The Differential Expression of BmGlcNAcase2 in Strains of Bombyx mori (Lepidoptera: Bombycidae) with Different Susceptibility to Bombyx mori (Lepidoptera: Bombycidae) Nucleopolyhedrovirus Infection

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RESEARCH

The Differential Expression of BmGlcNAcase2 in Strains of *Bombyx mori* (Lepidoptera: Bombycidae) With Different Susceptibility to *Bombyx mori* (Lepidoptera: Bombycidae) Nucleopolyhedrovirus Infection

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ABSTRACT. GlcNAcase is a glycosyl hydrolase located in the lysosomes of numerous organisms. Levels of the protein, β-N-acetylglucosaminidase 2 (GlcNAcase2), which is a member of the GlcNAcase family, are different in two strains of the silkworm *Bombyx mori* that have different resistance to *Bombyx mori* nucleopolyhedroviruses (BmNPVs). We identified six single-nucleotide differences in the GlcNAcase2 coding sequence between the 306 and NB strains. Five are silent changes, but one is a nonsynonymous mutation. Reverse transcription-polymerase chain reaction analysis showed that GlcNAcase2 mRNA levels in the NB strain were nearly 2.57 times higher compared with those in the 306 strain. In addition, GlcNAcase2 enzyme activity was much higher in the NB strain compared with that in the 306 strain. Together, these results indicate that GlcNAcase2 may be involved in variable BmNPV resistance in *B. mori*.

Key Words: *Bombyx mori,* β-N-acetylglucosaminidase2, resistance, enzyme activity, nucleopolyhedrovirus

The mulberry silkworm (*Bombyx mori*) has been used in agriculture for the last 5,000 years and has significant economic status. As a model in molecular biology and genetics, *B. mori* plays an important role in scientific research (Omenetto and Kaplan 2010). However, silkworm viral diseases, such as *B. mori* nucleopolyhedrovirus (BmNPV), can cause great loss in sericulture (Gomi et al. 1999).

BmNPV is a double-stranded DNA virus and is the first virus to be identified in insects (Hultmark 1994). Once infected by the virus, larva will die in 3 or 4 d, but the virus can spread to the mulberry leaves to infect other larvae. Thus, to control BmNPV disease, the mechanism of virus infection must be understood. By investigating all the silkworm genetic resources in China, Chen et al. (2003) identified a silkworm strain, named NB, with high resistance to BmNPV. Today, strains NB and 306 (the strain which is susceptible to BmNPV) are often used in comparative research of BmNPV resistance. In our previous two-dimensional gel electrophoresis study, our group determined that the expression of β-N-acetylglucosaminidase 2 (GlcNAcase2) is different between strains NB and 306 (Chang 2011). Here, we investigated whether the difference in resistance to BmNPV is related to GlcNAcase 2.

GlcNAcase is a major glycosidase located in the soluble fraction of lysosomes in many kinds of organisms (Nomura et al. 2010, Sarosiek et al. 2014). These enzymes can degrade various kinds of oligosaccharides and glycoconjugates; in particular, they can catalyze the hydrolysis of O-glycosidic bonds in nonreducing terminal N-acetylglucosamine (GlcNAc) residues in an oligosaccharide chain (Slamova et al. 2014). In insects, the enzymes are very important to the activity and stability of proteins (Kim et al. 2011). GlcNAcase2 shows broad substrate specificity. It can cleave terminal GlcNAc residues from the α-3 and α-6 branches of a biantennary N-glycan substrate and can also hydrolyzed chitotriose to chitobiose (Okada et al. 2007).

Materials and Methods

Insects, Cells, and Viruses. Three different strains of *B. mori* were used: NB (a strain that is resistant to BmNPV), 306 (a strain that is susceptible to BmNPV), and BC_8 (a strain that has a similar genetic background to 306 and also resistant to BmNPV). All larvae were reared with fresh mulberry leaves at 27°C under a 12:12 (L:D) h photoperiod.

Day 3 fifth-instar larvae were used for experiments. Tissues were dissected in cold phosphate-buffered saline and stored in RNA-free Eppendorf tubes at −80°C for later RNA isolation using Trizol (Life Technologies, State of California, USA) and protein extraction using RIPA Lysis Buffer (Aidiab, Beijing, China).

The BmN cell line was stored in our laboratory. These cells were maintained at 27°C in TC-100 insect medium (Gibco, Australia) supplemented with 10(v/v) fetal bovine serum (Gibco) using standard protocols.

Cloning *BmGlcNAcase2*. To obtain a full-length cDNA of *BmGlcNAcase2* by high-fidelity polymerase chain reaction (PCR), a pair of primers was designed using Primer premier 5.0 software. Forward primer: 5′-atgtttcgtctttttctttatttaaatattttag-3′ and reverse primer: 5′-ctaagcgcctaggcagaagc-3′. The PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min 30 s and final extension of 72°C for 10 min. A second pair of primers was designed for use in reverse transcription (RT)-PCR: forward primer: 5′-cgagagcaagtcaccagtta-3′, reverse primer: 5′-aagaagccgctgaccata-3′. The reference gene was *actin3*. The PCR reaction conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate and results were calculated using the delta-delta-C_T method (Livak and Schmittgen 2001).

Measurement of BmGlcNAcase2 Activity. The activity of BmGlcNAcase2 was measured according to the method described by Lin et al. (2003). 4-Nitrophenyl-N-acetyl-β-D-glucosaminide was used as the BmGlcNAcase2 zymolyte. Two milliliters of the enzyme activity system were mixed with 20 µl *B. mori* hemolymph and incubated at 37°C for 10 min; 2 ml of NaOH (0.5 mmol/l) was then added to terminate the reaction, and a microplate reader was used to read the absorbance at 405 nm ($OD_{405\ nm}$). Nitrobenzene is the reaction product with a molar extinction coefficient 8.8×10^3 M/cm. In this article, the definition of an active unit (U) is, under standard experimental conditions, the amount of enzyme needed to catalyze the 1 µM substrate to nitrobenzene in 1 min in a volume of 1 liter. And the definition of the

specific activity is the units of enzyme activity needed to catalyze 1 µl of *B. mori* hemolymph.

Preparation of a Polyclonal Antibody Against BmGlcNAcase2. For western blot analysis and subsequent studies, we prepared a polyclonal antibody against BmGlcNAcase2. A fragment of BmGlcNAcase2 was PCR amplified using the primer pair, forward 5′-aagaattcgaaccgggaccc-gaatatcc-3′ and reverse 5′-aaaagcttctacaaggtctcgtgataggctccc-3′, and inserted into the expression vector, pET-30 a, to create pET-BmG2s. The recombinant plasmid was transformed into *Escherichia coli*. Isopropyl-D-thiogalactoside (IPTG, 0.6 mM final concentration) was used to induce protein expression. After incubation for 4 h at 37°C, bacterial cells were harvested by centrifugation at 3,500 × *g* for 10 min at 4°C. SDS-PAGE was used to purify the protein. The gel slice containing recombinant BmGlcNAcase 2 was recovered and used as an antigen to inject a rabbit for polyclonal antibody production. After four rounds of injection, the blood of the rabbit was collected from the carotid artery. The blood was incubated at 37°C for 2 h and then kept at 4°C overnight. The antiserum, containing the polyclonal antibody, was collected by centrifugation at 8,000 × *g* for 10 min at 4°C and stored at −80°C for subsequent experiments.

Western Blot Analysis. Total protein was extracted from fat body, hemolymph, and midgut. After electrophoresis, protein samples were transferred to PVDF membranes (Bio-Rad, UK). The blots were then blocked in 5% skimmed milk powder (Karivita) in transblotting solution (TBS, 20 mM Tris, 0.9% NaCl, pH 7.2) for 12 h in at 4°C. The blots were washed (three times for 10 min) in TBS followed by incubation in 3% Tween transblotting milk solution (TBST, 20 mM Tris, 0.9% NaCl, 0.1% Tween-20, pH 7.2) containing 1/500 dilution of the polyclonal antibody for 3 h at room temperature. After subsequent washing (three times for 10 min in TBST), the blots were incubated in 3% TBST milk solution containing 1/10,000 dilution of horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (TAKARA, Dalian, China) for 3 h at room temperature. The bands were scanned into the computer, and the software ImageJ 1.48u was used to analyze the results.

Results

Cloning *BmGlNAcase2* From B. mori. To verify variations of *BmGlNAcase2* in different strains of *B. mori*, we designed a pair of primers to amply the *BmGlNAcase2* coding sequence from strains 306 and NB. After sequencing, we identified six nucleotide variations between the two sequences, at 579 bp, 770 bp, 959 bp, 1,292 bp, 1,314 bp, and 1,398 bp. Five of the variations (at sites 579 bp, 959 bp, 1,292 bp, 1,214 bp, and 1,314 bp) are silent differences, but the site of 770 bp is a nonsynonymous mutation with a threonine in the 306 strain mutated to an isoleucine in the NB strain (Fig. 1). Hence, we identified six nucleotide variations between the two strains at the cDNA level but only one mutation at the protein level. Therefore, coding sequence mutations of

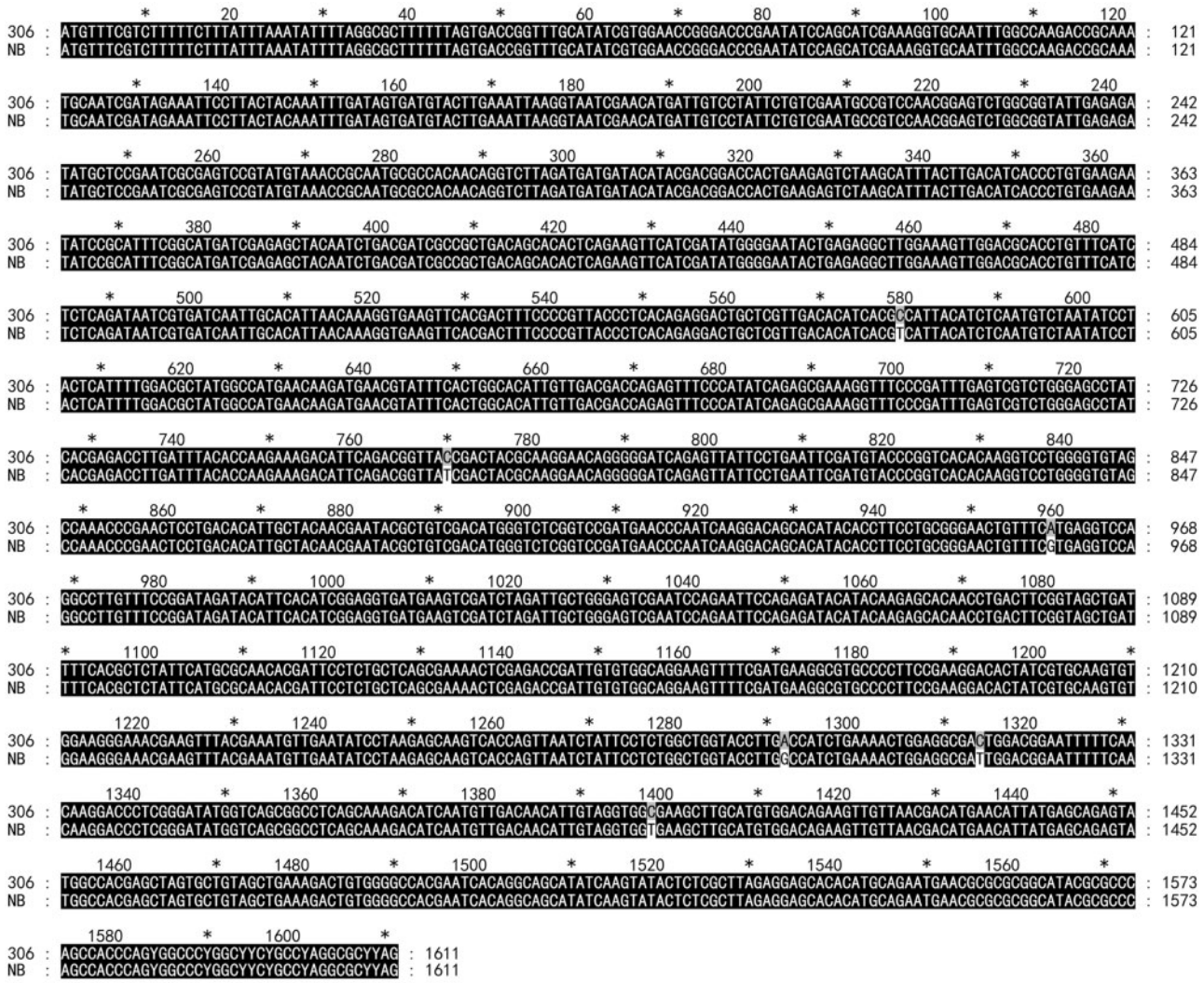

Fig. 1. Alignment of *BmGlcNAcase2* cDNA sequences from strain NB (resistant to BmNPV) and strain 306 (susceptible to BmNPV). Black background indicates identical sequence between the two cDNAs, whereas white background indicates the variant bases.

BmGlNAcase2 in different strains may not be the main reason for the different response to BmNPV in 306 and NB strains.

Transcription of the *BmGlNAcase2* Gene in the Midgut of 306 and NB. Quantitative RT-PCR was used to detect the relative amount of *BmGlNAcase2* mRNA in the midgut of day 3 fifth-instar larvae of 306, NB, and BC_8 strains. The amount of *BmGlNAcase 2* mRNA in BC_8 was about 6.87 times of that in 306 and 2.78 times of that in NB (Fig. 2). These results are consistent with previous observations (Liu et al. 2010).

Western Blot Analysis. Strains 306, NB, and BC_8 in day 3 fifth-instar larvae were dissected and the midgut, fat body, and hemolymph collected. At least 20 larvae were dissected for each group. Total protein was used for western blot analysis (Fig. 3). In the midgut, the level of BmGlNAcase2 protein was much higher in NB than in BC_8 and 306. In addition, the amount of protein in BC_8 was higher than that in 306. In the hemolymph, the BmGlNAcase2 protein level in NB and BC_8 was higher than that in 306. However, there was no significant difference between the levels in NB and BC_8. In the fat body, the level of BmGlNAcase2 protein was nearly equal between strains. These results indicate that levels of BmGlNAcase2 protein are significantly higher in the BmNPV-resistant strains, NB and BC_8, than in the BmNPV susceptible strain.

The Activity of BmGlNAcase 2 in Hemolymph. The activity of BmGlNAcase2 was investigated in two strains of *B. mori* with different resistance to BmNPV. In addition, changes in BmGlNAcase2 activity in different developmental stages were also assessed. Hemolymph was collected from day 1 fourth instar to day 3 fifth-instar larvae. BmGlNAcase2 activity in strain NB was significantly higher than that in strain 306 (Fig. 4). However, the activity of the enzyme was nearly the same across the 7 d analyzed in both NB and 306 strains. This indicates that BmGlNAcase2 activity is relatively stable in a single strain during this stage of development but that the BmNPV-resistant NB strain has higher levels of BmGlNAcase2 activity compared with those in the BmNPV susceptible 306 strain.

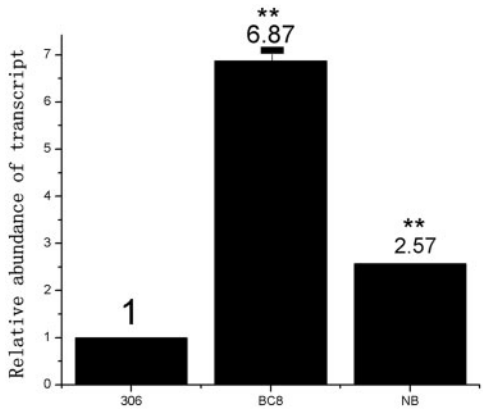

Fig. 2. RT-PCR analysis of BmGlNAcase2 mRNA levels among strains 306, NB, and BC_8. Data represent the mean ± SD. **Significant difference ($P < 0.05$) compared with strain 306.

Discussion

In a previous study, we compared the proteome of three strains of *B. mori*, NB, 306, and BC_8 (BC_8 is a near-isogenic line to NB and 306). We found a series of differentially expressed proteins, one of which was GlcNAcase2 (Liu et al. 2010). We then showed that GlcNAcase2 is expressed in the hemolymph, ovary, testes, fat body, trachea, midgut, and silk gland in fifth-instar larvae.

In this study, the full-length *BmGlcNAcase2* gene was amplified by PCR. We found six single-nucleotide differences between strains NB and 306. Five of them are silent changes, but one causes a threonine in 306 to change to an isoleucine in NB. There is no data indicating that the different resistance to BmNPV between these strains is related to any of these changes. However, the mutated amino acid site is not in the catalytic center; therefore, we suppose that the activity of BmGlcNAcase2 may not be affected by the amino acid change.

To further study GlcNAcase2, RT-PCR and western blot analyses were used to determine an overview of transcription and translation. The level of *BmGlcNAcase2*, at both the transcription and translation levels, was much higher in the NB strain than in the 306 strain (Figs. 2 and 3). High levels of transcription and translation of *GlcNAcase2* were observed in the midgut and hemolymph. We also measured the activity of BmGlcNAcase2 in hemolymph. The activity of GlcNAcase 2 in strain NB was nearly 1.5 times higher than

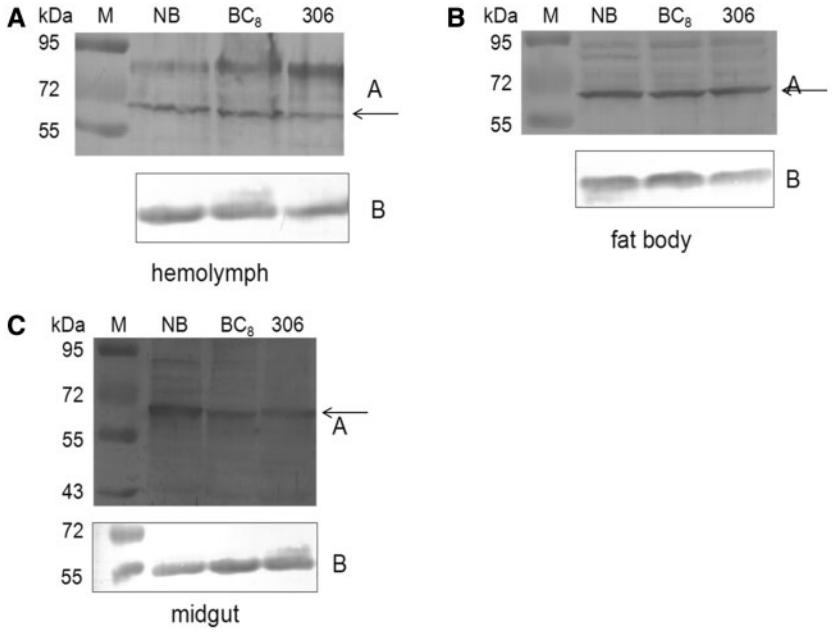

Fig. 3. Western blot analysis of BmGlcNAcase2 in different tissues of *B. mori*. (a), (b), and (c) show western blot analysis of BmGlcNAcase2 in the hemolymph, fat body, and midgut, respectively. Arrows point to the target protein. The pictures B indicate the reference protein, actin.

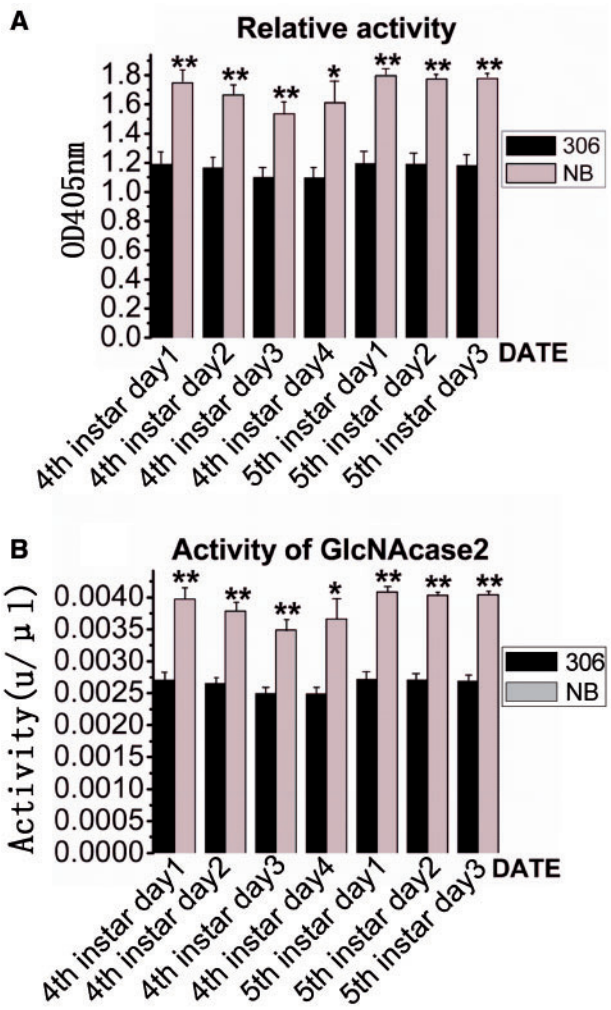

Fig. 4. The relative activity of BmGlcNAcase2 between 306 and NB. (A) The relative activity of the BmGlNAcase2. (B) The activity of BmGlNAcase2. The black column indicates strain 306 and the gray column indicates strain NB. **Significant difference ($P < 0.05$) compared with strain 306.

that in strain306 (Fig. 3). These results are highly consistent with previous studies. The fat body is critical to the metabolism of carbohydrate, lipid, and protein (Roma et al. 2010); however, there was no obvious difference in BmGlcNAcase2 activity in the fat bodies of the two strains.

The columnar epithelial cells of the midgut are the main target cells during primary infection of BmNPV (Zhang et al. 2014). After feeding *B. mori* larvae, a high dose of ODV, virion (BV) can be seen budding from the sides of epithelial cells, and infectious virus can be detected in the hemolymph after a few hours (Granados and Lawler 1981). Moreover, insect hemolymph is important in secondary infection because once the hemolymph is infected, BV can diffuse to the whole body via the open hemolymph circulation system.

N-glycosylation of viral glucoproteins is important for their intracellular transport and fusogenic activity, as well as for BV infectivity (Jarvis et al. 1998). We speculate that the high levels of β-N-acetylglucosaminidase expression in the hemolymph and midgut of resistant silkworms can disturb the N-linked glycans of viral glucoproteins, which are essential for initiating secondary infections. This would reduce the reproductive ability of infectious viruses. However, this hypothesis requires further study.

Differences in the level of GlcNAcase2 protein in different strains of *B. mori* may be one reason why these strains show different resistance to BmNPV. More studies are needed to further investigate the role of GlcNAcase2 in the silkworm response to BmNPV infection.

Acknowledgments

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