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Full Length Research Paper

Cloning and characterization of the densoviruses susceptible gene +$^{nsd-2}$ in the silkworm, *Bombyx mori*

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Recently, a silkworm gene responsible for the susceptibility to *Bm*DNV-2 (a parvo-like virus) has been discovered and designated as +$^{nsd-2}$, which encodes a 12-pass transmembrane protein. *Bm*DNV-Z, isolated in Zhenjiang of China, has a high homology with *Bm*DNV-2 in serological characteristics and genome structure. However, it is still uncertain whether +$^{nsd-2}$ is also responsible for susceptibility to *Bm*DNV-Z. In this study, we cloned +$^{nsd-2}$ gene from Chinese silkworm strain (HuaBa35) that is susceptible to *Bm*DNV-Z. DNA sequencing confirmed that +$^{nsd-2}$ from HuaBa35 is the same with that from NO.908 susceptible to *Bm*DNV-2. RT-PCR analysis showed that the gene +$^{nsd-2}$ was only expressed in larval midgut and widely expressed in every instar of larva. Bioinformatic study showed that +$^{nsd-2}$ is a putative amino acid transporter with three glycosylation sites and located on chromosome 11. In addition, +$^{nsd-2}$ gene was also expressed by baculovirus expression system in Sf9 cell. Our analysis will contribute to the detailed investigation on the infection mechanism of *Bm*DNV.

Key words: *Bm*DNV-Z, +$^{nsd-2}$, RT-PCR, transporter protein, baculovirus expression.

INTRODUCTION

Densoviruses (DNV) are autonomously replicating parvoviruses that are highly pathogenic for invertebrates and are commonly isolated from arthropod hosts (Bergoin and Tijssen, 2000). *Bombyx mori* densoviruses (*Bm*DNVs) multiply only in the nuclei of the columnar cells of the larval midgut epithelium (Seki and Iwashita, 1983), and was isolated from several regions, such as Ina isolate, Saku isolate, Yamanashi isolate and Zhenjiang isolate (China). Based on their serological characteristics and genome structure, Ina isolate belongs to the *Bm*DNV I type, whereas the Saku, Yamanashi isolate and China Zhengjiang isolate belong to *Bm*DNV II type. Previously, both Yamanashi isolate named *Bm*DNV-2 and Zhenjiang isolate named *Bm*DNV-Z had been assigned to Densovirinae in Parvoviridae. Recently, these two isolates were excluded from the family Parvoviridae. In contrast to the previously accepted character for the group, their genome is split into two molecules and contains its own DNA polymerase motif (Tattersall et al., 2005).

The DNV genome consists of a single-stranded linear DNA that is 4 – 6 kb in length (Tijssen and Bergoin, 1995; Bergoin and Tijssen, 2000). The most obvious characteristic of *Bm*DNV-2 is that it has 2 sets of DNA molecules (VD1, VD2) and each of them is encapsulated, respectively, in the form of single-stranded liner DNA. Furthermore, the genome of *Bm*DNV-2 is able to encode DNA polymerase itself (Bando et al., 1992, 1995; Hayakawa et al., 2000). Our group had completed the DNA sequencing for *Bm*DNV-Z in 2005. Comparing the genome sequence for *Bm*DNV-Z and *Bm*DNV-2, the homology for VD1 is 98.4%, VD2 is 97.7%, with about 228 bp substitutions, 11 bp deletion and 3 bp insertions in *Bm*DNV-Z (Wang et al., 2007).

Previous studies have shown that certain genes are responsible for non-susceptibility to *Bm*DNV. A dominant *Nid-1* gene control non-infection to *Bm*DNV-1 (Eguchi et al., 1986) and a recessive *nsd-1* gene control non-

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Abbreviations: DNV, Densoviruses; **BmDNVs,** bombyx mori densoviruses; **PCR,** polymerase chain reaction; **MCS,** multiple cloning sites; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **LB,** Lysogeny broth; **HTTM,** horizontally transferred transmembrane domain; **RFLP,** restriction fragment length polymorphism.

susceptibility to *Bm*DNV-1 (Eguchi et al., 1991). The resistance expressed in some silkworm strains against *Bm*DNV-2 is determined by a single gene, *nsd-2*, which is characterized as non-susceptibility irrespective of the viral dose (Seki, 1984). A linkage map of 30.6 cM was constructed for linkage group 17 with *nsd-2* mapped at 24.5 cM and three closely linked cDNA markers were also identified (Ogoyi, 2003).

Non-susceptibility to *Bm*DNV-Z was also found under the control of a recessive gene (*nsd-Z*). The *nsd-Z* gene was located on genetic linkage 15 (Qin and Yi, 1996). Li have constructed a SSR linkage map of 80.6 cM, with *nsd-Z* mapped at 30 cM and the closest SSR marker at a distance of 4.4 cM (Li, 2006). The standard linkage group corresponding to *nsd-Z* has not been identified yet.

Recently, Kidokoro has isolated *nsd-2* gene by positional cloning. The resistance to *Bm*DNV-2 in silkworm is caused by a 6-kb deletion in $+^{nsd-2}$ gene encoding a 12-pass transmembrane protein, which is predicted to be a member of an amino acid transporter family. Germ-line transformation with a wild type gene expressed in the mutant midgut restores susceptibility, showing that the defective membrane protein is responsible for resistance (Kidokoro et al., 2008). Their research showed that the complete membrane protein may be a receptor for *Bm*DNV-2.

Based on the high homology of *Bm*DNV-Z and *Bm*DNV-2 in serological characteristics and genome structure, we suspect that *Bm*DNV-Z and *Bm*DNV-2 might enjoy the same receptor. To confirm this hypothesis, we firstly cloned $+^{nsd-2}$ gene from silkworm strain HuaBa35, a susceptible silkworm to *Bm*DNV-Z. Transcription of $+^{nsd-2}$ gene were detected in different tissues and different developmental stages of the silkworm. Meanwhile, we carried out the bioinformatic analysis and expressed it in eukaryotic system.

MATERIALS AND METHODS

Animals and regents

Larvae of the silkworm (HusBa35) were reared with mulberry leaves at 25℃. Tissues were collected from midgut, Malpighian tube, ovary, silk gland, hemocyte, testis and fat body. Silkworms from different developmental stages (egg, larva and pupa) were also used. The RNAs were isolated from whole body of first and second-instar larva, midgut of third to fifth-instar. Total RNAs were extracted using Trizol reagent (Invitrogen). Restrictases, T4 DNA ligase, PCR reagents and pMD19-T were obtained from TaKaRa Company (China, Dalian); primer and other reagents were obtained from Shanghai Sangon Bio-technology Corporation. FITC-conjugated goat anti-rabbit IgG were from Sigma (USA).

Bioinformatic analysis of $+^{nsd-2}$

The full length of $+^{nsd-2}$ gene sequence was got from NCBI under the accession number AB365597. To search for sequence homology, a protein–protein basic local alignment tool (blastp) was used to search the NCBI protein database (http://www.ncbi._nlm. nih.gov/BLAST/). Function domain analysis was performed using the EXPASY web server (http://au.expasy.org/). ExPASy Translate tool (http://au. expasy. org/tools/dna. html) was used to identify glycosylation sites and Silkworm Genome Database (SilkDB) (http:// silkworm.genomics.org.cn/) for the gene location in chromosome.

RT-PCR analysis of $+^{nsd-2}$ gene expression

Total RNA was isolated from ~0.5 g sample tissues using RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). The extracted RNA was treated with RNase-free DNase (Promega, Madison, WI, USA) for first strand synthesis of cDNA using the SuperScript II™ reverse transcriptase (Promega) according to the manufacturer's instruction. To study the gene expression in different tissues and different developmental stages of silkworm, RT-PCR was carried out using SuperScript™ III One Step RT-PCR System with Platinums Taq DNA polymerase (Invitrogen).

The $+^{nsd-2}$ gene was amplified using a forward primer (5' - CGGATCCATGGATTCAAATGGGATAAATG- 3') containing a *BamH* □ restriction site (underlined) and a reverse primer (5'-CACTC GAGCCGCTTCCTGCGATACC- 3') containing a *Xho*I restriction site (underlined). PCR reactions were carried out with Taq polymerase for 36 amplification cycles (94℃ /30 s, 61℃/30 s, 72℃ /95 s), and was examined by electrphoresis in 1.2% agarose gel with the ethidium bromide staining.

Construction of expression plasmid

The PCR product was ligated into pMD19-T vector using T4 DNA ligase and *Escherichia coli* (DH5α strain) was used for plasmid amplification, and MiniBEST Plasmid Purification Kit (Takara) was used for purification. The DNA sequencing was performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.

For the $+^{nsd-2}$ gene expression, the pFasBacHTb baculovirus transfer vector was used. The fragment obtained above was inserted between *Bamhl* and *Xho*l sites in the multiple cloning sites (MCS). The plasmid pFasBacHTb-$+^{nsd-2}$ was used to transform competent DH10B cells containing helper and *Ac*MNPV bacmid. The transformed cells were added to 800 microlitres of SOC and incubated at 37℃ for 4 h and then were cultured on LB agar plates containing gentamicin, kanamycin, tetracycline, IPTG and X-Gal at 37℃ overnight. After two rounds of color selection, white colonies containing $+^{nsd-2}$ gene were obtained and inoculated into 3 ml of LB medium. Recombinant DNA was isolated and confirmed by PCR using pUC/M13 primers.

Expression of $+^{nsd-2}$ in Sf-9 cells

The Bac-to-Bac system was used to express $+^{nsd-2}$ gene. Recombinant AcMNPV bacmid-$+^{nsd-2}$ and wild type bacmid were transfected into Sf-9 cells, respectively. TC-100 insect medium was supplemented with 10% (v/v) fetal bovine serum (Gibco, USA). Cells were harvested at 72 h post-infection of recombinant virus. Then the cells were pelleted and resuspended in phosphate buffered saline (PBS, pH 7.4), lysed in SDS–polyacrylamide loading buffer and analyzed by SDS–PAGE.

After electrophoresis, proteins were transferred onto polyvinylidene fluoride membrane (Millipore). The membrane was blocked in 5% skimmed milk powder in PBST for 3 h followed by incubation with the anti-His tag antibody at a dilution factor of 1:500 for 1.5 h at room temperature. Subsequently, the membrane was incubated with peroxidase-conjugated goat anti-mouse IgG diluted 1:5000 for 1.5 h at room temperature. The signal was detected with a DAB substrate solution.

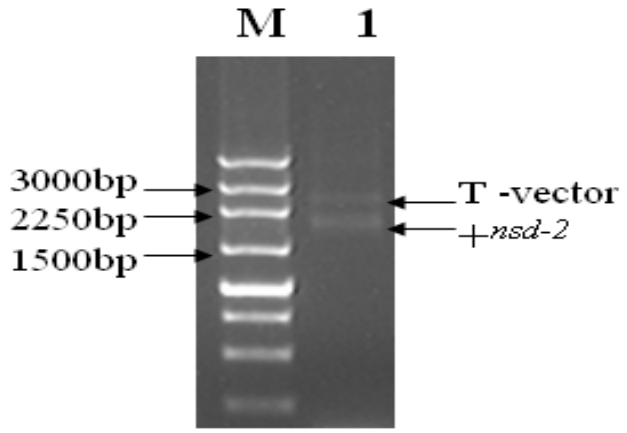

Figure 1. Identification of the constructed plasmid pMD19-T/+$^{nsd-2}$. Lane M, DNA maker; Lane 1: pMD19-T/+$^{nsd-2}$ digested with *BamH*I and *Xho*I generated two fragments: pMD19-T (2.7 Kbp) and +$^{nsd-2}$ (1900 bp).

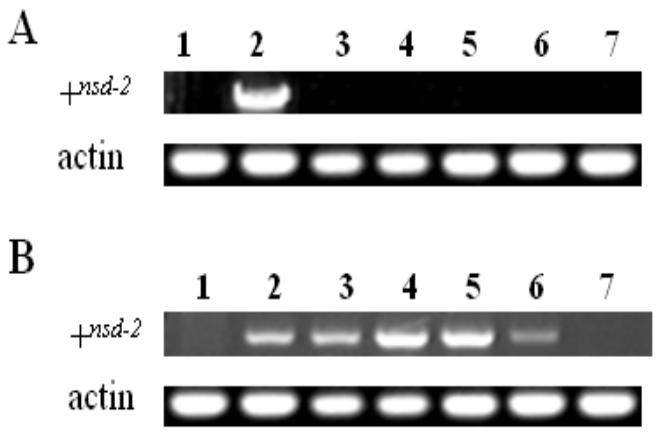

Figure 2. Expression profile of +$^{nsd-2}$ of by RT-PCR. (A) cDNAs isolated from different larval tissues of silkworm. Lane 1: Malpighian tube, Lane 2: midgut, Lane 3: ovary, Lane 4: silk gland, Lane 5: hemocyte, Lane 6: fat body and Lane 7: testis. A β-actin clone was used as the positive control. (B) cDNAs isolated from silkworm (HuaBa35) in different developmental stages. Lane 1: egg, Lane 2: first instar, Lane 3: second instar, Lane 4: third instar, Lane 5: fourth instar, Lane 6: fifth instar and Lane 7: pupa.

RESULTS

Cloning of +$^{nsd-2}$ gene from HuaBa

The PCR fragment from HuaBa susceptible to *Bm*DNV-Z was cloned into pMD19-T vector. Recombinant plasmid was verified by restriction enzyme digestion (*BamH I* and *Xho I*) (Figure 1, lane 1). The size of the PCR fragment is about 1900 bp. Sequencing of the PCR fragment showed that the number of nucleotides is 1878 bp and the PCR product from HuaBa is the same with that of +$^{nsd-2}$ from NO.908, which is susceptible to *Bm*DNV-2.

Transcription pattern of +$^{nsd-2}$ in different tissues and stages

The transcription of gene +$^{nsd-2}$ was detected by RT-PCR using cDNAs derived from total RNAs of midgut, Malpighian tube, ovary, silk gland, hemocyte, testis and fat body as templates, respectively, and β-actin mRNA was used as the positive control. The result showed that +$^{nsd-2}$ mRNAs existed only in larval midgut (Figure 2A). To compare the expression of +$^{nsd-2}$ in different stages, RNAs isolated from egg, larva (the first to fifth instar) and pupa were used for RT-PCR. The positive signals only existed in larva stage (Figure 2B).

Function domain and chromosome location of +$^{nsd-2}$ gene

The +$^{nsd-2}$ gene includes an ORF encoding 625 amino acid residues. The ORF of +$^{nsd-2}$ gene was predicted to encode a 11-pass transmembrane protein and belong to an amino acid transporter family (Figure 3). The domain analysis of +$^{nsd-2}$ showed it contains a HTTM (Horizontally Transferred TransMembrane Domain) domain. N-glycosylated and O-glycosylated residues were also predicted, which is at 155, 178, 520 site of the protein, respectively (Figure 3). SilkMap analysis in SilkDB showed that +$^{nsd-2}$ gene is located on chromosome 11 as a putative transporter.

Using BLAST software of NCBI to search for homology in the GenBank database, the protein of +$^{nsd-2}$ gene shows an identity of 50, 63, 48, 49, 50, 50, 37, 37 and 36%, respectively, to the corresponding genes of *Aedes aegypti* (XP_001659215.1), *Manduca sexta* (AAC24190), *Apis mellifera* (XP_001121238.1), *Drosophila melanogaster* (AAM51989.1), *Anopheles gambiae* (XP_309840.2), *Tribolium castaneum* (XP_973741.1), *Mus musculus* (AAN11408.1), *Homo sapiens* (AF142501_1) and *Xenopus laevis* (NP_001154864.1). Using GENEDOC to analyze these amino acid sequences, the result was shown in Figure 3. The sequence alignment reveales conservated regions (motifs) that may include the active-site residues of HTTM Domain. The phylogenetic tree reveals +$^{nsd-2}$ gene from silkworm has high homology with transporter from *M. sexta* (Figure 4).

Expression of +$^{nsd-2}$ gene by baculovirus in Sf-9 cells

The recombinant Ac-bacmid-+$^{nsd-2}$ was confirmed by PCR using pUC/M13 primers. Recombinant bacmid has 4.3 kb PCR product. Meanwhile, wild-type bacmid has 300 bp PCR products (Figure 5A).

To examine that 6×His-+$^{nsd-2}$ fusion protein is expressed in the +$^{nsd-2}$-recombinant virus infected cells, antibodies against His was used to perform western blot analysis. As shown in Figure 5B, SDS-PAGE and Western blot were

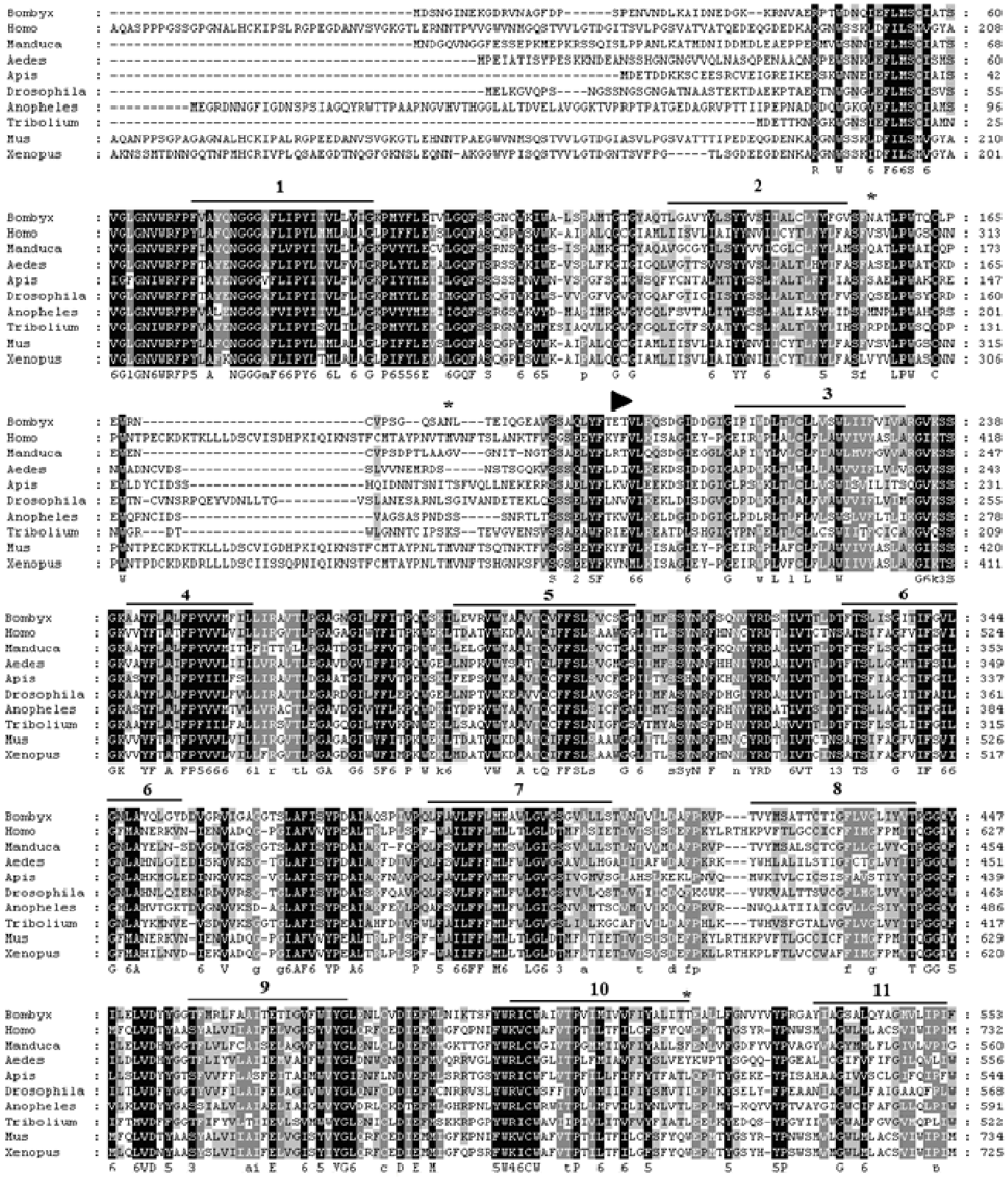

Figure 3. Transporter proteins sequence alignment. Multiple sequence alignment of amino acid transporter proteins from *A. aegypti* (XP_001659215.1), *M. sexta* (AAC24190), *A. mellifera* (XP_001121238.1), *D. melanogaster* (AAM51989.1), *A. gambiae* (XP_309840.2), *T. castaneum* (XP_973741.1), *M. musculus* (AAN11408.1), *H. sapiens* (AF142501_1), *X. laevis* (NP_001154864.1). Invariant residues are highlighted in black. Putative transmembrane domains are indicated with number 1 - 11. The deletion in *nsd-2* protein is beginning at black triangles to the end. Predicted N-glycosylated and O-glycosylated residues is indicated with the sign (*).

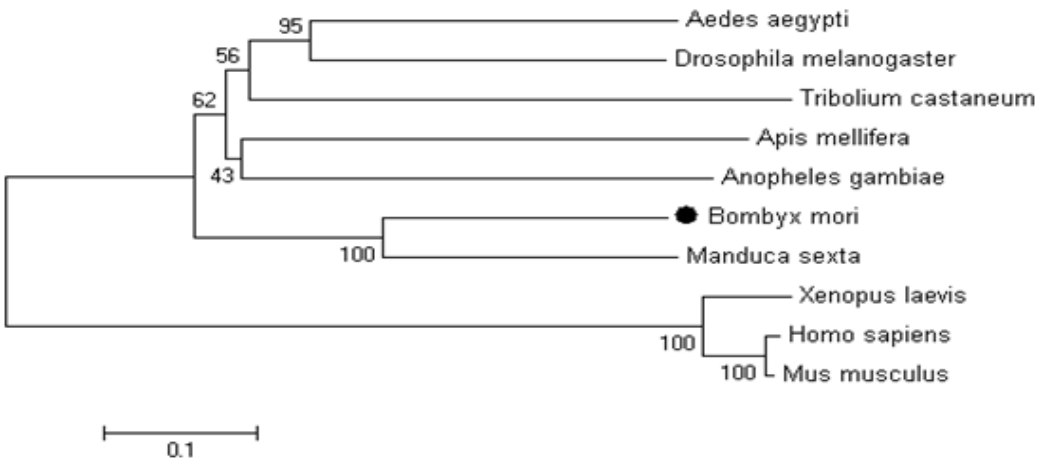

Figure 4. Phylogenetic analysis of transporter proteins NJ tree. Neighbour-joining phylogram showing the relationships between +$^{nsd-2}$ from *B. mori* and transporter proteins from other organisms. The tree distances were generated according to the ClustalW algorithm and the tree was constructed using MEGA 3.1.

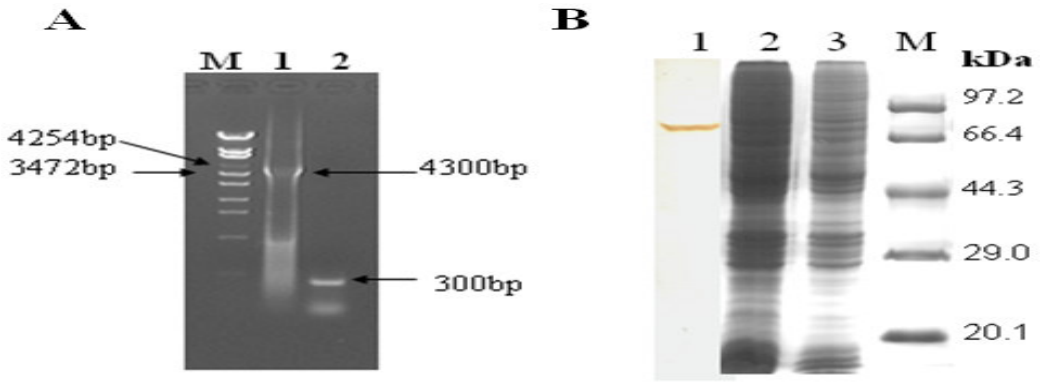

Figure 5. The expression of +$^{nsd-2}$ gene in Sf-9 cells. (A) PCR verification of recombinant baculovirus. M: DNA maker; Lane 1: 4.3 kb PCR product of recombinant bacmid; Lane 2: 300 bp PCR product of wild-type bacmid; (B) expression of +$^{nsd-2}$ gene in Sf9 cells. Cell extracts were separated by SDS-PAGE and Western blot was performed. Lane M: Protein molecular weight marker, Lane 1: Western blot using anti-His monoclonal antibody, Lane 2: proteins from Sf9 cells transformed with bacmid-+$^{nsd-2}$ plasmid and Lane 3: proteins from Sf9 cells transformed with wild bacmid plasmid.

performed to detect the expressed protein. Western blot indicated that the fusion protein was expressed with the size about 77 kDa.

DISCUSSION

Silkworm (*B. mori*) is an important economic insect and is regarded as a model insect of Lepidoptera. Studies on structures and functions of related genes in silkworm have attracted more and more attention.

The susceptibility to *Bm*DNV-2 in silkworm was certificated by gene +$^{nsd-2}$ encoding a 12-pass transmembrane protein with RFLP mapping and chromosome walking (Kidokoro et al., 2008). In this study, we analyzed the nucleotide sequences of +$^{nsd-2}$ gene cloned from HuaBa35 which is susceptible to *Bm*DNV-Z. The gene includes an ORF of 1878 bp encoding 625 amino acid residues. Nucleotide sequence comparison showed that there is no nucleotide difference between the PCR product from HuaBa and +$^{nsd-2}$ from silkworm strain NO908. The transcription pattern in different tissues and different developmental stages of silkworm HuaBa35 were analysed by RT-PCR. Our results revealed that +$^{nsd-2}$ gene was only expressed in larval midgut. It is not expressed in the stage of egg and pupa, but only expressed in the larva stage. Consistent with previous report, our data suggests +$^{nsd-2}$ may also be partially responsible for the *Bm*DNV-Z infection as receptor.

Previous researches have showed that *nsd-2* and *nsd-*

Z gene were located in molecular marker genetics linkage 17 and genetics 15, respectively. The position analysis of silkworm genome sequence showed that, $+^{nsd-2}$ gene located on chromosome 11 in this paper. The deduced amino acid sequences showed high identity to those from many other organisms, including protista to mammals, especially among the HTTM domain region. This indicates that transporter protein is conserved from invertebrates to mammals, which is also supported by the phylogenetic tree analysis.

It is still not clear how *Bm*DNV-2 interacts with the membrane protein. It would be great importance to determine the recognition sites of *Bm*DNV-2 and $+^{nsd-2}$. The cloning of $+^{nsd-2}$ gene and expression in baculovirus system will be very helpful for subsequent studies, such as characterization of the protein encoded by $+^{nsd-2}$ and identifiecation of the interacting proteins *in vivo* that may also be helpful for analysis of the infection mechanism in other densoviruses.

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