

Full Length Research Paper

Preliminary characterization of a death-related gene in silkworm *Bombyx mori*

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Accepted 29 April, 2009

In order to study the mechanism of programmed cell death (PCD) in silkworm *Bombyx mori*, we employed suppressive subtractive hybridization to screen associated genes. In this study, a novel gene was isolated from *B. mori*, which may be involved in PCD as analyzed by bioinformatics and it was named as DRP (death-related protein) gene. RT-PCR analysis showed and prokaryotic expression. Through RT-PCR analysis of death-related protein gene in different tissues and different developmental stage of *B. mori*, it showed the distributed condition of the gene. It was widely expressed in various tissues and mainly expressed in testis, malpighian vessels, posterior intestine, silk gland. Meanwhile, it was widely expressed in virous developmental stages and mainly expressed in egg, indicating its possible roles in development of programmed cell death. We also tested the expression of this gene in prokaryotic system, which may be useful for subsequent studies. This gene is registered in GenBank under the accession number FJ447481.

Key words: DRP, *Bombyx mori*, bioinformatics, prokaryotic expression, RT-PCR.

INTRODUCTION

Programmed cell death (PCD) is a genetically controlled process that functions in the development of multicellular organisms and in their responses to biotic and abiotic stresses. Many genes involved in PCD have been found in many organisms, for example, Apoptin, Ced-3, Dredd, DAP3 and so on. Apoptin, the product of the chicken anemia virus (CAV) gene, has been shown to be responsible for the apoptotic activity of the virus (Noteborn et al., 1994). Subsequent studies suggested that Ced-3 is the product of a gene which is necessary for programmed cell death in the nematode *Caenorhabditis elegans*. Dredd (the name stands for "Death related ced-3/Nedd2-like") protein is a Drosophila member of the caspase gene family; it encodes a 128 kDa nucleolar protein. In recent years, death-associated protein 3 (DAP3) is a GTP-binding protein that has been identified as a positive mediator in interferon (IFN)- α -induced cell death (Kissil

et al., 1995). Meanwhile, DAPK2 belongs to the family of DAPKs, is a Ca²⁺/calmodulin (CaM) regulated serine-threonine kinase, and functions as a positive mediator of PCD when overexpressed in several cancer cell lines (Kawai et al., 1999; Inbal et al., 2000). Recently, death-associated protein kinase (DAPK) has been found associated with HSP90 and inhibition of HSP90 with 17-alkylamino-17-demethoxygeldanamycin reduced expression of DAPK (Liguo Zhang and Patricia, 2007). Death-associated protein 5 (DAP5/p97) is a recently identified novel repressor of translation initiation and a member of the translation initiation factor G family (Imataka and Sonenberg, 1997; Imataka, 1997; Yamanaka et al., 1997). Although processes of some death-related protein have been investigated, only a few evaluations have been performed in lepidoptera species.

Here, we used a suppression subtractive hybridization (SSH) approach to identify novel genes from *B. mori*. One of genes that was identified as potentially involved in programmed cell death, named as *Bombyx mori* death-related protein gene (BmDRP), was cloned. Its expression pattern in different tissues and RT-PCR was analy-

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zed. We also showed it can be easily expressed in prokaryotic system.

MATERIALS AND METHODS

Materials

The silkworm *B. mori* and *Escherichia coli* (strain DH5 α and BL21) were maintained in this laboratory. Silkworm strain 306 and NB were used for this study. Restriction enzymes, T4 DNA ligase, PCR reagents and pMD18-T were obtained from Takara Company (Dalian). Primers and other reagents were from Shanghai Sangon Bio-technology.

Bioinformatic analysis of BmDRP

The full length of BmDRP gene was obtained by searching silkworm EST database. In order to establish the genomic organization, the cDNA sequence was BLASTed to the contigs of *B. mori* genome in GenBank. SIM4 (<http://pbil.univ-lyon1.fr/sim4.php>) was used to align the cDNA sequence with the genomic sequences to search potential introns. We also used another silkworm cDNA database BGI (<http://silkworm.genomics.org.cn/>), which analyze the ORF and obtain the map of location in chromosome. cDNA and protein analysis were performed using the EXPASY web server (<http://au.expasy.org/>). To search for sequence homology, a protein-protein basic local alignment search tool (blastp) was used with the NCBI protein database (<http://www.ncbi.nlm.nih.gov/BLAST/>). We used the ExPASy translate tool (<http://au.expasy.org/tools/dna.html>) to deduce amino acid sequence. SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) was used to predict the secondary structure. We used the PLOC tool (<http://www.genome.jp/SIT/plocdir/>) to predict the subcellular localization of BmDRP. n-gram indexing method and k-NN algorithm were used in this analysis.

RT-PCR analysis of BmDRP gene expression in different tissues

To study the gene expression in different tissues of *B. mori*, RT-PCR was carried out using superscript III one step RT-PCR system with platinum Taq DNA polymerase (Invitrogen). Total RNAs from various tissues of *B. mori* including testis, epidermis, malpighian vessels, neuroploca, headgut, hindgut, silk gland, ovary, fatbody, blood and midgut were extracted using Trizol reagent (Invitrogen). A segment of 306 bp of DRP gene was amplified using a forward primer (5'CGGAATTCATGTCGTCAACCGAGGAAA 3') containing a EcoRI restriction site (underlined), and a reverse primer (5'CCCTCGAGCTACTTTCTGGGCTGTTGGA 3') containing a XhoI restriction site (underlined). PCR reactions were carried out with Taq polymerase for 40 amplification cycles (94°C/20 s, 60°C/30 s, 72°C/30 s) and was examined by electrophoresis in 1.2% agarose gel with the ethidium bromide staining.

RT-PCR analysis of Bm DRP gene expression in different developmental stages

To study the DRP gene expression in different developmental stages of *B. mori*, RT-PCR was carried out using superscript III one step RT-PCR system with platinum Taq DNA polymerase (Invitrogen). Total RNAs from various tissues of *B. mori* including egg, 1 instar, 2 instar, 5 instar, pupa and imago were extracted using Trizol reagent (Invitrogen). The RT-PCR was performed as mentioned above.

Cloning and sequencing

The RT-PCR product was ligated into pMD18-T vector using T4 DNA ligase and *E. coli* (DH5 α strain) was used for plasmid amplification and MiniBEST plasmid purification kit (Takara) was used for purification. The sequencing was performed using an automatic sequence CEQ8000 (Beckman company).

Construction of expression plasmid

The plasmid pMD18 - T/BmDRP was digested with EcoRI and XhoI, and then purified fragment was ligated with the EcoRI-XhoI digested pET30a vector to construct pET30a-BmDRP. The transformants of pET30a-BmDRP in *E. coli* (BL21 strain) were confirmed by restriction enzyme analysis.

Expression of fusion protein in *Escherichia coli* and SDS-PAGE

For expression of recombinant protein, a single clone was cultured in LB medium supplemented with Kanamycin (100 μ g/ml) overnight at 37°C with shaking. This culture was added into fresh LB medium and cultured at 37°C with vigorous shaking to A₆₀₀ about 0.6. Then, the IPTG (final concentration of 1 mmol/L) was added to induce protein expression. 15% SDS polyacrylamide gel was used to analyze the recombinant protein. After electrophoresis, the gel was stained with coomassie brilliant blue G250 to visualize the protein bands.

RESULTS

Nucleotide sequence analysis

Using BLASTN database, we searched against silkworm EST fragments with BmDRP open reading frame, then, we assembled these EST fragments and obtained the full-length cDNA sequence of BmDRP. The ORF begins with the initiation codon ATG at position 32 bp and ending with TAG at 417 bp. The full-length cDNA also includes PolyA signal (Figure 1). Using silkworm cDNA data of BGI to search the location of BmDRP in chromosome, which is on the chromosome 20 in *B. mori* (Data not shown). BLAST revealed that contig32647 (GenBank accession no. BABH01032647) having a very high similarity. Using SIM4 to align the cDNA sequence with contig32647, we found 2 exons and three introns were in the relevant DNA sequence (Figure 2).

Analysis of the deduced amino acid sequence

Using BLAST software of NCBI to search for homology in the GenBank database, the deduced amino acid sequence showed an identity of 57, 51, 55, 55, 53, 50, 52, 51, 47 and 44% to the corresponding genes of *Drosophila melanogaster* (NP_610676), *Apis mellifera* (XP_392446), *Bos Taurus* (AAX08640), *Canis familiaris* (XP_849018), *Homo sapiens* (EAX08056), *Anopheles gambiae* (XP_001238360), *Culex quinquefasciatus*

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ATTCTCATATTCACAGGCCGCAATTGACACACGAAGAATTTGTGACGTTG
CATTGCATGATAAACTTCCAATATCGTAAATAAAT TAAATTCAAAATTATCA
AACGATA
1  ATGTCGTCAACCGAGGAACTTCACAACATAAAAGCTGGACATCCTCCTGCAGTAAAAGCT
M S S T E E T S Q L K A G H P P A V K A
61 GGAGGTATGAGGATCACCCAACATAAACTCCTCACTCAAAGACAGCAAAGAACCAGCC
G G M R I T Q H K T P H S K D S K E P A
121 AATGAAGACTTGACTGGTCTCTCTGGGCCCTCTCCCGTCCGTCGAATCCGATCTCCATT
N E D L T G L S G P S P V P S N P I S I
181 GCCGGAGCTCCAAACAAGGTAATGCAGACTTCACTCCAGAAGCAGCTCAAGTGGCCAC
A G A P N K G N A D F T P E A A Q V A H
241 AGTCCTAAGCCTCCAGCGCACATTAATCTCAGACCAAGCCAAACATCCAACAGCCAGA
S P K P P A H I N L R P S P N I Q Q P R
301 AAGTAG
K .
AATGCTGCACATTAACTACTAAAAGGTTGATACATAATAACGRAATAGAAAA
TGTTAGGCAATCATAAAATTCAAAATATAAATTATGTATAAATATAAAAATATA
GTATAATTGTTGGAATGTTTACCATAGCAATAGACTGGCTGTGCCCTGGCCC
CCCTGTCTTCATGAGCAATGTAGAGCATGTGTTGCTGTGTACCTCAAAGAC
ATAAAACATGCTTCTTGTTGTCTTCCTTTCACGCAGCAGTGACCCAATTT
TACACAGAGTAAGATTATGGACTGCAGAGTGACGTAGACCACTTTATAAGAA
AGAAAGCAATGACATTC AAGTT

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Figure 1. Nucleotide sequence and deduced amino acid sequence of the death-related gene. The open reading frame consists of 306 bp encoding a protein of 101 amino acid residues. The predicted amino acid sequence is represented by the one letter code underneath the nucleotide sequence. The initiation codon and the stop codon are framed. The polyA signal is underlined.



Figure 2. DNA sequence frame of the BmDRP gene. Gray showed exon, bias showed intron.

(XP_001848329), *Mus musculus* (NP_666169), *Gallus gallus* (NP_001026174) and *Xenopus tropicalis* (NP_001017164) respectively, which is shown in (Figure 3). Sequence alignment revealed these proteins are very conservative. The phylogenetic tree of BmDRP was constructed with MEGA3 (Figure 4). We also used PLOC tool to predict the subcellular localization of BmDRP, which is the nucleus (Data not shown). The secondary structure of BmDRP was also predicted by SOPMA, which is shown in (Figure 5).

RT-PCR analysis of *Bm DRP* gene expression in different tissues

Tissue expression of BmDRP gene was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). BmDRP gene was dominantly expressed in testis, malpighian vessels, posterior intestine, silk gland, mildly

expressed in epidermis, neuroploca, headgut, fatbody, midgut, ovary and slightly expressed in blood (Figure 6).

RT-PCR analysis of Bm DRP gene expression in different developmental stages

BmDRP gene expression was analyzed at different developmental stage by reaction (RT-PCR). BmDRP gene dominantly expressed in egg, mildly expressed in ovary, 3 instar, 5 instar, pupa and imago and slightly expressed in 1 instar (Figure 7).

Construction of expression plasmid

The RT-PCR product of BmDRP was subcloned into plasmid pMD18 to construct pMD18 T/BmDRP and was subcloned into pET30a vector to obtain pET30a-BmDRP,

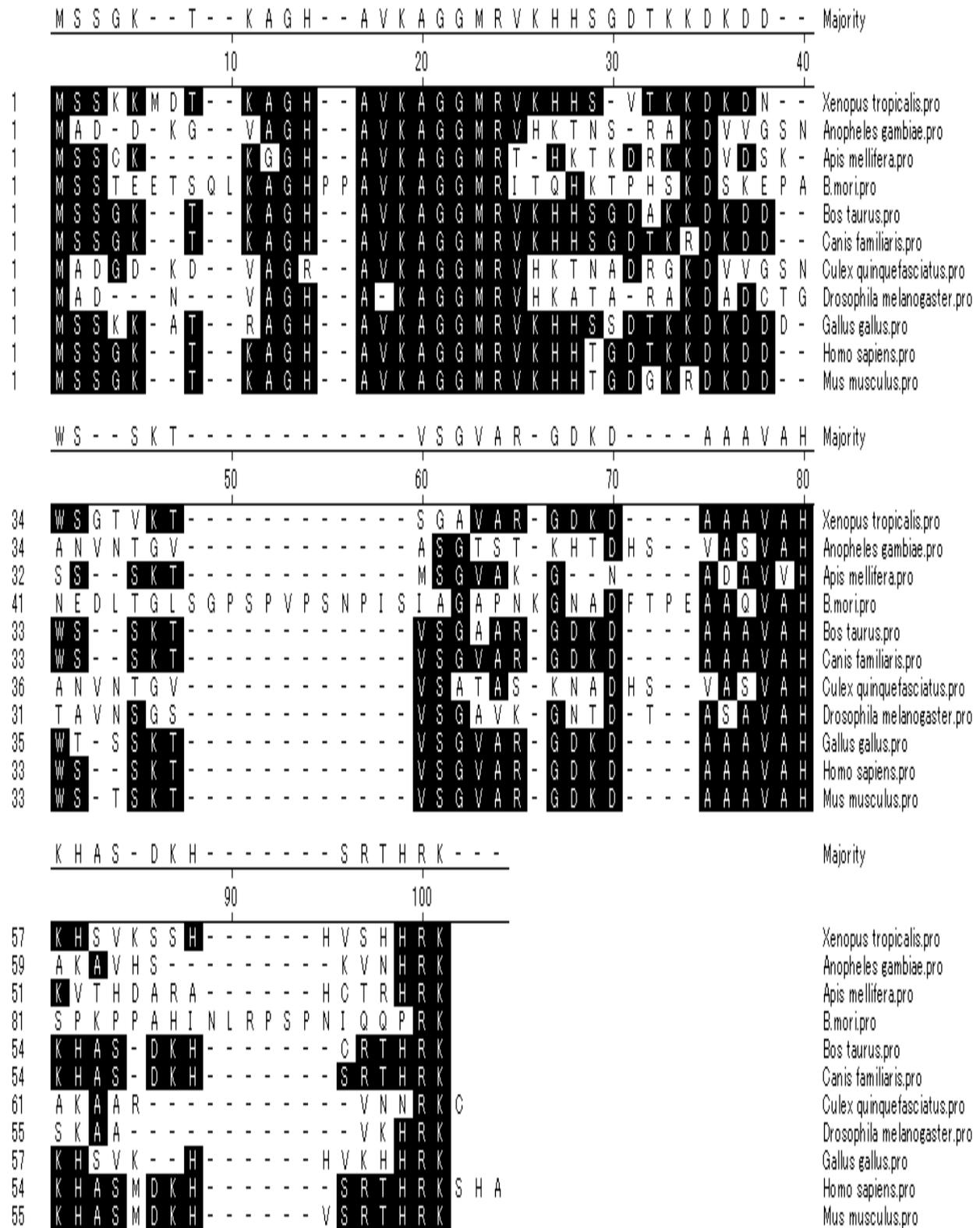


Figure 3. Amino acid sequence of *B. mori* DRP was aligned with sequences from *Drosophila melanogaster* (NP_610676), *Apis mellifera* (XP_392446), *Bos taurus*(AAX08640), *Canis familiaris*(XP_849018), *Homo sapiens*(EAX08056), *Anopheles gambiae*(XP_001238360), *Culex quinquefasciatus*(XP_001848329), *Mus musculus* (NP_666169), *Gallus gallus*(NP_001026174) and *Xenopus tropicalis*(NP_001017164). The sequences were aligned using DNASTar program. The identical residues are shaded in black, while the other residues are in white.

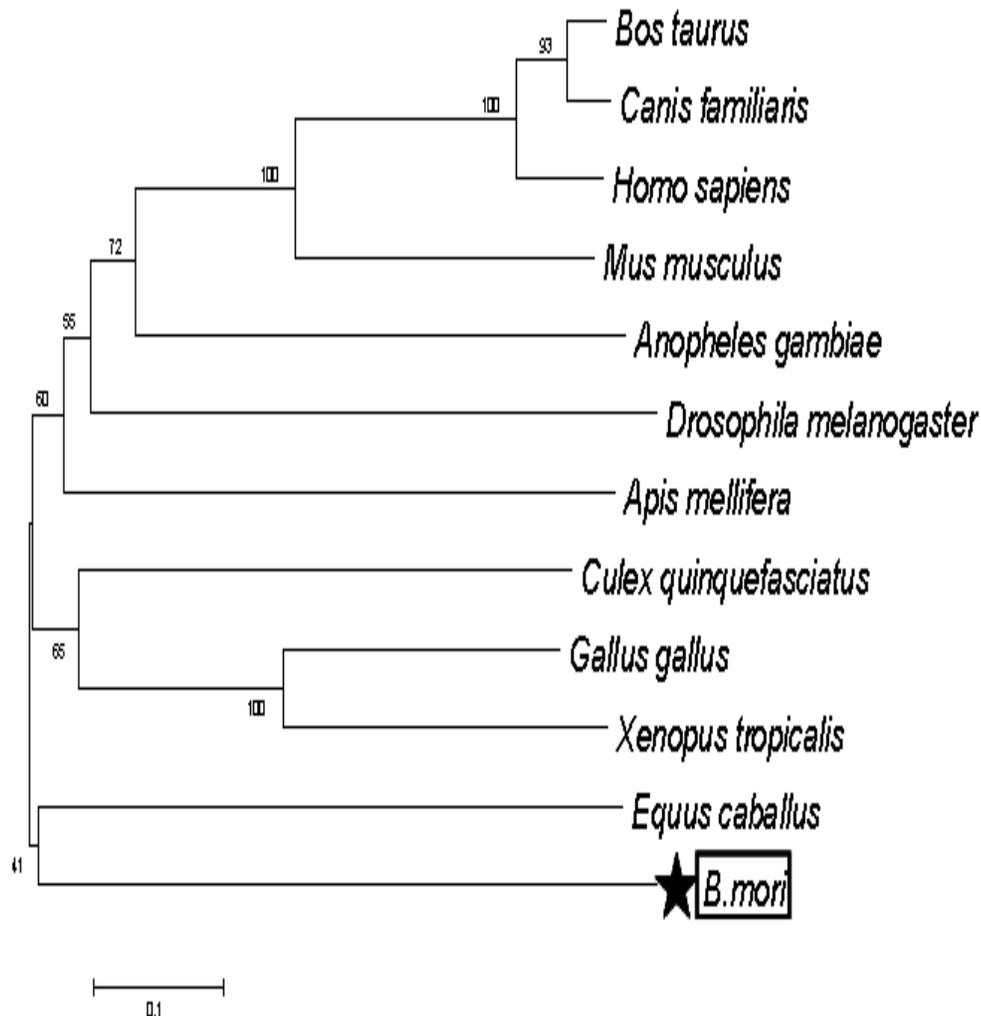


Figure 4. Phylogenetic analysis of BmDRP NJ tree was produced by MEGA3.

which is shown in (Figure 8).

Expression of fusion protein in *E.coli* and SDS-PAGE

pET30a/BmDRP was transformed into *E. coli* BL21 to plasmid to express the His-BmDRP recombinant fusion protein. SDS-PAGE analysis showed that as expected this fusion protein is of about 21 kDa, which is shown in (Figure 9).

DISCUSSION

Virtually all eukaryotic cells are capable of activating an intrinsic cell death program. During embryonic development and early postnatal life apoptosis, about 30 - 80% neural cells was reported to undergo programmed cell death (PCD) (Kajta, 2004). As far as we know, beyond our previous report in various species, there are no

previous references of lepidoptera. In the study, we successfully identified a novel *B. mori* DRP gene, which may be involved in PCD in *B. mori*. The BmDRP gene and encoded protein shared high degree of homology with the respective mammalian, drosophila and other lepidopterous homologues, suggesting that these genes are evolutionarily conserved and may have important biological functions, from the phylogenetic tree reconstructed by MEGA3 and we found that the *B. mori* DRP was not similar to the other species, suggesting that BmDRP may have unique functions in *B. mori*.

Furthermore, we performed RT-PCR and confirmed this gene is expressed mainly in testis, malpighian vessels, hindgut, silk gland and egg, which may indicate a possible role in the development of programmed cell death and the expression product *in vitro* should be approximately 21 kDa and the RT-PCR analysis in different tissues and developmental stages provided some evidences for further study. The result showed that it was dominantly expressed in testis, malpighian vessels,

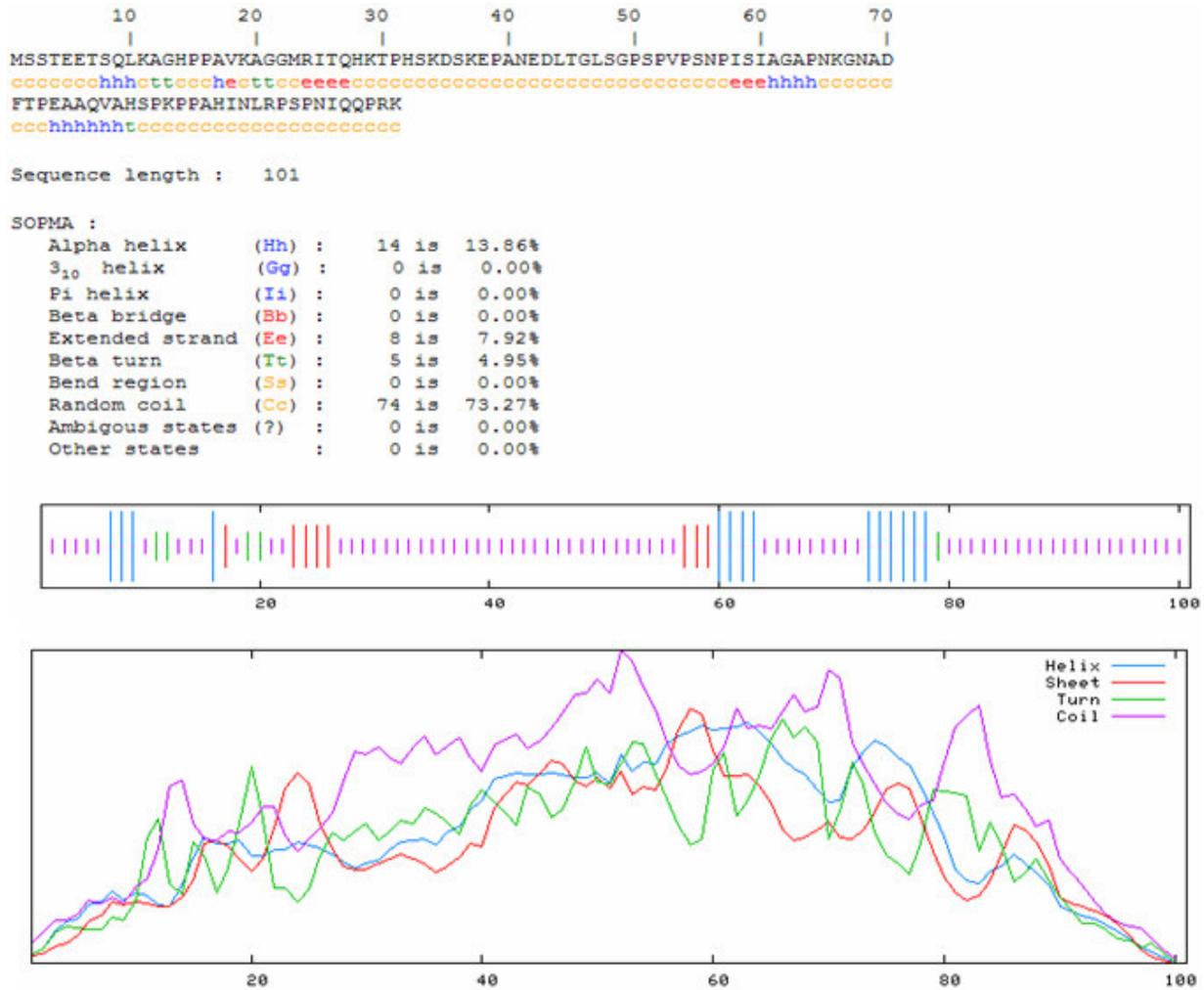


Figure 5. The secondary structure of BmDRP. Helix: Alpha helix (blue); Sheet: Extend strand (red); Turn: Beta turn (green); Coil: Random coil (purple).

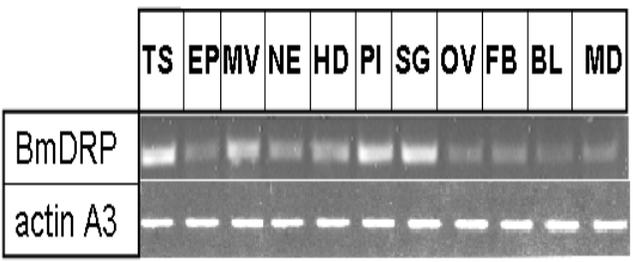


Figure 6. The RT-PCR analysis of death-related gene in different tissues of *B. mori* larvae; TS, testis; EP, epidermis; MV, malpighian vessels; NE, neuroploca; HD, headgut; PI, posterior intestine; SG, silk gland; OV, ovary ; FB, fatbody; BL, blood; MD, midgut.

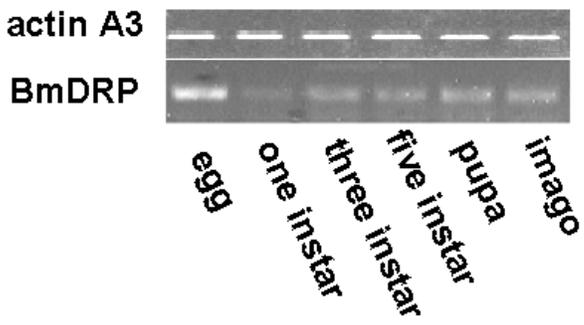


Figure 7. The RT-PCR analysis of death-related gene in different developmental stage of *B. mori* larvae, including egg, one instar, three instar, five instar, pupa, imago.

posterior intestine, silk gland and egg, mildly expressed in epidermis, neuroploca, headgut, fatbody, midgut,

ovary, 3 instar, 5 instar, pupa and imago and slightly expressed in blood and 1 instar. We also expressed this

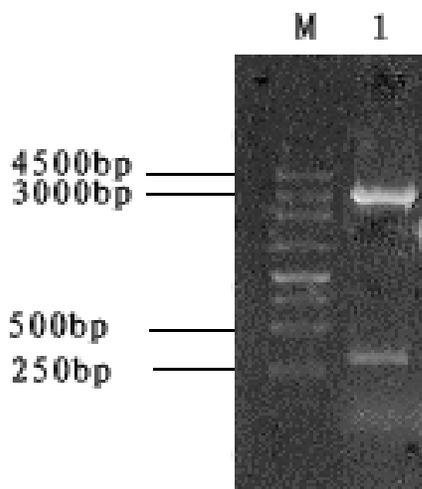


Figure 8. Identification of recombinant plasmid digested by EcoRI and XhoI. M: DNA molecular mass marker; Lane1: Identification of pET30a- BmDRP.

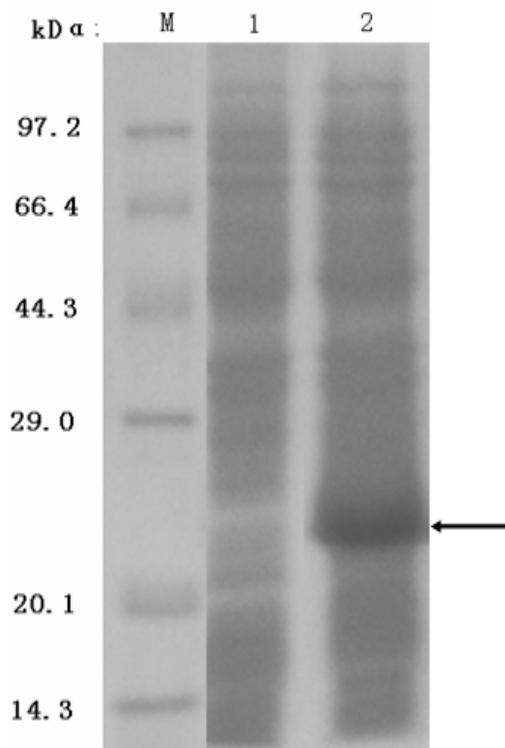


Figure 9. The expression of bmdrp in *E. coli*. The pet was transformed into BL21, and protein expression was induced by IPTG and analyzed by SDS-PAGE. M. Protein molecular mass marker; 1. lysate of *E. coli* BL21 contained pET30a induced by IPTG; 2. lysate of *E. coli* BL21 contained pET30a/BmDRP induced by IPTG.

BmDRP protein in *E. coli*, which will be useful for future studies.

ACKNOWLEDGEMENTS

This study was supported by the National Basic Research Program of China (No.2005CB121000) and grants from Jiangsu Sci-Tech Support Project - Agriculture (No. BE2008379) and China National "863" Project (No. 2008AA10Z145) and by grants from the Foundation of Jiangsu University (No: 1293000418).

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