV infection without differential expression in P50. Therefore, the decreased expression of these four proteins in the resistant strain highlighted the roles of these proteins in the viral replication process.

Energy metabolism-related proteins were also differentially expressed in BC9 following BmNPV infection. Ndufs8 is a subunit of mitochondrial NADH, which is primarily involved in the binding of two of the six to eight iron-sulfur clusters of a complex. Suszynska-Zajczyk et al. reported that Ndufs8 is down-regulated in mice with hyperhomocysteinaemia (HHcy) disease and is involved in energy metabolism53. The up-regulation of miR-101 directly repressed herpes simplex virus-1 (HSV-1) replication by negatively regulating ATP5B expression levels54. Based on our results, Ndufs8 and ATP5B expression levels in BC9 following BmNPV infection were confirmed to be down-regulated 2.58- and 6.05-fold, respectively, but without notable changes in P50. These two proteins also interacted closely with V-APTase subunit A, CTP1, and alpha-tubulin (Fig. 7). Therefore, we speculated that the host had to decrease energy metabolism to repress BmNPV infection by inhibiting the connection of Ndufs8 and ATP5B with the three proteins mentioned above. PD is a multienzyme complex member that provides the link between glycolysis and the TCA cycle55. However, the increased expression level of PD in BC9 following BmNPV infection could not be explained here and requires further study.

Some proteins exhibited up-regulation in BC9 following BmNPV infection, we speculated that these proteins were involved in the resistance to BmNPV infection according to related references. eIF3 plays an important role in promoting mRNA binding to the ribosomal 40S subunit, which is essential for translation initiation. Gao et al. reported that eIF3 could mediate virus resistance in several plant-potyvirus interactions56. LDH is an oxidative enzyme that widely exists in cell membrane and cytoplasm and converts lactate into pyruvate during glycolysis. Baba et al. reported that LDH expression levels were enhanced following bovine viral diarrhea virus (BVDV) infection in vitro57. MSD as the member of aldehyde dehydrogenases plays an important role in repressing white spot syndrome virus replication58. We found that these three proteins were not differentially expressed in P50 following BmNPV infection but changed significantly in BC9. Additionally, these proteins could indirectly interact with each other based on the PPI analysis (Fig. 7). Thus, the up-regulation of these proteins in a resistant silkworm strain BC9 indicated that these proteins were involved in response to BmNPV infection.

In addition to the adaptive immune system, apoptosis plays a vital role in resisting virus infection in Lepidoptera59. In the current study, two proteins related to apoptosis were found in BC9 following BmNPV infection: TPx and PEBP. Powell et al. reported that the higher expression of TPx in a scaleless wing mutant of silkworm was responsible for the delayed apoptosis of the scale cells59. TPx up-regulation was potentially involved in further repressing viral infection via the apoptosis of infected host cells. PEBP is a novel member of the PEBP family and functions as an anti-apoptotic molecule60. In our study, the expression levels of TPx and PEBP in BC9 following BmNPV infection were significantly down-regulated, without notable changes in P50. Therefore, we speculated that the down-regulation of these two proteins could induce enhanced apoptosis to repress the ability of a virus to infect other cells.

In summary, this report is the first to study DEPs in silkworm different resistant strains following BmNPV infection using a comparative subcellular proteomics analysis. Our data provide useful proteomic information of the silkworm midgut response to BmNPV and establish a foundation to clarify the molecular mechanism of silkworm resistance to BmNPV infection.

References
1. Goldsmith, M. R., Shimada, T. & Abe, H. The genetics and genomics of the silkworm, Bombyx mori. Annu Rev Entomol. 50, 71–100 (2005).
2. Shao, Q. M. et al. Hindgut Innate Immunity and Regulation of Fecal Microbiota through Melanization in Insects. J Biol Chem. 287, 14270–14279 (2012).
3. Bao, Y. Y. et al. Gene expression profiling of resistant and susceptible Bombyx mori strains reveals nucleopolyhedrovirus-associated variations in host gene transcript levels. Genomics. 94, 138–145 (2009).
4. Nakazawa, H. et al. Antiviral activity of a serine protease from the digestive juice of Bombyx mori larvae against nucleopolyhedrovirus. Virology. 321, 154–162 (2004).
5. Pommel, K. M. et al. A lipase isolated from the silkworm Bombyx mori shows antiviral activity against nucleopolyhedrovirus. J Virol. 77, 10725–10729 (2003).
6. Pommel, K. M., Nithya, K., Sirigineedi, S., Awasthi, A. K. & Yamakawa, M. In Vitro Antiviral Activity of an Alkaline Trypsin from the Digestive Juice of Bombyx mori Larvae against Nucleopolyhedrovirus. Arch Insect Biochem Physiol. 81, 90–104 (2012).
7. Setol, R. et al. Identification of a soluble NADPH oxidoreductase (BmNOK) with antiviral activities in the gut juice of Bombyx mori. Biochim Biophys Acta. 1760, 200–5 (2007).
8. Kang, L. Q. et al. Arginine kinase is highly expressed in a resistant strain of silkworm (Bombyx mori, Lepidoptera): Implication of its role in resistance to Bombyx mori nucleopolyhedrovirus. Comp Biochem Physiol Mol Biol. 158, 230–234 (2011).
9. Liu, X., Yao, Q., Wang, Y. & Chen, K. Proteomic analysis of nucleopolyhedrovirus infection resistance in the silkworm, Bombyx mori (Lepidoptera: Bombycidae). J Invertebr Pathol. 105, 84–90 (2010).
10. Cheng, X., Wang, X. Y., Hu, H., Killiny, N. & Xu, J. P. A Hypothetical Model of Crossing Bombyx mori Nucleopolyhedrovirus through Its Host Midgut Physical Barrier. PLoS One 9(9), 123–137 (2014).
11. Khan, R. et al. Protein expression profiling of nuclear membrane protein reveals potential biomarker of human hepatocellular carcinoma. Clin Proteomics. 10, 6 (2013).
12. Schumacker, P. T., Jr. et al. Mitochondria in lung biology and pathology: more than just a powerhouse. Am J Physiol Lung Cell Mol Physiol. 306, L962–1974 (2014).
13. Wu, X. et al. Mitochondrial proteome analysis of human host cells infected with H3N2 swine influenza virus. J Proteomics. 91, 136–50 (2013).
15. Sofra, V. et al. Antigen-loaded ER microsomes from APC induce potent immune responses against viral infection. Eur J Immunol. 39, 83–95 (2009).
16. Wang, S. Q. et al. Imaging microdomain Ca2+ in muscle cells. Circ Res. 94, 1011–22 (2004).
17. Perham, R. N. Swinging arms and swinging domains in multifunctional enzymes: Catalytic machines for multistep reactions. *Annu Rev Biochem*. 69, 961–1004 (2000).
18. Nandi, D., Tahlilani, P., Kumar, A. & Chandu, D. The ubiquitin-proteasome system. *J Biocat.* 31, 137–55 (2006).
19. Yao, Q. et al. Screening of molecular markers for NPV resistance in *Bombyx mori* L. (Lep., Bombycidae). *J. Appl. Entomol.* 127, 134–136 (2003).
20. Rahman, M. M. & Gopinathan, K. P. Systemic and in vitro infection process of *Bombyx mori* nucleopolyhedrovirus. *Virus Res.* 101, 109–18 (2004).
21. Cheng, Y., Wang, X. Y., Du, C., Gao, J. & Xu, J. P. Expression analysis of several antiviral related genes to BmNPV in different resistant strains of silkworm, *Bombyx mori*. *J Insect Sci.* 14 (2014).
22. Qin, Y., Lu, G., Kepping, C. & Zhigang, H. Detection of proliferation of *Bombyx mori* nucleopolyhedrovirus in its host by fluorescence quantitative PCR. *Acta Entomologica Sinica.* 48, 871–875 (2005).
23. Zou, Z. H. et al. Comparative proteomic maps of subcellular protein fractions of the Asian citrus psyllid *Diaphorina citri*, the vector of citrus huanglongbing. *Physiol Entomol.* (2016).24. Iwashov, V. A. et al. Lipidome and proteome of lipid droplets from the methylobactrophic yeast *Pichia pastoris*. *Biochim Biophys Acta.* 1831, 282–290 (2013).
25. Iwashov, V. A. et al. Lipidome and proteome of lipid droplets from the methylobactrophic yeast *Pichia pastoris*. *Biochim Biophys Acta.* 1831, 282–290 (2013).
26. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72, 248–54 (1976).
27. Lu, Z. J. et al. Proteomic analysis of the host response in the bursa of Fabricius of chickens infected with Marek’s disease virus. *Virus Res.* 153, 250–257 (2010).
28. Steiner, S. et al. Proteomics to display lovastatin-induced protein and pathway regulation in rat liver. *Electrophoresis.* 21, 2129–37 (2000).
29. Barabasi, A. L., Gulbahce, N. & Loscalzo, J. Network medicine: a network-based approach to human disease. *Nat Rev Genet.* 12, 56–68 (2011).
30. Zhou, Z. H. et al. Comparative Proteomic Analysis between the Domesticated Silkworm (*Bombyx mori*) Reared on Fresh Mulberry Leaves and on Artificial Diet. *J Proteome Res.* 7, 5103–5111 (2008).
31. Huber, L. A., Pfäfer, K. & Victer, I. Organelle proteomics: implications for subcellular fractionation in proteomics. *Circ Res.* 92, 962–8 (2003).
32. Pradet-Balade, B., Boumel, F., Beug, H., Mullner, E. W. & Garcia-Sanz, J. A. Translation control: bridging the gap between genomics and proteomics? *Trends Biochem Sci.* 26, 225–229 (2001).
33. Kvitinsnadze, N. et al. Peroxidase processes in mitochondria and microsome of human prostate tissues at different pathology. *Eur Urol.* 8, 616–616 (2009).
34. Long, G., Pan, X. Y., Kormelink, R. & Vlak, J. M. Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. *J Virol.* 80, 8830–8833 (2006).
35. Kingsley, D. H., Behbahan, A., Rashbain, A., Blissard, G. W. & Zimmerberg, J. A discrete stage of baculovirus GPsE-mediated membrane fusion. *Mol Biol Cell.* 10, 4911–200 (1999).
36. Hinton, A., Bond, S. & Forsgac, M. V-ATPase functions in normal and disease processes. *Pflugers Arch.* 457, 589–98 (2009).
37. Beyenbach, K. W. & Wieczorek, H. The V-type H⁺-ATPase: molecular structure and function, physiological roles and regulation. *J Exp Biol.* 209, 377–89 (2006).
38. Forsgac, M. V-ATPase: rotary proton pumps in physiology and pathophysiology. *Nat Rev Mol Cell Biol.* 8, 917–29 (2007).
39. Lin, T. Y. et al. Carbonic anhydrase 2-like a and a5a are involved in acid-base regulation and Na⁺ uptake in zebrafish H⁺-ATPase-rich cells. *Am J Physiol Cell Physiol.* 294, C1250–60 (2008).
40. Volkmann, L. E. Baculovirus infectivity and the actin cytoskeleton. *Curr Drug Targets.* 8, 1075–83 (2007).
41. Gunning, P. W., Ghoshdastider, U., Whitaker, S., Popp, D. & Robinson, R. C. The evolution of compositionally and functionally distinct actin filaments. *J Cell Sci.* 128, 2009–19 (2015).
42. Fang, M. G., Nie, Y. C. & Theilmann, D. A. AcMNPV EXON0 (AC141) which is required for the efficient egress of budded virus nucleocapsids interacts with beta-tubulin. *Virology.* 385, 496–504 (2009).
43. Jolly, C., Mitar, I. & Sattentau, Q. J. Requirement for an intact T-cell actin and tubulin cytoskeleton for efficient assembly and spread of human immunodeficiency virus type 1. *J Virol.* 81, 5547–5560 (2007).
44. Radlke, K., Dohner, K. & Sodeik, B. Viral interactions with the cytoskeleton: a hitchhiker’s guide to the cell. *Cell Microbiol.* 8, 387–406 (2006).
45. Guo, Z. J. et al. Characterization of aggregate/aggresome structures formed by polyhedrin of *Bombyx mori* nucleopolyhedrovirus. *Sci Rep.* 5, 14601 (2015).
46. Pashe, T., Saarikkutti, J., Isomaki, P., Yang, J. & Silvennoinen, O. Expression analysis of Tudor-SN protein in mouse tissues. *Tissue Cell.* 45, 21–31 (2013).
47. Su, C. et al. Tudor Staphylolococal Nuclease (Tudor-SN), a Novel Regulator Facilitating G(1)/S Phase Transition, Acting as a Co-activator of E2F-1 in Cell Cycle Regulation. *J Biol Chem.* 290, 7208–7220 (2015).
48. Taterka, J., Sutcliffe, M. & Rubin, D. H. Selective reovirus infection of murine hepatocarcinoma cells during cell division. A model of viral liver infection. *J Clin Invest.* 94, 553–60 (1994).
49. mouse, V. et al. Chaperonin TRiC/CCT participates in replication of hepatitis C virus genome via interaction with the viral NS5B protein. *Virology.* 410, 38–47 (2011).
50. Zhang, J. Y. et al. Cellular Chaperonin CCT gamma Contributes to Rabies Virus Replication during Infection. *J Virol.* 87, 7608–7621 (2013).
51. Zhang, J. Y. et al. The chaperonin CCT alpha is required for efficient transcription and replication of rabies virus. *Microbiol Immunol.* 58, 590–599 (2014).
52. Amaya, M. et al. The Ubiquitin Proteasome System Plays a Role in Venezuelan Equine Encephalitis Virus Infection. *PLoS One.* 10, (2015).
53. Suszyńska-Żajczyk, J., Sikora, M. & Jakubowski, H. Paraoxonase 1 deficiency and hyperhomocysteinemia alter the expression of mouse kidney proteins involved in renal disease. *Mol Genet Metab.* 113, 200–206 (2014).
54. Zheng, S. Q., Li, Y. X., Zhang, Y., Li, X. & Tang, H. MiR-101 regulates HSV-1 replication by targeting ATP5B. *Antiviral Res.* 89, 219–226 (2011).
55. Bhandary, S. & Aguan, K. Pyruvate dehydrogenase complex deficiency and its relationship with epilepsy frequency–An overview. *Epilepsy Res.* 116, 40–52 (2015).
56. Gao, L. et al. Nla-Pro of Papaya ringspot virus interacts with Carica papaya eukaryotic translation initiation factor 3 subunit G (PcIF3G). *Virus Genes.* 50, 97–103 (2015).
57. Baba, C., Yanagida, K., Kanzaki, T. & Baba, M. Colorimetric lactate dehydrogenase (LDH) assay for evaluation of antiviral activity against bovine viral diarrhea virus (BVDV) in vitro. *Antivir Chem Chemother.* 16, 33–9 (2005).
58. Lin, Y. R. et al. The Role of Aldehyde Dehydrogenase and Hsp70 in Suppression of White Spot Syndrome Virus Replication at High Temperature. *J Virol.* 85, 3517–3525 (2011).
59. Shi, X. F. et al. Proteomic analysis of the phenotype of the scaleless wings mutant in the silkworm, Bombyx mori. J Proteomics. 78, 15–25 (2013).

60. Li, H. et al. Phosphatidylethanolamine-binding protein 4 is associated with breast cancer metastasis through Src-mediated Akt tyrosine phosphorylation. Oncogene. 33, 4589–4598 (2014).

Acknowledgements
This work was supported by the National Natural Science Foundation of China (31472148), Seed Engineering Fund of Anhui Academy of Agricultural Sciences (16D0608), and Natural Science Fund of Chongqing (cstc2015jcyjA80014). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions
J.P.X. conceived and designed the experiments. X.Y.W., S.Z.Z., D.Y., M.H.L., and L.L.W. performed the experiments. X.Y.W. and J.P.X. analysed the data and wrote the manuscript. H.Z.Y. revised the manuscript. All the authors reviewed and approved the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing Interests: The authors declare no competing financial interests.

How to cite this article: Wang, X.-Y. et al. Comparative Subcellular Proteomics Analysis of Susceptible and Near-isogenic Resistant Bombyx mori (Lepidoptera) Larval Midgut Response to BmNPV infection. Sci. Rep. 7, 45690; doi: 10.1038/srep45690 (2017).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017