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Full Length Research Paper

Cloning and characterization of peptidylprolyl isomerase B in the silkworm, *Bombyx mori*

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Peptidylprolyl isomerases (PPIases) play essential roles in protein folding and are implicated in immune response and cell cycle control. Our previous proteomic analysis indicated that *Bombyx mori* PPIases may be involved in anti- *Bombyx mori* nucleopolyhedrovirus (BmNPV) response. To help investigate this mechanism, we cloned a *B. mori* PPIase gene PPIB and characterized it by bioinformatic and experimental analysis. We found that the *B. mori* PPIB gene contains 4 exons and its cDNA is about of 618 bp, encoding a protein of 205 amino acid residues (21474.41 Da) with an isoelectric point of 8.05. PPIB contains conserved and unique cyclophilin domain and belongs to cyclophilin superfamily. Its transcription could be detected by PCR in all the *B. mori* tissue samples, which is consistent with normal PPIase expression pattern and their essential roles. It is localized in cytoplasm revealed by fluorescence microscopy. We also successfully expressed this protein in *E. coli* and characterized it by SDS-PAGE and Mass Spectrometry. The cloned DNA sequence was submitted to GenBank (EU583493).

Key words: Bioinformatics, transcription analysis, prokaryotic expression, mass spectrometry, subcellular localization.

INTRODUCTION

Peptidylprolyl isomerases (PPIases) are ubiquitously expressed in prokaryotic and eukaryotic organisms (Mitra et al., 2006; Edvardsson et al., 2003; Yeh and Klesius, 2008). They can catalyze the *cis–trans* isomerization of the folding of newly synthesized proteins, which is a critical step in protein folding (Rahfeld et al., 1994; Fanghänel and Fischer, 2004). They also act as molecular chaperones *per se* or co-operate with other chaperones (Miele et al., 2003; Barik, 2006). Recently, an interesting discovery that phosphorylation-dependent prolyl *cis-trans* isomerization could cause significant and physiological conformational changes, which could further regulate many signal transduction pathways, greatly inspired the “Gold-Mining” in this field (Lippens et al., 2007; Isakov, 2008; Wulf et al., 2005). Thus, it is not surprising that they have been found to play essential roles in a variety of biological processes, including protein quality control, protein trafficking, immune system, virus infection/replication and Ca_2^+-mediated intracellular signaling (Göthel and Marahiel, 1999; Galat, 2003; Yao et al., 2005).

The PPIases are usually divided into three classes based on drug specificity: cyclosporin A-binding cyclophilins, FK 506- binding proteins (FKBPs) and parvulins (Göthel and Marahiel, 1999; Shaw, 2002; Galat, 2003; Kang et al., 2008). Cyclophilins were firstly discovered as receptors for cycloporin A (CsA), which is an undecapeptide produced by many fungi, such as *Tolypocladium inflatum*. Later on, cyclophilins were demonstrated to be the same proteins as PPIases. Cyclosporin A and FK 506 are immunosuppressants that are widely used in transplantation and they also have strong neuroprotective effects (Setkowicz and Ciarach, 2007). These drug-bound protein complexes can bind to phosphatase calcineurin and inhibit the transport to the nucleus of a cyto-

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Abbreviations: PPIase, Peptidylprolyl isomerase; **Cyps,** cyclophilins; **FKBPs,** FK 506 binding proteins; ***B. mori*,** *Bombyx mori*; **BmNPV,** *Bombyx mori* nucleopolyhedrovirus.

solic component of the transcription factor NF-AT in T cells (Miele et al., 2003), indicating a role in immune system. The third class of PPIases-parvulins are discovered later. They are not sensitive to immunosuppressants and have been shown to be involved in cell cycle progression (Rippmann et al., 2000). Recently, Pin1, one notable member of parvulins, has been intensively explored for its specific phosphorylation-dependent isomerase activity and is implicated in numerous cellular processes and pathogenesis, including mitosis control, chromosome condensation, immune reaction, tumorigenesis, Alzheimer's disease and asthma (Uchida et al., 2003; Xu et al., 2007; Esnault et al., 2008; Finn and Lu, 2008; Lu et al., 2007a, b; Tang and Liou, 2007; Shaw, 2002).

Cyclophilins (Cyp) can be divided into four groups, based on sequence similarity, subcellular localization and PPIase activity (Pelle et al., 2002). CypA is the most abundant and conserved cytosolic protein (Pelle et al., 2002); CypB and CypC are localized in ER (Denys et al., 1998; Page and Winter, 1999). CypD is an integral part of mitochondria permeability transition complex and has been isolated from the filarial (Page and Winter, 1999; Waldmeier et al., 2003). Interestingly, CypA and CypB have been demonstrated to be required for HIV-1 and human HCV virus infection (Luban et al., 1993; Yang et al., 2008; Watashia et al., 2005; Saphire et al., 2000), suggesting novel approaches to design anti-virus drugs. CypB have been reported to be isolated from milk (Mariller et al., 1996), yeast (Koser et al., 1990) and arthropod (Takaki et al., 1997), but up to date there is no report about silkworm CypB.

BmNPV is one of the major silkworm pathogens that can usually cause devastating consequence in sericulture. To facilitate the discovery of new approachs to control BmNPV infection, we have been interested in exploring the interaction mechanism between *Bombyx mori* and BmNPV, as well as cellular factors involved in this process. Our previous proteomic analysis showed that PPIase B (PPIB) expression was affected in *B. mori* strains with different levels of anti-BmNPV activity, suggesting a possible role for PPIB in immune response against virus invasion (unpublished data; Liu et al., 2008). The PPIB belongs to cyclophilin B (CypB) protein family and contains one cyclophilin domain (Tryon et al., 2007). As a first step towards understanding the role of PPIB in silkworm anti-BmNPV response, we started the cloning and characterization of *B. mori* PPIB.

In this paper, we firstly identified *in silicon* the PPIB gene from *B. mori* genome database and analyzed it by bioinformatics. We showed *B. mori* PPIB belongs to CypB family and expressed it in *E. coli*. We further characterized it by mass spectrometry, tissue transcription and subcellular localization analysis. Our data provided the first report about characterization of *B. mori* PPIB, which greatly facilitates the following investigation about its physiological functions and possible roles involved in host-virus interaction.

MATERIALS AND METHODS

Materials

The silkworm *B. mori* is inbred in our lab. All tissue samples were obtained from fifth instar larva of silkworm strain 306 and immediately used for RNA extraction. Restriction enzymes, T4 DNA ligase, PCR reagents and pMD18-T were obtained from TaKaRa Company (China, Dalian). Primers and other reagents were obtained from Shanghai Sangon Bio-technology Corporation.

Bioinformatic analysis

The DNAstar software was used to locate the open reading frame (ORF) for *B. mori* PPIB. Poly-A signal was predicted by POLYAH (http://www.softberry.com/cgi-bin/programs/promoter/polyah.pl). In order to assemble the genomic DNA sequence, we blasted the cDNA sequence against the contigs of *B. mori* genome in GenBank. Another silkworm cDNA database BGI (http://silkworm.genomics.org.cn/) was also used. Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/) was used to detect introns, exons and potential splicing sites. Homology-modeling was performed by SWISS-MODEL (http://swissmodel.expasy.org//SWISS-MODEL.html).

The *B. mori* PPIB protein sequence was deduced by ExPASy Translate tool (http://au. expasy. org/tools/dna. html) and similarity analysis was performed by GENEDOC. Phylogenetic tree was constructed by MEGA 3.1. PLOC (http://www.genome.jp/SIT/plocdir/) was used to predict the subcellular location and TMHMM (<http://www.cbs.dtu.dk/se rvices/TMHMM-2.0>) was used to predict the transmembrane structure.

PPIB cloning and transcription analysis

The primers were designed based on the sequence of *B. mori* PPIB ORF. The forward primer (5'GGGGATCCATGGGTACCCTTACAA TGGC 3') contains a BamH1 site and the reverse primer (5′ GGCTCGAGTTAGTGAGCACTCTCTTTTGTGAC 3′) contains a Xho1 site. The cDNA was synthesized by RT-PCR using *B. mori* blood-lymph RNA as template and then was used for amplification of PPIB ORF. PCR was carried out with Taq polymerase for 35 cycles (94°C/30 s, 58°C /30 s, 72°C /1 min). The PCR product was ligated into pMD18-T vector using T4 DNA ligase to construct pMD18-T/PPIB and then was transformed into *E. coli* (TG1) for plasmid preparation. The plasmid pMD18-T/PPIB was digested with BamH1 and Xho1 and the purified PPIB fragment was ligated into similarly digested pET30a vector to construct pET30-PPIB, which was transformed into *E. coli* BL21 (DE3) for protein expression.

To detect the tissue transcription of PPIB, PCR was performed using cDNA samples from various tissues, including testis, ovary, fat body, midgut, silk gland, embryo and tuba Malpighii. 1% Agarose gel electrophoresis and ethidium bromide staining were used to check the PCR products.

Protein expression in *E. coli*

pET30-PPIB was transformed into *E. coli* BL21(DE3) and a single colony was incubated in LB medium supplemented with 50 μg/ ml kanamycin for overnight at 37°C with shaking, then the starter culture was transferred into 100 mL fresh LB medium and cultured at 37°C with shaking. When A_{600} reached to about 0.6, the culture was induced with 8 μg/ ml IPTG (final concentration) at 27°C for 10 h. 12% SDS-PAGE and Mini-Protein System (Bio-Rad, USA) were used to analyze the expression of recombinant protein. The gel was stained with Coomassie Brilliant Blue R250 to visualize the protein

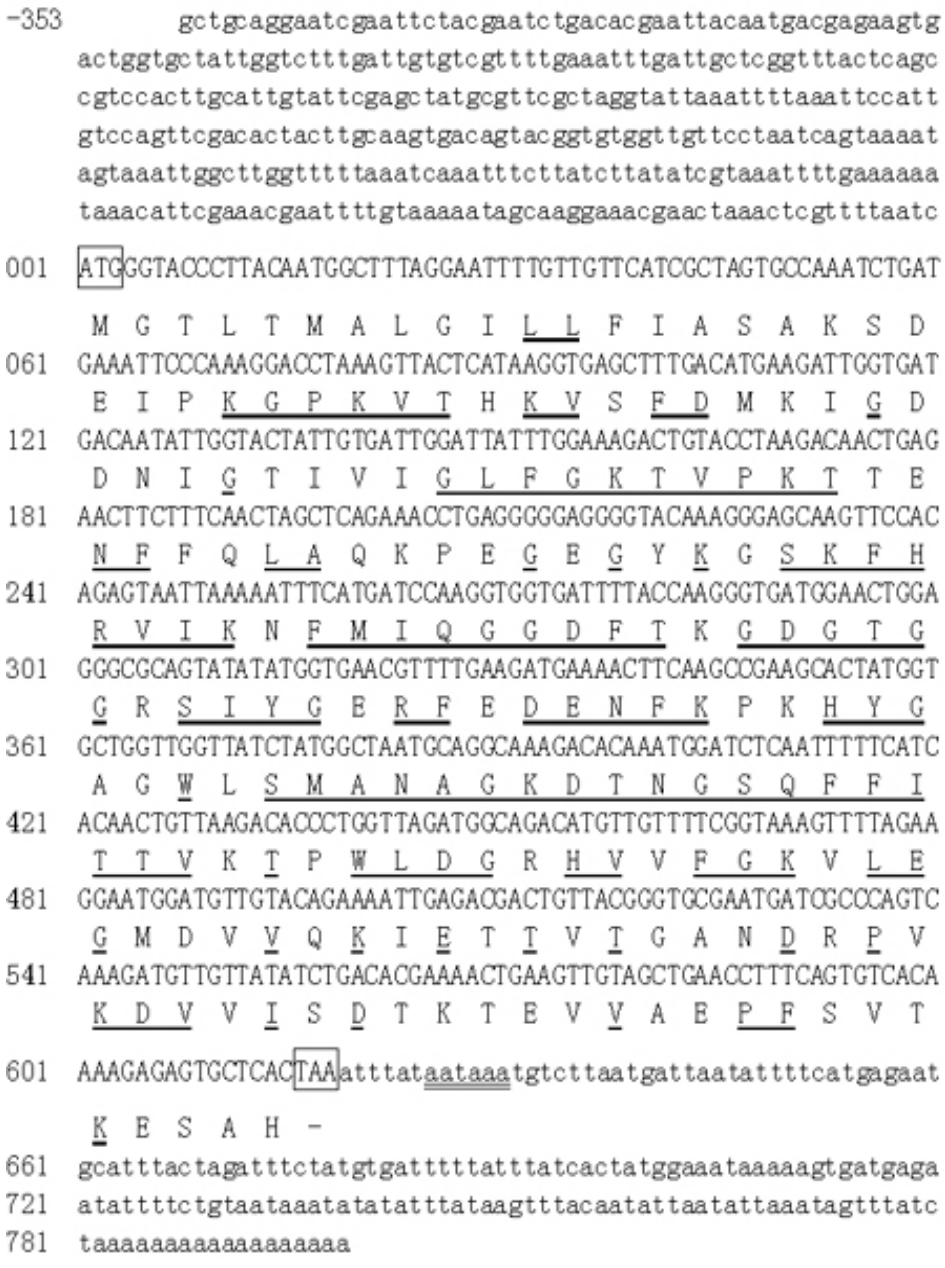

Figure 1. Nucleotide and deduced amino acid sequence for *B.mori* PPIB. The amino acid is indicated by the one letter designation below the nucleotide sequence. The conserved amino acids are underlined. The initiation codon ATG at position 354 and the stop codon TAA at position 969 are boxed. The Poly-A signal (aataaa) is double-underlined.

bands.

Mass spectrometry analysis

The gel piece for PPIB was cut from SDS-PAGE, washed twice with 50 µl ddH_2O, and destained by a 1:1 solution (50 µl) of 50 mM ammonium bicarbonate and 50% acetonitrile. The gel was dehydrated by 50 µl acetonitrile, dried in SpeedVac for 5 min, treated with 10 mM DTT (50 µl) and incubated at 56℃ for 1 h. 55 mM IAM (20 µl) was added into the dried gel and incubated without light. The gel was washed with 25 mM NH_4HCO_3, 50% (v/v) acetonitrile and acetonitrile, respectively. After dried in SpeedVac for 5 min, the gel was incubated with 2 µl of trypsin solution (0.01 µg/µl in 25 mM NH_4HCO_3) in ice bath for 30 min. Then the gel was incubated in 25 mM NH_4HCO_3 (10 µl) at 37℃ for overnight. 2% TFA was added (final concentration of 0.1%) to stop the reaction. Samples were applied onto a target module that was preincubated with a-cyano-4-hydroxy-cinnamic acid matrix thin layer. Finally, the treated sample was analyzed by ultraflex II MALDI TOF/ TOF MS (Bruker, Gemany). Mascot (http://www.matrixscience.com) was used for analysis.

Construction of recombinant virus vBm-PPIB-EGFP and expression of PPIB-EGFP fusion protein in BmN-4 cells

The primers were designed as following: the forward primer (5'GGGGATCCATGGGTACCCTTACAATGGC 3') contains a BamH1 restriction site and the reverse primer (5'GGCTCGAG GTGAGCACTCTCTTTTGTGACACT 3') contains a Xho1 restriction site. The PCR fragment was inserted into pFasBacHTb baculovirus transfer vector that contains an ie-1 promoter. The plasmid pFasBacHTb-PPIB-EGFP was used to transform competent DH10B cells containing helper and BmNPV bacmid to obtain recombinant bacmid pBm-PPIB-EGFP. The transformed cells were incubated with 800 ml SOC medium at 37°C for 4 h and then were applied onto LB agar plates containing gentamicin, kanamycin, tetracycline, IPTG and X-Gal at 37°C overnight. After two rounds of selection, white colonies containing PPIB-EGFP gene were obtained and inoculated into 3 ml of LB medium. Plasmid DNA was isolated and confirmed by PCR using puc/M13 primers. BmN-4 cells were transfected with pBm-PPIB-EGFP (approximately 200 ng) using Lipofectin (Invitrogen) method to produce the recombinant baculovirus vBm-PPIB-EGFP and to express the PPIB protein with a GFP tag. To examine the subcellular localization of PPIase B, we used the DeltaVision Personal microscopy system (Applied precisim).

RESULTS

Nucleotide and amino acid sequence

The nucleotide and amino acid sequence of the full-length *B. mori* PPIB gene were shown in Figure 1. This gene contains an open reading frame (ORF), beginning with the initiation codon ATG at position 354 and ending with a termination codon TGA at position 969. A polya-denylation consensus sequence (aataaa) was found in 3' region.

Bioinformatic analysis

We found four exons for the *B. mori* PPIB gene, the size of which was of 93, 208, 185 and 132 bp, respectively. The map of corresponding genomic region was shown in Figure 2A. Using SilkMap of BGI, we found this gene was located in Scaffold 2767 of Chromosome 19, as shown in Figure 2B.

B. mori PPIB gene encods a peptide of 205 amino acid residues with molecular weight of 21474.41 Da and the isoelectric point of 8.05. It was predicted to contain no transmembrane structures and to be a cytoplasmic protein, which is confirmed by our subcellular localization analysis (shown below). It contains cyclophilin super-family domain, as shown in Figure 2C. As revealed by multiple sequence alignment (Figure 2D), it shows high degree of identity to those PPIases from *Bos Taurus* (identity = 69%), *Equus caballus* (identity = 70%), *Gallus gallus* (identity = 70%), *Homo sapiens* (identity = 67%), *Ictalurus punctatus* (identity = 69%), *Mus musculus* (identity = 68%), *Pan troglodytes* (identity = 68%), *Rattus norvegicus* (identity = 67%) and *Xenopus laevis* (identity = 71%). Cyclophilins contain a characteristic eight-stranded β-barrel, which is highly conserved in Cyp family (Wang et al., 2005). We constructed a 3-D structure model for PPIB (Figure 2F), which shows clearly the unique cyclophilin conserved β-barrel. We also used MEGA 3.1 to construct a phylogenetic tree (Figure 2E), which shows the close evolution relationship among these PPIases. Taken together, our data suggests that *B. mori* PPIB belongs to cyclophilin super-family.

Transcription analysis by PCR

As shown in Figure 3, semi-quantitative PCR revealed that PPIB is widely expressed in *B. mori* blood-lymph, fat body, silk gland, midgut, tuba Malpighii, embryo, ovary and testis, indicating its important roles in *B. mori*.

Expression of recombinant protein and mass spectrometry analysis

B. mori PPIB was expressed in *E. coli* BL21(DE3) with a His6 tag. The yield is amazingly high. Although most protein was found to form inclusion bodies, still significant amount of proteins were soluble. As expected, the calculated molecular mass for PPIB is about 27 kDa (Figure 4). We obtained a peptide mass fingerprint with high signal-noise ratio and peak value (Figure 5) by mass spectrometry, which indicates the target protein is *B. mori* PPIB with a highly convincing score of 112 and the sequence coverage is 51% by Mascot analysis. The matched peptide fragments are located at the residues 38 - 53, 58 - 75, 58 - 78, 85 - 95, 103 - 117, 118 - 131, 145 - 151, 168 - 181 and 182 - 189 of the protein (Table 1), which strongly supports our determination.

Expression of PPIB-EGFP fusion protein in BmN-4 cells

To characterize PPIase B further, we constructed the recombinant virus vBm-PPIB-EGFP that allows the production of PPIB-EGFP fusion protein in BmN-4 cells. Localization of PPIase B protein was examined by Delta Vision Personal microscopy system. Our data clearly showed (Figure 6) that PPIB-EGFP is located in the cytoplasm, suggesting *B. mori* PPIB is a cytosolic protein.

DISCUSSION

Peptidylprolyl isomerases catalyze the peptidyl-prolyl *cis-trans* isomerization, the rate-limiting step of protein folding, which is essential for newly synthesized protein to acquire its correct 3-D shape. They can also catalyze the *trans-cis* isomerization that has recently been shown to be critical for some normal physiological processes (Lu

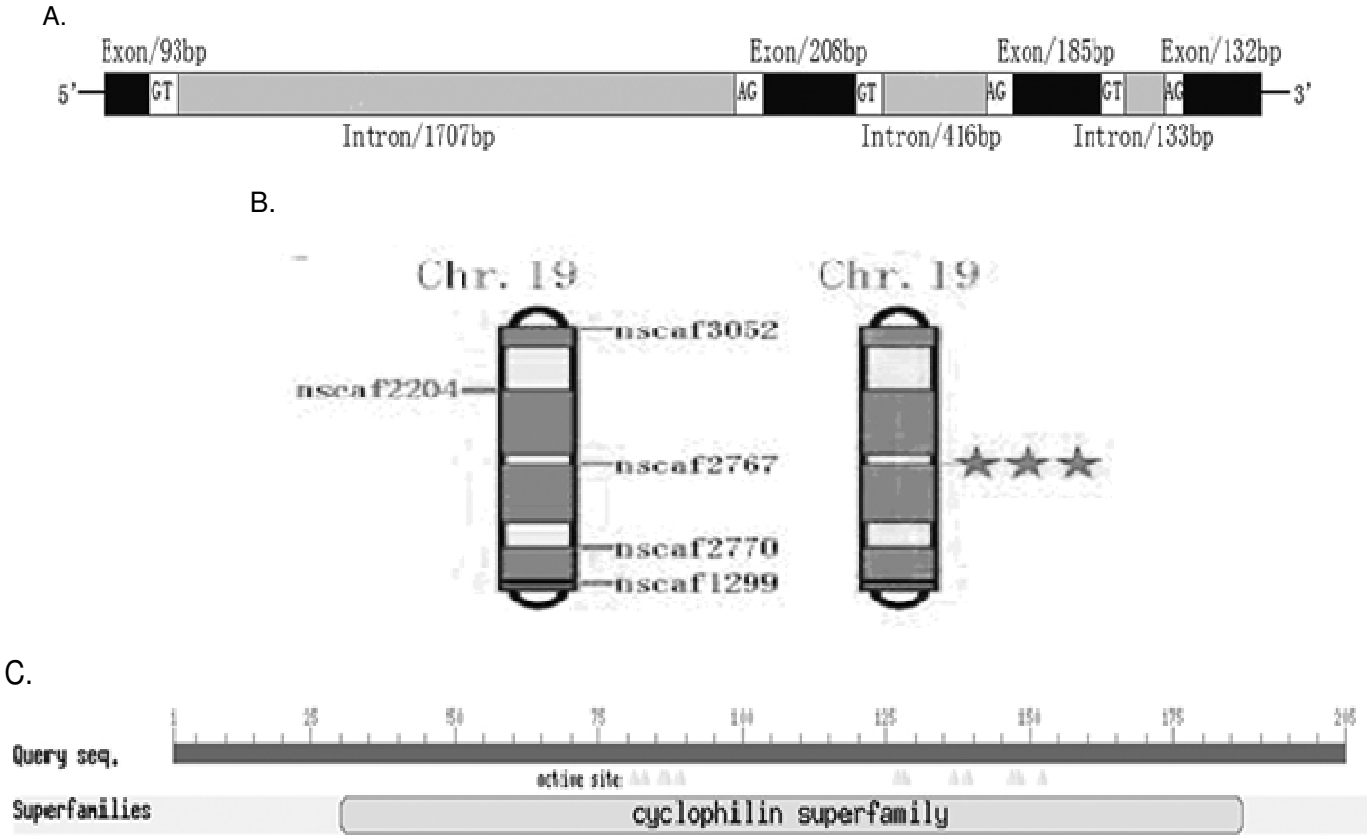

Figure 2. Bioinformatic analysis of *B.mori* PPIB gene and protein. (A) Genome structure. Black box, extrons; Gray box, introns. Splicing sites (exon/GT-intron-AG/exon) are also indicated. This gene spans 2874 nucleotide from 5' untranslated region to 3' untranslated region. The lengths of exons are 93, 208, 185 and 132 bp, respectively. The introns are of 1707, 416 and 133 bp in size. (B) Scaffold and Chromosome mapping. This gene was predicted to be located in Scaffold 2767 in Chromosome 19, as indicated by three pentacles. (C) conserved cyclophilin superfamily domain. (D, E) Multiple sequence alignment and Phylogenetic tree analysis. D, The black region indicates the completely conserved amino acids. *Bos Taurus* (identity = 69%, NP_776577), *Equus caballus* (identity = 70%, NP_001093231), *Gallus gallus* (identity = 70%, NP_990792), *Homo sapiens* (identity = 67%, CAG33110), *Ictalurus punctatus* (identity = 69%, ABC75555), *Mus musculus* (identity = 68%, NP_035279), *Pan troglodytes* (identity = 68%, XP_001174161), *Rattus norvegicus* (identity = 67%, NP_071981) and *Xenopus laevis* (identity = 71%, NP_001080505). E, MEGA was used to construct the Phylogenetic tree. (F) Three-dimensional structure model. Homology-modeling was performed by SWISS-MODEL server. Red, helix; Orange, strands; Green, loops.

et al., 2007a, b; Isakov, 2008; Wulf et al., 2005). More interestingly, some PPIases have been shown to be involved in virus infection, which may lead to exploration of novel anti-virus drug designs (Yang et al., 2008; Watashia et al., 2005; Saphire et al., 2000). Although there are no reports about *B. mori* PPIases, our previous comparative proteomic analysis showed that the expression of PPIases were up-regulated in certain silkworm strains resistant to BmNPV, yet the mechanism of which remains unresolved.

As the first step to delineate the role of PPIases in *B. mori*-BmNPV interaction, in this work we identified a *B. mori* PPIase B gene, then carried out tissue transcription, prokaryotic expression and subcellular localization analyses. The *B. mori* PPIB gene contains a 618 bp ORF encoding a peptide of 205 amino acids and the protein contains highly conserved cyclophilin superfamily domain and belongs to cyclophilin family. We further characterized it by *in vitro* expression in *E. coli* and mass spectrometry analysis. Although most of expressed proteins formed inclusion bodies, significant amount of soluble proteins could still be obtained for characterization. The *B. mori* PPIB was widely expressed in all the tissues examined by PCR, which is consistent with its essential roles. The subcellular localization analysis indicated it is a cytosolic protein. We did not obtain data for the peptidylprolyl isomerase activity for *B. mori* PPIB due to technical difficulties. However, based on the sequence alignment and homology-modelling, it is most likely that *B. mori* PPIB also has the activity of peptidylprolyl isomerase.

To our knowledge, this is the first report about cloning and characterization of *B. mori* PPIB, which enables us to carry out mutagenesis, RNAi and *in vivo* overexpression in silkworm cells to examine the effect on BmNPV infection. Taken together, our data presented here provides

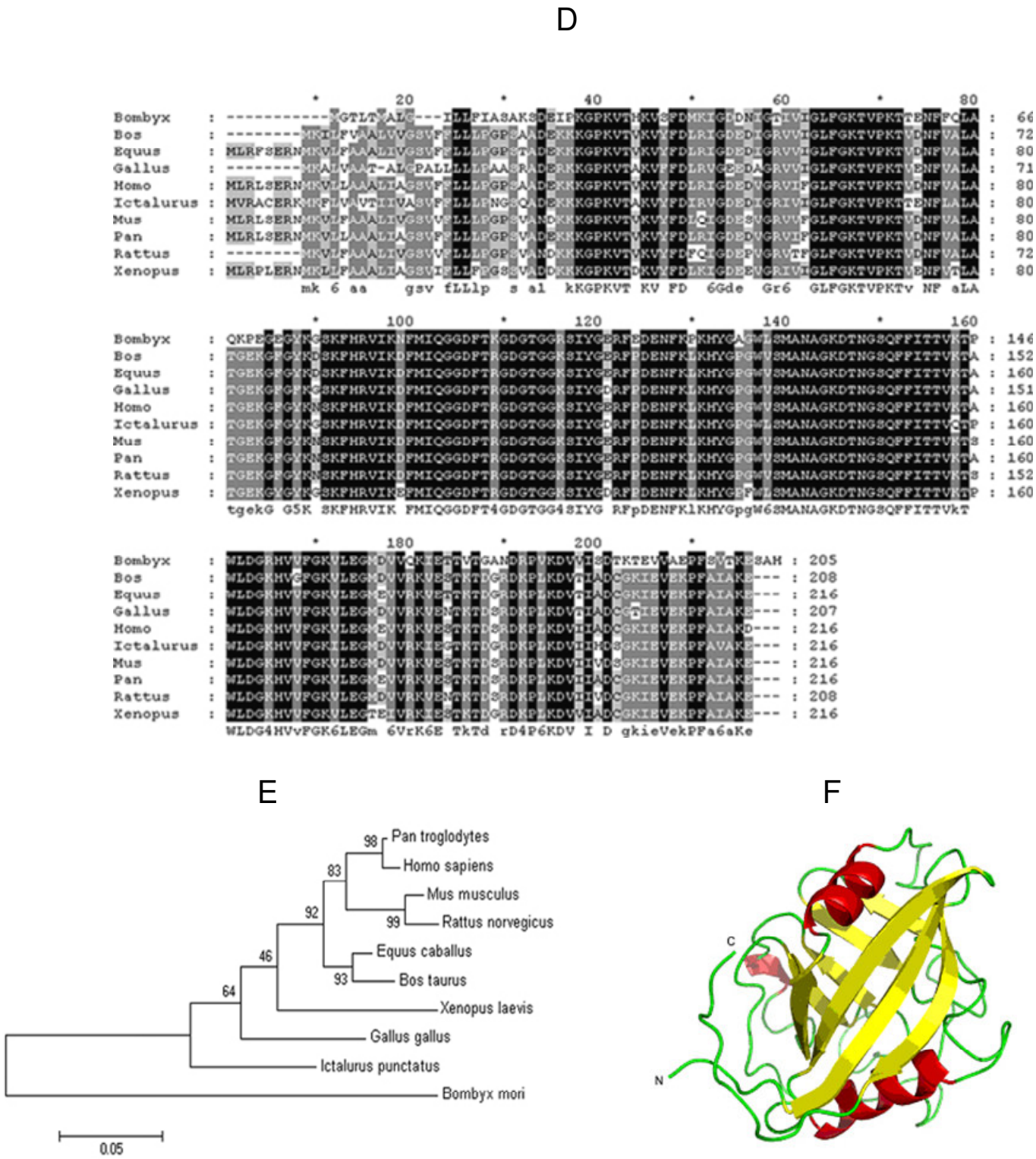

Figure 2. Contd.

good foundation for the following investigation into the role of PPIB in anti-BmNPV response and other cellular physiological roles in silkworm, as well as further studies on cyclophilin superfamily.

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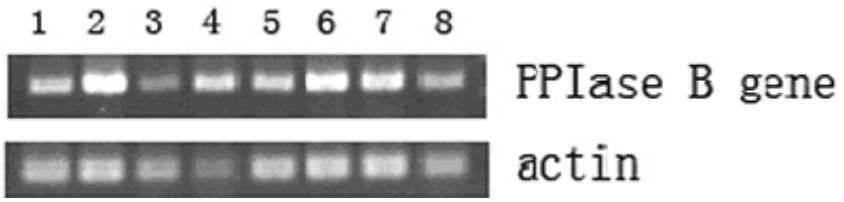

Figure 3. Tissue transcription analysis of PPIB gene by PCR. 1. blood-lymph; 2. fat body; 3. silk gland; 4. midgut; 5. tuba Malpighii, 6. embryo; 7. ovary; 8. testis. *B.mori* actin gene A3 was used as the control.

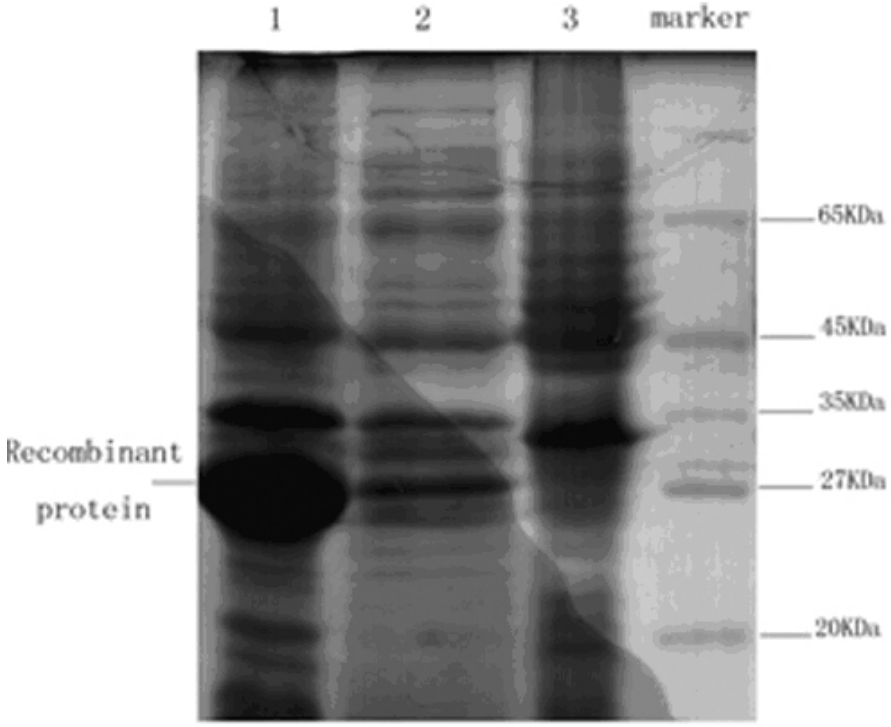

Figure 4. Expression of *B. mori* PPIB in *E. coli*. 1. Pellet; 2. Supernatant; 3. pET-30a vector as control. PPIB was subcloned into pET-30a vector and was expressed in *E. coli* BL21(DE3), and significant amount of soluble proteins could be obtained.

Table 1. List of identified peptides by mass spectrometry.

Start - End	Observed mass (Da)	Calculated mass (Da)	Sequence
38 - 53	1631.9441	1630.893	KIGDDNIGTIVIGLFGKT
58 - 75	2087.1704	2086.0007	KTTENFFQLAQKPEGEGYKG
58 - 78	2359.3547	2358.1492	KTTENFFQLAQKPEGEGYKGSKF
85 - 95	1257.6545	1256.586	KNFMIQGGDFTKG
103 - 117	1859.0244	1857.8897	R.SIYGERFEDENFKPKH
118 - 131	1462.7448	1461.6823	KHYGAGWLSMANAGKD
145 - 151	844.4857	843.4239	KTPWLDGR.H
168 - 181	1500.8069	1499.7944	KIETTVTGANDRPVKD
182 - 189	876.4802	875.46	KDWISDTKT

The sequence coverage is 51%. Observed mass indicates the actual molecular mass and Calculated mass indicates the deduced molecular mass. TheNCBI nr database was searched to identify these sequences with Carbamidomethyl (C) as fixed modifications, Oxidation (M) as variable modification and Trypsin as cleavage enzyme.

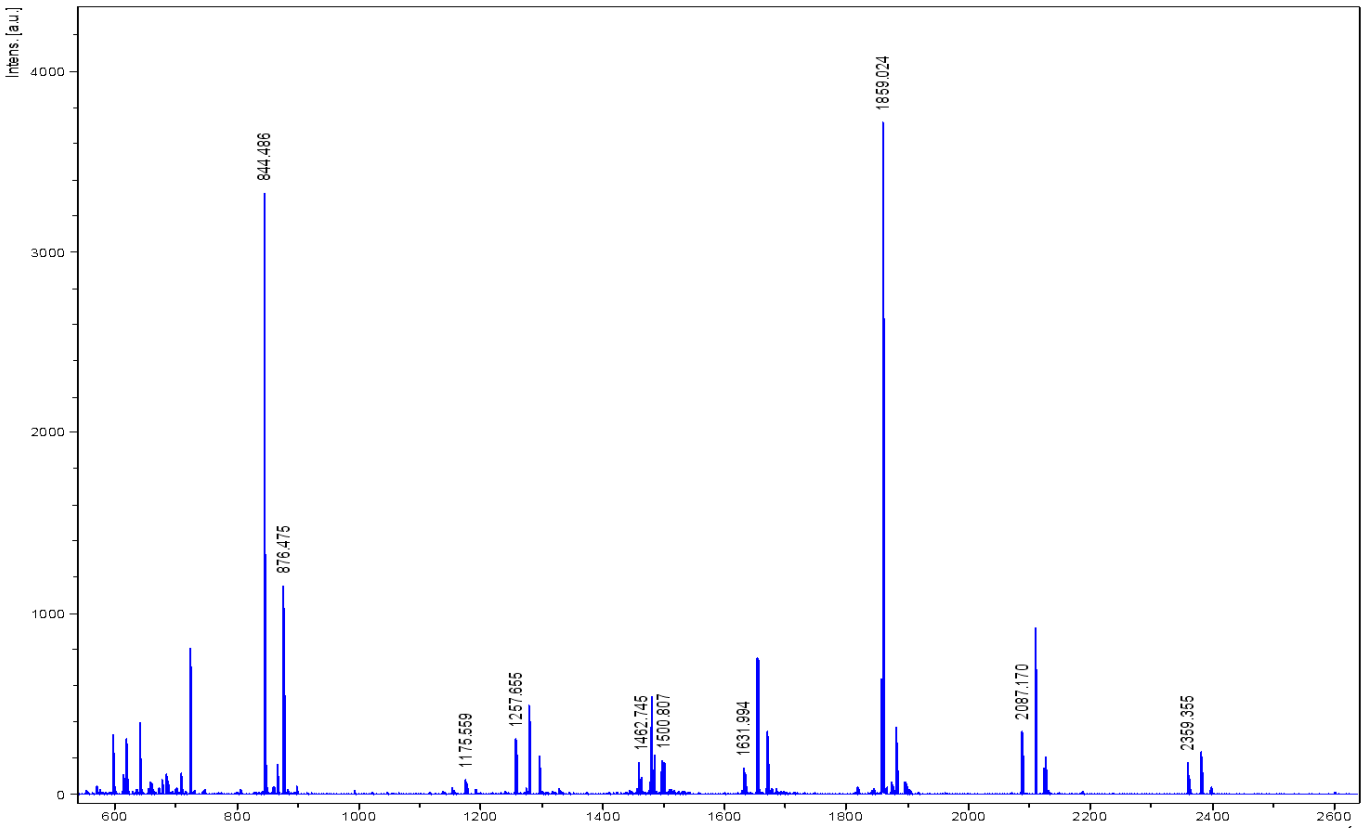

Figure 5. The peptide mass fingerprint of PPIase B. Mass spectrometry analysis was performed by ultraflex II MALDI TOF/ TOF MS. This peptide mass fingerprint has high signal-noise ratio.

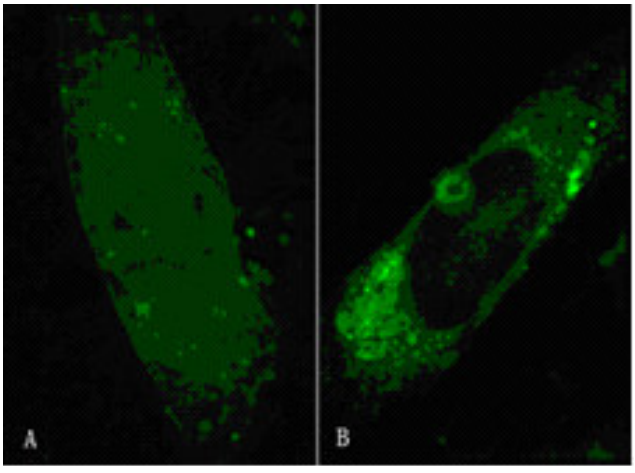

Figure 6. The subcellular localization of PPIase B in *B.mori* cells. A, The cells were transfected with vBm-EGFP plasmid as control; B, The cells were transfected with vBm-PPIB-EGFP plasmid. The GFP fluoresence was detected by DeltaVision Personal microscopy system.

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