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Full Length Research Paper

Identification and characterization of *Bms3a* in *Bombyx mori* L.

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Using fluorescent differential display (FDD) technique, we analyzed the differential expression of genes related to BmNPV resistance in highly resistant silkworm strain NB, highly susceptible silkworm strain 306 and near isogenic line 306NNZZ. Based on the differential display bands, a 609 bp fragment named $C18_{609}$ was cloned and confirmed by Northern blot hybridization. The sequence was then electric extended by identified in NCBI ESTs. A novel gene was characterized and revealed to encode a putative BmS3a protein. Becasue it has high homology to some insect S3a protein, it was named s3a. S3a protein have been known to play crucial roles in protein synthesis, and is related to apoptosis. It is differentially expressed in silkworm high resistance strain, high susceptible strain and BmNPV treated silkworms. Therefore, it is conceivable that BmS3a is involved in silkworm BmNPV resistance.

Key words: BmNPV, Resistance, Bms3a, fluorescent differential display.

INTRODUCTION

Baculoviridae is a family of enveloped, double-stranded DNA viruses that infect arthropods. *Bombyx mori* nuclear polyhedrosis virus (BmNPV) was the first virus discovered in the past studies of insect virology (Lu, 1998). Silkworm nuclear polyhedrosis virus (NPV) disease is a highly infective disease, caused by BmNPV, which has led to the world's silkworm raising countries suffer great loss. There is remarkable difference among strains in the silkworm resistance to BmNPV. The heredity of silkworm resistance to NPV is relatively complicated because it is controlled both by major dominant genes and multiple micro-effect genes (Chen et al., 2003). Though significant progress has been made in breeding highly resistant strains (Chen et al., 1991, 1996), and some molecular markers have been identified for NPV resistance in *B. mori* (Yao et al., 2003), little about the NPV infection pathways and resistance mechanism has been clarified so far. The systemic infection process of BmNPV *in vitro* has recently been reported (Rahman and Gopinathan, 2004). Some proteins such as serine protease and lipase from silkworm digestive juice were reported to have anti-viral activity to BmNPV (Nakazawa et al., 2004; Ponnuvel et al., 2003). Some silkworm strains exhibit high resistance to BmNPV, but the immune mechanisms of *B. mori* against BmNPV remain obscure.

In order to identify genes related to silkworm resistance and susceptibility to BmNPV, we used the fluorescent differential display technique to identify genes linked to silkworm resistance and susceptibility to NPV disease using highly resistant silkworm strain NB bred by Chen et al. (1991, 1996), highly susceptible silkworm strain 306 and near isogenic line BC_8 (8^{th} generation of backcross) of 306NNZZ, bred with 306 and NB. We have obtained some differentially expressed sequences linked to silkworm resistance to BmNPV disease in mRNA level ((XU, 2005a, b). Another gene that was differentially expressed in NB, 306 and 306NNZZ was identified and characterized. The results are hereby reported.

MATERIALS AND METHODS

Experimental animals

Highly resistant silkworm strain NB (LC_{50} = 8.25 polyhedra/mL), highly susceptible silkworm strain 3069 (LC_{50} = 4.95 polyhedra/mL)

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and near isogenic line 306NNZZ (LC_{50}=8.23 polyhedra/mL) bred by NB and 306 were used for this study. Near isogenic line 306NNZZ was prepared in accordance with Yao et al. (2003), which was obtained by continuously using the females of cyclical backcross parent, 306, to cross the males of strain NB which was used as the donor of resistant genes. The backcrosses were conducted for eight generations followed by two generations of self-crossing. BmNPV virus was administrated to larvae of each generation to screen the individuals carrying resistant genes.

All larvae of three silkworm strains, NB, 306 and the near isogenic line 306NNZZ, were raised on artificial diet to the fifth instar. For each strain, 50 newly ecdysis 5th instar larvae were taken and fed with artificial diet treated with 3.6×10^8 BmNPV polyhedra (BmNPV T3 strain), and the controls were fed with artificial diet treated with the same volume of water. 48 hpi after the virus administration, from each treatment, 30 larvae midguts were sampled for isolating RNAs to eliminate the difference caused by heredity background. The samples were labeled R1, S1 and N1 for strains NB, 306 and 306NNZZ, respectively. Those of controls were labeled R2, S2 and N2, respectively. Additionally, NPV treatment groups and the controls were designed in the same conditions for the incidence of disease investigation 4 days later.

mRNA fluorescent differential display (FDD)

Total RNAs were isolated from silkworm midgut samples using Trizol (Invitrogen Co.) and treated with RNase-free DNase (Promega, WI, Madison, USA). For FDD (GenHunter, RNAspectra Kit, Tennessee, USA) analysis, 0.2 μg of total RNAs was reverse-transcribed with MMLV reverse transcriptase and 3'-anchored oligoH-T_{11}C primer to yield the first strand of cDNA. Subsequently, 2 μl of first-strand cDNA was used for PCR reaction. The reaction mixture contained 1 unit Taq polymerase (Quagen, Valeucia, CA, USA), 50 μM of each dNTP, 10 pmol of arbitrary primer, 10 pmol of fluorescein isothiocyanate-labeled 3'-anchored oligoH-T11C (FH-T_{11}C) primer and 2.0 μl of 10×PCR buffer. The thermal cycling profile was as follows: 94ºC 3min, 40 cycles of 94ºC 30s, 40ºC 2 min and 72ºC 1 min, followed by final extension at 72ºC for 5 min. Each PCR product was electrophoresed (Life Technologies, Carlsbad, CA, USA) in a 6% denatured polyacrylamide gel in 1×TBE buffer. FDD gel was scanned with the FMBIO system (Hitachi Genetics, Tokyo, Japan).

Molecular cloning of the bands of interest

The differential bands of interest were cut and extracted to recover cDNA. The cDNA was re-amplified, and the positive bands identified through Northern blotting were cloned into T-vectors followed by α-complementation clone selection and DNA sequencing.

Northern blot analysis

Thirty μg of total RNA was separated on 1.2% agarose gel containing 0.22 M formaldehyde and ethidium bromide, and subsequently transferred onto a nylon membrane (hybond N^+ Amersham, Buckinghamshire, UK). The differential cDNA band was labeled with [α^{32}-P]-dCTP using a random primed DNA labeling kit (Takara, Shiga, Japan). Nylon membrane was pre-hybridized for 4 h followed by the addition of a radio-labeled probe for 18 h at 45℃ in 5 × SSPE (1× SSPE = 180 mM NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA) containing 50% formamide, 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. After hybridization, the membrane was washed with 0.2 × SSPE at 45℃ and finally exposed to X-ray film for 24 h at –70℃.

Sequence analysis and gene obtained

The FDD sequence was used to search (BLAST) GenBank and Silkbase, and high homology of the sequence was acquired,. Though EST extension of the sequence, a full cDNA which had a deducible code was obtained. A pair of specific primer was designed for the RT-PCR based extension sequence. Forward: 5'-GAA CAT GGC GGT CGG GAA-3', and Reverse: 5' -GAA CGG GAG GCT CGT AG-3'. The PCR products were examined by electrophoresis in 1% agarose gel with ethidium bromide staining. PCR fragment was cloned and sequenced. The cloned full cDNAs sequence was to search GenBank and Silkbase and the deduced amino acid sequence was compared to known proteins using Clustal W program. Finally, the DNA sequence of the gene was obtained by searching the cDNA sequences in silkworm genome (SilkDB).

RESULT

Resistance identification of NB, 306 and 306NNZZ

For each strain, 50 newly metamorphosed 5th instar larvae were taken and fed with artificial diet treated with 3.6×10^8 BmNPVs, and the controls were fed with artificial diet treated with the same volume of water, Incidence of disease investigation suggested that, the average incidence of S1 is 99.3%, and the others are all nearly 0 (Table 1). The results indicated that the resistant silkworm strain NB, susceptible silkworm strain 306 and near isogenic line NN have great difference in susceptibility to BmNPV, and imply that through 8 generation back-cross and 2 generation self-cross the near isogenic line 306NNZZ possess homogeneous resistant genes from NB.

FDD analysis of mRNAs isolated from NB, 306 and 306NNZZ

To identify genes specifically related to BmNPV resistance, the mRNAs of strains NB, 306 and 306NNZZ were compared by FDD. To perform FDD-PCR, we used 12 arbitrary primers, paired with Oligo$(dT)_{11}$C, to amplify each first-strand cDNA sample from all the treatments. The results showed that there were 60 differential bands among all the treatments.

After Northern blotting (Figure 1), a band of 609 bp (C_{609}) was obtained, revealing that the result obtained from FDD (Figure 2) was positive bands. The result also confirmed that the expression of the corresponding transcript had been faithfully reflected on FDD fingerprints correlating to silkworm resistance to BmNPV, which had high expression level in NB_2, 306_2 and NN_2, and low expression level in NB_1, NN_1 and 306_1, with 306_2 being the lowest.

The sequence of C_{609} was used to search Silkbase, and the result indicated that it has high similarity in EST Base; specifically, it has 100% similarity with the sequence AV405804, *B. mori* wing disk C108, 5th-instar day

Table 1. Incidence of disease of R1, R2, S1, S2 and N1, N2.

Material	Treatment area	Examination number	Death number	Survival number	Incidence disease	Average incidence disease
R1	1	50	1*	49	2%	0.6%
	2	50	0	50	0	
	3	50	0	50	0	
R2	1	50	0	50	0	0.6%
	2	50	0	50	0	
	3	50	1*	49	2%	
S1	1	50	50	0	100%	99.3%
	2	50	50	0	100%	
	3	50	49	1**	98%	
S2	1	50	0	50	0	2.6%
	2	50	3*	47	6%	
	3	50	1*	49	2%	
N1	1	50	0	50	0	0
	2	50	0	50	0	
	3	50	0	50	0	
N2	1	50	0	50	0	0.6%
	2	50	0	50	0	
	3	50	1*	49	2%	

*Indicate that those dead not for BmNPV.
**Indicate that those survived which not find polyhedra in hemolymph. R1, S1, N1 represented treatments of NB, 306, 306NNZZ with BmNPV, respectively. R2, S2, N2 represented those of controls, respectively.

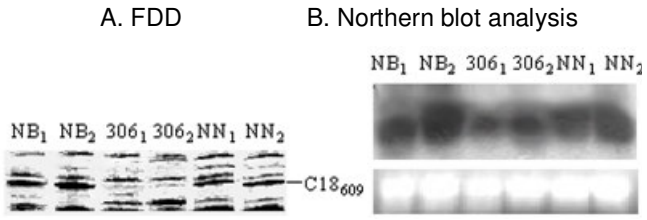

Figure 1. (**A**) Expression pattern of S3a (Band $C18_{609}$ was from silkworm midgut. NB_1, 306_1, NN_1 represented treatments of NB, 306, 306NNZZ with BmNPV, respectively. NB_2, 306_2, NN_2 represented those of controls, respectively. (**B**) Ribosomal RNAs stained with ethidium bromide were shown in the lower part as the control.

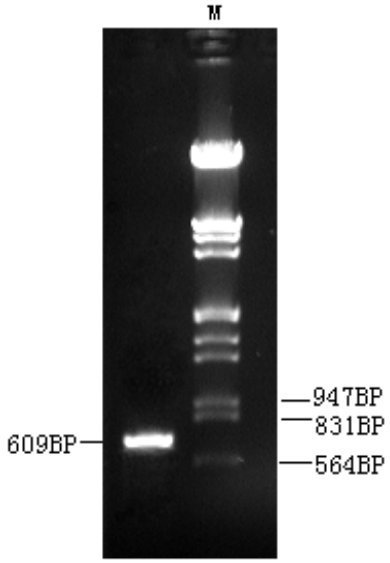

Figure 2. C_{609} PCR products.

-3 larva *B. mori* cDNA clone wdV30257 T3 mRNA sequence; identities = 590/590 (100%). After joining it with FDD sequence, the full length reached 851 bp, which has a whole ORF. Subsequently, by PCR amplification using a pair of specially designed primers, a band of 782 bp (Figure 3) length was acquired, then cloned and sequenced. The full nucleotide sequence and the deduced amino acid sequence are showed in Figure 4. Sequence identification showed that the fragment is a newly isolated gene, and the sequence had been registered in GenBank with the name of *Bms3a* (accession number: AY705974) for its deduced amino acid sequence has high similarity with RPS3a protein of other species. The coding region starts at 5 nt and ends at 794 nt. The full length coding region is 789 bp long. The deduced amino acid length has 374 aa. The full length sequence contains a polyadenyation signal, AATAA, 11 bases up-

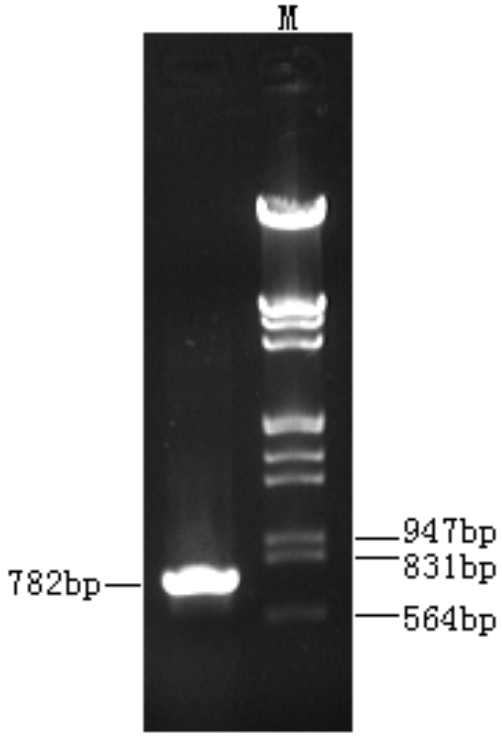

Figure 3. Bms3a PCR products.

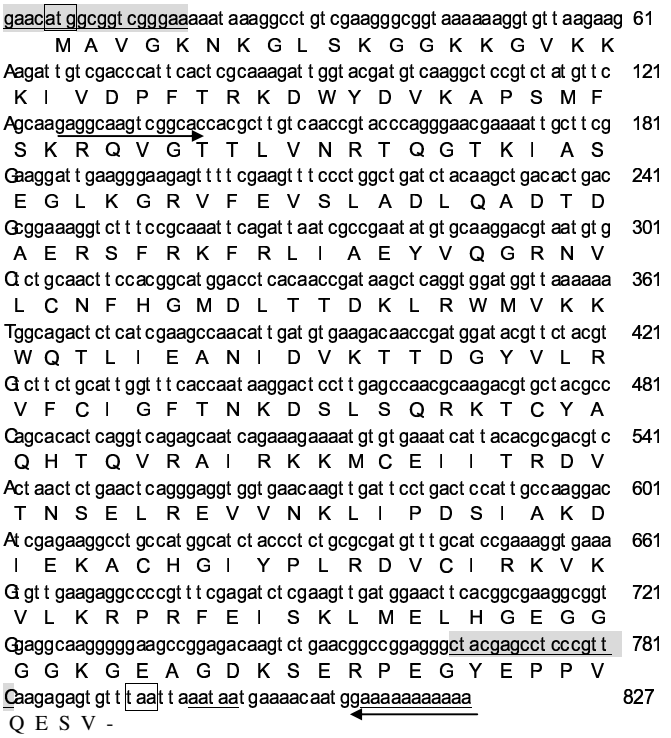

Figure 4. Bombyx mori ribosomal protein *S3a* cDNA sequence and deduced amino acid sequence. The first ATG and the stop codon are denoted by box. The polyadenylation signal, AATAA, and the poly(A) tail in the 3' untranslated region of cDNA are underlined. The sequence arrowhead area from 125 to 827 bp is FDD sequence.

stream of poly(A) addition site, and has 406 bp sequence of 3' non-coding region. From the deduced amino acid sequence, it appears that the molecular weight of the protein is 29753.49. The protein has 55 basic and 33 acidic amino acid residues. The predicted isoelectric point of the protein is 9.66.

Isolation and sequence analysis of *Bms3a* gene

A comparison of amino acid sequence of BmS3a protein deduced from the newly isolated cDNA to that of the RPS3a protein of other species showed that it had similarities of 97.7, 94.0, 75.3 and 74.5% to *Spodoptera frugiperda*, *Heliothis virescens*, *Drosophilamelanogaster* and *Anopheles gambiae* RPs3a protein respectively (Figure 5, 6 and Table 2).

To isolate the gene, we aligned the cDNA sequence in SilkDB (Silkworm Knowledgebase, Xia et al., 2004). The genomic structure of *Bms3a* gene is shown in Figure 7. The gene is about 4.5kb in length and comprises six exons and five introns. The exons share an identical sequence with the cDNA.

DISCUSSION

We have, for the first time, isolated and characterized *Bms3a* gene relating to BmNPV resistance by using the method of FDD. The ribosomal protein, S3a, is one of the proteins constructing the small subunit of the ribosomal complex, 40S. Immunoelectron microscopy suggested that the ribosomal protein S3a binds to initiation factors eIF-2 and eIF-3 in the 40S subunit. mRNA and met-tRNA also bind to ribosomal protein S3a. These data support the theory that, as a part of the ribosomal complex, S3a takes part in the initiation of protein synthesis (Lutsch et al., 1990). Some researches have indicated that RPs3a is involved in regulating cell growth, transformation, apoptosis and give rise to increasing speculation that components of the translational apparatus can act as multifunctional proteins. It has been found that this induction of apoptosis was not specific to apparently transformed cells, as cells at low confluence, which likewise expressed RPS3a at enhanced levels but exhibited no morphological transformation, underwent apoptosis when RPS3a expression was inhibited. These results support a role for RPS3a in the apoptotic process, but not as an oncoprotein per se (Naora et al., 1998). Later researches showed that ribosomal protein S3a (RPS3a) plays important roles in cell transformation and death, whereby constitutively or transiently enhanced

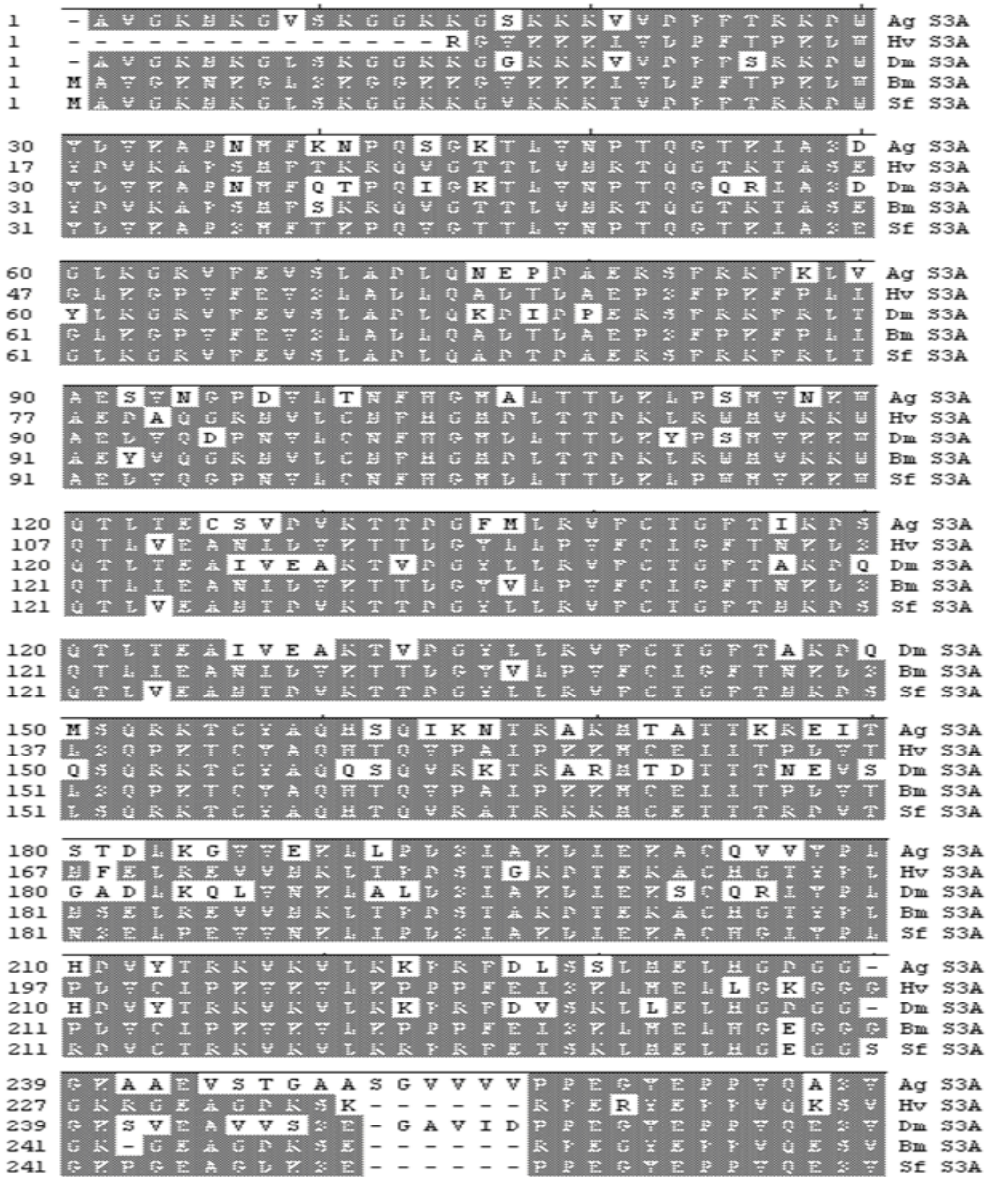

Figure 5. Comparison of deduced amino acid sequence of several species. The sequences were aligned with DNA Star and manually adjusted to give best fit. Consensus sequences are shaded. GenBank accession number (Ag: *Anopheles gambiae*; CAA66861; Dm: *Drosophila melanogaster;* P55830: *Spodoptera frugiperda;* AAL26579: *Heliothis virescens:* AAK59927; Bm: *Bombyx mori*, AY763110).

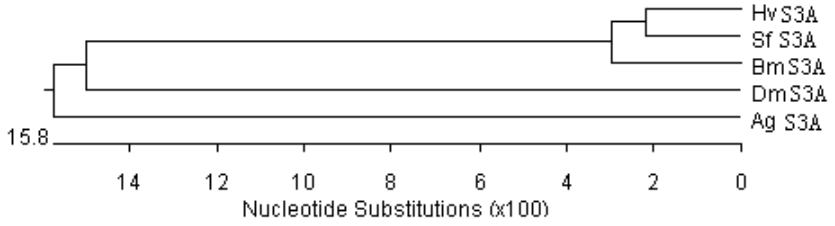

Figure 6. Phylogenetic tree of S3A among different species (Ag: *Anopheles gambiae;* CAA66861; Dm: *Drosophila melanogaster;* P55830: *Spodoptera frugiperda;* AAL26579*; Heliothis virescens;* AAK59927; Bm: *Bombyx mori*, AY763110).

Table 2. Pair distances of deduced amino acid sequence of Bms3a and several species by ClustalW.

	Ag S3A	Hv S3A	Dm S3A	Bm S3A	Sf S3A
Ag S3A	***	71.6	74.5	74.9	73.9
Hv S3A		***	71.2	94.0	96.0
Dm S3A			***	75.3	74.6
Bm S3A				***	97.7

Percent Similarity in upper triangle. Ag: *Anopheles gambiae*; CAA66861; Dm: *Drosophila melanogaster*; P55830: *Spodoptera frugiperda;* AAL26579: *Heliothis virescens*; AAK59927; and Bm: *Bombyx mori*, AY763110.

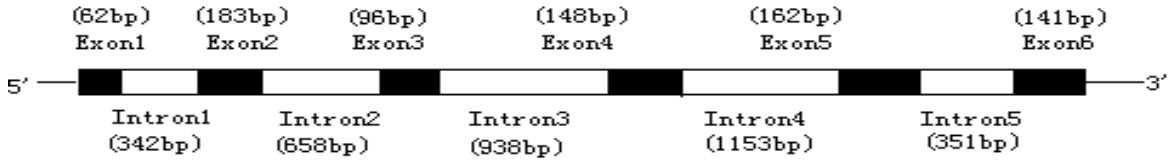

Figure 7. The sketch map of *Bms3a* gene (The DNA sequence of Bms3a is located in the region contig92407, contig178523 and contig477496 of silkworm genome).

RPS3a expression can be regarded as 'priming' a cell for apoptosis and suppression of such enhanced expression as execution (Naora et al., 1999). A causal relationship between the suppression of enhanced RPS3a expression and apoptotic induction has been strongly implicated that apoptosis could be induced by incubation with RPS3a antisense oligomers and by transient expression of RPS3a antisense sequences in cell lines (Naora et al., 1996, 1998).

Song et al. (2002) have demonstrated that S3a acts as a bridge protein to mediate the interaction between Bcl-2 and PARP (apoptosis regulator poly (ADP-ribose) polymerase) and that Bcl-2 facilitates the inhibition of PARP activity by S3a. Since Bcl-2 failed to inhibit PARP activity in the absence of S3a, he suggested that Bcl-2 together with S3a prevents apoptosis probably by inhibiting PARP activity. Kashuba (2005) observed a clear induction of Fte-1/S3a in freshly EBV-infected cells. The Fte-1/S3a induction by EBV was observed also by immunostaining. Fte-1/S3a levels were increased dramatically in the EBV-infected, EBNA-5-positive cells.

Cell apoptosis is not only self-defence mechanism but also an important resistant mechanism of insect to virus infection. The defence mechanism to virus infection is to confine the virus infection and replication by cell self-destruction apoptosis. It is also a survival method in order to preserve individual and variety (Clem et al., 1991; Koyama et al., 1998; Manji et al., 2001; Pei et al., 2002). It is reasonable to note that silkworm body performs self-defence resulting in cells apoptosis due to BmNPV infection. Experimental results showed that $C18_{609}$ expression in resistance variety and near-isogenic line (with heredity background of 99.9% comparable with susceptible variety, but differently expressing the resistant gene) is higher than in susceptible 306 strain, and $C18_{609}$ show higher expression when induced by BmNPV. So it is conceived that Bms3a is related to silkworm apoptosis and caused some level of resistance to virus infection.

Our study showed that *Bms3a* gene was differentially expressed in NB, 306 and 306NNZZ. It is proved that the expression was especially active in strain NB infected by BmNPV; contrarily the expression level was low in strains 306. Therefore, it is conceivable that Bms3a is involved in silkworm BmNPV resistance.

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