Int. J. Indust. Entomol. Vol. 13, No. 1, 2006, pp. 31~35

International Journal of *Industrial Entomology*

BmNPV Infection Enhances Ubiquitin-conjugating Enzyme E2 Expression in the Midgut of BmNPV Susceptible Silkworm Strain

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(Received 23 May 2006; Accepted 31 August 2006)

The ubiquitin conjugating enzyme 2 (E2) is core component of ubiquitin proteasome pathway (UPP) which represents a selective mechanism for intracellular proteolysis in eukaryotic cells. The E2 has been implicated in the intracellular transfer of ubiquitin to target protein. We show here the involvement of E2 in antiviral immune of Bombyx mori to *Bombyx mori* nuclear polyhedrosis virus (BmNPV). In this study, mRNA fluorescent differential display PCR (FDD-PCR) was performed with BmNPV highly resistant silkworm strain NB and susceptible silkwrm strain 306. At 24 h post BmNPV infection, FDD-PCR with the arbitrary primer AP34 showed that one cDNA band was downregulated in the midgut of resistant strain, but highly expressed in susceptible strain. The deduced amino acid sequence of this cDNA clone share 99% identity with the recently published *B.mori* ubiquitin conjugating enzyme E2 (Genbank NO: DQ311351). Fluorescent quantitative PCR corroborated downregulation of E2 in resistant strain. We there conclude that BmNPV infection evokes strong response of susceptible strain including activation of UPP. BmNPV may evolve escape mechanisms that manipulate the UPP in order to persist in the infected host. In addtion, the identification of downregulation of E2 in resistant strain, as well as structure data, are essential to understanding how UPP operates in silkworm antiviral immune to BmNPV disease.

Key words: *Bombyx mori*, Resistant, BmNPV, Ubiquitin conjugating enzyme, Fluorescent differential display

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Introduction

Bombyx mori is one of the most important economic insect. But it is unfortunate that the cultivation of silkworm has been seriously affected due to the outbreak of *Bombyx mori* nuclear polyhedrosis virus (BmNPV) disease, one of the most prevalent of insect virus disease. The lack of effective therapeutics for BmNPV-caused viral diseases underscores the importance of searching for antiviral compounds. Main sericiculture countrys focuses their attention on controlling this disease. Additionally, silkworm and BmNPV are good model organism to study interaction between insect and virus. So the understanding of the molecular responses and defense mechanisms in silkworm against invading BmNPV also facilitate the control of agricultural and forestry pest. To date, some antiviral molecules have been reported in the gut juice of silkworm (Nakazawa *et al.*, 2004; Ponnuvel *et al.*, 2003). However, many molecular events associated with virus infection process are still not well understood. Much interaction between baculoviridae and host remains to be found.

Comparison of gene expression profiles in silkworm lines exposed to BmNPV can provide information on the molecular basis of silkworm antiviral immune. In former research, a silkworm strain NB that exhibits high resistance to BmNPV disease was screened from Chinese silkworm library (Chen *et al.*, 1996). By using fluorescent differential display technique (FDD-PCR), we had isolated two genes related to silkworm resistance to NPV disease from silkworm NB, *Bms3a* and *Bmsop2* (Xu *et al.*, 2005a, 2005b). BmNPV resistance is complex immune process involving many physiological regulation. Obviously, it is likely that a number of other so far unknown molecules and genes will play an important role in BmNPV resistance. So further searching for BmNPV infection-related genes remains to be done.

Ubiquitin conjugating enzyme (E2) is key enzyme of the

ubiquitin-proteasome proteolytic pathway. As ubiquitin carrier, E2 mediate transfer of ubiquitin to the target protein. Here, we report our findings that expression of E2 decreased in the midgut of silkworm strain NB as compared with highly susceptible silkworm 306 at 24 h post BmNPV infection.

Materials and Methods

Baculovirus and animals

Highly resistant silkworm strain NB, highly susceptible silkworm strain 306 was used in this experiment. All larva of newly metamorphosed 5th instar were orally administered with same dose of BmNPV (1×10^6 BmNPVs/larvae), which is a local isolate (strain T3). They were reared on mulberry leaves at 27°C. 50 larva for each strain were dissected at 24 h post infection to collect midgut, and immediately placed in liquid nitrogen until use.

RNA extraction and cDNA synthesis

Total RNAs were obtained from NB and 306 respectively, using Trizol (Invitrogen) reagent. 2 μg of each RNA was reverse transcribed with 200 units MMLV reverse transcriptase (promega) in the presence of 50 μM 3'-anchored oligo (dT)18 primer in 20 μl RT buffer (1 × PCR buffer, 2.5 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP), at 65°C for 5 min, followed by cooling to 37°C for 60 min. Reverse transcriptase was inactivated at 75°C for 5 min.

Fluorescent differential display

For FDD-PCR (GenHunter, RNAspectra Kit), PCR amplification was carried out using 2 μl of the cDNA, primed with mixed fluorescein isothiocyanate labeled 3'-anchored oligo-T11A primer and a 10-mer arbitrary primer. The cycle parameters were 94°C for 1 min, and then 40 cycles of 94°C for 30s, 40°C for 2 min, 72°C for 2 min. A final extension was carried out at 72°C for 5 min. The resultant products were run on a 6% polyacrylamide gel electrophoresis and FDD gel was scanned on the FMBIO II (Hitachii Genetic System). Differentially upregulated band bands were excised from the gel. Reamplified cDNA product was cloned into T-vectors (Takara), followed by α-complementation clone selection and sequencing (CEQ8000, Beckman Company).

BLAST Searching of *B. mori* ESTand Genomic database

cDNA fragments were used as a probe to search the *B. mori* EST and Genomic database for homologous clone. Overlapped DNA sequences were assembled using the SeqMan II sequence analysis package (DNAstar 6.0).

Quantitative real-time RT-PCR analysis

The primers were designed according to the sequences of *Bombyx mori* Ubc E2 (Genbank NO: DQ311351). The sense primer for E2 was 5'-TACAGAACGGCGACATTGATCT-3' and the antisense primer, 5'-ATTAGCCTGTAGCCACCTCACC-3'. *B. mori* housekeeping gene, *actin A3*, was meantime amplified as control with the same templates. The upstream/downstream primers of *actin A3* (Genbank NO: U49854) was 5'-GGATGTCCACGTCGCAC-3' and 5'-GCGCGGCTACTCGTTCACTACC-3'. Realtime PCR was carried out using the Mx3000PTM PCRinstrument (Stratagene). The amplifications were performed in a 96-well plate using a 20 μl reaction volume containing 10 μl of 2 × SYBR Green mix (Takara), 1 μl of 1:10 diluted cDNA from each pool as template. Final concentration of primers is 0.2 μM. The thermal profile was 94°C for 0.5 min followed by 40 cycles of denaturation (94°C, 20s), annealing (60°C, 30s) and extension (72°C, 30s). The specificity of PCR amplification was determined by constructing a melting curve after the PCR amplification. Melt curve analysis was performed over the range 55~95°C by monitoring SYBR Green fluorescence with 0.5°C increment. Each sample was conducted in triplicate. Sterile water was added in place of template as a negative control. The relative expression ratios of gene were calculated relative to the housekeeping gene, *B. mori actin A3*, using the equation by (Livak *et al.*, 2001) . where R is the relative expression ratio. Statistical analysis of differences between the groups was carried out using the t-test. P values of <0.05 were considered significant.

$$R = 2^{Ct\text{actin} - Ct\text{target gene}}$$

Analyses of deduced amino-acid sequence.

Translation into amino-acid sequence was performed with ExPASy Translate tool (http://www.expasy.ch/tools/dna.html). The multisequence alignment was performed by the CLUSTAL W program, using the following sequences: *D. melanogaster* (GenBank, AAM49914), H.sapiens (GenBank, AAH58924), *T. nigroviridis* (GenBank,CAF95616), *A. thaliana* (GenBank, AAM19897).

Results

Identification of EST Clone containing *B. mori* E2 Gene

For BmNPV infection, time-course studies revealed that significant difference of virus copies between strain NB and 306 were detected in the midgut after 24 h postinfection (data not shown). So gene expression profiles of midgut was analyzed at 24 hpi. After FDD-PCR was per-

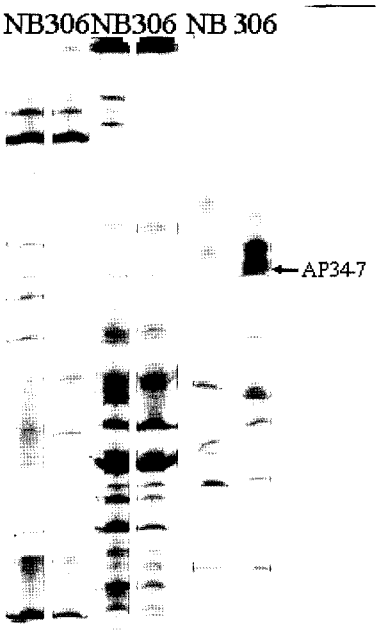

Fig. 1. mRNA fluorescent differential display of BmNPV resistant silkworm strain NB and BmNPV susceptible silkworm strain 306. Arrow indicates the recovered band. AP34-7.

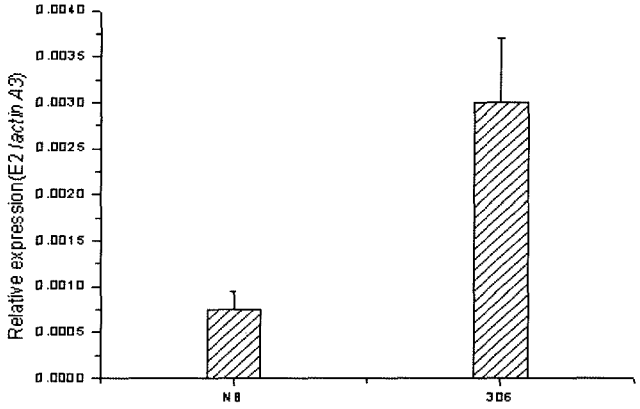

Fig. 2. E 2 expression in resistant strain and susceptible strain. The levels of each E2 mRNA and actin A3 weremeasured by real-time PCR in silkworm midgut. Each column and bar represent the average and S.E. of three amplification reactions. Each sample is normalized using actin A3 and ratios of E2 and actin A3 mRNA expression levels are shown. E2 mRNA of susceptible strain 306 was significantly higher than that of resistant strain NB ($P<0.05$).

formed, only those differentially expressed bands were further investigated. With the arbitrary primer AP34, One cDNA fragments of about 600 bp was found downregulated in strain NB at 24 h post BmNPV infection (Fig. 1). Databases searching showed that this cDNA exhibited a sequence similarity to *B. mori* EST clone CK534225 and CK487465. By assembling these nucleotide sequences, we found that it was 936 bp in length plus a polyadenylation. The deduced amino acid sequence of this DNA share 99% identity with the recently published *B. mori* ubiquitin conjugating enzyme E2.

Analysis of ubiquitin conjugating enzyme gene by Real-time PCR

To comfirm that the results from differential display, we carried out fluorescent Real-time PCR assay. The more relible real-time PCR results showed that the level of mRNA expression for E2 of strain 306 increased by 4 times over strain NB upon BmNPV challenge ($P<0.05$), similar to the FDD-PCR result (Fig. 2). It appears that the E2 gene of highly susceptible silkworm 306 responded more strongly to the virus invasion than that of strain NB at the early phase of infection.

Identification of deduced amino-acid sequence

To confirm further that the newly identified EST clone belongs to the silkworm homolog of human E2, the deduced amino acid sequence of this clone was used to compare E2 derived from various species. As shown in Fig. 3, E2s from different species shows considerable identity within the E2 core. The region around Cys^{111} of *B. mori* E2, a active-site, shows extremely high conservation across species without any large insertion or deletions. Based on this fraction of sequence conservation, we expect that *B. mori* E2 will have similar tertiary structure to other E2s. Much structure work has been performed on E2 protein. E2 is an α/β protein that β-sheet is established as core domain with flanking α-helix. E2 structure appears to be relatively inflexible since few amino acide changes are observed among various E2s. Taken together, we designate this differentially expressed gene as E2 of *B. mori*.

Discussion

Over 25 years ago, it is well known that the ubiquitin proteasome pathway (UPP) can tag abnormal or short half-life protein for selective degradation in eukaryotic cells. Activation of UPP involves the sequential activity of enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin-protein ligase (E3) and 26S proteasome. As core enzyme of UPP, E2 facilitate transfer of the ubiquitin moiety to substrate proteins. Although many E2s have been identified, catalytic motif of E2 is highly conserved. Active-site Cys and neighbouring residues forms a shallow groove and links ubiquitin by thioester bond (Fig. 4). Highly conserved ubiquitin protein has six lysine residues. The canonical ubiquitin

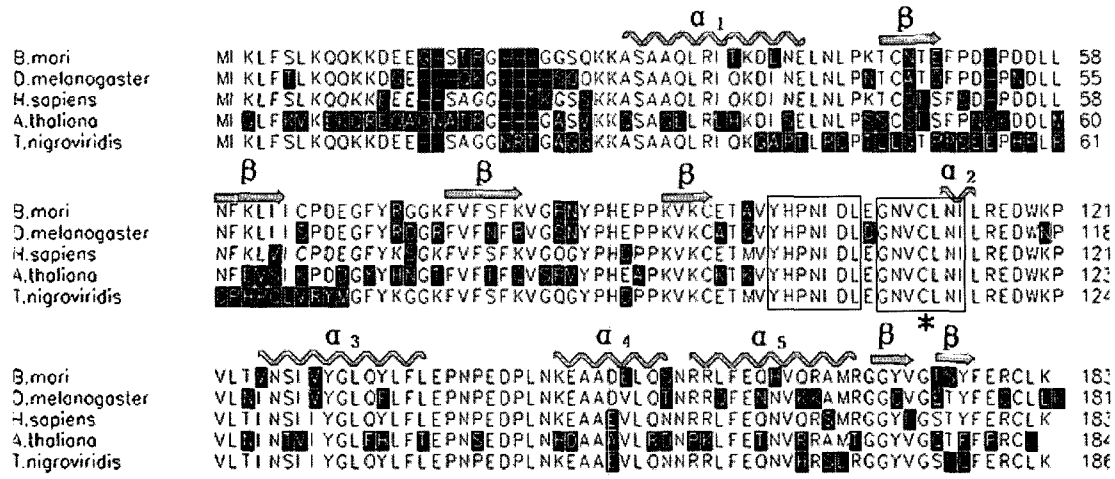

Fig. 3. Amino acid sequences of *B. mori* E2, *D. melanogaster* E2, *H. sopiens* E2, *A. thaliana* E2 and *T. nigroviridis* E. Secondary structure assignments based on the E2 structure are noted above the residues. The active-site cysteine in each sequence corresponds to Cys^{111} in *B. mori.* The conserved sequence and active-site Cys are indicated by box and asterisk respectively.

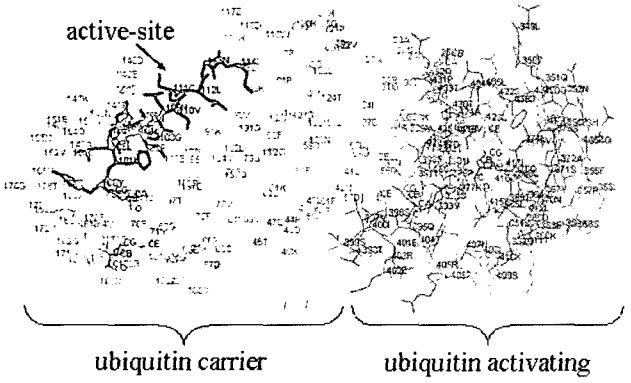

Fig. 4. Wireframe drawings of *H. sopiens* E2. *H. sopiens* E2 comprises two parts: ubiquitin carrier and ubiquitin activating. Red residue Cys^{111} is active catalytic site. Blue wireframe is conserved sequence around Cys^{111}. The conserved motif forms a shallow groove and links ubiquitin by thioester bond. This figure was prepare using the program WebMol.

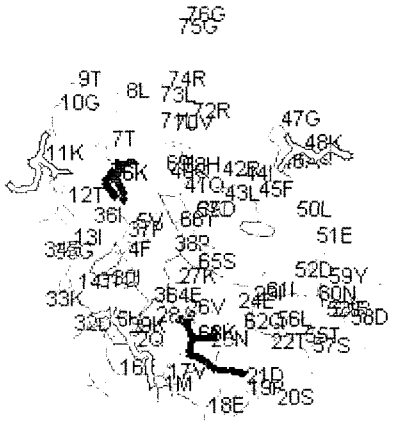

Fig. 5. Wireframe drawings of *H. sopiens* ubiquitin. Ubiquitins are high conserved across various species. Five lysine function as a linkage for poly-ubiquitin chains. Three residues of these (Lys^{11}, Lys^{29} and Lys^{48}) are indicated by red. Black residues indicates another two Lys, Lys^{6} and Lys^{63} respectively.

signal, Lys^{11}, Lys^{29} and Lys^{48}-linked poly-ubiquitin chains may target proteins to degradation by the 26S proteasome (Baboshina *et al.*, 1996). On the other hand, Lys^{6} and Lys^{63}-linked poly-ubiquitin chain suggest a nonproteolytic role in the direct modification of the structure and function of proteins (Schnel *et al.*, 2003) (Fig. 5).

Recent studies on the UPP have suggested that UPP plays a fundamental role in immune response and antigen presentation. By selective protein degradation, proteasomes regulate MHC class I antigen processing and present viral antigenic peptides to the immune system (Sijts *et al.*, 2001). Many viruses developed escape mechanisms that manipulate the ubiquitin-proteasome system in order to persist in the infected host. For example, nuclear antigen 1 encoded by Epstein-Barr virus contains Gly-Ala repeat domain which may affect MHC I restricted responses by inhibiting antigen processing via the ubiquitin-proteasome pathway (Levitskaya *et al.*, 1997). APOBEC3G, a cytidine deaminase, can induce G to A hypermutation in newly synthesized viral DNA. The Vif protein of HIV-1 counteracts the antiviral activity of APOBEC3G by targeting it for destruction by the ubiquitin-proteasome pathway and establish HIV infection (Mehle *et al.*, 2004). Meanwhile, the cellular ubiquitin-

proteasome machinery is involved in the budding process of enveloped virus such as VSV and RV. Inhibition of ubiquitination of virus protein can remarkably infect viral titers (Harty *et al.*, 2001). Additionally, ubiquitylation also regulate other activity such as transcription of viral gene, chromatin remodeling and virus replication. So many evidences indicate that UPP plays a role in the life cycle of virus.

Besides utilizing host UPP, some virus can encode ubiquitin and ubiquitin-related enzyme. So far, african swine fever virus (ASFV) is only known virus that can encode E2 to catalyze ubiquitylation of itself protein (Hayakawa *et al.*, 1999). In addition, AcMNPV and BmNPV can encode ubiquitin homology which show high similarity to host ubiquitin. BmNPV IE2 has ubiquitin ligase activity that is dependent on the RING finger domain (Imai *et al.*, 2003). Previous study has shown that IE2 is involved in the formation of nuclear foci in host cells (Krappa *et al.*, 1995). At these foci, IE2 ubiquitylates its substrate to support infection of NPV and then is gradually removed by autoubiquitylation and the proteasomal degradation system (Imai *et al.*, 2005).

Although the functions of this up-regulated protein in susceptible strain can be speculated, the exact natures and its relation to the immune response remains to be further investigated. Regardless, identification and elucidation of this gene provided an insight into the molecular basis for BmNPV disease. Meanwhile, the structure predicted here may therefore contribute to successfully design novel drug for controling the outbreak of BmNPV disease.

Acknowledgements

This project was surported by the Program of China under grant (NO.2005CB121000).

References

Baboshina, O. V. and A. L. Haas (1996) Novel multiubiquitin chain linkages catalyzed by the conjugating enzymes E2-EPF and RAD6 are recognized by 26 S proteasome subunit 5. *J. Biol. Chem.* **271**, 2823-2831.

Chen, K.P., C.Q. Lin and Q. Yao (1996) Studies on the resistance to NPV and its heredity regularity in the silkworm (*Bombyx mori* L.). *Acta Sericologica Sinica.* **22**, 160-164.

Harty, R.N., M.E. Brown, J.P. McGettigan, G.L. Wang, H.R. Jayakar, J.M. Huibregtse, M.A. Whitt and M.J. Schnell (2001) Rhabdoviruses and the cellular ubiquitin-proteasome system: A budding interaction. *J. Virology.* **75**, 10623-10629.

Hayakawa, T., R. Ko, K. Okano, S.I. Seong, C. Goto and S. Maeda (1999) Sequence analysis of the xestia c-nigrum granulovirus genome. *Virology.* **262**, 277-297.

Imai, N., N. Matsuda, K. Tanaka, A. Nakano, S. Matsumoto and W. Kang (2003) Ubiquitin ligase activities of *Bombyx mori* nucleopolyhedrovirus RING finger proteins. *J. Virol.* **77**, 923-930.

Imai, N., S. Matsumoto and W.K. Kang (2005) Formation of *Bombyx mori* nucleopolyhedrovirus IE2 nuclear foci is regulated by the functional domains for oligomerization and ubiquitin ligase activity. *J. General Virology.* **86**, 637-644.

Krappa, R., R. Roncarati and D. Knebel-Morsdorf (1995) Expression of PE38 and IE2, viral members of the C3HC4 finger family, during baculovirus infection: PE38 and IE2 localize to distinct nuclear regions. *J. Virol.* **69**, 5287-5293.

Levitskaya, J., A. Sharipo, A. Leonchiks, A. Ciechanover and M. Masucci (1997) Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. *Proc Natl Acad Sci.* USA. **94**(23), 12616-12621.

Livak, K.J. (2001) ABI Prism 7700 Sequence Detection System. User bulletin 2, PE Applied Biosystems.

Mehle, A., B. Strack, P. Ancuta and C. Zhang (2004) Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. *J. Biol. Chem.* **279**(9), 7792-7798.

Nakazawa, H., E. Tsuneishi, K.M. Ponnuvel, S. Furukawa, A. Asaoka, H. Tanaka, J. Ishibashi and M. Yamakawa (2004) Antiviral activity of a serine protease from the digestive juice of *Bombyx mori* larvae against nucleopolyhedrovirus. *Virology.* **321**(1), 154-162.

Ponnuvel, K.M., H. Nakazawa, S. Furukawa, A. Asaoka, J. Ishibashi, H. Tanaka and M. Yamakawa (2003) A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus. *J. Virol.* **77**(19), 10725-10729.

Schnell, J. D. and L. Hicke (2003) Non-traditional functions of ubiquitin and ubiquitin-binding proteins. *J. Biol. Chem.* **278**, 35857-35860.

Sijts, A., D. Zaiss and P. M. Kloetzel (2001) The role of the ubiquitin proteasome pathway in MHC class I antigen processing: implications for vaccine design. *Curr. Mol. Med.* **1**(6), 665-76.

Wang, H. W., Z. F. Zhang, Q. L. Xiao, W. G. Li and J. He (2001) LInsect juvenile hormone enhancing gene expression in silkworm baculovirus vector system. *Sheng Wu Gong Cheng Xue Bao.* **17**(5), 590-593.

Xu, J. P., K.P. Chen, Q.Yao, M.H. Liu, G.T. Gao and Y. Zhao (2005a) Identification and characterization of an NPV infection-related gene *Bmsop2* in *Bombyx mori* L. *J. Appl. Entomol.* **129**(8), 425-431.

Xu, J.P., K.P. Chen, Q. Yao, Q.G. Xu, X.Y. Liu and G.T. Gao (2005b) Fluorescent diferential display analysis of the gene s 3 a related to NPV resistance in *Bombyx mori* L. *Acta Entomologica Sinica.* **48**(3), 347-352.