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Characterization and regulation of *Bacillus thuringiensis* Cry toxin binding aminopeptidases N (APNs) from non-gut visceral tissues, Malpighian tubule and salivary gland: Comparison with midgut-specific APN in the moth *Achaea janata*

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ABSTRACT

Bacillus thuringiensis (Bt) crystal proteins (Cry) bind to aminopeptidase N (APN) receptors on insect midgut membrane leading to pore formation and subsequent death. However, evolution of insect resistance to Bt toxins threatens their long-term application. Therefore, search for new targets which could function as Cry toxin receptors is an immediate mandate. In the present study, two full-length APN cDNAs were cloned from Malpighian tubule and salivary gland tissues of the moth, *Achaea janata*. Both these APNs showed 99% and 32% sequence homology with fat body and midgut APNs respectively. Tissue distribution analysis revealed the presence of two different APN isoforms, one predominant in non-gut visceral tissues while the other exclusively expressed in the midgut. Immunofluorescence and western blot analyses showed cross-reactivity in Malpighian tubule and salivary gland when probed with anti-fat body APN antiserum. These results clearly indicated the presence of non-gut (AjAPN1) and gut-specific (AjAPN4) isoforms in this moth. The expression of both the isoforms steadily increased during the larval development. Hormonal studies indicated regulation of the APN genes by the morphogenetic hormones, 20-hydroxyecdyone and juvenile hormone. Further, *in vitro* ligand-blotting studies demonstrated binding of Cry toxins to APNs in Malpighian tubule and salivary gland indicating their potential as alternate targets.

1. Introduction

Aminopeptidases N (APNs) are a class of zinc-dependent endoproteases that cleave the N-terminus of polypeptides to release single amino acids (Pigott and Ellar, 2007). In insects, these ectoenzymes are located primarily on the brush border membrane of midgut epithelial cells attached through a glycosylphosphatidyl-inositol (GPI) anchor (Garczynski and Adang, 1995). Lepidopteran APNs are widely studied not only for their role in dietary protein digestion (Terra and Ferreira, 1994) but also for their involvement in the binding to the *Bacillus thuringiensis* (Bt) crystal insecticidal toxins, also called Cry toxins or Cry proteins. Cry proteins are produced as protoxins, which upon ingestion are solubilized and proteolytically activated in the midgut of susceptible insects (Gill et al., 1992). The activated toxin then binds to specific receptors in the brush border of midgut epithelial cells and subsequently creates pores in the cell membrane leading to cellular swelling, lysis and eventually death (Bravo et al., 2007; Schnepf et al., 1998). One of the most interesting features of Cry toxins is their insect specificity which is largely determined by the specific binding of these toxins to surface proteins located in the microvilli of larval midgut cells. In case of lepidopteran insects, Cry1 binding proteins have been identified as cadherin-like proteins (Vadlamudi et al., 1995), GPI-anchored APN (Knight et al., 1994), GPI-anchored alkaline phosphatase (ALP) (Jurat-Fuentes et al., 2004), a 270 kDa glycoconjugate and a 250 kDa protein named P252 (Pigott and Ellar, 2007). In addition, glycolipids were proposed to act as Cry toxin receptors in the nematode *Caenorhabditis elegans* (Griffitts et al., 2005).

APN is the most extensively studied Cry toxin receptor with different isoforms from more than 20 lepidopteran species but the number of APN genes for each single species is uncertain (Angelucci et al., 2008; Herrero et al., 2005; Nakanishi et al., 2002; Pigott and Ellar, 2007; Simpson et al., 2008; Wang et al., 2005). So far, the phylogenetic analysis has shown that the APNs within a particular species can be clustered into eight different clades (Crava et al., 2010). However, the characteristic motifs essential for their enzymatic activity and zinc-binding have been conserved in all the cloned lepidopteran APNs.

Several *in vitro* as well as *in vivo* studies provide direct evidences of the participation of APNs in the Bt pathogenesis. The transgenic expression of the APN from *Manduca sexta* generated susceptibility to Cry1Ac toxin in tolerant *Drosophila melanogaster* larvae (Gill and Ellar, 2002) and the RNAi mediated reduction of the expression of an APN of *Spodoptera litura* resulted in a reduced susceptibility of the larvae to Cry1C (Rajagopal et al., 2002). Herrero et al. (2005) described the lack

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of APN expression in a Cry resistant *Spodoptera exigua* strain. More recently Zhang et al. (2009) showed a deletion mutation in the *APN* of a Cry1Ac resistant *Helicoverpa armigera* strain.

Cry toxins have been shown to be a valuable tool for insect control, especially with the development of transgenic plants expressing these proteins. This technology has been successful in reducing the use of chemical insecticides (James, 2009). However, reduction in the binding properties of Cry toxins to midgut cell receptors in different insect species due to mutations and/or decrease in the number of receptor molecules such as APN have been cited as the main reasons for the development of resistance to Cry toxins in agricultural systems that pose foremost threat to sustained application of Bt technology (Gahan et al., 2001; Herrero et al., 2005; Jurat-Fuentes et al., 2004; Morin et al., 2003; Zhang et al., 2009). Despite success stories of 'gene stacking method', only a handful of GM crops with three or more stacked genes have only gained regulatory approval (Bravo et al., 2011; Halpin, 2005). Therefore, there is an immediate need to widen the horizon of search for novel Cry toxin targets in insects. Non-gut visceral tissues as possible Cry toxin targets could provide a good alternative in this regard.

In general, all the reported lepidopteran APN sequences have been obtained from midgut cDNA analysis (Pigott and Ellar, 2007). Limited information is available on the molecular targets of Bt toxins in the insect larval body cavity. Cheon et al. (1997) have demonstrated pore forming cytolytic ability of Cry toxins on the *in vitro* cultured fat body cells indicating the presence of Cry binding proteins in this tissue. A report by Hall (1986) demonstrated the occurrence of APNs in other insect organs in addition to the midgut. Budatha et al. (2007a,b)) first reported the complete characterization of APN from fat body of *A. janata* and *S. litura*. In the present study, we extended our search for APN in larval Malpighian tubule and salivary gland of the economically important pest *A. janata* (Castor semilooper), a noctuid moth that causes serious damage and loss of castor crop in Asia.

In this study, we demonstrated the presence of APNs in the larval Malpighian tubule and the salivary gland and cloned their corresponding cDNAs. These non-gut visceral tissue APNs showed fairly low sequence homology (only 32%) with the midgut-specific APN cDNA. Further, we established the existence of two APN isoforms in *A. janata* (i) *AjAPN1*, which is predominantly expressed in non-gut visceral tissues and (ii) midgut-specific *AjAPN4*. These isoforms manifested a variation in their expression with respect to developmental stages and hormonal treatments. Further, *in vitro* interaction of Malpighian tubule and salivary gland APNs with various Cry toxins has been demonstrated.

2. Materials and methods

2.1. Insect culture

Achaea janata larvae were obtained from Directorate of Oilseeds Research (DOR), Hyderabad, India. These larvae were reared on fresh castor leaves (*Ricinus communis*) as diet under a photoperiod of 14:10 h (light:dark), 60–70% relative humidity at 26 ± 2 °C. The larval development proceeds through five instars and the life cycle is completed in 45–50 days. Each instar from first to fourth, lasts for 2 days while the fifth or last instar duration is 4–5 days long. The fifth instar is further classified into early (5E) and late (5L) stages followed by the non-feeding pre-pupal (PP) stage. The larvae were narcotized on ice for 15 min and tissues were dissected out in ice-cold insect Ringer's solution (130 mM NaCl, 0.5 mM KCl and 0.1 mM $CaCl_2$) and processed immediately.

2.2. APN enzymatic activity

The APN activity (EC 3.4.11.2) was determined as described by Garczynski and Adang (1995). Briefly, protein preparations from Malpighian tubule and salivary gland tissues (20 μg) were incubated with APN specific substrate leucine *p*-nitroanilide (1 mM in 50 mM Tris–Cl, pH 7.3) (Sigma–Aldrich) for different time points at 30 °C. The hydrolyzed product *p*-nitroaniline was monitored by measuring the increase in absorbance at 405 nm (A_{405}) (Malik et al., 2001). The specific activity was expressed as μmoles of *p*-nitroaniline released/min/mg protein. Spectrophotometric values for midgut as well as fat body extracts were used as control.

2.3. Molecular cloning of APN cDNAs from Malpighian tubule and salivary gland of A. janata

Total RNA was isolated from Malpighian tubules and salivary glands of early fifth instar larvae using TRI® reagent (Sigma–Aldrich) following manufacturer's protocol. The concentration was assessed by NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies) and the quality was checked by formaldehyde agarose gel electrophoresis. Total RNA (5 μg) was reverse transcribed to first strand cDNA using Superscript® III following the manufacturer's protocol (Invitrogen). Using Lasergene software (release 3.05; DNASTAR), a set of degenerate primers (MTSGF1- 5′ YTT CYR CAT ACY TGS TMG CTT TYM W 3′ and MTSGR1- 5′ GYW WSG TCA RCM SAR TSW GTY MMR AAG TA 3′) were designed and synthesized based on the alignment of reported insect APN sequences. Using these primers, partial cDNA fragments were amplified from Malpighian tubule as well as salivary gland, cloned into pTZ57R/T vector (MBI Fermentas) and sequenced. Based on the sequence information, gene specific primers were designed to obtain 5′ and 3′ regions of the sequences using GeneRacer™ kit (Invitrogen) following the manufacturers' instructions. The primers designed for 5′ RACE of Malpighian tubule and salivary gland APNs were MTSGR2- 5' CAG GAA TGG CTG CTT GCT GCA TG3' (specific) and MTSGR3-5' GCC ACT CGC TCG TAG TGA GAG ACG A3' (nested). For the 3′ RACE of Malpighian tubule APN, the primers used were MTF2- 5' GGA GCT ATG GAG AAC TGG GGT CTG T 3' (specific) and MTF3-5'GCT TGG TGG GAT AAC CTC TGG CTA A3' (nested). For the 3′ RACE of salivary gland APN, the gene specific primers used were SGF2- 5' GGA GCT ATG GAG AAC TGG GGT CTG T 3' and nested SGF3- 5' GCT TGGTGG GAT AAC CTC TGG CTA A 3'; SGF4- 5′ CCG TGC TCA GAT TGT CGA CGA CG3′ and nested SGF5-5′ GCA CCC TGG GAT GCC GCT ATC 3′. The amplified RACE products obtained were cloned into pTZ57R/T vector, sequenced and aligned using NCBI-BLAST to obtain the respective full-length cDNA sequences.

2.4. In silico analysis of Malpighian tubule and salivary gland APN nucleotide sequences

Primers were designed using Lasergene and Beacon Designer 7 programs. The corresponding amino acid sequences of both APN cDNAs were deduced using the translation tool at the ExPASy Proteomics website (http://expasy.org/translate/). Amino acid sequences were aligned using ClustalW2 program (http://www.ebi.ac.uk/tools/msa/clustalw2/) and the results were visualized as colored classification using Jalview software. The phylogenetic tree was constructed using PhyML using bootstrapping procedure (500 replicates). The presence and location of signal peptide cleavage site in the amino acid sequences were determined using SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The presence of GPI-anchor in the amino acid sequences was predicted using PredGPI software (http://gpcr.biocomp.unibo.it/predgpi/pred.htm). The presence of potential N-, C- and O-linked glycosylation sites was analyzed using NetOGlyc2.0 program (http://www.cbs.dtu.dk/services/netOGlyc). The computation of the theoretical isoelectric point (pI) and molecular weight (MW) was carried out with Compute pI/MW program (http://www.expasy.org/compute_pi/).

2.5. qRT-PCR analysis of APNs for tissue distribution, developmental pattern and hormonal regulation

Transcript abundance of APN was quantified by real-time RT-PCR of total RNA isolated from the Malpighian tubule, salivary gland, fat body

and midgut tissues collected during early fifth instar (5E) for tissue distribution and hormonal regulation studies. However, for the study of developmental regulation of APN gene, tissues from third, fourth, fifth and prepupal stages were used. Absence of genomic DNA contamination in the total RNA was confirmed by using non-reverse transcribed samples as templates. Absence of DNA in total RNA was also ensured by treating with *DNase* I before proceeding for first strand cDNA synthesis. The gene expression levels were quantified by relative qRT-PCR using TaqMan MGB probes (Applied Biosystems). Specific primers and TaqMan MGB probe for *AjAPN1* and *AjAPN4* (forward primer1: 5′ AGG AAT ACA CAG GCT ATC CGT ACT 3′, reverse primer1: 5′ GGC TGC TTG CTG CAT GAT 3′ and probe1: 5′ CAA TGA CCG AGA ACA TC 3′; forward primer4: 5′ TGC TCA GTC TAG TCT GTA 3′, reverse primer4: 5′ GCC CTG TTC AAA TAG TGA 3′ and probe: 5′ TCC TCT CAC GAT TGC TGC TCT T 3′) were custom designed using primer express 2.0 software (Applied Biosystems). Insect specific *18S rRNA* was used as an internal reference to normalize the APN transcript expression levels. Primers used for *18S rRNA* were- forward primer: 5′ GCT ACC ACA TCC AAG GAA GGC AGC 3′ and reverse primer: 5′ CGG CTG CTG GCA CCA GAC TTG 3′. Reverse transcription was carried out with 1 μg total RNA and random hexamer primers using Superscript®III (Invitrogen) according to manufacturer's protocol. Quantitative PCR was carried out in 25 μL reaction volume in triplicates containing the following components: 1 μL of cDNA template (1:10 diluted), 1 μL of Taqman probe mix and 12.5 μL of Fast TaqMan master mix (Applied Biosystems). A two-step PCR reaction was performed in ABI-7500 fast real-time PCR machine (Applied Biosystems) for 40 cycles under the following condition: initial hold at 95 °C for 20 s followed by two-step RT-PCR for 40 cycles at 95 °C (30 s) and 60 °C (30 s). Dissociation or melting curve analysis was performed for *APN* and *18S* to check for specific amplification. The amplification efficiency for *APN* was 95% with slope of the curve being −3.3 and for *18S* efficiency was 99% with the slope value of −3.7. During each cycle of the PCR, fluorescence accumulation resulting from DNA amplification was analyzed and converted into Cycle threshold (Ct) by the ABI Fast 7500 sequence detection system software. Ct values were obtained from the exponential phase of PCR amplification. The transcript levels were calculated using relative quantification method wherein the expression of *APN*s was normalized against the expression of *18S rRNA*, generating a ΔCt value (ΔCt = Ct value of *AjAPN1/4* − Ct value of *18S rRNA*). For tissue-specific expression, relative mRNA for each sample group was then expressed according to the equation $2^{-\Delta CT}$. For hormonal regulation of *AjAPN1* and *AjAPN4* gene expression analysis, the results are represented as change in the transcript levels relative to the control using the target gene Ct values normalized to that of *18S rRNA* Ct values using the $2^{(-\Delta\Delta Ct)}$ method described by Livak and Schmittgen (2001).

2.6. Western blot analysis of APN

The membrane protein fractions of fat body, Malpighian tubule and salivary gland were prepared according to method described by Kiran Kumar et al. (1997). Briefly, each tissue was homogenized on ice in buffer A (5 mM HEPES, pH 8.5 and 0.1 mM $CaCl_2$), centrifuged at 30,000 *g* for 30 min at 4 °C and the pellet thus obtained was washed once with buffer A followed by suspension in a small volume of buffer B (5 mM HEPES, pH 7.0 and 0.1 mM $CaCl_2$). The midgut brush border membrane vesicles (BBMVs) were prepared as described by Wolfersberger et al. (1987). The protein preparations were resolved on 7.5% SDS-PAGE and electroblotted onto nitrocellulose membranes (Pall-Life Sciences) using trans-blot apparatus (Bio-Rad) according to the procedure of Towbin et al. (1979). Non-specific binding sites were blocked with 5% skim milk (w/v) and incubated with fat body APN polyclonal antibody (1:10,000 dilutions) of *A. janata* (Budatha et al., 2007a). This was followed by incubation with ALP-conjugated goat anti-rabbit IgG (Bangalore Genei, India) and developed with NBT-BCIP substrate (Sigma–Aldrich).

2.7. Immunolocalization of APN in tissue sections

The Malpighian tubule, salivary gland, fat body and midgut tissues of fifth instar larvae were fixed in 4% paraformaldehyde (PFA), dehydrated and embedded in paraffin blocks and sectioned at 5 μm thickness using a rotary microtome (Leica). The paraffin sections were deparaffinized, processed through 10 mM citrate buffer (pH 6) and then permeabilized using 0.1% Triton X-100 in the same buffer. Endogenous peroxidases were inhibited with 3% H_2O_2 in methanol, blocked with 10% goat serum and incubated with fat body APN polyclonal antibody (1:1000 dilutions) in 2% rabbit serum. Sections incubated separately with pre-immune serum were used as controls. Sections were washed in PBS with 0.1% Tween 20 (PBST) for 10 min to remove any unbound antibody and further incubated with Alexa Flour 594-labeled goat anti-rabbit IgG (Invitrogen) (1:1000 dilutions) and nuclear counter stained with diamidino-2-phenylindole (DAPI, Pierce). The images were captured with laser-confocal microscope (Leica, TCS STED).

2.8. Hormone treatments

Thorax ligation of early fifth instar larvae was performed as described by Ashok and Dutta-Gupta (1988). 20-Hydroxyecdysone (20E) (Sigma–Aldrich) was dissolved in ethanol (1 mg/mL) diluted in insect Ringer's solution as per requirement. Methoprene, a JH analog (Sigma–Aldrich) was dissolved in acetone. 24 h post-ligation, each ligature was injected with 0.5, 1, 5 or 10 μg of 20E using a Hamilton micro syringe. The final concentration of ethanol did not exceed 0.05%. Similar concentrations of methoprene in acetone were topically applied on the dorsal side of each of ligated larvae. Control larvae were either injected or topically applied with equal volume of the corresponding carrier solvent. The injection sites were dressed with traces of antibiotic mixture (streptomycin: puromycin in 1:6 ratio) and sealed with bee wax.

2.9. Preparation of biotinylated activated Cry toxins

Cry1Aa, Cry1Ab and Cry1Ac protoxins were prepared from recombinant *Escherichia coli* JM103 strains ECE52, ECE53 and ECE54 harboring *cry1Aa*, *cry1Ab* and *cry1Ac* genes respectively (Lee et al., 1992) which were supplied by *Bacillus* Genetic Stock Centre (Ohio State University, USA). Further, Cry protoxin(s) from a local Bt isolate DOR5 was prepared by differential ultracentrifugation (Thomas and Ellar, 1983) in a Beckman L8-80M ultracentrifuge using SW 50.1 rotor at 42,000 rpm for 4 h at 4 °C. The toxins were activated (Lee et al., 1992), purified by gel filtration on Sephadex G-100 column and biotinylated using a kit (Bangalore Genei, India).

2.10. Ligand blot analysis

The Malpighian tubule and salivary gland membrane protein fractions (30 μg each) were separated by 7.5% SDS-PAGE and electrotransferred to nitrocellulose membranes (Pall-Life Sciences). The membranes were blocked in a blocking buffer (3% (w/v) BSA in 0.01 M Tris-buffered saline, pH 7.4) for 1 h, followed by incubation in a blocking buffer containing individual biotinylated Cry1Aa, Cry1Ab, Cry1Ac or DOR5 Cry toxins (200 ng/mL) for 1 h. Following this, the blots were washed with TBS (pH 7.4) supplemented with 0.1% Tween-20 (TBST), and then incubated for 2 h in a blocking buffer containing streptavidin-ALP conjugate (1:1000 dilutions). Cry toxin-bound proteins were detected using the ALP substrate, NBT-BCIP (Sigma–Aldrich).

2.11. Statistical analysis

Data are expressed as mean ± SEM of three independent experiments ($n = 3$). Differences between groups were analyzed for statistical

significance by One-Way ANOVA followed by Students–Newman–Keuls' post hoc test using SigmaPlot 11.0 software (Systat Software Inc., USA). A probability of $P < 0.05$ is considered statistically significant.

3. Results

3.1. Cloning and characterization of APN cDNAs from Malpighian tubule and salivary gland tissues

Significant APN activity detected in Malpighian tubule and salivary gland tissues indicates the expression of APN gene (Supplementary Fig. 1). Based on these results, partial fragments of APN cDNA were cloned from Malpighian tubules and salivary glands using degenerate primers (MTSGF1 and MTSGR1) and subsequently a full-length cDNA of APN from both tissues was obtained using 5′ and 3′ RACE. Malpighian tubule APN cDNA is 3095 bp long with an open reading frame (ORF) of 2982 bp which encodes a putative 994 amino acid protein of 111 kDa theoretical molecular mass and a calculated pI of 4.83. On the other hand, salivary gland APN cDNA is 3125 bp long and contains an ORF of 2994 bp, encoding a putative 998 amino acid protein with an estimated molecular mass of 112 kDa and pI of 4.83. The gluzincin APN motif (GAMENWG) and the consensus zinc-binding/gluzincin motif ($HEXXHX_{18}E$) found in all known APNs are present in the obtained sequences as well (Fig. 1A). The predicted N-terminal signal peptide cleavage site is located between alanine (20th amino acid) and phenylalanine (21st amino acid) in both the sequences. Further, a putative C-terminal GPI-anchor signal, several potential *O*-glycosylation sites and one *N*-glycosylation site were identified in both the sequences. Based on the presence of the signature motifs, APNs from Malpighian tubule and salivary gland were classified under gluzincin family of aminopeptidases. Although both these sequences displayed typical APN features, they revealed only 32% sequence identity with midgut APN (**AjABH07377**). However, the two sequences shared nearly 99% sequence identity with fat body APN (**AjABE02186**) indicating the presence of a unique isoform in non-gut visceral tissues. Phylogenetic analysis of APN sequences from Malpighian tubule and salivary gland of *A. janata* with the reported lepidopteran insect APNs showed that these sequences from non-gut visceral tissues (Malpighian tubule and salivary gland) are clustered under Class 1 along with fat body APN, while APN specific to midgut of *A. janata* belonged to Class 4 (Fig. 1B). Hence, we designated the APNs from non-gut visceral tissues as "AjAPN1" and midgut-specific APN as "AjAPN4". The Malpighian tubule and salivary gland APN nucleotide sequences have been deposited in the GenBank®/EBI data base (KF537663 and KF537664, respectively).

3.2. Tissue-specific expression of AjAPN1 and AjAPN4

The tissue specificity of *AjAPN1* and *AjAPN4* was determined by quantitative real-time analysis using specific TaqMan probes and *18S rRNA* as internal control. *AjAPN1* was expressed in almost equal levels in Malpighian tubule and fat body while in salivary gland the expression decreased to almost half. A low level expression was also detected in the midgut (Fig. 2A). In contrast, *AjAPN4* was specifically expressed in the gut tissue at high levels (Fig. 2B). This study clearly indicated the presence of at least two different isoforms of APN in *A. janata* and their differential expression in various larval tissues.

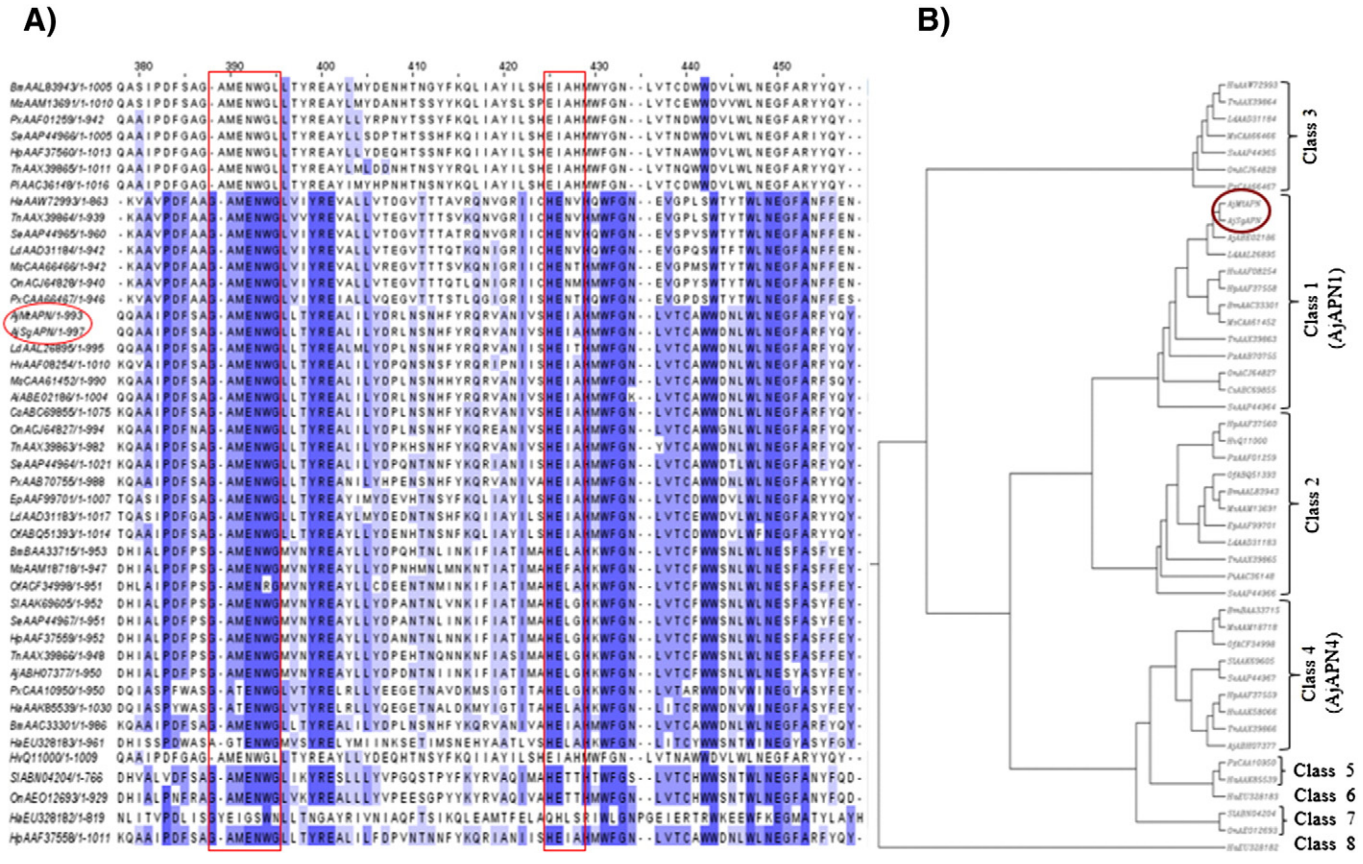

Fig. 1. A) Comparison of amino acid sequences of *A. janata* larval APNs with reported lepidopteran insect APNs aligned by multiple sequence alignment tool. Red boxes indicate the conserved aminopeptidase activity motif 'GAMENWG' and Zn^{++} binding motif "$HEXXHX_{18}E$" in all the sequences. **B**) Phylogenetic relationship of *A. janata* AjAPN1 (from Malpighian tubule and salivary gland) with other lepidopteran insect APNs. Full-length amino acid sequences were aligned using ClustalW to generate a phylogenetic tree. Bootstrap analysis with 500 replicates was used to assess the strength of nodes in the tree. Accession numbers of each APN amino acid sequence attached to the abbreviated species names are represented in the tree. Aj: *A janata*, AjMtAPN: *A. janata* Malpighian tubule AjAPN1, AjSgAPN: *A. janata* salivary gland, AjAPN1, Px: *Plutella xylostella*, Ms: *Manduca sexta*, Ld: *Lymantria dispar*, Hv: *Heliothis virescens*, Tn: *Trichoplusia ni*, Ha: *Helicoverpa armigera*, Bm: *Bombyx mori*, Hp: *Helicoverpa punctigera*, Sl: *Spodoptera litura*, Se: *Spodoptera exigua*, On: *Ostrinia nubilalis*, Of: *Ostrinia furnacalis*, Ep: *Epiphyas postvittana*, Pi: *Plodia interpunctella*, Cs: *Chilo suppressalis*.

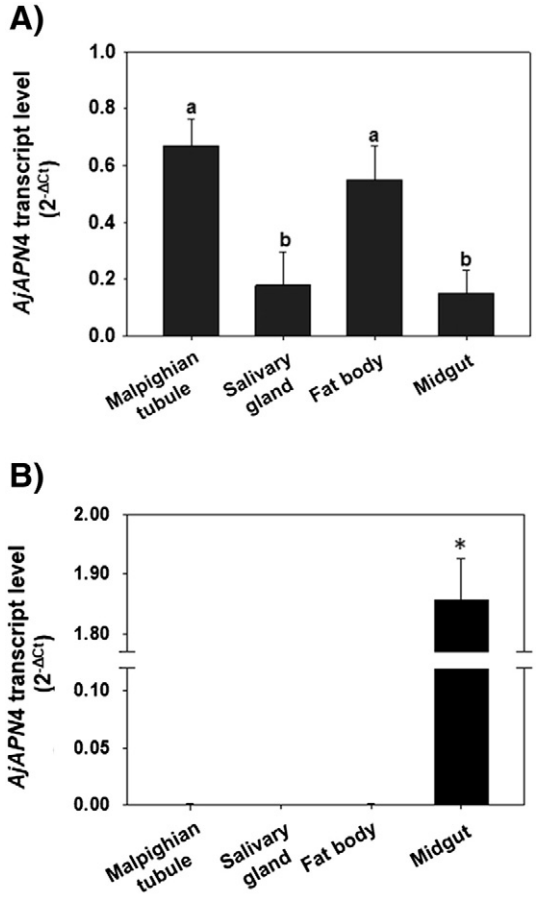

Fig. 2. qRT-PCR analysis of **A**) *AjAPN1* and **B**) *AjAPN4* transcript expression in tested tissues. Data is expressed as mean ± SEM of three independent experiments ($n = 3$) normalized with *18S rRNA* expression. Values labelled with different letters and '*' indicate statistical significance ($P < 0.05$).

3.3. Western and immunolocalization analyses of AjAPN1 expression in Malpighian tubule and salivary gland

As the *AjAPN1* sequences of Malpighian tubule and salivary gland showed almost 99% sequence homology with that of fat body APN, anti-fat body APN antibody was used for western and immunolocalization analyses. The specificity of the antibody was confirmed by preincubation with commercially synthesized APN antigenic peptides (Supplementary Fig. 2). The anti-fat body APN antiserum recognised a 113 kDa membrane protein in Malpighian tubule, salivary gland, fat body and midgut. In addition, the antibody showed weak cross-reactivity with a 170 kDa protein in the midgut (Fig. 3A). Immunolocalization analysis using fat body AjAPN1 polyclonal antibody detected flourescence of Alexa Flour 594 along the basement and luminal border of the epithelial cells of Malpighian tubule and on the epithelial surface of salivary gland. Fat body tissue sections probed with the antibody showed the presence of AjAPN1 expression primarily on the membrane of cells (adipocytes) forming the surface of the tissue. Midgut sections revealed cross-reactivity primarily on the brush border membrane of the epithelium (Fig. 3B).

3.4. AjAPN1 and AjAPN4 expression during larval development

Studies were carried out to monitor the expression of *AjAPN1* (Fig. 4A) and *AjAPN4* (Fig. 4B) during larval development. The mRNA transcripts for both *AjAPN1* and *AjAPN4* were detected during larval developmental cycle. The expression gradually increased from 3rd instar to the early 5th instar. Thereafter, transcript levels declined during late 5th instar larval and pre-pupal stages. Expression was undetected towards the end of larval–pupal transition.

3.5. Effect of 20E and methoprene on AjAPN1 and AjAPN4 expression

Quantitative RT-PCR analysis using specific primers designed from the conserved region of APN demonstrated that the expression of both the isoforms, *AjAPN1* and *AjAPN4* was under the influence of insect morphogenetic hormones, 20-hydroxyecdysone (20E) and juvenile hormone (JH). The transcript levels were monitored after 24 h treatment of 20E (0.5, 1, 5 or 10 μg/larva). Lower concentrations of 20E (0.5, 1 and 5 μg/larva) did not affect the gene expression. However, significant reduction in *APN* transcript levels was observed with high dose of 20E (10 μg/larva). The transcript levels in all the tissues declined significantly at this concentration of 20E (Fig. 5A). Thus, the effect of 20E on *APN* expression appears to be dose-dependent. On the contrary, topical application of methoprene displayed an inductive effect on the transcript levels of *APN*. Both low and high doses of methoprene (0.5, 1, 5 and 10 μg/larva) significantly elevated the transcript levels of both the genes in the tested tissues (Fig. 5B).

3.6. Cry toxin binding to 113 kDa membrane protein of Malpighian tubule and salivary gland

The interaction of different Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac and DOR5) with the 113 kDa membrane protein of Malpighian tubule and salivary gland was studied by ligand blot analysis. The membrane protein profiles of Malpighian tubule and salivary gland were visualized by Coomassie Blue staining. The absence of any signal in the control blots where membrane preparations were incubated with the corresponding unlabelled Cry toxins prior to probing with streptavidin–ALP conjugate suggests a specific interaction between 113 kDa membrane protein and the Cry toxins. In addition, stronger immunoreactivity was visualized in Malpighian tubule (Fig. 6A) compared to salivary gland (Fig. 6B). Further, DOR5 Cry toxin preparation (Supplementary Fig. 3) exhibited strong signal in both the tissues compared to individual Cry toxin. DOR5 isolate comprising of *cry1Aa*, *cry1Ab*, *cry1C*, *cry2A* and *cry2B* was detected by exclusive-PCR (E-PCR) method using the primers and conditions as described by Juarez-Perez et al. (1997). Family primer pairs for *cryI* genes [I(+) and I(−)] and *cryII* genes [II(+) and II(−)] were designed from two highly conserved regions among all *cryI* and *cryII* genes respectively. The specific primer pair for a *cry* type gene belonging to a given family was designed to specifically detect the hypervariable region of the given *cry* type. The presence of a gene of a particular type was determined by including a type specific primer along with the family primer pair to form a triplex PCR. The generation of a single PCR product (~1.5 kb) confirms the presence of a particular *cry* gene family and the appearance of a second band indicates the presence of a specific *cry* gene type (Supplementary Fig. 3; B & C). The protoxins were activated with trypsin as described by Lee et al. (1992). The activated toxin (~60–65 kDa) was purified by gel filtration on Sephadex G-100 column and biotin-labelled.

4. Discussion

Insect midgut APNs as Cry toxin receptors are well characterized (Pigott and Ellar, 2007). In view of emerging insect resistance, search for alternate Cry toxin targets has gained momentum. At least two different isoforms of APN, one predominant in non-gut visceral tissues Malpighian tubule, fat body and salivary gland and the other, midgut-specific, are present in the moth *A. janata*. Based on their canonical features and position in the phylogenetic tree, these isoforms are conveniently classified as AjAPN1 and AjAPN4. Thus, the two APN genes cloned from Malpighian tubule and salivary gland of *A. janata* could be alleles of the one already reported from fat body of the same species (Budatha et al., 2007a). Different lepidopteran APNs were initially

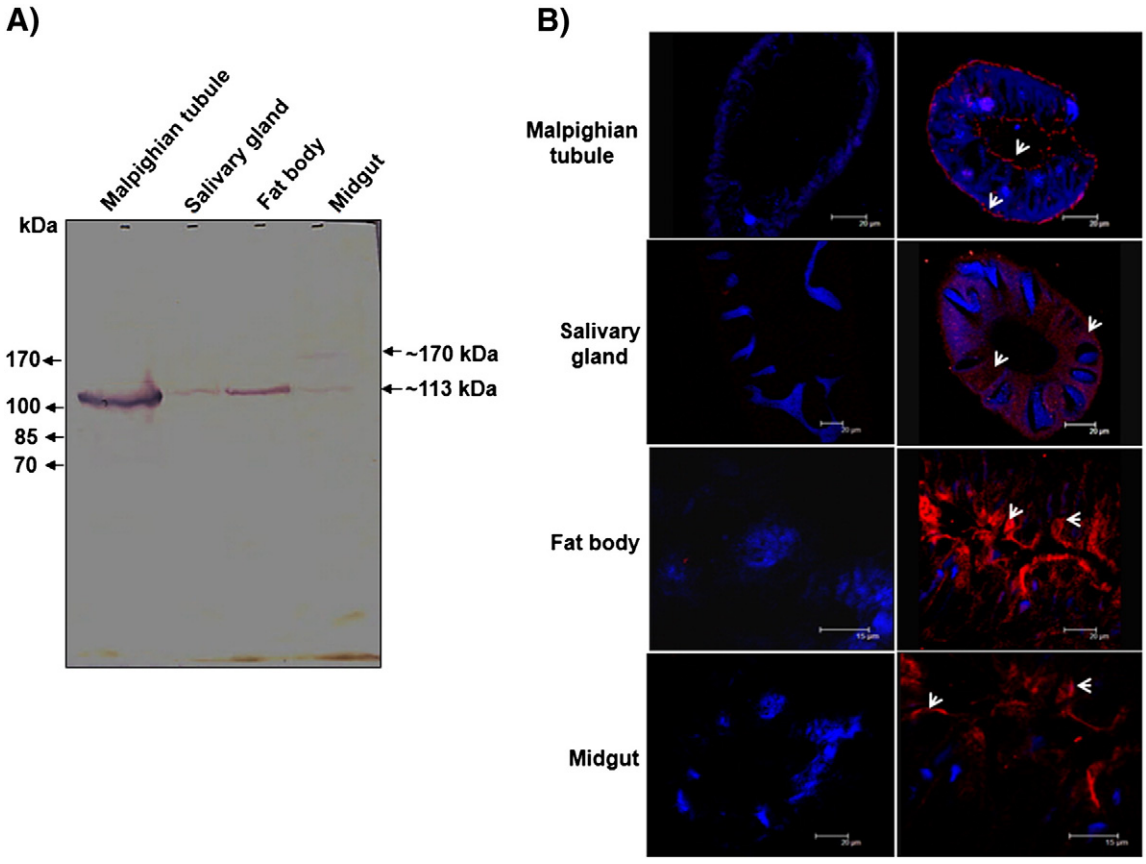

Fig. 3. A) Western analysis using fat body APN antiserum. Tissue protein preparations (30 μg each) separated by 7.5% SDS-PAGE, electro-transferred onto nitrocellulose membrane and probed with the polyclonal antibody. Lane 1: Malpighian tubule, lane 2: salivary gland, lane 3: fat body and lane 4: midgut. Note the presence of signal at 113 kDa and ~170 kDa (depicted by arrows). **B)** Immunolocalization of AjAPN1 using fat body APN antiserum. Panel 1 Malpighian tubule, Panel 2 salivary gland, Panel 3 fat body and Panel 4 midgut. Nuclei were stained blue with DAPI (left micrographs in all panels). Immunofluorescence was visualized in red color (right micrographs in all panels) with Alexa Flour 594-conjugated secondary antibody using confocal microscopy. Arrows (→) indicate the site of immunoreactivity. Magnification is indicated as scale bars in the respective micrographs.

classified into four homology groups following phylogenetic analysis of the sequences (Nakanishi et al., 2002; Oltean et al., 1999). Later, Herrero et al. (2005) proposed the presence of five classes of APNs. Currently, with the addition of new sequences to GenBank, an analysis clearly revealed the presence of at least 8 different classes of APNs in Lepidoptera (Crava et al., 2010). The multiple APN isoforms existing in insects are believed to have either arisen as a consequence of gene duplication events (Chang et al., 1999) or linked to the components of the diet (Chougule et al., 2005).

The possibility of fat body AjAPN1 being exploited as a Cry toxin receptor in *A. janata* and *S. litura* earlier demonstrated by our laboratory (Budatha et al., 2007a,b) further opened up the search for additional avenues. In the last eight years, different groups have demonstrated APN expression in fat body and/or Malpighian tubules of cabbage looper, *T. ni* (Wang et al., 2005), tortricid moth, *Epiphyas postvittana* (Simpson et al., 2008), European corn borer, *Ostrinia nubilalis* (Crava et al., 2010) and silk moth, *B. mori* (Crava et al., 2010). However, none of these earlier studies have reported the presence of APN in salivary glands. Consistent levels of *O. nubilalis APN8* were expressed in both Malpighian tubule and midgut. However, low expression levels of *APN 3a* were found only in Malpighian tubule and fat body but not in midgut (Crava et al., 2010). Microarray data analysis of *B. mori* APN expression profiles reveals midgut-specific transcripts (*Bm APN2*, *Bm APN3* and *Bm APN4*) and a Malpighian tubule-specific transcript (*Bm APN1*) (Xia et al., 2007). In *E. postvittana*, the expression of APN belonging to groups 1 to 5 was detected in all the tested tissues (midgut, fat body, Malpighian tubule and carcass) (Simpson et al., 2008). Differential expression of various gut enzymes including aminopeptidases from *H. armigera* has been demonstrated in larvae fed with different diets (Chougule et al., 2005).

Western analysis identified the presence of 113 kDa membrane protein in all the tested tissues using anti-fat body APN antisera which could be AjAPN1 as the theoretical molecular masses are in accordance with the size of the membrane protein detected in the present study. The additional ~170 kDa protein detected in the gut recognized by anti-fat body APN was also observed in our earlier study demonstrating the release of GPI by phospholipase C treatment which was detected by anti-CRD antibody (Budatha et al., 2007a). Lu and Adang (1996) reported a similar phenomenon for GPI anchored 120 kDa toxin-binding APN in *Manduca sexta*. Later, Oltean et al. (1999) cloned a 170 kDa Cry1Ac receptor from *Heliothis virescens* larval midgut which encoded a protein with deduced molecular mass of only 112.21 kDa, but possessed a stretch of poly(threonine) residues at the C-terminus, which they assumed was most likely glycosylated to give rise to the 170 kDa protein. However, in the present study, AjAPN cDNAs from fat body, Malpighian tubule and salivary gland did not show the presence of any poly(threonine) stretch. Immunolocalization analysis revealed abundant expression of APN on the apical brush border membranes of the columnar epithelial cells of midgut as was observed in other insect species (Chen et al., 2005; Valaitis, 2011) and along the membranes of the Malpighian tubule, salivary gland and fat body. This expression pattern could prove significant in providing easy access for Cry toxins to interact with the membrane-associated APNs in these tissues, which float freely in the larval hemocoel.

The mRNA transcripts for *AjAPN1* and *AjAPN4* in the present study were detected throughout the larval developmental cycle in all the tested tissues. In *E. postvittana*, abundant expression of aminopeptidases was detected in all larval stages and lower levels in pupae (Simpson et al., 2008). Northern blot analysis revealed a developmental regulation of

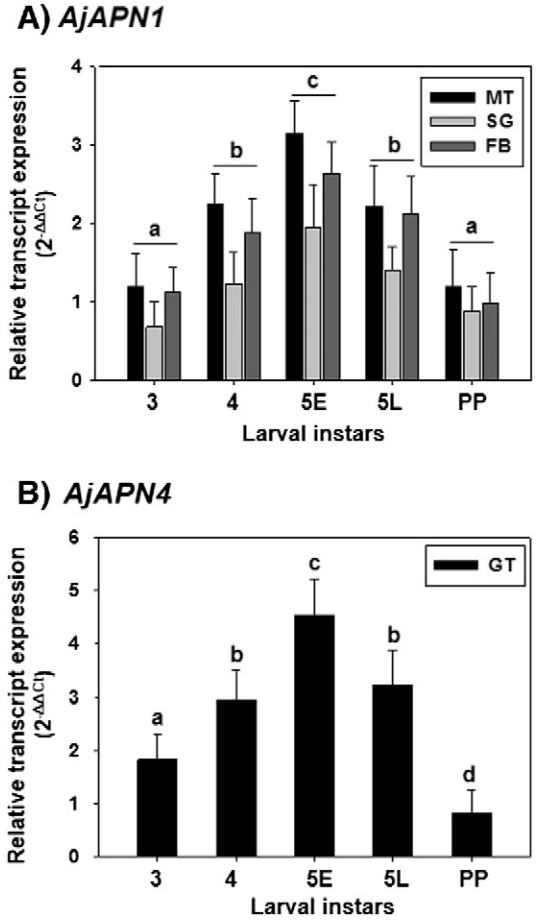

Fig. 4. Developmental expression profile of **A)** *AjAPN1* and **B)** *AjAPN4* transcripts. 3-third instar, 4-fourth instar, 5E-early fifth instar, 5L-late fifth instar, PP-prepupa tissues were used for the analysis. Changes in *AjAPN1* and *AjAPN4* transcript expression are shown relative to 5E stage. Data is expressed as mean ± SEM of three independent experiments ($n = 3$) normalized with endogenous *18S rRNA* expression. Values labelled with different letters differ significantly ($P < 0.05$).

Class 1 APN in *H. punctigera* (Emmerling et al., 2001). The APN expression throughout the larval cycle probably facilitates metabolism leading to storage of macromolecules as well as metabolites that support growth and metamorphosis. In insects, fat body is the principle metabolic tissue that synthesizes, stores or releases molecules central to the prevailing nutritional requirements and/or metamorphic events and is also involved in homeostatic maintenance of hemolymph proteins, lipids and carbohydrates. During metamorphosis, APNs might be involved in the catabolism of larval storage proteins including hexamerins that are sequestered by the fat body (Budatha et al., 2011; Burmester and Scheller, 1999; Haunerland, 1996). Thus, the expression of APN during late larval stages when fat body is functionally most active indicates a significant physiological role in the tissue. As Malpighian tubules are known to collaborate with the larval gut in the absorption of nutritive substances, the APN expression in this tissue could resemble their prevalent expression in the corresponding mammalian tissues i.e., the intestine (where APNs act as digestive enzymes) and the kidney (where APNs play a role in hydrolysis of small peptides in the glomerular filtrate of kidney) (Vlahovic and Stefanovic, 1998). However, the exact function of APNs in insect Malpighian tubule is unclear. Likewise, the physiological role of APN in salivary glands that synthesize and secrete silk proteins for cocoon formation essential for larval–pupal–adult transition is unknown (Chaitanya et al., 2011; Sehnal and Akai, 1990). Fat body, Malpighian tubule as well as salivary gland were shown to sequester intact hexamerins from hemolymph during pre-pupal stage and utilize them during pupal–adult transformation (Damara et al., 2010; Tang et al., 2010). However, more in-depth investigations are required to understand the physiological significance of APN expression in the larval non-gut visceral tissues.

The hormonal regulation of insect APNs is not known. Developmental expression of *AjAPN1* and *AjAPN4* transcripts observed in the present study further warranted investigation of the role of insect morphogenetic hormones, 20E and JH that are known to regulate post-embryonic development in holometabolous insects. Present study showed down-regulation of *AjAPN1* transcript expression in Malpighian tubule, salivary gland and fat body and *AjAPN4* in midgut by high dose of 20E. On the contrary, JH analog, methoprene induced *AjAPN1* and *AjAPN4* transcript expression in these tissues. The opposite action of JH and 20E has also been reported in the regulation of heat shock (Berger et al., 1992) and silk protein genes (Chaitanya et al., 2013). In *Aedes aegypti*, methoprene blocked midgut remodeling by interfering with 20E action (Wu et al., 2006). The action of 20E and JH mediated through EcR and USP is well established (Yao et al., 1992). However, the mechanism of hormonal regulation of APN remains to be elucidated.

Earlier studies demonstrated Cry toxin binding to fat body cells thereby indicating the presence of these proteins in fat body (Budatha et al., 2007a; Cheon et al., 1997). In the present study, binding of Cry1Aa, Cry1Ab, Cry1Ac and DOR5 Cry toxins to 113 kDa membrane proteins of Malpighian tubule and salivary gland on membrane blots is demonstrated. The increased interaction of 113 kDa protein with DOR5 Cry toxin preparation (mixture) could either be due to higher binding property of specific toxin or binding of many toxins present in DOR5 Bt isolate. The weak signals detected could be attributed to low expression levels of APN in these tissues.

Intrahaemocoelic delivery of Cry toxins was shown to illicit toxic response in *Lymantria dispar* (Lepidoptera) (Cerestiaens et al., 2001). Earlier studies have demonstrated Cry toxin penetration across midgut epithelium into the hemolymph where they can interact with different tissues/cell types in the body cavity and alter their function including reproductive organs. However, the mechanism of entry was not established (Hussein et al., 2006). Further, several large proteins and fusion peptides/proteins have been reported to cross the insect midgut epithelium and reach the hemolymph through diet (Brandt et al., 2004; Jeffers et al., 2005; Trung et al., 2006). Although, insecticide uptake in larval forms is primarily through oral ingestion, a significant amount also penetrates through cuticle directly into the hemocoel (Shah et al., 1983) that can interact with non-gut visceral tissues. The binding sites of several Cry toxins have been located on the fat body membrane and Malpighian tubule epithelium of some insect species; however, their role in toxicity has not been fully characterized. Cheon et al. (1997) have demonstrated pore forming cytolytic ability of Cry toxins on the *in vitro* cultured fat body cells. Maddrell et al. (1989) reported important changes in the trans-epithelial potential difference of *Rhodnius prolixus* Malpighian tubules after treatment with *Bt* toxin and Reisner et al. (1989) located *B. thuringiensis kurstaki* δ-endotoxins on the Malpighian tubules of *Calpodes ethlius* larvae and described its effect as inhibitory of fluid secretion causing cytological alterations and eventually cellular lysis and epithelial damage. These reports along with our current findings further signify the role of alternate Cry toxin targets in various insect tissues.

5. Conclusion

For the first time, we characterized APN genes from Malpighian tubule and salivary gland of the moth *A. janata* which revealed the presence of at least two different isoforms *AjAPN1*, which is predominantly expressed in non-gut visceral tissues (Malpighian tubule, salivary gland and fat body) and *AjAPN4*, specifically expressed in midgut. *AjAPN4* and *AjAPN1* genes are primarily expressed during larval instars and are under the control of hormones, 20E and JH. Their expression might have significant biochemical and physiological roles in these tissues and contribute to overall growth, development and metamorphosis of *A. janata*. These APNs could possibly be involved in Cry toxin interaction. However, further detailed

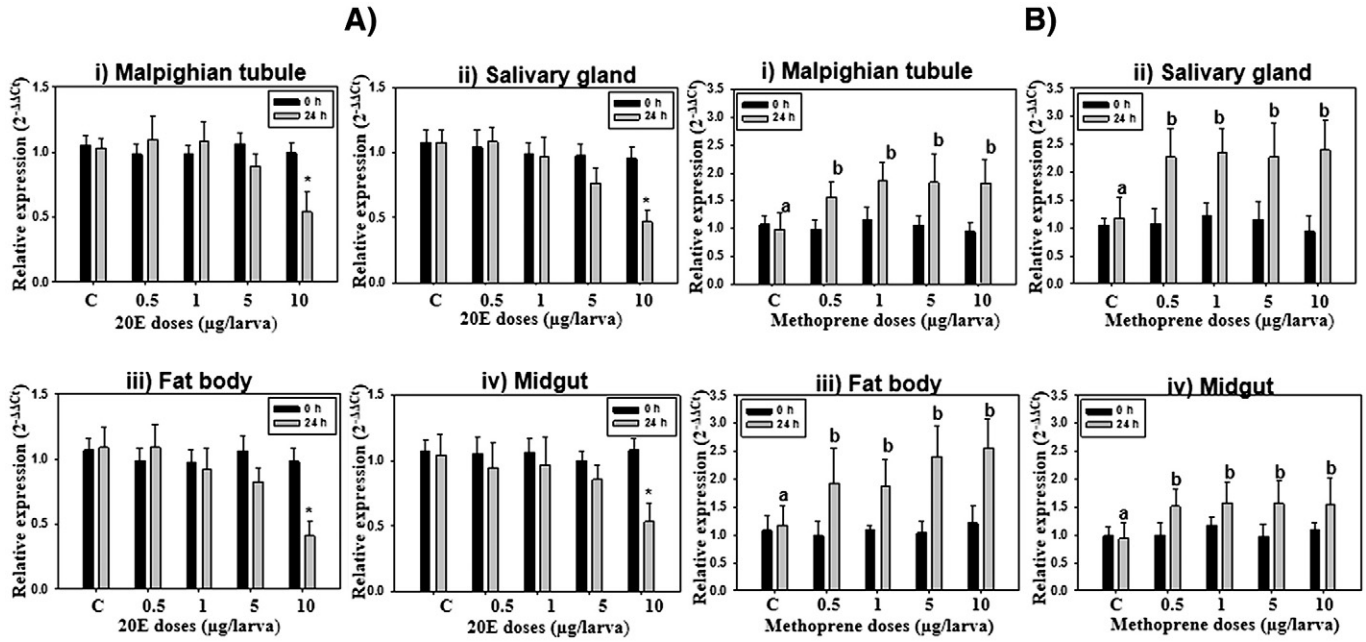

Fig. 5. A) Effect of 20E on *A. janata* i) Malpighian tubule ii) salivary gland iii) fat body and iv) midgut *APN* expression. Effect of 20E (0.5 or 1 or 5 or 10 µg/larva) on *APN* expression of various tissues during 5E stage after incubation for 24 h. Data is reported as fold change relative to 0 h control normalized with 18S rRNA expression calculated using $2^{(-\Delta\Delta Ct)}$. * denotes the value is significantly different ($P < 0.05$). **B)** Effect of methoprene on *A. janata* i) Malpighian tubule ii) salivary gland iii) fat body and iv) midgut *APN* expression. Effect of methoprene (1 or 10 µg/larva) on *APN* expression of various tissues during 5E stage after incubation for 24 h. Data is reported as fold change relative to 0 h control normalized with *18S rRNA* expression calculated using $2^{(-\Delta\Delta Ct)}$. Values labelled with different letters differ significantly ($P < 0.05$).

studies are essential to decipher the physiological functions of Malpighian tubule and salivary gland APNs and elucidate the mechanism of their hormone action in order to enhance the significance of them being used as alternate targets for designing efficient pest control strategies.

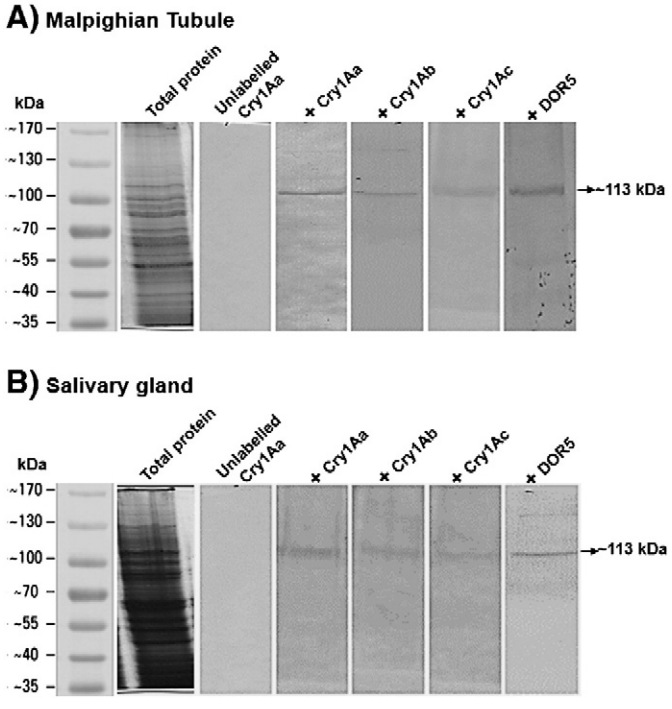

Fig. 6. Cry toxin binding assay with **A)** Malpighian tubule and **B)** salivary gland membrane proteins. The blots were separately incubated with any one of the biotin-labelled activated Cry toxin or DOR Cry toxins and detected with streptavidin–ALP conjugate. Lane 1 protein molecular mass markers, Lane 2 coomassie blue stained membrane proteins, Lane 3 protein blot incubated with unlabelled Cry1Aa as control, Lanes 4–7 protein blots incubated with biotinylated activated Cry toxins Cry1Aa (lane 4), Cry1Ab (lane 5), Cry1Ac (lane 6) and DOR5 Cry toxins (lane 7).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpb.2013.09.005.

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