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Differentially expressed genes in resistant and susceptible *Bombyx mori* strains infected with a densonucleosis virus

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ABSTRACT

We investigated variations in the gene expression of *Bombyx mori* following infection with a densonucleosis virus (BmDNV-Z). Two *B. mori* near-isogenic lines, *Jingsong* and *Jingsong.nsd-Z.NIL*, which are highly susceptible and completely resistant to BmDNV-Z, respectively, were used in this study. The infection profiles of BmDNV-Z in the midguts of the *B. mori Jingsong* and *Jingsong.nsd-Z.NIL* larvae revealed that the virus invaded the midguts of both of these strains. However, its proliferation was notably inhibited in the midgut of the resistant strain. By using the suppression subtractive hybridization method, three cDNA libraries were constructed to compare BmDNV-Z responsive gene expression between the two silkworm lines. In total, 151 differentially expressed genes were obtained. Real-time qPCR analysis confirmed that 11 genes were significantly up-regulated in the midgut of the *Jingsong.nsd-Z.NIL* strain following BmDNV-Z infection. Our results imply that these up-regulated genes might be involved in *B. mori* immune responses against BmDNV infection.

1. Introduction

Unlike the immune responses of insects against bacteria or fungi, their responses to viral infections are poorly understood. Insect resistance to viruses has received extensive attention because it plays an important role in both the biological control of pests through the use of viral agents and the breeding of resistant strains of beneficial insects.

The silkworm, *Bombyx mori*, exhibits high intraspecific variability in its resistance to viral diseases (Watanabe, 2002). The *Bombyx* densonucleosis virus (BmDNV) is a major pathogen that causes flacherie disease in the silkworm. Certain *Bombyx* strains are highly susceptible, while other strains exhibit complete resistance to BmDNV infection (Abe et al., 1998).

BmDNVs are classified into two major types, type I and type II, based on their genomic characteristics. The genome of BmDNV type I, such as BmDNV-1 (Ina isolate), contains single-stranded complimentary DNA molecules that are 5.048 kb in length (Bando et al., 1990). In contrast, 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) that contains two ssDNA molecules, which are 6.542 and 6.032 kb in length (Bando et al., 1992, 1995), and BmDNV-Z (Zhenjiang isolate) that contains two ssDNA molecules, which are 6.543 and 6.022 kb in length (Wang et al., 2007). In addition, BmDNV type I is more pathogenic to the silkworm than type II (Watanabe, 2002).

Genetic analysis has revealed that several major genes contribute to the complete resistance of *Bombyx* strains to BmDNV infection. A dominant *Nid-1* (Eguchi et al., 1986) and a recessive *nsd-1* gene (Watanabe and Maeda, 1978) appear to be responsible for conferring resistance to BmDNV-1, while two other recessive genes, *nsd-2* (Seki, 1984) and *nsd-Z* (Hu et al., 1984), are likely to be functional against BmDNV-2 and BmDNV-Z infection. However, to date, the genes mentioned above have not been cloned from *B. mori*. The details of the mechanisms by which BmDNV specifically infects its host and the manner in which *Bombyx* strains effectively defend themselves against BmDNV infection remain unclear.

To gain a better understanding of the mechanisms underlying resistance to BmDNV in some *Bombyx* strains, we first investigated the infection profiles of BmDNV-Z in the midguts of *B. mori Jingsong* and *Jingsong.nsd-Z.NIL* larvae by performing real-time quantitative PCR (qPCR) at different time points. Our results revealed that BmDNV-Z invaded the midgut tissue of both the *Jingsong* and *Jingsong.nsd-Z.NIL* strains. However, viral proliferation in the midgut of the resistant strain was inhibited by unknown mechanisms. In the susceptible *Jingsong* strain, the process of BmDNV-Z

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infection was demonstrated in the latent phase (3–24 h pi) and exponential phase (24–96 h pi), implying that the expression variations of genes that are involved in the inhibition of BmDNV-Z viral proliferation may occur during the latent phase. Based on our results and the understanding that BmDNV-Z exclusively invades the columnar cells of larval midgut epithelium at an early stage of infection (3–24 h pi) (Wang et al., 2007), we focused on clarifying the differentially expressed genes that respond early to infection in the midgut of *Bombyx* strains and chose 12 h pi as a time point to determine up-regulated genes. Then, we explored resistance-related genes by performing suppression subtractive hybridization (SSH), an effective technique by which differentially expressed cDNA fragments can be selectively amplified (Diatchenko et al., 1996). Two near-isogenic lines (NILs), *B. mori Jingsong* ($+^{nsd\text{-}Z}/+^{nsd\text{-}Z}$) and *Jingsong.nsd-Z.NIL* (*nsd-Z*/*nsd-Z*), which are highly susceptible and completely resistant to BmDNV-Z, respectively, were used for comparing differentially expressed genes. Three subtracted cDNA libraries were constructed using a BmDNV-Z-infected and phosphate-buffered saline (PBS)-treated *Jingsong* strain, a BmDNV-Z-infected and PBS-treated *Jingsong.nsd-Z.NIL* strain, and BmDNV-Z-infected *Jingsong.nsd-Z.NIL* and *Jingsong* strains. A total of 151 cDNA clones that were expressed in response to BmDNV-Z infection were obtained from the libraries. Real-time qPCR analysis confirmed that 11 genes were significantly up-regulated following BmDNV-Z infection in the midgut of the *Jingsong.nsd-Z.NIL* strain when compared with the *Jingsong* strain.

The aim of this study is to identify *B. mori* genes with potential anti-BmDNV-Z function in the larval midgut and to use these genes to understand the antiviral mechanisms involved in the immune responses of insects.

2. Materials and methods

2.1. Silkworm strains

The *B. mori Jingsong* ($+^{nsd\text{-}Z}/+^{nsd\text{-}Z}$) and *Jingsong.nsd-Z.NIL* (*nsd-Z*/*nsd-Z*) strains were provided by the Sericultural Research Institute of the Chinese Academy of Agricultural Sciences, Zhenjiang, China.

The NILs are a group of strains that are genetically identical, except at one locus or a few loci. NILs resistant to BmDNV-Z were bred *via* successive backcrosses, using the *B. mori L10* strain (*nsd-Z*/*nsd-Z*), which is resistant to BmDNV-Z as the donor parent and the *B. mori Jingsong* strain ($+^{nsd\text{-}Z}/+^{nsd\text{-}Z}$), which is susceptible to BmDNV-Z, as the recurrent parent. In each generation, the individuals that were obtained were similar to the recurrent parent, except with regard to the *nsd-Z* gene, and were selected and backcrossed with the recurrent parent. Following 12 successive backcrosses, the traits of the NIL obtained were observed to be similar to those of the recurrent parent, i.e., the *Jingsong* strain, and this line was designated as *Jingsong.nsd-Z.NIL* (Li et al., 2007).

All *B. mori Jingsong* ($+^{nsd\text{-}Z}/+^{nsd\text{-}Z}$) and *Jingsong.nsd-Z.NIL* (*nsd-Z*/*nsd-Z*) larvae were reared on fresh mulberry leaves at 27 °C. The newly exuviated fifth instar larvae were used for this experiment.

2.2. Virus

BmDNV-Z was propagated in the fifth instar larvae of the susceptible *B. mori Jingsong* strain. The midguts were removed and dried; those that were observed to contain BmDNV-Z were homogenized with distilled water, and the homogenate was filtered through gauze and centrifuged at 3500 rpm (3479*g*) for 20 min. The supernatant was supplemented with an equal volume of 7% acetic acid, the solution obtained was incubated at 25 °C for 40 min, and its pH was then adjusted to 7.0 by using 1% $NaCO_3$. This BmDNV-Z supernatant was diluted with distilled water to attain a 5% inoculum. Next, 100 μl of kanamycin (50 mg/ml) and 100 μl of gentamicin (7 mg/ml) were added to the viral suspension. Each newly exuviated fifth instar larva of the susceptible and resistant strains was orally administered 10 μl of the BmDNV-Z viral suspension by using an Eppendorf pipette, while the susceptible and resistant control larvae were treated with 10 μl of PBS. Oral administration of this viral volume produced 100% infection in the susceptible *Jingsong* strain.

2.3. Midgut collection

The BmDNV-Z-infected fifth instar larvae were dissected, and the midguts were collected at different time points post infection (pi) (3, 12, 24, 48, 72, and 96 h pi). The midguts were quickly washed in diethylpyrocarbonate (DEPC)-treated PBS solution (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4 (pH 7.4)) and immediately frozen in liquid nitrogen.

2.4. Investigation of BmDNV-Z proliferation by performing real-time qPCR

Total DNA was extracted from the midguts of the BmDNV-Z-infected *B. mori Jingsong* and *Jingsong.nsd-Z.NIL* larvae at 3, 12, 24, 48, 72, and 96 h pi as well as from the PBS-treated larvae by using the Universal Genomic DNA Extraction Kit Ver 3.0 (TaKaRa). DNA samples obtained from five larvae per treatment group were used as the template for PCR amplification.

The BmDNV-Z genome is composed of two kinds of different single-stranded linear DNA molecules (VD1 and VD2). VD1 (viral DNA 1) consists of 6543 nt including inverted terminal repeats (ITRs) of 224 nt, and VD2 (viral DNA 2) consists of 6022 nt including ITRs of 524 nt (Wang et al., 2007).

Two pairs of primers were designed based on the ORF1 region of the BmDNV-Z VD1 genomic sequence (GenBank accession no. DQ017268) and the ORF2 region of the BmDNV-Z VD2 genomic sequence (GenBank accession no. DQ017269). The *β-actin* gene of *B. mori* was used as an internal control (Table 1). The specificity of the primers was confirmed using NCBI BLAST (BLASTN) algorithms.

Real-time qPCR was conducted on an iCycler iQ instrument (BIO-RAD) using the SYBR *Premix Ex Taq* Kit (TaKaRa) according to the protocol prescribed by the manufacturer. Total DNA and specific BmDNV-Z primers were used for amplifying the ORF1 and ORF2 sequences of the viral VD1 and VD2 genome. Each amplification reaction was performed using a 25 μl reaction mixture under the following conditions: denaturation at 95 °C for 1 min, followed by 40 cycles of treatment at 95 °C for 10 s and at 60 °C for 20 s. The fluorescent signals yielded by the PCR products were detected by subjecting the products to a heat-dissociation protocol (temperature range, 60–95 °C) during the last step of each cycle. Following amplification, melting curves were constructed, and data analysis was performed using the iCycler iQ optical system software

Table 1
Primers used in real-time qPCR for determination of BmDNV-Z proliferation

Target	Accession no.	Antisense primer	Sense primer	Size (bp)
BmDNV-Z VD1 ORF1	DQ017268	5′ GACTTCATCTGCTGCTTTCC 3′	5′ ACATCTCATCTCCCTCAACG 3′	109
BmDNV-Z VD2 ORF2	DQ017269	5′ GACCTGCACCAGATGGAAT 3′	5′ CCTCGTTATGCTCAAGAAG 3′	115
Bombyx mori β-actin	AF422795	5′ AATGGCTCCGGTATGTGC 3′	5′ TTGCTCTGTGCCTCGTCT 3′	150