

Paralytic Peptide Binding Protein (PP-BP) Gene Expression During Egg Diapause and Its Multi-Gene Organization in the Silkworm *Bombyx mori*.

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Abstract

Paralytic peptide binding proteins (PP-BP) are 30KP proteins that show similarity to ENF binding proteins. The ENF-BP act as active regulators of ENF peptides. ENF peptides are multifunctional insect cytokines. The comparison of gene expression in diapause induced and non-diapause eggs at different time intervals after oviposition showed an upregulation of PP at 18h as well as PP-BP at 12 and 18h after oviposition along with few other genes. The current study has been taken up to investigate the role of PP as well as PP-BP in diapause induction in polyvoltine silkworms and to study the multigene organization of PP-BP in the *Bombyx mori* genome. The tissue specific expression analysis revealed that, PP-BP is highly expressed in fat body followed by egg and brain while no expression was observed in mid-gut. The expression levels of PP and PP-BP in diapause and non-diapause eggs from 0h to 48h after oviposition, validated through realtime PCR revealed that PP is highly expressed at 18 and 24h while PP-BP expression is higher at 12 and 18h time intervals suggesting their possible role in diapause induction. The whole genome survey of the PP-BP paralogous sequences revealed a total of 46 *B. mori* PP-BP homologs that are classified into 3 categories viz., ENF-BP, Typical 30KPs and serine/threonine rich 30KPs. These paralogous sequences are distributed on chromosomes 7, 20, 22 and 24, all 30KP and S/T rich 30KP proteins are present in the same locus of chromosome 20.

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Introduction

During insect development the central nervous system regulates growth, metamorphosis, diapause and reproduction by secreting peptides. Insect hemolymph contains various proteinaceous components viz.,

transport proteins, storage proteins, peptide hormones which are essential for growth and development, and defensive peptides while induced by bacterial invasion or injury. Injection of the hemolymph obtained from larvae of other *Manduca* species into that of *Manduca sexta*, the latter become paralyzed. This fac-

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tor from the hemolymph was isolated from several kinds of lepidopteran hemolymph and characterized as 23 amino acid peptides which were named as Paralytic peptide (Ha *et al.*, 1999).

This ENF peptide family shares similar amino acid sequence and conserved at C-terminal region. It is named after the N-terminal-conserved three amino acids (Glu-Asn-Phe) (Strand *et al.*, 2000). ENF family proteins are synthesized as inactive precursors that are activated by a serine protease cleavage. Mature ENF family proteins, including *Bombyx mori* paralytic peptide (BmPP), have been reported to show multiple effects, such as induction of morphological changes of plasmatocytes, inhibition of larval growth, promotion of cell growth and local muscle contraction (Ha *et al.*, 1999; Sasagawa *et al.*, 2001; Miura *et al.*, 2002; Nakahara *et al.*, 2003).

In *B. mori* the embryonic diapause is normally induced by the diapause hormone (Bommo-DH), which is produced in the pupae from the suboesophageal ganglion. Injection of *Antheraea yamamai*-paralytic peptide (Antya-ParP) into non-diapausing pupae of *B. mori*, however, converted the pupae into diapauses egg producers. Even extirpation of brain along with SG had no influence on the induction of diapausing egg production by Antya-ParP, which demonstrated that Antya-ParP does not act via endogenous *B. mori* diapause hormone (Bommo-DH) but mimics its action at the level of target tissues. No homology exists between the primary sequences of the Bommo-DH and ENF peptide families, but this does not exclude that one can mimic the action of the other (An *et al.*, 2007).

In *B. mori*, 30K proteins are synthesized by the fat body and then secreted into hemolymph during the last instar larval stage, where they get accumulated to a high concentration and then are gradually absorbed into the oocyte (Chen and Yamashita, 1990). About 35% of the total yolk proteins consists of 30K proteins (Zhu *et al.*, 1986). These proteins have a variety of biological functions, providing a source of nutrients for embryogenesis (Zhong *et al.*, 2005), binding to glucan to defend fungal infection (Ujita *et al.*, 2002;

2005) and inhibiting apoptosis (Kim *et al.*, 2003; Park *et al.*, 2003).

There are several 30KPs as well as their homologs reported in silkworm *B. mori*. Ten 30KP genes have been identified in the silkworm (Sun *et al.*, 2007), and more genes show sequence homologous to 30KPs like *B. mori* larvae serum protein (LSP) and LSP-T protein that are homologs of 30KPs (Fujiwara and Yamashita, 1992; Miyagawa *et al.*, 2004). Microvitellogenin of *Manduca sexta* and the C terminus (170th–430th amino acids) of growth-blocking peptide binding protein (GBP-BP) of *Pseudaletia separata* also show some similarity to 30KPs (Matsumoto *et al.*, 2003; Wang *et al.*, 1989). Both Growth blocking peptide (GBP) and PP belong to ENF peptide family in view of the consensus N-terminal sequence (Glu-Asn-Phe). A cytokine-like factor, *B. mori* paralytic peptide (BmPP), which was purified from the silkworm hemolymph, belongs to the ENF peptide family. Like other Paralytic peptides, the Paralytic peptide of *B. mori* (BmPP) is also multifunctional ENF-peptide (Ha *et al.*, 1999). The C-terminal amino acid sequence (190th–430th amino acids) of *B. mori* paralytic peptide binding proteins is homologous to 30KPs (Hu *et al.*, 2006). Growth-blocking peptide binding protein was reported to function for silencing the GBP action (Matsumoto *et al.*, 2003)

In a recent study by Zhang *et al.*, (2012), 73 genes of Lipoprotein_11 family were retrieved from 12 lepidopteran species and the structural analysis showed that these genes could be divided into three distinct subfamilies according to their differential N-terminal domain. A novel subfamily of Lipoprotein_11 was reported for the first time in the study and named serine/threonine-rich 30KP according to its exclusive domain.

The abundant level of 30KPs expression suggests that they may serve as storage proteins (Fujiwara and Yamashita, 1992; Izumi *et al.*, 1981; Wang *et al.*, 1989). Not much is known about the molecular mechanisms controlling the terminal process in the cellular immune response. The expression of PP and PP-BP has not been studied till date in diapause induced eggs

of multivoltine silkworm *B. mori*.

In this study we report a high expression of the paralytic peptide and paralytic peptide binding protein in the diapause induced eggs of multivoltine silkworm *B. mori* which may suggest that these proteins might also play a role in regulation of diapause induction.

Materials and Methods

Insect rearing

The multivoltine strain MW13 (Indian origin) was selected for the study. The larvae were reared as per the standard rearing method of Krishnaswami., (1978) up to last instar to obtain non-diapausing eggs, The late stage (4th & 5th instars) larvae were then reared under low temperature (18°C) and photoperiod (6L: 18D) up to cocooning stage and the moths were made to lay diapausing eggs at normal room temperature (25°C) (Saravanakumar *et al.*, 2008).

RNA isolation

After oviposition, the diapause and non-diapause egg samples were collected from 0 to 48h at every 6h time interval, while, other tissues were collected from the day 3 of 5th instar larvae. Total RNA was extracted from the diapause and non-diapause eggs using TRIzol reagent (Invitrogen, USA), denatured in formaldehyde, formamide and electrophoresed in 2.0% agarose gels.

cDNA preparation

The first strand cDNA was synthesized utilizing RNA (2 µg) treated with 0.5µl of DNase buffer and 0.5 µl of DNase (Invitrogen, USA) for 15 minutes. Then, the reaction was terminated by heating at 75°C for 10 minutes, to the above DNase treated sample, 1µl 10 mM dNTP, 1µl oligo (dT)18 (0.01mM) (Eurofin India Pvt Ltd, Bangalore) was added followed

by incubation at 65°C for 5 min. Finally 1X reverse transcriptase buffer (4 µl), 5 mM DTT (1 µl) and 1µl of M-MLV Superscript III reverse transcriptase (Invitrogen, USA) was added to obtain a final volume of 20 µl. The reaction was terminated by heating at 75°C for 10 min according to the manufacturer's protocol.

Microarray experiment and data analysis

A genome wide oligonucleotide microarray containing 24,924 probes were used to investigate the gene expression profiles of diapause induced and non-diapause eggs of multivoltine silkworm *B. mori* at 18 and 30hrs after oviposition. The complete sets of raw and normalized data from this study have been deposited in the NCBI Gene Expression Omnibus (GEO) repository (accession number GSE35622).

Reverse transcription polymerase chain reaction (RT PCR) analysis for tissue specific gene expression

PCR amplification was performed in a 25 µl reaction mixture containing 2.0 µl of 10X reaction buffer (100 mM Tris-HCL, pH 8.3, 500 mM KCl), 0.2 mM dNTPs, 1.5 mM MgCl₂, 10 picomoles of forward and reverse primers, 0.3 U of Taq DNA polymerase (MBI Fermentas) with 1µl first strand cDNA as template. β actin (FP 5'cactgaggctccccctgaac 3' and RP 5'ggagtgcgtatcccctcgtag 3') (Eurofins, Bangalore) was used as an internal standard. The PCR amplification was carried out under the following conditions: 94°C for 3min followed by 27 cycles of 94°C for 30s, 54°C for 30s, 72°C for 2 min and a final extension of 7 min at 72°C.

Real time PCR analysis

One µl of first strand cDNA synthesized from diapause and non-diapause eggs from 6 to 48h after oviposition was used as template for qPCR analysis in a 25µl reaction mixture containing SYBR green mas-

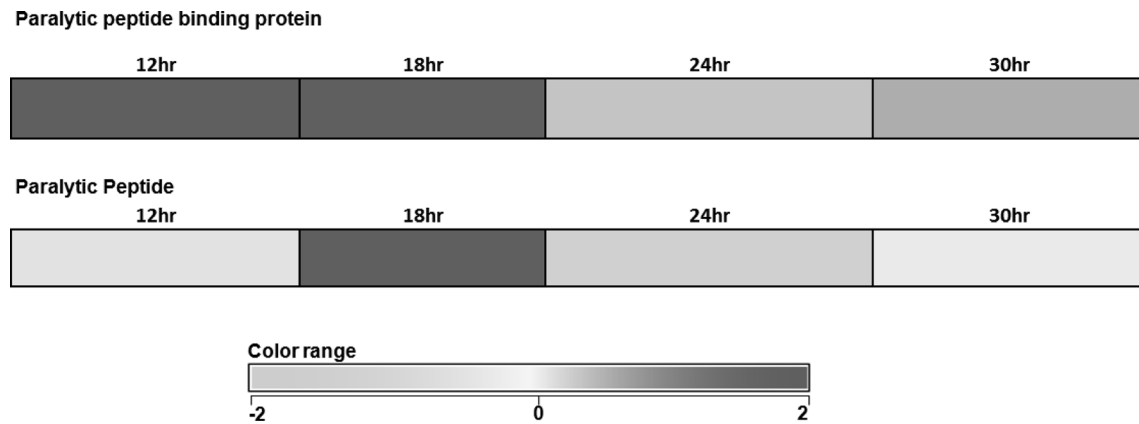


Fig. 1. Microarray expression profile of paralytic peptide and paralytic peptide binding protein in diapause induced eggs of silkworm *B.mori* at different time intervals. Fold change gene expression values are provided as log base 2. The fold change considered for upregulation was ≥ 0.8 and for down regulation was ≤ -0.8 .

termix (ABI, CA, USA) and the specific primers designed for Realtime PCR (qPCR). The reactions were conducted on a STRATAGENE Mx 3005P realtime PCR system. The experiment was performed in triplicate and results were standardized to the expression level of the constitutive β actin gene. A Non-template control (NTC) sample was also run to detect contamination if any.

Identification of paralogous gene sequences in *B.mori*

The *B.mori* cDNA sequences were BLAST searched with Silkworm Genome Database (<http://silkworm.genomics.org.cn>) to identify paralogous multigene family. Using the silkworm database, the functional annotation of genes, paralogous gene sequences, gene products and chromosome mapping were determined. Further, the tools provided in the database were utilized to perform specific genomic BLAST search as well as Map view (a visualization tool that provides a graphical view of selected genes). The organization of paralogous 30KP multigene family on individual scaffold was also analyzed using BLAST search with Gene ID. Phylogenetic analyses were performed with the multiple sequence alignment using ClustalW through MEGA 4 (Kumar *et al.*, 2004). The Bootstrap consensus NJ tree for

30KP paralogous gene sequences was constructed with the Bootstrap values. Signal peptide cleavage sites were predicted using SignalIP algorithm (www.cbs.dtu.dk/services/SignalIP), based on the Neural Network and Hidden Markov Model.

Results

Oligonucleotide microarrays containing 24,924 probes were used to investigate the gene expression profiles of diapause induced and non-diapause eggs of multivoltine silkworm *B.mori* at 18 and 30hrs after oviposition. The complete sets of raw and normalized data from this study have been deposited in the NCBI Gene Expression Omnibus (GEO) repository (accession number GSE35622).

A total of 638 genes were upregulated and 1136 genes were down regulated at 18 h after oviposition, whereas, 675 genes were found to be upregulated and 595 genes down regulated at 30 h after oviposition. Further, genes whose expression was detected in diapause induced and non-diapause silkworm eggs were classified into two groups, stably expressed and variably expressed genes at both 18 and 30 h. 115 genes were stably upregulated, while 117 genes stably down regulated at both 18 as well as 30 h. Up and down regulated genes showing fold change of 0.8 and

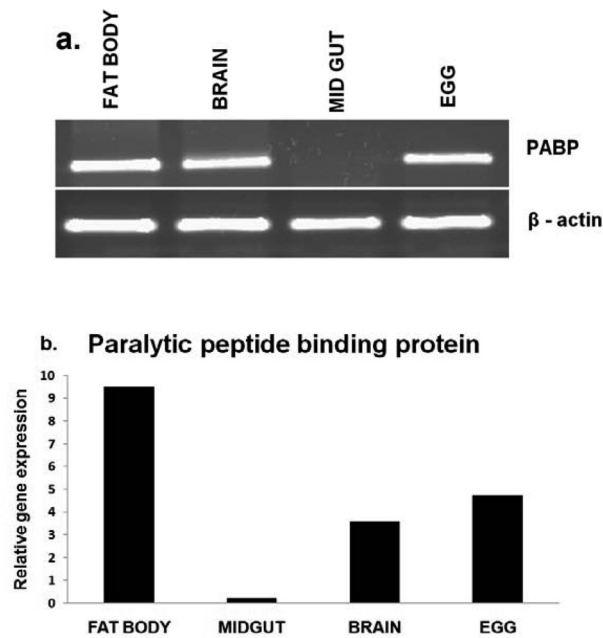


Fig. 2. Expression profiles of paralytic peptide binding protein in different tissues. *B. mori* using (a) semi quantitative RT-PCR and (b) real time PCR (qPCR), β -actin was used as internal control.

above were considered as significantly expressed. 315 genes were found to be variably upregulated at 18 h and 259 genes were found to be variably up regulated during 30 h of diapause-induced eggs (Unpublished data). Among those upregulated genes PP and PP-BP were significantly up-regulated at 12 and 18 hours (Fig. 1). Further these two gene expressions were analyzed in diapause induced eggs at different time intervals after oviposition.

PP-BP gene expression in different tissues

RT-PCR analysis was performed to determine the tissue specific expression of the *B. mori* PP-BP gene. Different tissues viz., fat body, brain, mid gut of 5th instar 3rd day larvae as well as, diapause induced eggs were analyzed for tissue distribution of PP-BP transcripts. The results indicated that PP-BP was highly expressed in fat body followed by brain and eggs, while no expression was observed in mid gut tissue (Fig. 2. a). *B. mori* β -actin gene was used as an inter-

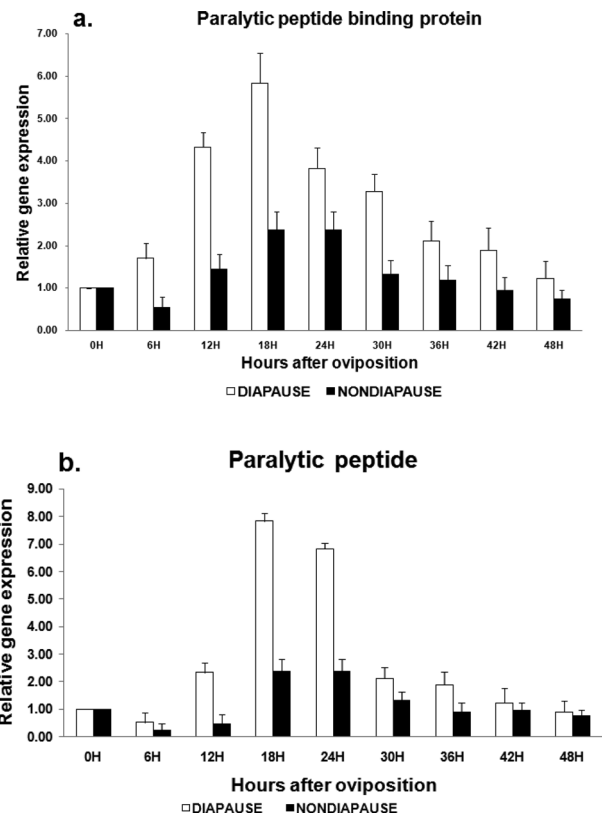


Fig. 3. Relative gene expression patterns of (a) paralytic peptide binding protein gene and (b) paralytic peptide gene upregulated during diapause.

nal control. Further the results validated through real-time PCR analysis (Fig. 2. b) revealed that the PP-BP gene expression was higher in diapause induced eggs, compared to the non-diapause eggs.

Differential expression of *B. mori* PP-BP and PP in diapause induced eggs

The expression level of PP-BP gradually increased from 0 to 24 h, where, the expression was at maximum, followed by a very steep decrease from 30 to 48 h in diapause induced eggs. The gene expression in non-diapause eggs was very low from 6 to 48 h time intervals compared to diapause eggs. The expression level of paralytic peptide was almost similar to PP-BP in diapause eggs while in non-diapause eggs they were slightly higher compared to PP-BP. (Fig. 3a, 3b).

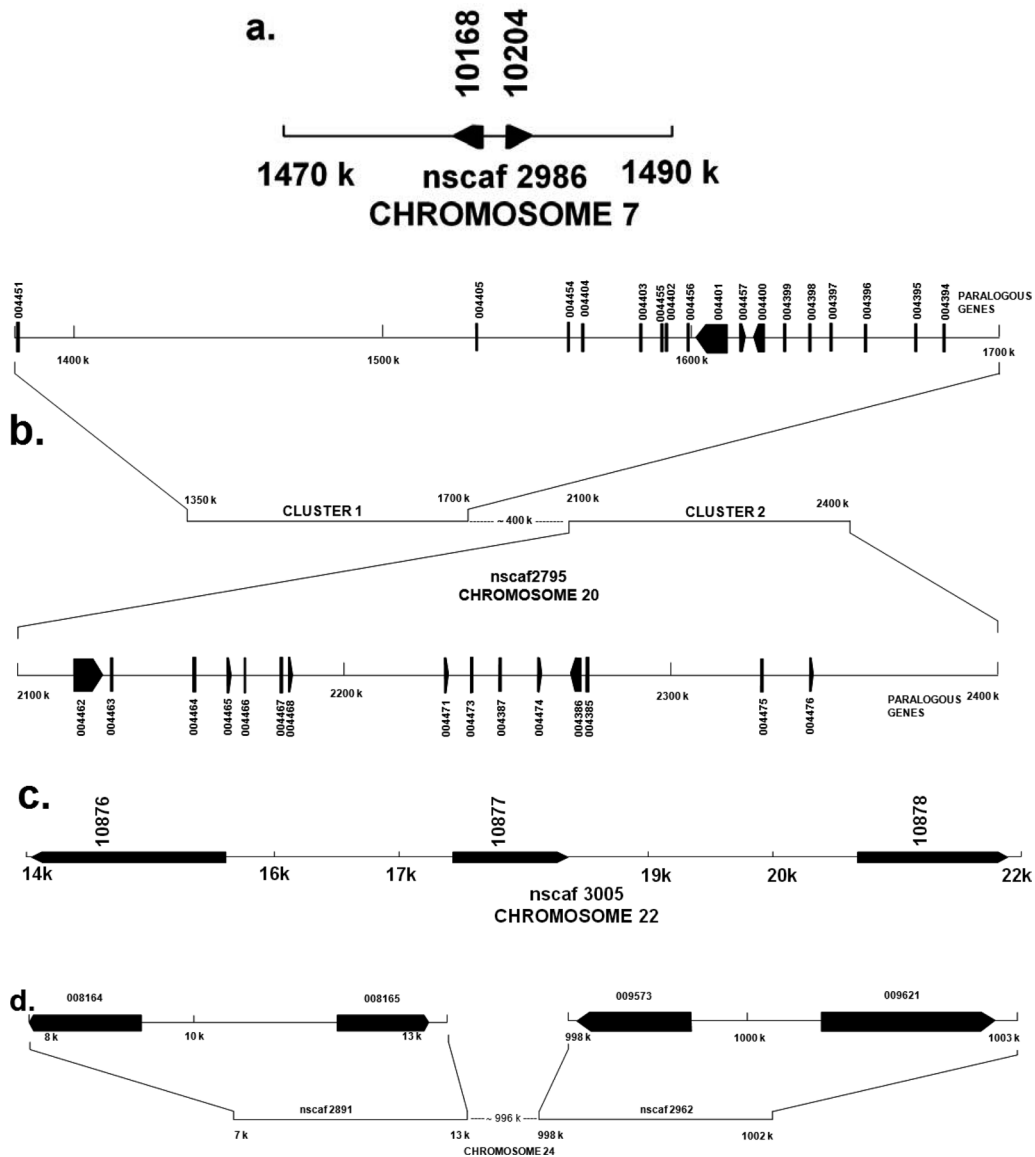


Fig. 4. Schematic representation of gene clusters arranged on different chromosomes of *B. mori*. (a) The two paralogous genes of ENF-BP located in nscf 2986 between 1470 kb and 1490 kb on chromosome 7. (b) The two major gene clusters of 30 KP and S/T rich 30 KP located in nscf 2795. Cluster one flanked from 1350 kb to 1700kb and cluster two from 2100 kb to 2400 kb on chromosome 20 with intergenic region of 400 kb. (c) Three paralogous genes of ENF-BP gene located in nscf 3005 between 14000 kb to 22000 kb on chromosome 22. (d) Two clusters of ENF-BP gene located in nscf 2891 and nscf 2962. Cluster one flanked from 1350 kb to 1700 kb and cluster two from 2100 kb to 2400 kb on chromosome 24 with intergenic region of 996 kb.

Genomic organization of *B. mori* PP-BP paralogous genes

The cDNA sequence of *B. mori* PABP was subjected to blast analysis and several paralogous contigs were

identified. A total of 46 paralogous sequences were identified which were distributed on chromosomes 7, 20, 22 and 24 (Fig. 4. a, b, c, d). Based on the structural characteristics of the above identified 46 sequences they have been classified into ENF-BP, typical 30KP, and

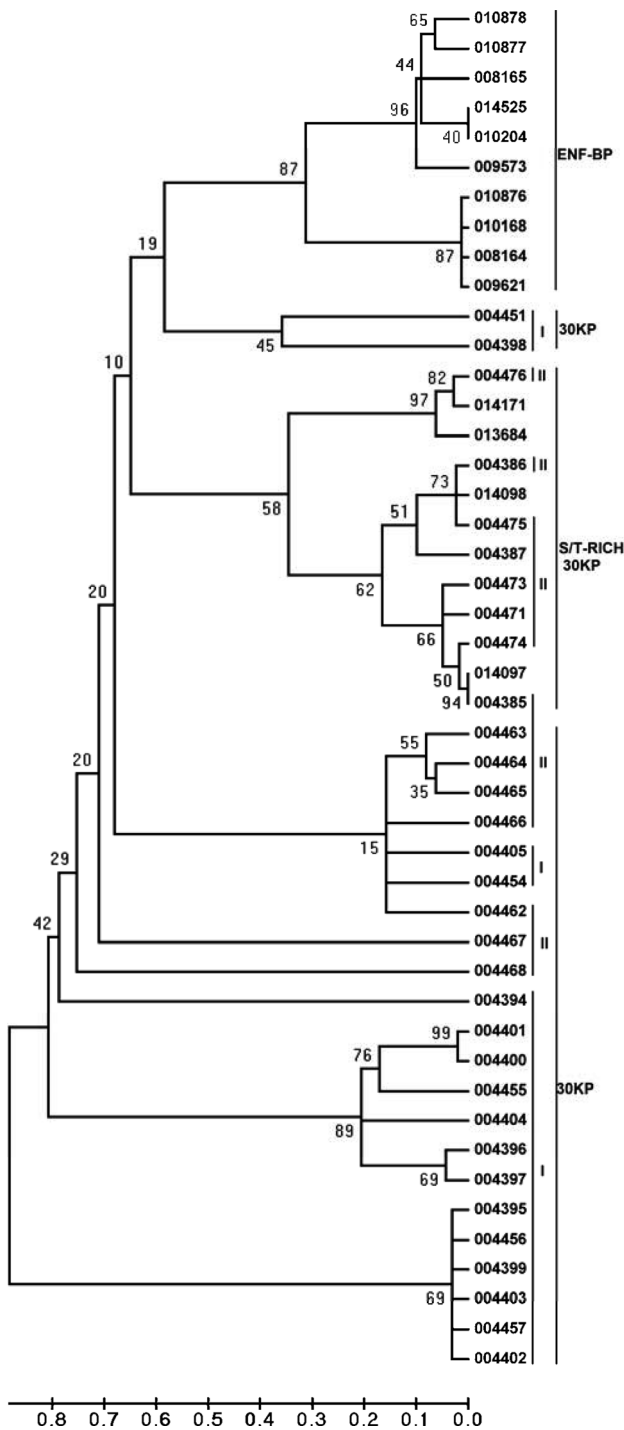


Fig. 5. Neighbor-joining tree of paralogous genes of paralytic peptide binding protein, percentage of bootstrap values (based on the 1000 replication) for the main branching nodes are shown on the tree. The paralogous gene sequences retrieved from silkworm genome database are indicated by Gene ID number.

serine/threonine-rich 30KP. ClustalW analyses were performed with the multiple sequence alignment using ClustalW through MEGA 4 which revealed that these paralogous sequences formed three major clusters. Of the 46 sequences identified, 10 fall under the category of ENF-BP cluster, 12 fall under S/T-rich 30KP cluster and 24 of them fall under typical 30KPs clusters (Fig. 5).

Discussion

Since the finding of the first member of the ENF peptide family, eleven lepidopteran species including *B. mori* have been found to have such peptides (Ha *et al.*, 1999). Antya-ParP when injected in to the non-diapause egg producing pupae, the pupae are converted into diapause egg producers even lacking sub esophageal ganglion. Even though Bommo-DH and ENF peptides share no homology in the primary sequences, the later can mimic the action of the former. ENF peptides are regarded as insect cytokines and have multiple biological activities, including mitogenic, paralytic, hemocyte-spreading, and growth blocking activities (Aizawa *et al.*, 2002; Kamimura *et al.*, 2001). However, limited information is available on diapause eggs

In the present study, the gene expression levels of PP and PP-BP were compared in diapause and non-diapause eggs after oviposition. The expression of PP was higher at 18 h similarly the expression of both PP and PP-BP genes was higher at 12 and 18 h along with few other genes in microarray experiment. These genes were taken up for further study, which were validated through q-PCR.

Most of the reports emphasize the role of ENF peptides in cellular immunity and cell proliferation. An *et al.*, (2007) proved that *Antheraea yamamai* paralytic peptide induces egg diapause even in pupae where subesophageal ganglion had been extirpated and also causes a rapid and rigid larval paralysis in *B. mori*. It was also inferred that AnyParP interacts with other pathways to excite diapause rather than those involved in paralysis. It was also speculated that AnyParp and

homologs act on the same channel in the cascade of diapause egg induction as the DH.

The structural and phylogenetic analyses of paralogous sequences revealed that these sequences can be categorized into three categories ENF-BP, typical 30k proteins and S/T rich 30K proteins. The 30k proteins are involved in the diapause mechanism through regulating the action of paralytic peptide.

In a study by Zhang *et al.*, (2012), the ENF-BP genes were also expressed in the gonads (ovary and testis), fat body, head, integument, silk gland, etc which was different from the hemocyte specific expression pattern of *P. separate* ENF-BP. The Bm ENF peptides, also show extensive tissue expression profiles, such as in gonads, fatbody, integument, central nervous system, etc. (Aizawa *et al.*, 2002; Kamimura *et al.*, 2001). Similarly in the present study it is found that the PP-BP is highly expressed in fat body followed by eggs and brain while, no expression is observed in midgut. As ENF peptides are reported to play a role in regulation of the cell proliferation as growth factors, it can also be assumed that ENF-BPs may be involved in development by regulating the expression or activities of ENF peptides.

Multigene organization of *B. mori* PP-BP gene

Genes that have originated by gene duplication retained a certain degree of similarity forming multigene families. The multigene family members are often arranged in a compact cluster due to chromosomal rearrangements subsequent to gene duplications. The members of a multigene family can be functional or nonfunctional which are known as pseudo genes. Multigene families containing paralogous gene sequence that evolve in different ways, and assumption of the multigene family evolution is essential for estimation of its phylogeny. It is assumed that members of a multigene family may evolve at a faster rate and such members are designated as fast evolving genes. This phenomenon takes place when one gene member of a multigene family takes on a novel function, which is essential for survival and thus encounters significantly different selective

pressure from other multigene family members. Another usual assumption of molecular tree of multigene family is that, each branch of the tree evolves independently from other branches. These families often show coincidental evolution, either indirectly through biased mutational and selective force or directly by mechanism such as gene conversion (Roach *et al.*, 2005).

A total of forty six *B. mori* paralytic peptide binding protein homologs were retrieved from the silkworm genome database (<http://silkworm.genomics.org.cn>). These forty six paralogous gene sequences, belonging to PP-BP gene family diverged into three different groups, viz., ENF-BP, S/T rich, Typical 30KP. Ten sequences belonged to ENF-BP, twelve to S/T rich 30KP proteins and Twenty four to typical 30KPs. The PP-BP has conserved domain similar to ENF-BP and hence, ENF-BP is also considered as paralytic peptide binding protein. The twenty four 30KP paralogous genes are organized in a single locus of chromosome 20; however, two paralogous genes of 30KPs formed a separate cluster from the remaining 30KP gene family (Fig. 5). The 30KPs can be divided into 2 groups one as typical 30KP and S/T rich 30KP. The S/T rich 30KP have a serine/threonine rich domain between signal peptides and N-terminal domain, while the N terminal of typical 30KPs is composed of the signal peptides and an un-conserved region. Twenty four 30KPs as well as Twelve S/T rich 30KPs formed a major cluster located in nscaf 2795 of chromosome 20 (Fig. 4B). This locus was grouped into two clusters with intergenic region of 400K. The cluster 1 consists exclusively of 30KP; however, the cluster 2 consists both 30KP and S/T rich 30KP. Though a major cluster is formed, the 30KP and S/T rich 30KP grouped independently in this major cluster. The paralogous gene 04451 was located approximately 150kb from the remaining cluster, indicating, that this paralogous gene has diverged from the single ancestral gene prior to multigene family formation, to further support this hypothesis the gene 4451 formed a separate cluster in the phylogenetic tree. Comparison of the molecular distance of the paralogous genes within the species indicates the amount of coincidental evolution (Walsh

et al., 1995). The presence of S/T rich subgroup in the 30KP group indicates that both the groups have evolved recently from the ancestral gene. The original gene might have originated from the common ancestors by gene duplication and later the individuals within the multigene family were formed during the process of evolution. Unlike the typical 30KP and ST rich 30KPs the multigene cluster of ENF-BP was spread over different chromosomes viz., chromosome number 7, 22 and 24. The long intergenic regions between two paralogous gene sequences as well as presence of these genes in different chromosomes indicate that the multi gene family evolved much ahead of typical 30KP and S/T rich 30KPs.

Similar phenomenon was observed for cecropin multigene family in *B. mori*. Cecropin B locus on genomic DNA consists of 6 paralogous gene groups as well as Cecropin D and E (Ponnuvel *et al.*, 2010). However, the maximum intergenic region of 23Kb was observed between Cecropin D and remaining Cecropin B family indicates that the two gene families were distantly evolved. The ENF-BP located on chromosome 24 had 4 paralogous gene sequences in a single locus of opposite orientation. These 4 genes of ENF-BP located on the same position of nscaf 3005 on chromosome 22 indicates that these 3 genes were closely evolved. Similarly, 2 genes present in nscaf 2986 of chromosome 27 also formed a subcluster from the remaining group. It was considered as 30KPs only based on the conserved sequence present in the c terminal region of the protein.

In the current study it is observed that paralytic peptide is specifically up-regulated at 18h time point suggesting its possible role in induction of diapause along with other factors that contribute to the same. Excessive amount of PPs cause serious damage to insect self organization. Paralytic peptide binding proteins are also up-regulated during the diapause period, which suggests that PP-BP act as active regulators of the paralytic peptide (Matsumoto *et al.*, 2003), as even the over expression of the paralytic peptide might cause damage to the cells.

Paralytic peptide and paralytic peptide binding protein interaction has to be further exploited to find out whether it is an alternative mechanism of diapause in-

duction and also its possibility to design novel growth regulators in other insects.

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