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 characterized in insects (Hulmark 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 study (Gomi et al. 1999), 5 min, followed by 40 cycles of 95° C for 10 min, a second pair of primers was designed for use in reverse transcription (RT)-PCR:

- Forward primer: 5'-ctaacagccagcagacc-3'
- Reverse primer: 5'-gcaaggtctgctttaataatttgttg-3'

The PCR reaction conditions were 95° 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'-caagagagcaagcagcagtcatta-3'
- Reverse primer: 5'-ggagagagcaagcagcagtcatta-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 (OD405 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. 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'-aagaattcgaaccgggagccgaatctt-3' and reverse 5'-aaaagcttctacaaggtctcgtgataggctccc-3', and inserted into the expression vector, pET-30a, 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 BmGlcNAcase2 From B. mori.** To verify variations of BmGlcNAcase2 in different strains of *B. mori*, we designed a pair of primers to amplify the BmGlcNAcase2 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
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 BC₈ strains. The amount of BmGlNAcase 2 mRNA in BC₈ 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 BC₈ 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 BC₈ and 306. In addition, the amount of protein in BC₈ was higher than that in 306. In the hemolymph, the BmGlNAcase2 protein level in NB and BC₈ was higher than that in 306. However, there was no significant difference between the levels in NB and BC₈. In the fat body, the level of BmGlNAcase2 protein was nearly equal between strains. These results indicate that levels of BmGINcase2 protein are significantly higher in the BmNPV-resistant strains, NB and BC₈, 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 BmGINcase2 activity is relatively stable in a single strain during this stage of development but that the BmNPV-resistant NB strain has higher levels of BmGINcase2 activity compared with those in the BmNPV susceptible 306 strain.

Discussion

In a previous study, we compared the proteome of three strains of B. mori, NB, 306, and BC₈ (BC₈ 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 GlcNAcase2 in strain NB was nearly 1.5 times higher than...
compare with strain 306.

which are essential for initiating secondary infections. This would disturb the N-linked glycans of viral glucoproteins, (Jarvis et al. 1998). We speculate that the high levels of cellular transport and fusogenic activity, as well as for BV infectivity because once the hemolymph is infected, BV can diffuse to the whole body via the open hemolymph circulation system.

Moreover, insect hemolymph is important in secondary infection of BmNPV (Zhang et al. 2014). After feeding the hemolymph after a few hours (Granados and Lawler 1981). ODV, virion (BV) can be seen budding from the sides of epithelial cells, and infectious virus can be detected in the hemolymph. Slamonová, K., N. Kulík, M. Flála, J. Krejzová-Hofmeisterová, R. Ettrich, and V. Kren. 2014. Expression, characterization and homology modeling of a novel eukaryotic GH84 beta-N-acetylglucosaminidase from Penicillium chrysogenum. Protein Expr. Purif. 104: 143–152.

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.

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