Comparative Proteome Analysis of Silkworm in Its Susceptibility and Resistance Responses to *Bombyx mori* Densonucleosis Virus

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Key Words

Silkworm • Densovirus • Resistance mechanism •
2-D electrophoresis • MALDI-TOF-TOF MS

Abstract

Bombyx mori densonucleosis virus (*BmDNV*) is one of the most disastrous viruses in cocoon production. Silkworm resistance to *BmDNV* has been examined previously using a number of traditional biochemical and molecular techniques. In this study, a near isogenic line, BC₆, was constructed to eliminate the difference in inherited background, which has 99.9% identity with the susceptible strain but carries a resistant gene. We utilized a proteomic approach involving two-dimensional differential gel electrophoresis and mass spectrometry to examine changes in the midgut proteins from the susceptible and resistant silkworm larvae infected with *BmDNV*. The protein profiles were compared and 9 differentially expressed proteins were identified by mass spectrometry. In the resistant strains, the heat-shock 70-kDa protein cognate, cytochrome P450, vacuolar ATP synthase subunit B, arginine kinase, vacuolar ATP synthase subunit D and glutathione S-transferase sigma were strongly upregulated and α -tubulin was downregulated. Our results imply that these upregulated genes and the downregulated genes might be involved in *B. mori* immune responses against *BmDNV*-Z infection.

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Introduction

Bombyx mori densovirus (*BmDNV*), a virus that impacts the sericultural industry by destructive damage throughout the rearing of larvae in many farms, has long been seen as a serious problem. The midgut columnar cells infected by this virus contain hypertrophied nuclei where the virus multiplies. The infected susceptible larvae become flaccid and develop diarrhea as midgut tissue is destroyed, a condition known as flacherie, and finally die [1, 2].

BmDNV belongs to Parvoviridae. *BmDNVs* are classified into two major types, type I and type II, based on their genomic characteristics. *BmDNV* type II possesses a split genome comprising two types of single-stranded linear DNA molecules. *BmDNVs* of this type include *BmDNV*-2 (Yamanashi isolate) [3, 4] and *BmDNV*-Z (Zhenjiang isolate) that contains two ssDNA molecules [5]. Li et al. [6] have shown that a recessive gene, *nsd*-Z, controls non-susceptibility to *DNV*-Z. This gene is located on chromosome 15 [7] and the closest SSR marker linked to *nsd*-Z was mapped at a distance of 4.4 cM from *nsd*-Z [6, 8]. Although this gene has been mapped with phenotypic or DNA markers, neither of them has been

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0300–5526/12/0551–0021\$38.00/0

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Fig. 1. Establishment of near isogenic lines BC₆. BC₁ and BC₂ stand for the first and second backcrosses and so forth. R = Dominant gene; r = recessive gene.

isolated from *B. mori* yet. The details of the mechanisms by which *BmDNV* specifically infects its host and the manner in which *B. mori* strains effectively defend themselves against *BmDNV* infection remain unclear.

The silkworm response upon DNV infection is of particular interest, especially the response of resistant lines in comparison to susceptible lines. Some studies on resistance have been undertaken successfully on the gene and transcription level [9], but few studies have been performed to investigate the silkworm response upon DNV infection on the translation level. Proteomic techniques provide powerful tools for studying *B. mori* stress responses and possible mechanisms of *B. mori* resistance to *BmDNV*-Z. Using a two-dimensional gel electrophoresis (2-DE)-based protein separation method, a global protein expression profile can be generated and compared. The objective of this study was to identify differentially expressed proteins between *BmDNV*-resistant and *BmDNV*-susceptible strains under infected conditions, as well as describe the possible mechanisms of resistance in *B. mori*.

Materials and Methods

Silkworm Strains

The silkworm strain Qiufeng (*nsd-Z/nsd-Z*), which is resistant to *BmDNV*-Z, and Huaba35 strain (*+nsd-Z/+nsd-Z*), which is susceptible to *BmDNV*-Z, were maintained by single pair matings.

We constructed the near isogenic line BC₆, which was obtained by continuously using the females of cyclical backcross parent, Huaba35, to cross the males carrying resistant genes of strain Qiufeng that was used as the donor of resistant genes. First we used Huaba35 to cross the males of strain Qiufeng. Then the F₁ generation was self-crossed. *BmDNV*-Z virus was inoculated to larvae of each self-crossed generation to screen the individuals carrying resistant gene. Subsequently, we used the individuals carrying resistant genes to cross the strain Huaba35. The backcrosses were conducted for 6 generations (fig. 1). Thus the BC₆ strain also carries the recessive gene (*nsd-Z/nsd-Z*). In theory, the inherited background of BC₆ has 99.9% identity with Huaba35, the susceptible strain [10].

In order to investigate the mortality of silkworm, DNV-Z was fed to the second instar larvae of three silkworm strains, Huaba35, the near isogenic line BC₆ and Qiufeng. For each strain, 100 larvae were taken and the controls were fed with fresh mulberry leaves treated with the same volume of water.

Sample Preparation

All larvae of three silkworm strains, Huaba35, BC₆ and Qiufeng, were raised on fresh mulberry leaves to the fifth instar. For each strain, 15 newly metamorphosed fifth instar larvae were taken and fed with fresh mulberry leaves treated with 0.1% *BmDNV*-Z. All of the infected silkworms were checked for DNV by immunoperoxidase staining methods [11].

Midguts of the larvae were extracted, frozen in liquid nitrogen and ground immediately. The samples were directly placed in a lysis buffer consisting of 8 M urea, 4% CHAPS, 40 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 0.2% BioLyte 3/10 ampholyte. Fresh 1 mM dithiothreitol (DTT) and a protease inhibitor mixture containing 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml pepstatin A, and 10 mg/ml aprotinin were added to buffers prior to use. Finally, the sample was centrifuged under 12,000 rpm, 4° for 30 min. Protein concentrations in the samples were determined by an RC/DCTM protein assay after rehydration. All chemicals and kits were supplied by BioRad unless otherwise specified.

Two-Dimensional Gel Electrophoresis

The manufacturer's recommended volume (185 µl, containing approx. 600 µg proteins) of recovered proteins was applied to 11-cm IPG strips (Immobiline DryStrips), pH 4–7, for overnight active rehydration. The IPG strips were then subjected to isoelectric focusing using a Protean IEF Cell. Focusing was performed as follows: 250 V for 30 min, 1,000 V for 30 min, 8,000 V for 4 h and then up to 40,000 V for approximately 3 h. Current did not exceed 50 mA per strip.

After isoelectric focusing, the IPG strips were equilibrated for 15 min in equilibration buffer I (6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol and 2% (w/v) DTT) followed by 15 min in buffer II (same as buffer I but containing 2.5% iodacetamide instead of DTT).

For the second dimension, IPG strips were placed across a Criterion Precast Gel and overlaid with agarose. Electrophoresis was run with a constant voltage, 120 V, until the bromophenol blue dye front had completely migrated out of the bottom of the gel.

Gels were fixed overnight in a solution of 50% ethanol and 10% acetic acid, stained with Coomassie (BioRad) for at least 1 h and washed in water. Three independent samples were analyzed for each treatment.

Image Analysis

Gel images were scanned with a BioRad SanMaker 9700XL imaging system and image analysis was performed using PD-Quest (version 7.2.0; BioRad). The differential spots were first compared between Huaba35 and BC₆. Only the spots in BC₆, which are consistent with Qiufeng, were chosen. Protein spots detection and matching were performed automatically and matches reviewed manually and edited where necessary in order to remove false spots, include missed spots, and to distinguish spots within clusters. The Total Quantity in Valid Spots normalization method was used to compensate for any non-expression-related variations in spot intensity. Only those spots that were reproducible in all three biological replicates were included in the analysis. Student's t test ($p < 0.05$) was used to identify differentially expressed spots (at least twofold change in expression).

In-Gel Digest

Bands were trimmed as close as possible to minimize background polyacrylamide material. The gels were washed in 50% methanol and 5% acetic acid for several hours. The gel bands were dried with acetonitrile and reconstituted with DTT solution to reduce the cysteines. Iodoacetamide was added to alkylate the cysteines, and the gel was washed again with cycles of acetonitrile and ammonium bicarbonate. Trypsin was added and digested at room temperature overnight. After they were dried in a SpeedVac for 10 min, peptide extraction was performed with 10 ml of 1% TFA. Extracted peptides were directly applied onto a target (AnchorChip™; Bruker Daltonics, Bremen, Germany) that was loaded with α -cyano-4-hydroxycinnamic acid matrix thin layer.

MALDI-TOF/TOF Mass Spectrometry

Protein spots that were found to have interesting patterns of differential expression in the susceptible and resistant strains 2-D maps were subjected to identification of proteins using peptide mass fingerprinting. Mass spectrometry (MS) measurements were carried out on an ultraflex II TOF-TOF (Bruker Daltonics) with delayed ion extraction. The instrument was operated in the reflector for MALDI-TOF peptide mass fingerprint mode for MALDI-TOF/TOF with a fully automated mode using the FlexControl™ software. An accelerating voltage of 25 kV was used for PMF and mass spectra were obtained in a mass range of 500–6,000 Da. External calibration was performed with Peptide Mass Standard kit (Perspective Biosystems). The matrix and the autolytic peaks of trypsin served as internal standards for mass calibration. After MS acquisition, the six strongest peptides per spot were selected automatically for the analysis.

Protein Identification and Database Searching

Protein identification using peptide mass fingerprinting was performed by the MASCOT search engine (<http://www.matrix-science.com>) against the NCBI protein database and Chinese *B. mori* genome database (<http://silkworm.genomics.org.cn/>). Mass fingerprints were compared with known proteins from *B. mori* and *Drosophila melanogaster* as well as from other insects and non-redundant protein databases. Identification criteria were based on the number and coverage of matched peptides. The following parameters were used in all searches: the maximum number of missed cleavages allowed, 1; the mass tolerance, 0.2 Da; minimum peptides required to match, 4, and the monoisotopic masses of observation peaks were used to match the calculated

Table 1. Results of inoculation of silkworm larvae with *BmDNV-Z* and its control

Treatment/strain	Number inoculated	Number infected with <i>BmDNV-Z</i>	Survivors, n	Incidence of disease, %
Inoculation				
Huaba35	100	98	2	98
BC ₆	100	0	97 ¹	0
Qiufeng	100	0	96 ¹	0
Controls				
Huaba35	100	4	96	6
BC ₆	100	0	97 ¹	0
Qiufeng	100	0	100	0

¹ The number of survivors decreased because some were infected with other diseases.

monoisotopic fragment masses for protein identification. Possible covalent modifications considered in the search procedure were carbamidomethylation of cysteine, conversion of peptide N-terminal glutamine to pyroglutamate, oxidation of methionine, and acetylation of protein N-terminus.

Results

Resistance Identification of Huaba35, the Near Isogenic Line BC₆ and Qiufeng

For each strain, 100 second instar larvae were taken and fed with 0.1% *BmDNV-Z*, and the controls were treated with the same volume of water. Normally, silkworms that are susceptible to *BmDNV-Z* develop very slowly and cannot survive through the third instar. The incidence of disease investigation suggested that the average incidence of Huaba35 is 98%, and the others are all zero (table 1). This is consistent with previous results showing that resistance to DNV-Z is recessive [1].

Protein Differences between Susceptible and Resistant Strains

To compensate the variability of gel electrophoresis, three replicate gels were run for each silkworm strain. Therefore, a total of 9 gels in three groups were subject to statistical analysis using PDQuest software. Based on an automated spot-detection algorithm provided by PD-Quest, means of 350 ± 3 , 360 ± 2.5 , and 365 ± 2 protein spots were detected in the gels of Huaba35, BC₆, and Qiufeng, respectively (fig. 2).

Since BC₆ has a genetic background similar to Huaba35, comparison of the location and volumes associated with each spot revealed that the level of the most midgut

Fig. 2. 2-D electrophoresis maps of midguts from **a** Huaba35, **b** BC₆ and **c** Qiufeng. Proteins were separated by isoelectric focusing in the first dimension, pH 4–7, 11 cm, then by size (15–100 kDa) in the second dimension and stained with Coomassie blue. Maps were analyzed with PDQuest software. Selected spots were numbered 1–16.

proteins remains virtually unchanged in the susceptible strain Huaba35 and the near isogenic line BC₆. Those spots that were only consistent regulated between BC₆ and Qiufeng but opposite to Huaba35 were identified. Comparison of 2-DE images from three biological replicates indicated there were both qualitative and quantitative differences between protein profiles from Huaba35 and BC₆. Sixteen spots of 350 total spots in Coomassie-stained samples showed twofold or higher differences between the two samples.

Only the relatively abundant proteins, those stained with Coomassie and having at least a twofold difference between susceptible and resistant strains in all 3 replicates, were selected for identification. These spots, 6 from

a susceptible strain and 10 from a resistant one, are indicated in figure 2.

Identification of Differential Proteins

Most protein peptide mass fingerprints did not match entries in the public databases indicating that there are considerable variations of the trypsin hydrolytic peptide masses for homologous proteins among organisms. Consequently, only those proteins identified are indicated by arrows in figure 2 and are listed in table 2. The expression levels of 16 of the 350 proteins were significantly different in the susceptible and resistant insects. Samples of resistant strains contained 1 unique and 9 upregulated protein spots, and 6 downregulate protein spots. Spot volume

Table 2. Identification of resistance-related midgut proteins in *B. mori*

Spot No.	Accession No.	Species	Protein ID	Predicted mass, kDa/pI	Mowse score ¹
<i>Downregulated proteins</i>					
1	17136564	<i>D. melanogaster</i>	α -tubulin	49,876/5.00	94
2–6	–	–	unknown	–	–
<i>Upregulated proteins</i>					
7	112984012	<i>B. mori</i>	heat-shock 70-kD protein cognate	73,166/5.13	137
8	157111182	<i>Aedes aegypti</i>	cytochrome P450	58,309/8.5	83
9	148298717	<i>B. mori</i>	vacuolar ATP synthase subunit B	54,667/5.25	89
10	112983926	<i>B. mori</i>	arginine kinase	40,308/5.87	190
12	112983926	<i>B. mori</i>	arginine kinase	39,966/5.87	95
13	112983926	<i>B. mori</i>	arginine kinase	39,966/5.87	96
14	114053249	<i>B. mori</i>	vacuolar ATP synthase subunit D	27,443/9.59	85
16	160333678	<i>B. mori</i>	glutathione S-transferase sigma	23,382/5.85	74
11/15	–	–	unknown	–	–

¹ Mowse score >74 indicates identity or extensive homology ($p < 0.05$); higher scores indicate higher confidence of identity.

comparisons of the selected spots (fig. 3) indicated pronounced differences in the amounts of specific proteins present in the midgut of susceptible and resistant larvae. The proteins identified by peptide mass fingerprinting include α -tubulin, heat-shock 70-kDa protein cognate, cytochrome P450, vacuolar ATP synthase subunit B, arginine kinase, vacuolar ATP synthase subunit D and glutathione S-transferase sigma (table 2).

Discussion

In this study, two non-susceptible strains displaying significant resistance to *BmDENV-Z* as well as a susceptible strain were analyzed using 2-DE in combination with MALDI-TOF MS. The protein database for *B. mori* and other lepidopterous insects is poor, thus the task of identifying the proteins with certainty remains a challenge. In this study, nine of these proteins have been identified, which may play a role in *BmDENV-Z* resistance. Most spots in the gel figures displayed a reasonable correlation of the theoretical pI and M_r values with the experimental data.

Insects are among the earliest and most successful group of animals that exist in a myriad of environments where the potential for infection by microorganisms and parasites is great. Insects have also demonstrated considerable ability to develop resistance to viruses. Though cellular reactions like phagocytosis, nodulation and encapsulation and humoral reaction through secretion of

antibacterial proteins are known for fungus and bacterial invaders, little is known about insect defense against insect virus infection [12]. Compared to vertebrates, insects do not possess the ability to produce antibodies (immunoglobulins) and do not use immunoglobulin as recognition molecules in the classical sense, against foreign antigens and hence antigenic memory appears to be lacking. Insects possess innate immunity which is characterized by non-specific immune reactions against foreign materials. Non-specific immunity consists of structural and passive barriers like cuticle, gut physicochemical properties, and peritrophic membrane.

The *BmDENV-Z* infection starts when the larva ingests the virions; the virions multiply only in the nuclei of the columnar cells of the larval midgut epithelium [13]. The role of the peritrophic membrane in the resistance mechanism against *BmDENV* has not been demonstrated, although this midgut cellular structure is considered an important gut barrier against insect viral infection [14]. The midgut pH may affect virus activity by changing and altering the rate and location of virion release in the midgut lumen [15]. Vacuolar ATP synthases (V-ATPases) are phylogenetically widespread, highly conserved, multi-subunit proton pumps [16]. This enzyme is present in the plasma membrane of columnar cells and is the sole energizer for secretion and absorption by serving as an H^+/K^+ transporter across the insect midgut epithelium [17]. In our study, the resistant strains contained increased V-ATPase. Perhaps an increased V-ATPase expression may rapidly acidifies the epithelial cells to a low pH level,

Fig. 3. Selected area of 2-D gels and corresponding spot volumes for selected spots identified in the Huaba35 and Qiu Feng strains. Image pairs and detection of protein spots with relative spot volumes were achieved using PDQuest software (BioRad). 2-DE = 2-D electrophoresis.

which accompanies the *Bm*DNV-resistant state of the silkworm larva, reflects the pH level across the midgut epithelium that may be necessary to combat the viruses and consequently to maintain resistance at the tissue level.

One of the major proteins that were expressed differentially between the susceptible strain and the resistant one was chaperone, heat-shock 70-kDa protein cognate (hsc70). hsc70 is a constitutive member of the heat-shock-induced hsp70 protein family that functions in normal cellular physiology. The proteins in this family are highly conserved nucleocytoplasmic ATPases which have been associated to a number of functions, including protein folding, translocation across biological membranes, and assembly and disassembly of oligomeric complexes. In response to different stress conditions, these proteins prevent the formation of protein aggregates by stabilizing unfolded intermediates which are subsequently refolded to the native state or degraded [18–20]. hsc70 and other heat-shock proteins have been reported to be present on the surface of tumor cell lines [21] and in cells infected with viruses [22], as well as in mammalian spermatogenic cells [4], monocytes and B cells [23]. hsc70 has been

detected more abundant on the surface of the rotavirus-resistant cells than the susceptible cells [24]. It has been shown that hsc70 protein was able to block rotavirus infectivity in a dose-dependent manner when preincubated with the viruses before infection. In this study, hsc70 was upregulated in the resistant strains, which further indicates that differential expression of hsc70 is correlated with resistance to *Bm*DNV.

The amount of glutathione S-transferase (GST) and was 10.1 times higher in the resistant strain, suggesting an increase in the use of reduced GSH and possible changes in the redox environment in the midgut epithelium of the resistant larva. GSTs are a family of phase II enzymes that catalyze the conjugation of glutathione to endogenous and exogenous electrophilic substrates. This converts these chemicals into water-soluble, less toxic metabolites that can be easily excreted from the cell. GSTs therefore play an important role in the detoxification of many potentially harmful compounds [25]. The presence of cytochrome p450 with 7.4 times higher in resistant strains nicely supports the previous evidence that abundance of GSTs have been found in chemical insecticide-resistant insect strains together with increased levels of

cytochrome p450s that are responsible for metabolic resistance to insecticides [26, 27].

Comparison of midgut proteins in both susceptible and resistant larvae also revealed a noticeable increase in the level of arginine kinase (AK). Previously, overexpression of the *Trypanosoma cruzi* improves the ability of the transfectant cells to grow and resist nutritional and pH stress conditions [28]. AK plays an important role as a regulator of energetic reserves and cell growth by catalyzing the reversible transfer of the phosphate group from MgATP to arginine, leading to production of phosphoarginine and MgADP [29]. Canonaco et al. [30] reported that recombinant yeast expressing AK showed improved resistance under stress challenges that drain cellular energy, which were transient pH reduction and starvation. Probably the increase in the level of AK in the resistant insects is required to replenish the energy in the resistant larval midgut cells that have a relatively higher utilization to adjust to the extreme conditions than the susceptible strain.

The tubulin gene family consists of six distinct but highly conserved subfamilies, α -, β -, γ -, δ -, ϵ -, and ζ -tubulins [31]. Heterodimers of these proteins are the primary constituents of microtubules, which are the main components of the cell cytoskeleton [32]. Microtubules have long been implicated in viral nucleocapsid movement since their close association was first observed by electron microscopy [33, 34]. In the previous studies, the involvement of microtubules in virus transport has been reported for a number of viruses [35–38]. In addition, quite a few specific viral proteins that interact directly with microtubules or with microtubule motor proteins have also been identified [39–42]. Previous studies indicated that virus could utilize actin microfilaments of the host for transport to the plasma membrane of infected cells [43, 44]. The decrease expression of α -tubulin in the resistant strain might inhibit the infection cycle of

BmDNV-Z in the host, which includes viral assembly, budding, and maturation of progeny virions. As we know, the antiviral mechanism occurring in resistant *B. mori* strains is not due to resistance against *BmDNV-Z* invasion, but due to the inhibition of *BmDNV-Z* proliferation in larval midgut cells. The pathways involved in these reactions are currently unclear. Our data provide a global view of host responses to viral infection and insights for further investigation of the complex interactions between *BmDNV-Z* and its host, *B. mori*.

Peptide mass fingerprinting, which involves MS coupled with bioinformatics and relies on current databases, is a useful proteomic technique for protein identification. With increased availability of genome information and predicted proteomes of insects, a comprehensive understanding of the resistant state of insects and their genomic and proteomic expression profiles will be facilitated. Furthermore, comparative proteomic analysis of susceptible and resistant insects should help understand how insects adapt to or resist environmental challenges. One of the drawbacks associated with the use of 2-DE is that the identities of some of the differentially expressed proteins seen in the present study remain unknown. This may have been the absence of protein information in the database. Although we were unable at this time to obtain specific antibodies to verify all of the differentially expressed proteins that we identified, we have described several targets that warrant further investigation utilizing functional studies.

Acknowledgements

This work was supported by the National High Technology Research and Development Program of China (No. 2008AA10Z145) and the National Natural Science Foundation of China (No. 30871826).

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