Larval mid-gut responses against sub-lethal dosages of Cry toxins in Lepidopteran pest, *Achaea janata*

A thesis submitted to University of Hyderabad
for the award of Ph.D. degree in
Department of Animal Biology

By

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(A Central University established in 1974 by an Act of Parliament)

DECLARATION

I, **Vinod Kumar Chauhan** hereby declare that this thesis entitled **"Larval mid-gut responses against sub-lethal dosages of Cry toxins in Lepidopteran pest, *Achaea janata*"** submitted by me under the guidance and supervision of Prof. Aparna Dutta Gupta is a bonafide research work which is also free from plagiarism. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled **"Larval mid-gut responses against sub-lethal dosages of Cry toxins in Lepidopteran pest, *Achaea janata*"** submitted by **Vinod Kumar Chauhan,** bearing registration number **12LAPH04** in partial fulfillment of the requirements for award of Doctor of Philosophy in the **Department of Animal Biology, School of Life Sciences** is a bonafide work carried out by him, under my supervision and guidance.

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Parts of this thesis have been:

A. Published in the following publication.

1. Frontiers in Physiology (Invertebrate Physiology), doi: doi.org/10.3389/fphys.2017.00662 (Chapters 1 & 2)
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Supervisor **Head of the Department** **Dean of the School**

This thesis is dedicated to
My sister and brother in law
For all their hardships to bring me till this stage

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Table of contents

Abbreviations i

General Introduction 1

General Materials and Methods 13

Chapter 1: Evaluation of physiological responses of *Achaea janata* larvae to sub-lethal dosages of Cry toxin exposure

1.1 Introduction 25

1.2 Materials and Methods 30

1.3 Results 36

1.4 Discussion 46

Chapter 2: Role of endogenous mid-gut arylphorin in cell proliferation/ tissue regeneration

2.1 Introduction 51

2.2 Materials and Methods 54

2.3 Results 58

2.4 Discussion 64

Chapter 3: Characterization of major receptor aminopeptidase N(s) in larval mid-gut and its expression profile during sub-lethal Cry toxin toxicity

3.1 Introduction 66

3.2 Materials and Methods 71

3.3 Results 79

3.4 Discussion 93

Summary and Conclusion 98

References 101

Publications from thesis

Anti-plagiarism Certificate

Abbreviations

ºC	Degree centigrade/Celsius
ABCC2	ATP-binding cassette transporter subfamily C2
ACP	Acid phosphatase
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
APN	Aminopeptidase N
AV	Annexin V
BBMVs	Brush border membrane vesicles
BCIP	Bromo-4-chloro-3-indolyl phosphate
BLAST	Basic Local Alignment Search Tool
BrdU	5-Bromo-2'-deoxyuridine
Bt	*Bacillus thuringiensis*
BSA	Bovine serum albumin
CHAPS	3-((3-Cholamidopropyl) dimethylammonio)-1-propane sulfonate
cDNA	Complementary DNA
cAMP	Cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate
dNTPs	Deoxy ribonucleoside triphosphates
DTT	1, 4-Dithiothreitol
DIGE	Differential gel electrophoresis
EDTA	Ethylen ediaminetetraacetic acid
EGTA	Ethylene glycol-bis (2-aminoethylether- N,N,N',N'-tetra acetic acid
FACS	Fluorescence-activated cell sorting
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HRP	Horseradish peroxidase
IgG	γ Immunoglobulin
kDa	Kilo Dalton

LB	Luria-Bert
LD	Light:Dark
mg	Milligram
mM	Millimolar
mRNA	Messenger ribonucleic acid
MALDI	Matrix Assisted Laser Desorption/Ionization.
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MS	Mass spectrometry
NaCl	Sodium chloride
NBT	Nitrotetrazolium blue
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nM	Nanomolar
OCT	Optimal Cutting Temperature
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PI	Propidium Iodide
pmol	Picomole (10^{-12} moles)
pH	Negative logarithm (base 10) of the molar concentration of dissolved hydrogen ions (H+)
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl fluoride
PNP	Para-nitrophenol
PP	Prepupa
RH	Relative humidity
RT	Room temperature
RNA	Ribonucleic acid
RPM	Revolutions per minute

SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
SEM	Standard deviation of the mean
SNK	Student-Newman-Keuls
TAE	Tris-acetate EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline Tween-20
TE	Tris-EDTA
TEMED	N,N,N',N', tetramethylethylenediamine
TOF/MS	Time-of-flight mass spectrometry
v/v	volume/volume
w/v	weight/volume
µg	Microgram
µM	Micromolar

General Introduction

Farming and pest management

Worldwide nearly 1.8 billion people are engaged in agriculture practice, while in India around 60-70% of its population practices agriculture. Variety of practices are being used to protect the agricultural fields from various pests. They range from traditional practices to pesticides. Pesticides which are categorized as herbicides (weed killer), fungicides (kill fungi and their spores), insecticides (kill insect pests) and others including rodenticides (kill rodents). In India most of the pesticides used are of chemical origin, and it is only less than 5% are of biological origin. When compared to world's per capita usage, Indian use of pesticides is much lower (0.6 Kg/Ha) than other countries (Fig. 1).

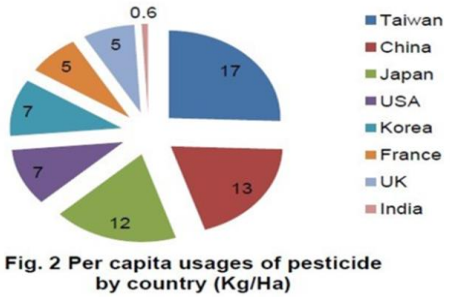


Fig. 2 Per capita usages of pesticide by country (Kg/Ha)

Fig. 1 Per capita usage of pesticides by various countries (Kg/Ha).
(Image adopted from http://www.ficci.com/)

Consumptions pesticides

India uses more than 80% of insecticides as compared to total world usage (De *et al.*, 2014). This is primarily due to the tropical climate conditions and the diversity of insects is more (Fig. 2).

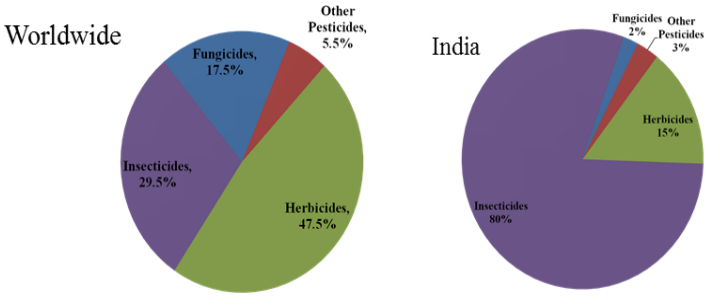


Fig. 2 Consumption of pesticides in world as well as in India. (Adopted from De *et al.*, 2014**).**

There is always an attempt to reduce the usage of deleterious chemical pesticides by implementing organic agricultural practices and incorporating alternate approaches including usage of biopesticides. Although the biopesticide market presently makes a very small component of total world market, i.e. $3 billion in 2016, but expected is expected to rise to ~$5.0 billion by 2024 (Fig. 3)

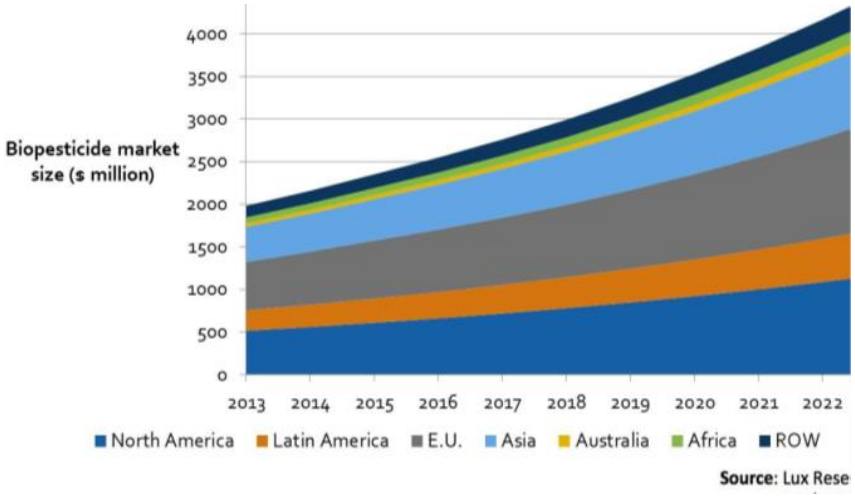


Fig. 3 Forecast of the global market for biopesticides.
(Image is adopted from http://www.luxresearchinc.com/)

Biopesticieds

Biopesticides, can be defined as the group of control agents derived from natural sources. These are the common alternatives to some of the most problematic chemical pesticides currently in use. Biopesticides are primarily classified into: (i) Phytochemicals and biochemical substances commonly present in plants are derived to control pests by various mechanisms, (ii) Microbial pesticides consisting of microorganisms such as bacteria (*Bacillus thuringiensis*), fungi (*Beauveria sp, Nomeria sp*), virus (Nucleopolyhydrous virus) as well as their active ingredients, which are target specific. For example, there are fungi that control certain weeds (*Colletotrichum, Phoma,* and *Sclerotinia sp)* and other fungi that kill specific insects (*Beauveria, Isaria, Hirsutella, Metarhizium* and *Nomaria sp)*. The most widely used microbial pesticide for the management of insects pests are subspecies and strains of *Bacillus*

thuringiensis (Bt) either in the form of formulations or *Bt* transgenic plants with their toxin genes.

Bacillus thuringiensis

Bacillus thuringiensis (Bt), a gram positive spore forming bacteria, produces vegetative insecticidal proteins (Vip's) during early growth phase and Crystal (Cry) inclusions as well as Cytolytic (Cyt) proteins during sporulation (Fig. 4) These inclusions are composed of δ-endotoxins (protoxins). The *Bt* strains produce Cry toxins in combinations which are product of various *cry* genes and are effective against Lepidopteran (moths), Coleopteran (beetles and weevils), Dipteran (flies and mosquitoes) and Hymenopteran (wasps and bees) order of insects. The advantage associated with *Bt* based biopesticies include target specificity (entomopathogenic), their ecofriendly nature which have very little or no side effects on mammals and non-target organisms and their high effectively as they are normally required in low dosage to show insecticidal activity. The use of *Bt* toxins in the form of *Bt* based spray formulations (mixtures of spores and crystals) as well as *Bt* transgenic plants where these effective protein(s) (*cry*) genes are introduced into economically important crops like *Bt* cotton, *Bt* corn, *Bt* maize, *Bt* brinzal etc. are widely being used for the effective management of insect pests.

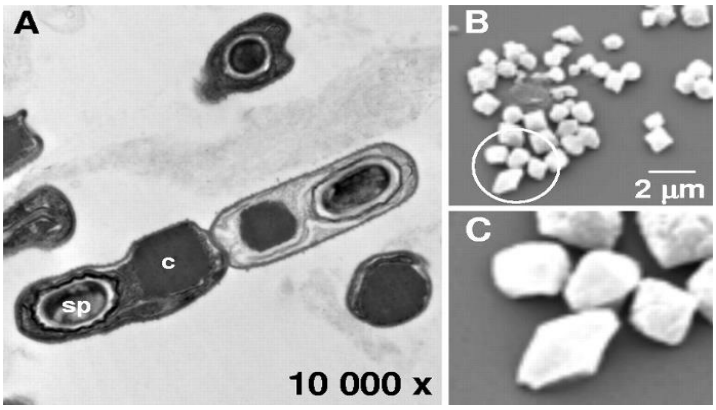


Fig. 4 Morphology of insecticidal crystals *B. thuringiensis* subsp. *thuringiensis.* (A) Transmission electron micrograph of sporulated cells, sp, spore; c, crystal (B) Scanning electron micrograph of purified crystals(circled in panel B). (C) Higher magnification crystals (Image is adopted from Swiecicka *et al.*, 2008)

Cry toxin and its binding/ receptor proteins

The crystal inclusions upon ingestion are solubilized and activated in the host digestive system, in larval mid-gut the active toxins bind to specific binding/ receptor proteins like glycosylphosphotidyl- inositol (GPI)-anchored aminopeptidase-N (APN) (Knight *et al.,* 1995), transmembrane cadherin-like proteins (Nagamastsu *et al.,* 1999), GPI-anchored alkaline phosphatase (ALP) (Jurat-Fuentes and Adang, 2004), or to a high molecular weight glycoconjugate (Valaitis *et al.,* 2001). In general all these aforesaid proteins play important physiological role in digestive function, however by default they also bind to the Cry toxins, and facilitate pore formation in gut cells and cause septicemia leading to death of the larvae.

i) Cadherins: The cadherin family proteins are diverse in nature and perform variety of functions like cell adhesion, migration, cytoskeletal organization and morphogenesis (Angst *et al.,* 2001). These are primarily transmembrane proteins, which possess a cytoplasmic domain as well as extracellular ectodomain with several repeats (Nagamastu *et al.,* 1999). Further they are located in the microvilli of larval mid-gut epithelial cells. Various studies have demonstrated that Cry toxins primarily interact with these proteins (Vadlamudi *et al.,* 1993), which promotes oligomerization. The oligomeric toxins later interact/bind to secondary receptors/binding proteins like aminopeptidase N and alkaline phosphatase present in the brush border membrane of mid-gut cells.

ii) Aminopeptidase N (APNs): The APN's are digestive enzymes that cleave the neutral amino acids from the N-terminal end of polypeptides. These proteins belong to the Zn^{++} binding metalloprotease family of peptides called gluzincins (Hopper, 1994) and have highly conserved aminopeptidase activity motif "GAMENWG" and Zn^{++} binding motif " $HEXXXHX_{18}H$" (Pigott and Elllar, 2007). Mid-gut APNs have been identified as Cry toxin receptor/binding proteins in various Lepidopteran insect pests (Soberon *et al.,* 2009).

iii) Alkaline phosphatase (ALP): These enzymes are mainly involved in remo`ving phosphate groups from organic molecules. ALPs are shown to play many biological functions in insects (Funk, 2001). They bind to Cry1Ac toxin, which points to the fact they might play a role in Cry toxin toxicity (Jurat-Fuentes and Adang, 2004).

iv) Glycolipids: They are known to act as putative Cry toxin receptor. It is hypothesized that both glycolipids and Cry toxin receptors may play role simultaneously or sequentially in the toxin insertion into the membrane bilayer of mid-gut cells. However, the exact function of these molecules in Cry toxicity is yet to be validated. However, an alteration in glycolipids levels in *Plutella xylostella* is believed to play role in evolution of resistance to Cry1Ac (Kumaraswami *et al.*, 2001).

Cry toxin - Mode of action

Two distinct and contrasting models have been proposed for the mode of Cry toxin action based on various studies carried out in Lepidopteran insects (Soberón *et al.*, 2009) (Fig. 5).

i) Pore formation model: This is a multistep (Bravo *et al.*, 2004) and well accepted model to describe the mechanism of Cry toxin toxicity (Fig. 5). Upon ingestion of Cry toxin present in crystal inclusions as δ-endotoxins (130 kDa protoxin), is solubilized and released in mid-gut lumen. The alkaline medium and reducing condition of the gut facilitates protoxins cleavage by gut proteases. They are converted into active form toxins which are 64-66 kDa proteins with three domain structure (Schnepf *et al.*, 1998). Similarly from the *Bt* transgenic plants which express the protoxin, upon feeding it is released in mid-gut lumen of larvae and gets activated. (Gasser and Fraley 1989, Fischhoff 1996). This active toxin binds to the receptor/binding protein present in inner apical brush border membrane vesicles (BBMV's) primarily with cadherin like proteins (Vadlamudi *et al.*, 1993; Gomez *et al.*, 2001, 2002), where one α helix

from domain 1 gets cleaved (Gomez *et al.*, 2002). They interact among themselves and give rise to highly effective oligomeric forms of Cry proteins (Peng *et al.*, 2010), which then further bind either to GPI-anchored receptors like aminopeptidase N (APNs) (eg. *Manduca sexta* (*Pacheco et al.*, 2009) or alkaline phosphatase (ALPs) (eg. *Heliothis virescences*) (Bravo *et al.*, 2004; Jurat-Fuentes and Adang, 2004). Binding of the oligomers to secondary receptors (APNs/ALPs) is followed by its insertion into the microdomains, which causes pore formation in the apical membrane epithelial cells, leading to septicemia and death of the larvae (Schenepf *et al.*, 1998; de Maagd *et al.*, 2003; Bravo *et al.*, 2004).

ii) Signal transduction model: This model proposes that toxicity of Cry toxin is through Mg^{++} - dependent signal cascade pathway, where the interaction of activated toxin with primary receptor (cadherin like proteins) activates a guanine nucleotide binding protein (G-protein), which in turn activates adenylyl cyclase and promotes increased production of intracellular cAMP. This causes protein kinase A (PKA) activation which promotes intracellular downstream pathway leading to cell death (Fig. 5). This model is derived entirely through *in vitro* studies carried out using Cry toxin receptor transfected ovarian cells (H5- High five) from cabbage looper *Trichoplusia ni* induced by Cry1Ab toxin (Zhang *et al.*, 2006).

For effective toxicity of Cry toxin, it is not necessary that all the proteins reported as Cry toxin binding/receptor proteins are involved at a given time and in different insect model. The mechanism varies from toxin to toxin and type of receptors involved in it differ significantly. However, at present aforesaid pore formation model is well accepted model for Cry toxin toxicity.

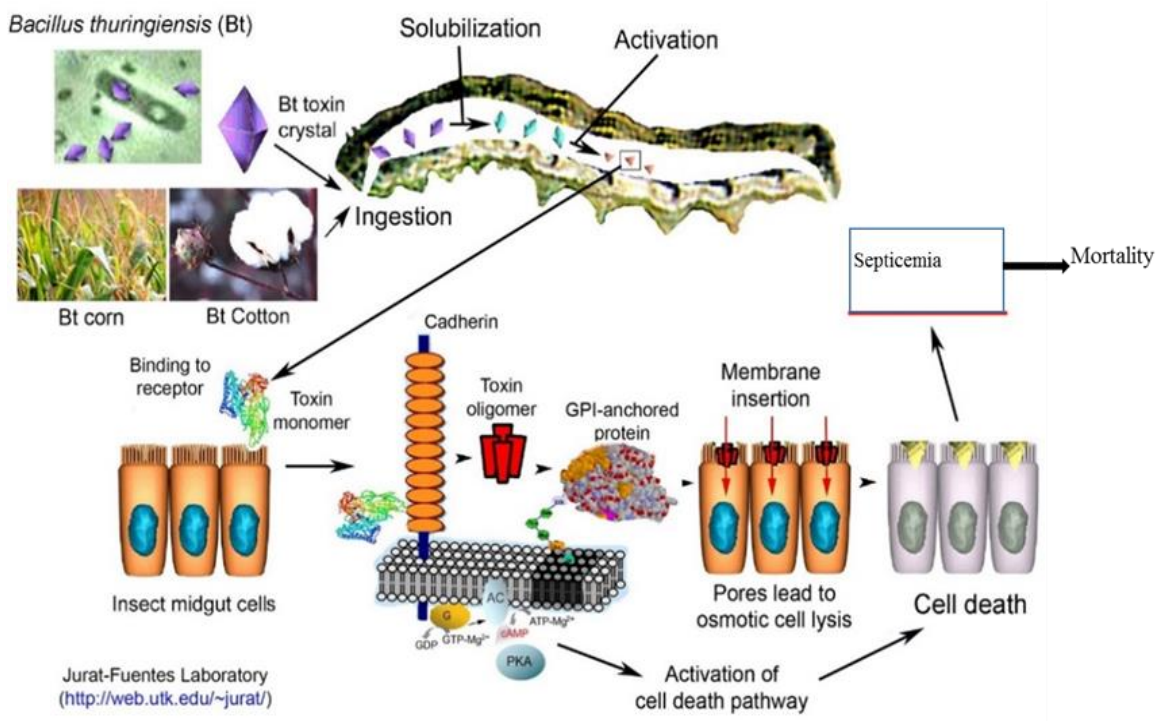


Fig. 5 Mode of action. This multi-step toxicity process of mode of Cry toxin action includes ingestion of the Cry protein, solubilisation, activation, interaction primarily with cadherin like proteins, oligomerization, binding to the secondary APN/ALP receptors, toxin insertion, pore formation, septicaemia and death of the larvae (Image is adopted and modified from Jurat-Fuentes laboratory, http://web.utk.edu/~jurat/).

Development of tolerance and resistance in Lepidopteran pests

Bt- based biopesticides are currently being used as an alternative to chemical insecticides for the effective management of economically important Lepidopteran pests in the form of either *Bt* based formulations or *Bt* transgenic plants. However, uncontrolled widespread use of *Bt* sprays and *Bt* crops in last two decades is a topic of concern because of its adverse effects on environment and human health (Shelton *et al.*, 2002). Further, development of resistance against Cry toxin based control strategies in insect populations is a serious matter that needs a careful

evaluation (Tabashnik *et al.*, 2013). Various reports from different insects such as diamond black moth; *Plutella xylostella*, Indian meal moth ; *Plodia interpunctella* and cabbage looper; *Trichoplusia ni* are the evidence for the evolved resistance to commercial *Bt* sprays, when used in open field and greenhouses (Ferre and Van-Rie 1991; Oppert et al., 1997; Janmaat and Myers, 2003). Recently, field-evolved pest resistance to variety of *Bt* transgenic crops such as *Bt* corn (maize stem borer- *Busseola fusca*, western corn rootworm- *Diabrotica virgifera virgifera*, and fall armyworm- *Spodoptera frugiperda*), *Bt* cotton (cotton bollworm- *Helicoverpa armigera* and pink bollworm- *Pectinophora gossypiella*) and *Bt* maize (Fall armyworm-*Spodptera frugiperda*) have also been reported (Storer *et al.*, 2010; Dhurua and Gujar 2011; Zhang *et al.*, 2012; Pereira *et al.*, 2015; Jakka *et al.*, 2015)

The insects were shown to develop resistance due to alteration in mid-gut binding sites leading to reduced interaction of *Bt* toxins to their receptor (Pigott and Ellar, 2007) and also mutation in toxin binding/interacting protein genes (Guo *et al.*, 2015). In addition variety of physiological responses which are primarily non-receptor mediated mechanisms could also interfere with steps common to more than one Cry toxin leads to resistant against a wide range of *Bt* toxins. One of the process was shown to be enhanced mid-gut regeneration response observed in resistant larvae (Martinez-Ramirez *et al.*, 1999; Castagnola and Jurat-Fuentes 2016). Dulmage *et. al.*, (1978) demonstrated that Lepidopteran larvae recovered from exposure to sub-lethal doses of Cry toxins and it was primarily due to mid-gut epithelial regeneration (Spies and Spence, 1985).

Experimental Model

Achaea janata

Achaea janata is a holometabolus insect. It is commonly known as castor semilooper, belongs to super family Noctudidae and order Lepidoptera. It is a widely spread agricultural pest of the tropical and sub-tropical region of the world. Although it is a polyphagous insect but primarily feeds on castor plant (*Ricinus communis)* and in absence of it, occasionally feeds on other plants belonging to *Euphorbiaceae, Brassica* and *Ficus* species.

Economic importance

Castor is an important non-edible oil seed crop grown in India, China and Brazil (Lakshminarayana and Raoof, 2005) and it provides completely biodegradable industrial oil (Gaur, 2013). India is the world leader in castor production (Shah and Anish, 2017). Major insect pests of this crop belongs to Lepidopteran species like *Spodoptera litura*, *Amascata albistriga* and *Achaea janata.* Among this *A. janata* is a serious castor pest which leads to 50-70% loss in India during the period of July to September (Gaikwad and Bilapte, 1992).

Life cycle

A. janata is a holometabolus insect pest. It completes its life cycle in 48 -50 days under laboratory conditions. It follows four different life stages consisting of egg, larval, pupal and adult (Karmawati, and Tobing, 1988) (Fig. 6). There are five instars during the larval development which are voracious feeders. The total larval period lasts between 15-20 days, the duration of first to fourth larval stages is of nearly three days, while the last instar takes 4-5 days. The full grown larvae are about 7 cm long. Pupation takes place either in soil or between the fallen leaves. The pupae are normally enclosed in loose silk cocoon. The pupal period extends between 10-15 days and gets prolonged to few months under unfavourable conditions (diapause). The duration of pupal stage is directly influenced by the temperature and humidity.

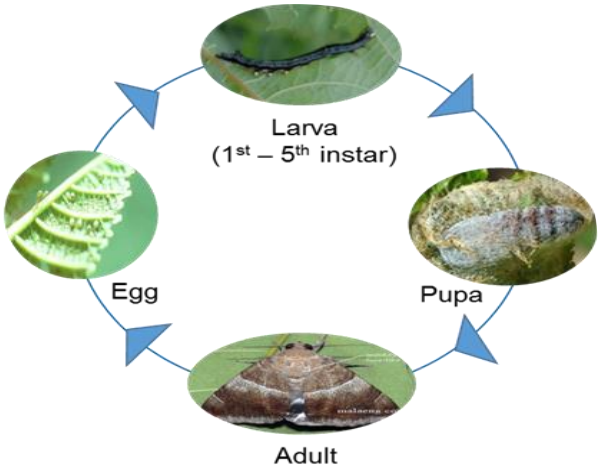


Fig. 6 The life cycle of *Achaea janata*. A holometabolous life cycle consisting of four distinct stages- egg, larva, pupa and adult.

Organs of *A. janata* larvae (used for the present study)

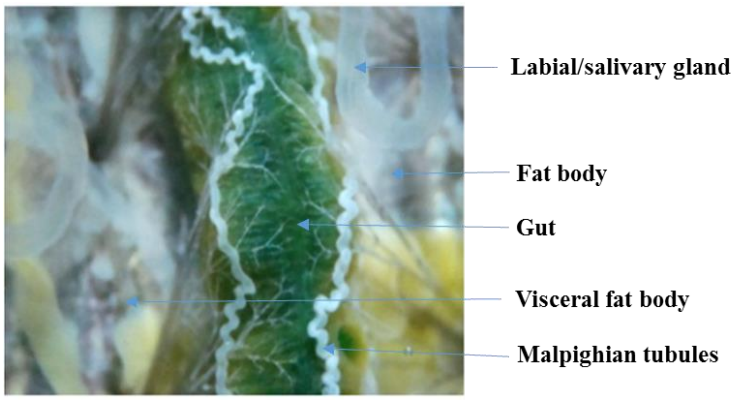


Fig. 7 Organs of *A. janata*. Abdominal cavity of 5th instar larvae showing gut, fat body, Malpighian tubules and Salivary gland (Labial gland).

i) Gut: This is primary organ which is involved in digestion and absorption. Larval gut is further divided into fore-gut, mid-gut and hind-gut. The mid-gut of Lepidopteran larva is the major region of digestive tract, which is not only involved in digestion but also in other physiological functions such as metabolism, immune response etc. It is composed of columnar epithelial cells with microvillar apical brush border and Goblet cells with large cavity, which connect the apical surface by inter digitated processes (Cavalcante and Cruz-Landim, 1999; Pinheiro *et al.*, 2008).

Presence of microvilli in mid-gut increases the surface area, which allows to exchange of molecules/ nutrients (Dow, 1987).

ii) Fat body: It is the major organ of metabolism, and nutrient storage in insects (Law and Wells, 1989), primarily composed of variety of cells like adipocytes, oenocytes, urocytes and bacteriocytes (Arrese and Soulages, 2010). It is known to be a multifunctional organ which co-ordinates the hormonal and nutritional signals and regulates insect development, metamorphosis and reproduction (Colombani *et al.*, 2005).

iii) Malpighian tubules: They are primary excretory and osmoregulatory organ of insects. These tubules spread throughout the surface of gut and visceral cavity. At the junction of mid-gut and hind-gut, they are connected with the gut lumen. The metabolic waste from the surrounding haemolymph as well as gut is excreted and released outside, by them to the exterior through hind-gut and/or rectum. They are composed of a single layer of epithelial cells.

iv) Larval salivary glands: These are a pair of tubular structure, which get specialized in Lepidopteran larvae and produce silk. Constitutive expression of silk protein genes is transient during the larval development. However, the amount of silk protein produced during prepual stage is copious and plays important role in cocoon formation.

v) Haemolymph: It is a circulatory fluid constitute of the plasma and cells, which are commonly known as haemocytes. The plasma of haemolymph has high concentrations of a variety of macromolecules including proteins, carbohydrates, lipids, free amino acids, fatty acids, organic phosphates etc. The larval haemolymph, shows the presence of large number of proteins and among them storage proteins (hexamerins) constitute 40-50% of it. These hexamerins are synthesised by fat body during feeding stage and released into the circulatory fluid (Telfer and Kunkel, 1991; Burmester, 1999). They are then sequestered back into the fat body during non-

feeding (prepupal) stage, and are utilized for pupal-adult transformation as well as in reproduction (Pan and Telfer, 1996).

Felid application of *Bt* based formulations

As *Bt* formulations are proteinaceous in nature, hence their efficacy/ activity gets affected by varied field conditions. Foliar spray of *Bt* formulation can result in the loss of toxicity from degradation through different ways such as UV light, wash-off by rain, drying, temperature and chemistry as well as soil acidity (Leong *et al.*, 1980; Beckwith *et al.*, 1987; Van Frankenhuyzen and Nystrom 1989). Uneven sprays of formulation further leaves the unexposed area of the plants, thus giving insects the chance to move from exposed to unexposed part of plants, even unexposed neighbouring fields. Under such conditions, there is always a possibility for the larvae to get exposed to sub-lethal doses of toxin, and exhibit a variable effect leading to survival of insects. Continuous exposure of Cry toxin(s) in sub-lethal dosage either in single generation or in the continuous generations is likely to promote the development tolerance towards this toxin which in turn might promotes insect pests resistance to *Bt* formulations.

Objectives

On the basis above concept as well as preliminary analysis carried out in our laboratory, the present study was designed with the following objectives to evaluate the effect of sub-lethal dosages of Cry toxins in Lepidopteran pest, *Achaea janata.*

i) Evaluation of physiological responses of *Achaea janata* larvae to sub-lethal dosages of Cry toxin exposure.

ii) Role of endogenous mid-gut arylphorin in cell proliferation/ tissue regeneration.

iii) Characterization of major receptor aminopeptidase N(s) in larval mid-gut and its expression profile during sub-lethal Cry toxin toxicity.

General Materials and Methods

Insect culture maintenance

Egg masses were collected from the agricultural fields of Telangana region where the crops were never sprayed with *Bt*- based biopesticides. Immediately after hatching, the neonates were transferred onto fresh sterile castor leaves. The larvae were maintained in insect culture facility under of 14:10 h (light: dark period), 70 ± 5% RH and 27 ± 2℃ temperature until pupation. The glass troughs containing sterile moist sand was used for maintaining the pupae which were allowed to emerge into adults. The adult moths (male/ female) were collected and transferred to breeding cages where they were fed with 10% honey (Pavani *et al.*, 2015). Adult female moths laid eggs on fresh castor leaves surface, which hatched in 4-5 days. Neonate larvae were used for maintaining generation wise cultures. In laboratory three continuous generations were maintained to get homogenous populations. Later based on the experimental requirement the larvae were exposed to various treatments and the cultures were parallely maintained throughout the study.

Total RNA isolation

Total RNA isolation from collected tissue was carried out using TRI reagent TM (Sigma Aldrich, USA) following the manufacturer's instructions. Tissue and Tri reagent were mixed gently (1 ml TRI reagent for 100 mg of tissue). After homogenization, chloroform was added (0.2 ml for 1 ml sample) and left for 10 min at RT followed by centrifugation at 15000 rpm at 4℃ for 15 min. Two layers were separated after centrifugation. The aqueous layer was carefully transferred in a fresh Eppendorf tube to which two volumes of 2-propanol was added, and kept at -20℃ for 60 min. The precipitated RNA was then pelleted down at 15000 rpm at 4℃ for 15 min and pellet was washed three times with 70% alcohol (DEPC treated) followed by centrifugation. The obtained pellet was air dried and dissolved in DEPC treated nuclease free water. Quantity and quality of isolated RNA was assessed using Nanodrop 1000 (Thermo Scientific, USA). A260/A280 ratio of ~2.0 was considered as a good quality of RNA.

RNA gel electrophoresis

Integrity of RNA was assessed by performing denaturing electrophoresis on a 1% formaldehyde agarose gel (Sambrook *et al.*, 1989). The total RNA (1-2 µg) was mixed with RNA denaturing buffer 25 µl (4 µl of formaldehyde (37%), 13.4 µl formamide (deionized), 3.6 µl of 10X MOPS gel buffer (0.2 M MOPS pH 7.0; 50 mM sodium acetate, pH 6.0; 10 mM EDTA, pH 8.0) 4 µl of DEPC treated water). It was boiled at 65℃ for 5 min, and then cooled on ice. Finally 2.5 µl of gel loading dye containing bromophenol blue and Xylene cyanol was added (Pavani *et al.*, 2015). 1% agarose gel was prepared (1g of agarose, 1X MOPS buffer, 12.3 M formaldehyde and 4 µl of ethidium bromide (10 mg/ml) and RNA samples were loaded on to it. Electrophoresis was carried out using 1X MOPS buffer until dye reached 3/4th portion, at a voltage of 5V/cm^2. Electrophoresed RNA was visualized using UV transilluminator.

DNase 1 treatment

DNase treatment was carried out using reaction mixture containing 1 µl of 10X DNase1 reaction buffer, 1 µl of DNase1 enzyme (Invitrogen, USA) and 2 µg of total RNA and total volume was made up to 10 µl using DEPC treated water. The reaction mix was incubated at 37℃ for 20 min. After incubation, DNase I enzymatic activity was inactivated by adding 1 µl of 25 mM EDTA at 65℃ for 10 min. This DNA free RNA was used for first strand cDNA synthesis.

First strand cDNA synthesis

First stand cDNA synthesis was carried out according to manufacturer's protocol (Invitrogen, Life Technologies, USA). The reaction mixture was set up by adding 1 µg of RNA, 1 µl of oligo dT primers (50 µM) and 2 µl of dNTP mix (10 mM). The volume was made upto 14 µl using nuclease free water in a PCR tube and incubated at 65^{o}C for 5 min. To this 4 µl of reaction buffer (5X), 1 µl of RT enhancer and 1 µl of reverse transcriptase (200 U/µl) were

added to carry out first strand cDNA synthesis at 42ºC for 60 min. The reaction was terminated at 85℃ using thermocycler. The synthesized cDNA was stored at -20℃ until further use.

Primer (short oligos) designing

As the whole organism genome sequence database for *A. janata* is not available, hence the gene sequence analysis was carried out using degenerate primer based strategy. Primers were designed based on the sequences similarity, reported for other related Lepidopteran species in NCBI data base (https://www.ncbi.nlm.nih.gov/). The retrieved sequences were aligned using multiple sequence alignment tool (http://www.ebi.ac.uk). The highly conserved region of the sequence in MulTalin was used for designing the degenerate primers. Primers were designed based on standard protocol (Chen, and Janes, 2002; Dieffenbach and Dveksler, 2003). The parameters considered were primer length (15-30 bp), primer annealing temperature, percentage of GC content (at least 50%), chances of hairpin as well as homodimer formation etc. All the designed primers used in the present study were analysed for the above basic parameters using Oligoanalyzer tool 3.1 (Integrated DNA Technologies Inc., USA).

Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out using thermocycler (Eppendorf, USA). The 10 µl reaction was setup using 5 µl of PCR master mix (2X) (Emerald Amp GT PCR master mix- Clonetech, USA), 4.5 µl nuclease free water, 0.5 µl forward primer (10 pmol), 0.5 µl reverse primer (10 pmol) and 0.5 µl cDNA template (dilutions of 1:10). Polymerase chain reaction was carried out for 94℃ for 2 min (initial denaturation); 94℃ for 30 sec (denaturation); *54℃ for 30 sec (annealing temperature * differ for various primers) and 35 cycles of amplification at 72℃ and final extension at 72℃ for 10 min. The amplification time for the cycles depended on the size of fragment (30 sec for below 500 bp; 1min for 1kb; 1.5 min for 1.5 kb etc.)

DNA gel electrophoresis

Agarose gel electrophoresis was used for the separation of DNA molecules (genomic DNA, plasmid DNA and PCR amplified DNA product) according to charge and size. Agarose gel was prepared using 1X TAE buffer diluted from 50X stock solution (24.2 g - Tris base, 5.7 mL - acetic acid and 10 ml 0.5 M EDTA made upto 100 ml with distilled water). For 1% agarose gel preparation 1g of agarose (Lonza, USA) was suspended in 1X 100 ml of TAE buffer and boiled using microwave. The solution was allowed to cool and 5 µl of ethidium bromide (10 mg/ml) was added and poured into gel casting boat with comb slotted for the loading of DNA samples. The DNA samples were mixed with 6X DNA loading dye (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cynol) and loaded in the wells. 4 µl of respective DNA marker (100 bp - SM0241, 1 Kb - SM0312, Thermo Scientific, USA) was loaded along with the samples (amplified DNA or Plasmid DNA or genomic DNA) was electrophored using 1X TAE buffer at 100 V for 30-40 min, and visualized and documented using gel documentation system (Bio-Rad, USA).

Elution of PCR amplified product

The desired amplified nucleic acid band was sliced using sterile blade and DNA extraction was carried out using commercial gel extraction kit (Qiagen, Germany) according to manufacturer's protocol. For this 100 mg of gel was suspended in three volumes of solubilisation buffer followed by incubation at 50℃ for 10 min (till it gets dispersed completely), to this one volume of isopropanol was added and mixed. The solution was loaded onto the silica gel column. It was centrifugation at 13,000 rpm for 1 min, then the column was washed with buffer provided in the kit. The bound DNA molecules were eluted using elution buffer (10 mM Tris-HCl, pH 8.5) in a fresh Eppendorf tube.

Construction of recombinant vector

The desired gene of interest (amplified DNA product) was inserted in pTZ57R/T (cloning vector; Thermo Fisher Scientific, USA) or pET-28a$^{(+)}$ (expression vector; Novagen, USA) using commercial kit (Thermo Fisher Scientific, USA). Insert/vector ratio (3:1 or 5:1) was calculated according to manufacturer's protocol. Ligation reaction (20 µl) was set up by adding vector pTZ57R/T (55 ng)/ pET-28a$^{(+)}$ 2 µl, 5X reaction buffer 4 µl, PCR product (variable), nuclease free water (up to 13 µl) and T4 DNA ligase (1 µl). The reaction mixture was vortexed, briefly centrifuged and was allowed to undergo ligation either at RT (22℃) for 1 h or at 4℃ for overnight.

Preparation of competent cells and transformation

Glycerol stock of DH5α or BL21 *E. coli* cells was streaked on to LB agar plates and incubated overnight at 37℃. A single colony from plate was inoculated in 5 ml of LB broth (Himedia, India) incubated overnight at 37℃ with 180 rpm shaking. The overnight primary culture was further inoculated, 1% in 10 ml of fresh LB broth and allowed to grow for 2 to 3 h till it reached 0.5-0.6 OD at 600 nm. This actively growing culture cells (0.5 OD) were further incubated on ice for 30 min and centrifuged at 6000 rpm for 10 min at 4℃. The pellet was resuspended in 10 ml of 0.1M $CaCl_2$ and incubated on ice for 1 h followed by centrifugation at 6000 rpm at 4℃. The prepared competent cells were aliquoted (100 µl) and used. For the long term storage the pellet was resuspended in 1ml cold 0.1 M $CaCl_2$ containing 15% (v/v) of glycerol and snap frozen in liquid nitrogen and stored at -80℃.

For transformation 10 µl of ligation reaction containing 50-100 ng of plasmid was added to 100 µl DH5α or BL21 *E. coli* competent cells and kept on ice for 30 min and subjected to heat shock at 42℃ for 90 sec followed by immediate incubation on ice for 5 min. 1ml of LB broth was added to transformed cells and incubated for 1 h at 37℃ with continuous shaking at 180 rpm. The actively grown cells were centrifuged at 3000 rpm for 5 min, 800 µl of the

supernatant was discarded and the pellet was resuspended in remaining 200 µl of cell culture media. To this 10 µl of IPTG (1M) and 20 µl of X-gal (for blue white screening) was added and the cells were plated on LB plate containing desired antibiotics (for the growth and selection of recombinant vector containing cells) and plates were incubated at 37℃ overnight/ maximum of 14 h.

Plasmid isolation

Plasmid was isolated from 10 ml overnight culture using Miniprep plasmid isolation kit (Qiagen, Germany) according to manufacturer's protocol. In brief, from the overnight culture cells were harvested at 8000 rpm for 3 min at RT. The pellet was resuspended in 250 µl of cold resuspension buffer (P1) containing RNase followed by addition 250 µl cell lysis buffer (P2) and mixed thoroughly by inverting the tube 4-6 times. To this 350 µl of neutralization buffer (N3) was added and immediately mixed by inverting tube 4-6 times, and centrifuged at 13000 rpm for 10 min. The supernatant was loaded onto QiAprep spin column and centrifuged at 13000 rpm for 1 min. The flow through was discarded and the column was washed twice with 750 µl wash buffer (PE). The bound plasmid was eluted using 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5). The concentration of plasmid was quantified using Nano drop and stored at -20℃. The same plasmid preparation was used for Sanger sequencing.

Gene expression analysis

For quantitative PCR analysis, a 20 µl reaction was carried out using a custom-made TaqMan gene expression assay (Applied Biosystems, USA). For this both forward and reverse primers of amplicon size (120-150 bp) were designed for various genes. Insect rS7 (KF984201) was used as an internal reference to normalize the transcript expression levels. The real-time expression analysis was performed in triplicates. Relative expression of various genes were calculated using $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).

Heterologous expression and purification of recombinant proteins

i) Construction of recombinant expression vector: The full length cDNA sequence (ORF) of the protein to be expressed was screened to identify the restriction for zero cutters (no cleavage site for this restriction enzyme was present in the ORF sequence but it was present in the expression vector pET28a+) (http://www.labtools.us/nebcutter-v2-0/). Based on the analysis, expression primers were designed with restriction site sequence at the 5' end. The same restriction enzyme was used for the digestion of both the amplified product (insert) and expression vector (pET28a^{+}) to create restriction site overhangs with sticky ends. This was followed by ligation reaction in which insert and vector (5:1 or 3:1) were ligated using T4 DNA ligase. Ligated recombinant vector mixture was used for transformation of BL21 *E. coli* expression host cells.

ii) Recombinant protein expression: Transformed single positive colony (BL21 *E. coli)* containing recombinant expression vector was picked up and inoculated in 10 ml of LB broth with desired antibiotics (Kanamycin (50 µg/ml)) and incubated at 37℃. This overnight culture was used for inoculating (1%) 100 ml LB broth with of kanamycin (50µg/ml) and allowed to grow at 37^{o} C till it reached an OD around 0.4-0.6 at 600 nm. This actively grown culture was induced with 0.5 mM IPTG and allowed to grow for 3 h at 37℃. It was centrifuged at 6000 rpm for 10 min. Pellet thus obtained was washed twice with PBS (137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 and 2.7 mM KCl, pH 7.4).

iii) Purification of recombinant protein using Ni-NTA column (His-tag proteins**):** The induced bacterial cell pellet obtained was resuspended in equilibration buffer (50 mM Tris, 250 mM NaCl and 10 mM imidazole, pH 7.4). Cell lysis was allowed by addling lysozyme (10 mg/ml), followed by sonication for 10 min on ice (31% amplitude, 10 sec on and 30 sec off). The lysate was centrifuged at 10,000 rpm for 30 min at 4℃. The supernatant was then

applied to equilibrated Ni-NTA column (Qiagen, Gemany) and allowed to pass through for binding. The column was further washed with 20 bed volumes of wash buffer (50 mM Tris, 250 mM NaCl and 40 mM imidazole, pH 7.4) and the bound histidine tagged protein was eluted using elution buffer (50 mM Tris, 250 mM NaCl and 250 mM imidazole, pH 7.4) in 1 ml fractions. Purity of the eluted protein fractions was assessed by SDS-PAGE.

Protein estimation

Protein concentration in various samples was estimated using colorimetric assay i.e, Bradford method (1976). For this procedure bovine serum albumin (BSA 10 mg/ml) was used for preparation of standard sample. An aliquot was taken and volume was adjusted to 100 µl with 10 mM Tris-HCl (pH 7.4). To this 900 µl 1X Bradford reagent (Bio-Rad, USA) was added and incubated for 10 min in the dark. Absorbance was then measured at 595 nm against blank using spectrophotometer (Shimadzu, Japan). The protein concentration of the sample was calculated using a BSA standard curve.

Profiling of proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): It was carried out using protocol of Laemmli (1970). For this 1/4th of 5% stacking gel (2 ml 30% acrylamide mix, 3 ml 0.5 M Tris-HCl pH 6.8, 6.76 ml distilled water, 120 µl 10% SDS, 50 µl 10% of APS and 6 µl of TEMED) and 3/4th of 10 % resolving gel (3.3ml 30% acrylamide mix, 2.5 ml 1.5 M Tris-HCl pH 8.8, 4.1 ml distilled water, 120 µl 10% of SDS, 150 µl 10% APS and 10 µl TEMED) was polymerised. The comb of desired volume size was inserted into stacking gel and allowed to polymerize. Samples were prepared by mixing known concentrations of protein and loading buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue). Proteins were electrophoresed at 60 V in stacking gel and at 100 V for resolving gel. The tracking dye was allowed to run into the tank buffer, and the gel was stained.

Visualization of separated protein/ peptides

i) Coomassie staining: The gel staining was carried out using protocol of Wilson *et al.*, (1979). Protein/polypeptides separated on gel were stained with staining solution (0.025% Brilliant blue- R 250 in 40% methanol and 7% acetic acid) for overnight by using a rocker. It was then destained with destaining solution (40% methanol and 10% glacial acetic acid) to clear the background leaving stained protein bands and molecular weight markers (Bio-Rad, USA). For storage the stained gel were kept in 5% glacial acetic acid solution.

ii) Silver staining: This is a more sensitive method when compared with coomassie. As per protocol of Blum *et al.*, (1987), the gel with separated protein/polypeptides was incubated in a fixative solution (50% methanol, 12% acetic acid and 0.05% formaldehyde) for 3 h and washed thrice in 50% ethanol. Sensitization was carried out using hypo solution (sodium thiosulphate - 20 mg/100 ml) for 30 -60 sec followed by rinsing three times with distilled water (1 min each). The gel was then incubated in silver nitrate solution (0.2% silver nitrate containing 0.076% of formaldehyde) with gentle agitation for 30 min. After incubation, it was rinsed three times with distilled water (1 min each). The gel was finally developed using developing solution (6% sodium carbonate containing 0.05% formaldehyde) till the clear protein bands were visible. Finally the reaction development was stopped by adding 12% acetic acid.

Generation of polyclonal antibodies

The polyclonal antibodies against DOR *Bt*-1and Cry1Ac proteins were raised in 6 weeks old mice (BALB/c) while APN antibodies were generated using 3 months old male rabbit (New Zealand variety) using standard protocol which was approved by Institutional Animal Ethics Committee. For antibody generation in mice, 100 µg of protein and in rabbit 200 µg of protein was emulsified with 500 µl of complete Freund's adjuvant and injected at multiple sites subcutaneously. Prior to injection, the lateral ear vein of rabbit and tail vein of mice was bled to collect pre immune serum. After14 days, first booster dose was given, which was followed

by a second booster dose (14 days after first injection), where 50 µg of protein was emulsified with incomplete Freund's adjuvant. Blood was collected after one week of second booster dose and left overnight at 4℃ for clotting. The serum was collected by centrifugation at 5000 X g for 20 min.

Antibody purification

The IgG fraction was purified by affinity chromatography using protein-A agarose column (Amersham, Germany) according to manufacturer's protocol. The serum protein was diluted (1:1) binding/wash buffer (20 mM sodium phosphate pH 7.4 with 150 mM NaCl) and was loaded on to the column pre-equilibrated with binding/wash buffer. This was followed with the washing of columns using the same buffer until the absorbance of elutes at 280 nm approaches the background level. The bound IgG fraction was eluted with 4 column volume of elution buffer (100 mM glycine-HCl, pH 3.0) in tubes containing 100 µl of neutralization buffer (1 M Tris-HCl, pH 9.0) for rapid neutralization of the eluted purified IgG fraction. The purified IgG fraction was aliquoted, lyophilized and stored at -20℃ till use.

Western blotting and immune detection

The proteins/ peptides from electrophoresed gel were transblotted to nitrocellulose membrane (Amersham, Germany) (Towbin *et al.*, 1979). The membrane as well as gel were immersed for 10 min, in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol). The gel and membrane along with filter paper were stacked into a cassette without air bubble and placed in transfer unit containing Towbin buffer. The transfer of proteins/peptides to the membrane was carried out at 25 V with 250 mA current limit at least for 14 h. Finally transfer of proteins to membrane was checked by Ponceau S (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) staining. It was followed with extensive washing with TBS (Tris buffered saline, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl). For immunoblotting above membrane was soaked in 5% blocking solution (5% skim milk powder (w/v) in TBS) or 3% BSA for 3 h to block nonspecific

regions on blot. Blot was washed three times (15 min each) with TBST (0.1% Tween- 20 in TBS (Sigma, USA)). Incubation of blot was carried out with desired primary antibody (1:1000 dilution) overnight at 4℃ followed by secondary antibody (1:4000 dilutions). Either alkaline phosphatase (ALP) or horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit IgG were diluted in 5% skim milk powder (w/v) in TBS and the blot was soaked for 2 h. The blot was washed to remove unbound antibodies and processed for blot development with BCIP/NBT (G-Biosciences, USA) for ALP or chemiluminiscence (Takara Bio Inc, Japan) for HRP conjugated antibodies. Finally blots were imaged with Kodak Photo Imager.

Histological procedures

i) Tissue fixation: The isolated tissues mid-gut, fat body, Malpighian tubules and salivary glands were fixed overnight in Bouin's fixative (saturated picric acid: formaldehyde: glacial acetic acid 15:5:1) at RT for a minimum of 12 h but not more than 36 h. The tissue was washed 3- 4 times with 70% ethanol and stored in same. It is recommended that the tissue should be used in a short period and the storage may not be more than one month for further processing.

ii) Paraffin embedding and sectioning: The fixed tissue was processed through a series of increasing alcohol concentrations (70%, 90% and 100%) for dehydration followed by clearance in xylene (5-10 min). The tissue was penetrated with molten paraplast (Sigma-Aldrich, USA) and finally embedded in the same as blocks. The blocks were sectioned (~5 µm) using a rotator microtome (Leica Microsystems, Germany). Wax strips containing tissue sections were placed on the Mayer's albumin coated glass slides containing some water on it. Stretching of the ribbon with sections was carried out by placing the slides hot plate (40℃) for 10-20 min. Water was drained and the slides were left overnight at 37℃.

iii) Haematoxylin and eosin staining: The slides containing sections were placed in coupling jars and processed through two changes of xylene (each of 5 min), and then rehydrated in series

of ethanol changes (2 changes in 100% for each of 10 min each; 90% for 5 min; 70% for 5 min; 50% for 5 min; 30% for 5 min) then transferred to distilled water for 5 min. The slides were stained with haematoxylin ($C_{16}H_{14}O_6$. xH_2O) for 20 min, followed by 10 min wash with distilled water. The slides were transferred to acid water to remove excess of staining and finally eosin ($C_{20}H_6Br_4Na_2O_5$) staining was carried out for 3-5 min. The stained sections were further dehydrated in increasing ethanol concentration (30%; 50%; 70% 90% and 100%) and cleared through xylene (3 changes; 5 min each). The slides were then mounted with DPX mountant (Sigma-Aldrich, USA), visualized using Olympus IX-81 microscope (Olympus Corporation, Japan) and photographed.

Statistical analysis

Presented data was validated using standard error of mean (SEM) (n=3), which passed homogeneity and normality tests. Statistical significance was verified using the Sigma Plot v12.3 software (SystatSoftware Inc., USA). Significance between groups was tested using One-Way ANOVA followed by Student-Newman-Keuls (SNK) test for pair wise multiple comparison analysis and p value was calculated for each set of experiments.

Chapter 1

Evaluation of physiological responses of Achaea janata larvae to sub-lethal dosages of Cry toxin exposure

1.1 Introduction

The toxins produced by *Bacillus thuringiensis (Bt),* are classified into three types (i) vegetative insecticidal toxins (Vips) (Warren *et al.,* 1996), (ii) Cytolytic (Cyt) toxins and (iii) Crystalline (Cry) toxins. Further the parasporal crystals produced during sporulation generally contain more than one proteins (Cyt and Cry toxins) (Schnepf *et al.,* 1998). Both Cyt and Cry toxin are known as pore forming toxins (PFT), they share common structural and functional features which facilitate their penetration in the cell membranes and formation of pores, which eventually cause cell lysis followed by insect death (González *et al.,* 2000, 2008; Feil *et al.,* 2010). Cry inclusion proteins are also referred as delta-endotoxins (Heimpel, 1967), which are primarily protoxin, activated in the mid-gut of target insects by proteolytic cleavage. Cry toxins have been shown toxic to the larval forms of Lepidopteran, Coleopteran, Hymenopteran and Dipteran order insects as well as towards soil nematodes (Hofte and Whiteley, 1989; Zhong *et al.,* 2000; Crickmore 2005; Likitvivatanavong *et al.,* 2011).

The nomenclature committee for *Bt* delta-endotoxin was set up in 1993 and proposed nomenclature of various toxins based on amino acid identity which allows closely related toxins to be ranked together. More than 700 *cry* genes have been classified and their products and grouped into 73 different Cry proteins (Cry1-Cry73) (Crickmore *et al.,* 2016). Further based on the host specificity Cry toxins have been grouped into: Group 1-Lepidopteran (Cry1, Cry9 and Cry15); group 2- Lepidopteran and Dipteran (Cry2); group 3-Coleopteran (Cry3, Cry7 and Cry8); group 4- Dipteran (Cry4, Cry10, Cry11, Cry16, Cry17, Cry19 and Cry20); group 5- Lepidopteran and Coleopteran (Cry1I); and group 6-nematodes (Cry6) specific. Further Cry2, Cry3, Cry10 and Cry11 toxins (73–82 kDa) are unique because they appear to be natural product of the larger Cry1 and Cry4 proteins (130–140 kDa). (de Maagd *et al.,* 2001; Pardo-Lopez *et al.,* 2012).

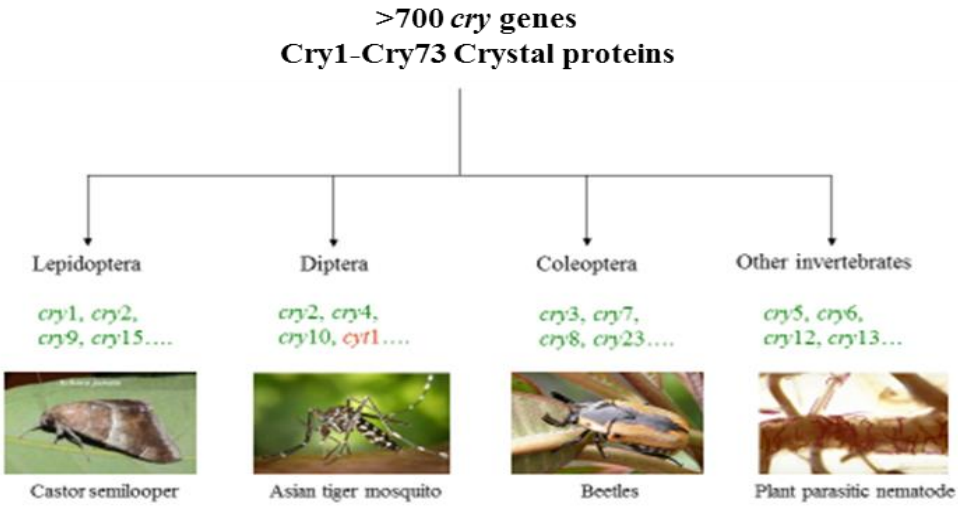


Fig. 8 Summarised spectrum of delta-endotoxin (Cry). Represents the different groups of toxin show its effect on different orders of phylum Arthropoda and other invertebrates including nematodes (Image adopted from http://www.btnomenclature.info/ and modified)

Cry toxins present in the parasporal crystal range between 120-130 kDa, are activated in the host digestive system by an array of proteases and form 64-67 kDa active toxins. X-ray crystallographic analysis of activated toxin revealed it three dimensional (3D) structure (Li *et al.*, 1991) which consisted of three domains. Domain I, a seven α helical structure, resembled to pore forming domain of colicin and was designated as pore-forming determinant and membrane insertion domain. Domain II which possessed to three anti-parallel β sheets, was suggested to help in receptor binding. Domain III consisted of two anti-parallel β sheets, played a role in receptor recognition and membrane insertion. Furthermore domains II and III lie at the same side while domain I is located on opposite side (Grochulski *et al.*, 1995). Both domain I and III have been demonstrated to play role in specificity and interactions with different gut receptors (Bravo *et al.*, 2007).

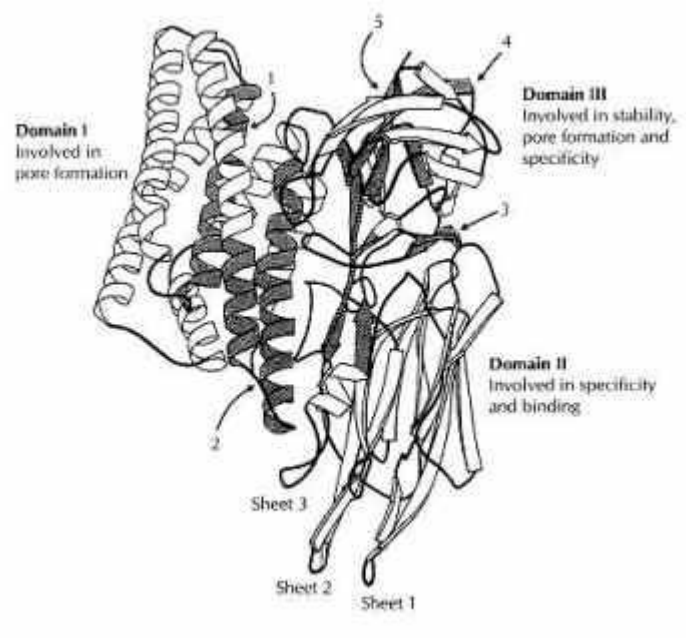


Fig. 9 Schematic representation of activated Cry1A toxin. Consists of three domains; domain I (seven α helices), domain II (three anti-parallel β sheets) and domain III (two anti-parallel β sheets). (Image adopted from http://www.mrclmb.cam.ac.uk/genomes/madanm/articles/dnashuff.htm).

For the mode of action of Cry *(Bt)* toxins two different models have been proposed. (i) The pore formation model is widely accepted one for the Cry toxin toxicity in *M. sexta* (Bravo *et al.*, 2004). In this model, several sequential steps like protoxin activation by gut proteases, oligomerization through cadherin like proteins, binding to the receptor proteins like APN/ALP, which lead to membrane insertion and pore formation. (ii) The other model is signal transduction model which is primarily based on *in vitro* transfection of cadherin receptor gene of *Menduca sexta* in High Five (H5) cells derived from ovarian cells of cabbage looper; *Trichoplusia ni.* Cry1Ab interaction activated Mg^{+2} dependant cellular signalling cascade which involved protein kinase A (PKA) pathway, and caused the cell death (Zhang *et al.*, 2006). This model further suggested that cleavage of α helix and oligomerization were not necessarily required for the pore formation (Soberaon *et al.*, 2009).

As the *Bacillus thuringiensis (Bt)* have potential insecticidal activity, they are formulated into various forms (powder/granules or liquid) for application to the agricultural/ horticultural fields as biological control agents, for the management of insect pests. More than 400 *Bt* based formulations are available in the market (Ahmedani *et al.*, 2008; Sanahuja, *et al.*, 2011) and applied in the form of sprays (Ali *et al.*, 2010). Majority of the sprayable formulations are based on natural stains of *Bt* like *B. thuringiensis var. kurstaki (Btk)* HD1 (Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa proteins), HD73 (Cry1Ac), HD137 (Cry1Aa, Cry1Ba, Cry1Ca and Cry1Da) and *B. thuringiensis var. tenebrionis* (Cry3Aa). *Btk* (HD1) was shown to produce Lepidopteran specific insecticidal proteins (Soberaon *et al.*, 2009). As the Cry toxins were found to be have potential insecticidal activity, plant biotechnologist took lead on to generating *Bt* based transgenic plants which expressed toxin encoding genes (Barton *et al.*, 1987; Walter *et al.*, 2010) and were resistant to variety of insect pests.

Continuous usage of *Bt* based formulations and *Bt* transgenic plants had always been a threat to the development of resistance in insect pests to these Cry toxins. Field evolved resistance against *Bt* was demonstrated in diamondback moth, *Plutella xylostella* (Sayyed *et al.*, 2004) and the same was shown in laboratory reared Europian corn borer, *Ostrinia nubilalis* (Pereira *et al.*, 2008) and pink bollworm, *Pectinophora gossypiella* (Fabrick *et al.*, 2009). Further studies revealed that some pests not only developed resistance to a particular toxin, but also exhibited resistance to a newer toxin(s) to which they were not exposed earlier. This kind of cross resistance was observed in *Plutella xylostella* (Gong *et al.*, 2010) and Asian corn borer, *Ostrinia nubilalis* (Xu *et al.*, 2010). Development of resistance against *Bt* toxin has been attributed to various reasons such as (i) reduced solubilisation of protoxin (ii) alteration of proteolytic activation of toxin (iii) precipitation in gut environment and toxin degradation (Bruce *et al.*, 2007) and (iv) reduced in binding of toxin to the receptors (Tiewsiri and Wang, 2011; Guo *et al.*, 2015).

Additional physiological responses from larvae like non-receptor mediated resistance mechanisms could also interfere with steps which are common to all Cry toxins and thereby result in cross-resistance to a wide range of *Bt* toxins. One such process is the enhanced mid-gut regeneration response observed in resistant larvae. It is documented that the Lepidopteran larvae could recover from exposure to sub-lethal Cry toxin doses (Spies *et al.*, 1985) by mid-gut epithelial regeneration (Forcada *et al.*, 1999). Cry toxin resistance involving an enhanced mid-gut healing response has only been reported in the tobacco budworm; *Heliothis virescens* so far (Martinez-Ramirez and Ferre, 1999; Blanco *et al*., 2009).

In this chapter a detailed study has been carried out decipher the probable mechanisms which facilitate repair of mid-gut epithelium upon Cry intoxication in *A. janata* larvae.

1.2 Materials and Methods

1.2.1 Bacterial strains

DOR *Bt*-1 (a *Bacillus thuringiensis thuringiensis* strain) was obtained from Indian Institute of Oilseeds Research, Hyderabad which exhibited high toxicity against larval forms of *A. janata* and as well as various other Lepidopteran pests. Recombinant Cry toxin (Cry1 Aa, Cry1Ab and Cry1Ac) containing JM103 *E .coli* strains were obtained from Bacillus Genetic Stock Centre (BGSC), Ohio State University, USA. The Cry toxins obtained from both local isolate and recombinant strains were used for the present studies.

1.2.2 Collection and purification of *Bt* crystal Spores

For preparation of *Bt* crystal spores (δ- endotoxin), the local DOR *Bt*-1 isolate was grown in Nutrient broth (Himedia, India) for 72 h with agitation (180 rpm/min) at 28℃ until sporulation and cell lysis was complete. The parasporal crystal and spores were harvested from the above culture by centrifugation at 1100 X g for 10 min at 4℃ followed by 3 times washing with distilled water. Pure crystals thus obtained were stored at 4℃ till use. Crystal incisions obtained from DOR *Bt*-1 were suspended in water and smeared on microscopic stub. The samples were air dried, gold coated and examined under Jeol scanning electron microscope (Jeol Inc, USA).

For preparation of recombinant toxins, a standard protocol was followed (Lee *et al.*, 1992), where JM103 cells were grown and harvested (10000 X g, 10 min) and the pellet was suspended in buffer (50 mM Tris-HCl, 50 mM EDTA, 15% Sucrose, pH 8.0). Cell suspension was sonicated on ice (10 min, 10 sec on and 30 sec off) and centrifuged at 15000 X g for 10 min. The pellet thus obtained was washed three times with ice cold 0.5 M NaCl containing 2% Triton X-100, five times with 0.5 M NaCl and finally two times with distilled water.

1.2.3 Solubilisation and activation of protoxin

The crystal pellet was solubilized in 50 mM sodium carbonate/bicarbonate buffer (pH 9.5) containing 10 mM DTT and incubated at 37℃ in water bath for 2 h. After solubilization the fraction was dialyzed to remove DTT. The solubilized protoxins were digested with trypsin in a trypsin/protoxin ratio of 1:50 (by mass) at 37℃ for 2 h. To this 1mM PMSF was added, to stop the proteolytic reaction. The activated toxins were purified using Sephadex G-100 (Sigma-Aldrich, USA) through gel permeation chromatography and profile of the digested toxin was assessed by SDS-PAGE. Presence of active Cry protein was confirmed by immunoblotting using antibody generated against recombinant Cry1Ac toxin.

1.2.4 *Bt* toxin activity evaluation

Feeding bioassay was performed where specific area of the upper leaf surface was coated with various concentrations of activated Cry toxin(s) (2, 4, 6, 10, 12, and 14 ng/cm2). Control were maintained on buffer coated leaves. Two days old (neonates) *A. janata* larvae (20 no's) were allowed to feed on both toxin coated and normal (buffer coated) leaves. Triplicates were maintained for each treatment and larval mortality was recorded at every 24 h interval for 5 days. LC_{50} dose (toxin concentration at which 50% larval lethality/mortality obtained) was calculated, using Probit analysis with the help of Finney'S table (Finney, 1978).

1.2.5 Feeding/ exposure of 3rd instar larvae with (DOR *Bt*-1) formulation

The reported LC_{50} value of DOR *Bt*-1 formulation was 247.52 µg/ml for seven-day old or 3rd instar larvae (Vimaladevi *et al.*, 2006). For the present study various dosage of toxin formulation was coated on fresh castor leaves. Laboratory standardized sub-lethal dosage (1/10th of the LC_{50}) of DOR1 *Bt-1* formulations coated leaves were air dried and used for feeding to a fairly large number of 3rd instar larvae (150 no's) in a restricted environment. For each feeding assay triplicates were maintained. The feeding was carried out for various time periods, the insects (30 no's) were removed after 24 h , 48 h and 72 h of toxin exposure

and were transferred to normal uncoated castor leaves and allowed to recover. Rest of the insects (60 no's) were continuously maintained on toxin coated leaves. For each time period age matched control larvae were maintained on normal (buffer coated) leaves. Furthermore the data was collected on mortality and morphological changes during development were recorded in toxin-exposed, recovered insects, and control insects for the comparative study.

1.2.6 Tissue isolation

Third instar larvae exposed to toxin coated leaves were collected at every 12 h interval till 72 h, along with appropriate age matched control insects. The larvae were briefly narcotized on ice and dissected in insect Ringer solution (130 mM NaCl, 0.5 mM KCl and 0.1 mM $CaCl_2$). An incision was made in the abdominal region and extended through the length of the larvae. The gut content was removed and the mid-gut region was isolated. After removal of the gut from the insect, the yellowish-white perivisceral fat body was collected. The isolated tissues were used for histology, *in-situ* hybridization, genomic DNA as well as total RNA isolation and protein analysis.

1.2.7 Histological analysis of mid-gut

The detailed protocol have been described in the General Materials and Methods Section. The isolated mid-guts were fixed overnight in Bouin's fixative, processed through a series of increasing alcohol concentration, cleared in xylene and embedded in molten paraplast (Sigma-Aldrich, USA). The tissue was sectioned (5 µm) using a rotator microtome (Leica Microsystems, Germany) and stained with hematoxylin-eosin or toluidine blue (Sousa *et al.*, 2010), visualized and photographed using Olympus IX-81 microscope (Olympus Corporation, Japan).

1.2.8 Confocal analysis

The larval mid-gut tissue from both control and toxin exposed larvae were dissected out and placed in Grace's insect culture medium (Thermo Fisher Scientific, USA). They were incubated in presence of both Propidium iodide and Annexin V staining solutions (AnnexinV-FITC Apoptosis Detection kit, Abcam pk, UK) for 10 min in dark and analysed using confocal microscope LSM 880 (Carl Zeiss,Germany).

1.2.9 FACS (Fluorescence-activated cell sorting) analysis

Single cell suspension prepared from mid-gut of both normal and toxin exposed larvae using 70 µm mesh size MACS® smart strainer (MiltenyiBiotec, Singapore) and phosphate buffer saline (PBS) solution (pH 7.4). Cell suspension collected through funnel was centrifuged at 1500 rpm for 5 min at 4ºC, and the cells were immediately processed for FACS analysis. It was carried out using Apoptosis Detection Kit according to the manufacturer's instruction (BD Biosciences, UK). AnnexinV (AV) and Propidium Iodide (PI) staining solutions were added to cell suspension, which was incubated at room temperature in the dark for 15 min. This was followed by addition of binding buffer and then analyzed using a FACS LSR Fortessa^{TM} flow cytometer (BD Biosciences, UK) and BD FACS Diva Software 6.0. For each sample, 10,000 events were acquired. The percentage of live cells versus cells undergoing death was plotted and for analysis data was represented in four quadrant (Q1: non-viable, PI^+ (necrotic); Q2: both PI^+ (necrotic)/ AV^+ (apoptosis); Q3: PI^-/ AV^- (viable cells) and Q4: AV^+ (apoptotic) cells.

1.2.10 Genomic DNA isolation and DNA fragmentation analysis

Genomic DNA from mid-gut of both control and toxin exposed insect larvae was isolated using standard protocol (Lagisz *et. al.*, 2010) with slight modifications. Homogenization of mid-gut tissue was carried out in 500 µl of lysis buffer (100 mM Tris-HCl (pH8.0), 50 mM EDTA (pH 8.0), 1.5 mM NaCl, 2% CTAB and 0.1% Mercaptoethanol).

The sample was incubated at 65℃ for 45 min with intermittent mixing. The phenol: chloroform: isoamyl alcohol (25:24:1) mix was added with vigorous mixing for 5 min, followed by centrifugation at 15000 rpm for 15 min. The supernatant was transferred to a fresh Eppendroff tube and two volumes of pre chilled 2-propanol was added along with 5 µl RNase A (10 mg/ml) and centrifuged at 10000 rpm for 10 min. The obtained pellet was washed thrice with 70% ethanol. The pellet was dried and dissolved in TE buffer. Quality of genomic DNA was analyzed using Nanodrop spectrophotometer. DNA fragmentation analysed using 0.8% of agarose gel electrophoresis.

1.2.11 BrdU labelling and detection

Streptavidin-biotin based BrdU staining (Invitrogen Corporation, USA) was used for monitoring the cell proliferation in the mid-gut during toxin-induced damage following manufacturer's protocol. Larvae were injected with BrdU labelling reagent (10 µl/g body weight) 4 h prior to isolation of toxin exposed larval mid-gut using a Hamilton syringe. Tissue was fixed in Bouin's fixative and processed (as mentioned in the General Materials and Methods Section).Tissue sections were adhered on poly-L-lysine coated slides (Sigma-Aldrich, USA) and incubated overnight at 37°C, following which deparaffinization was carried out using xylene and passed through a series of decreasing ethanol concentrations. Peroxidase quenching was performed using 30% H_2O_2 and absolute methanol (1:9). Denaturation and blocking steps were carried out using the kit based reagents prior to incubation with antibody. Biotinylated mouse anti-BrdU antibody was overlaid and the slides were incubated at room temperature. After incubation, streptavidin peroxidase (Invitrogen Corporation, USA) was added to detect the incorporation. Colour development was monitored after the addition of secondary antibody mixture provided by the manufacturer. Slides were counterstained and mounted using histomount TM (Invitrogen Corporation, USA) and visualized under Olympus IX-81 microscope.

1.2.12 Propidium Iodide (PI) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide staining (MTT) of fat body tissue

Fat body was collected from the sub-lethal dosage toxin exposed insects and analysed for the effects of Cry toxin using PI and MTT staining. The tissue was placed in 500 µl of TC-100 insect culture medium (Sigma-Aldrich, USA) containing trace amounts of streptomycin sulphate. For PI staining tissue was washed twice in PBS (pH 7.4) and incubated with 100 µl of 1X PI staining solution (BD Biosciences, USA) for 15 min, in dark at room temperature. After this 400 µl of 1X binding buffer was added and tissue was visualized under microscope. While for MTT 100 µl of staining solution (5 ng/ml) was added and incubated at 25°C with gentle shaking for 30 min in dark. The tissue was then washed twice in PBS (pH 7.4) and mounted on slide and visualized under microscope.

1.2.13 Hemocoelic injection of Cry toxin

Third instar larvae were narcotized on ice for 15 min and were then injected with activated Cry toxins i.e. 200 ng/0.2 g body weight through the mid-dorsal region using a Hamilton microsyringe. Control larvae received the same volume of the carrier solvent. Immediately after injection, the wound was sealed with bees wax and the larvae were placed on ice for 15 min before transferring them back to the rearing dishes/troughs provided with fresh castor leaves. Pathological effects of Cry toxin on various larval tissues were analysed using histological sections stained with haematoxylin-eosin staining.

1.3 Results

1.3.1 Preparation and purification of Cry toxins from local isolate

The scanning electron microscopic analysis clearly showed the presence of crystal proteins of various shapes in DOR *Bt* -1 preparation, which is suggestive of the expression of more than one *cry* gene in this local isolate (Fig. 10A). The *Bt* crystal spores solubilized in sodium carbonate buffer (pH 9.5) and activated by trypsinization showed the presence of both protoxin (120 kDa) and active toxin (64 kDa) bands (Fig. 10B), which is further purified with gel permeation chromatography using (Sephadex G-100) beads (Fig. 10C). Presence of toxin was confirmed by using Cry1Ac antibody generated against recombinant Cry1Ac protein purified from BGSC clones (Fig. 10D).

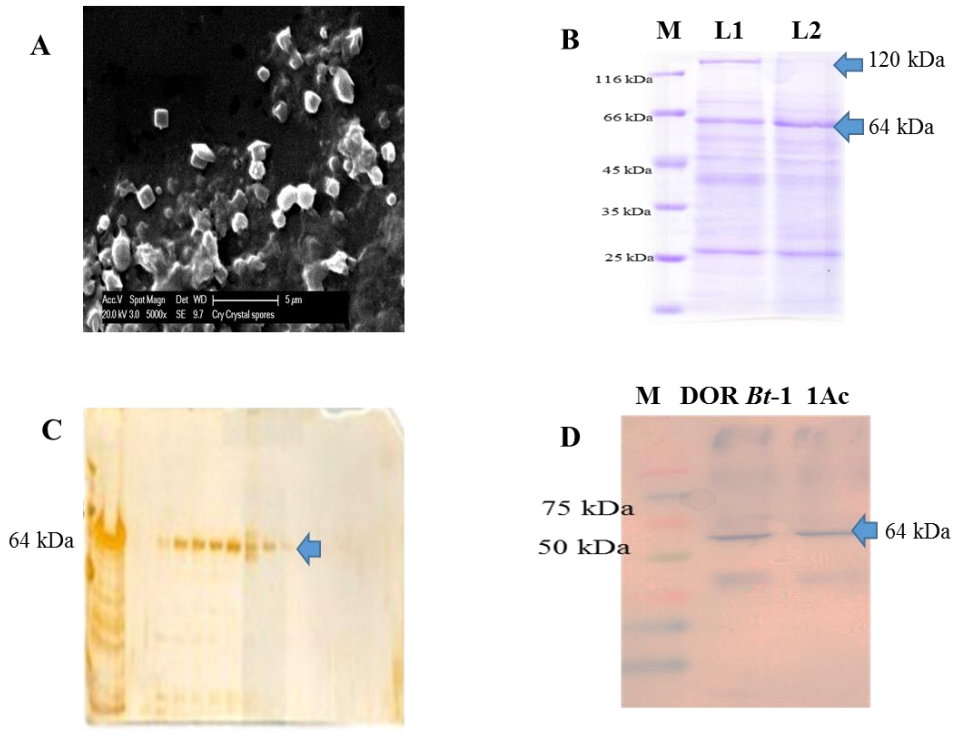


Fig. 10 Purification of Cry toxins from local isolate (DOR *Bt*-1). (A) Scanning electron micrograph of DOR *Bt*-1 crystal spores. (B) SDS-PAGE profiling of solubilized protoxin (⇨120 kDa) and active toxin(s) (⇨64 kDa). (C) Purification of active Cry toxin through gel permeation chromatography (Sephadex G-100). (D) Confirmation of purified activated Cry toxin(s) (⇨64 kDa) with recombinant Cry1Ac antibody.

1.3.2 Toxicity effects of Cry toxin

Feeding bioassay with 2 days old *A. janata* larvae showed that feeding of local isolate (DOR *Bt*-1) and recombinant Cry toxin(s) i.e. Cry1Aa, Cry1Ab, and Cry1Ac caused larval mortality. However, DOR *Bt*-1 Cry toxins showed much higher insecticidal activity with fairly low LC_{50} (4.1 ng/cm^2 of leaf surface) (Table 1). Furthermore DOR *Bt*-1 were reported to be free from any exotoxin activity (Vimaladevi *et al.*, 2006).

Toxin	LC_{50} (ng/cm^2 of leaf surface)
Cry1Aa	107.56
Cry1Ab	47.83
Cry1Ac	35.87
DOR *Bt*-1	4.010

Table 1 Evaluation of insecticidal activity of Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac and DOR *Bt*-1) against *A. janata* larvae after oral ingestion. Feeding assay was performed using neonate larvae (2 days old). The specific area of castor leaf was coated with various concentrations of activated proteins (2, 4, 6, 8, 10, 12 and 14 ng/cm^2). The experiment was performed in triplicates. Larval mortality was recorded at 24 h intervals for 5 days. LC_{50} was calculated based on area of feeding and mortality rate of insects and analysed using probit analysis using Finney's table (Finney, 1978).

1.3.3 Cry toxin altered larval growth and development

The larvae which were fed on toxin-coated leaves exhibited reduced growth in a time-dependent manner (24-72 h). The 24 h toxin-exposed larvae could recover and grow when transferred to normal leaves, whereas the 48 h and 72 h toxin-fed groups often showed slow growth as well as poor recovery (Fig. 11A). Eventually also resulting in defective metamorphosis and premature death (Fig. 11B). The 24 h toxin-exposed larvae could recover and normally develop when transferred onto uncoated leaves, whereas the 48 and 72 h toxin-fed groups could not recover from the effect of *Bt* and exhibited defective molting. Further,

the body weight of the toxin exposed larvae was fairly low and was only 50% of that of the age matched control groups after 72 h of recovery, when the larvae were exposed to toxin for 48 h or 72h (Fig. 11C).

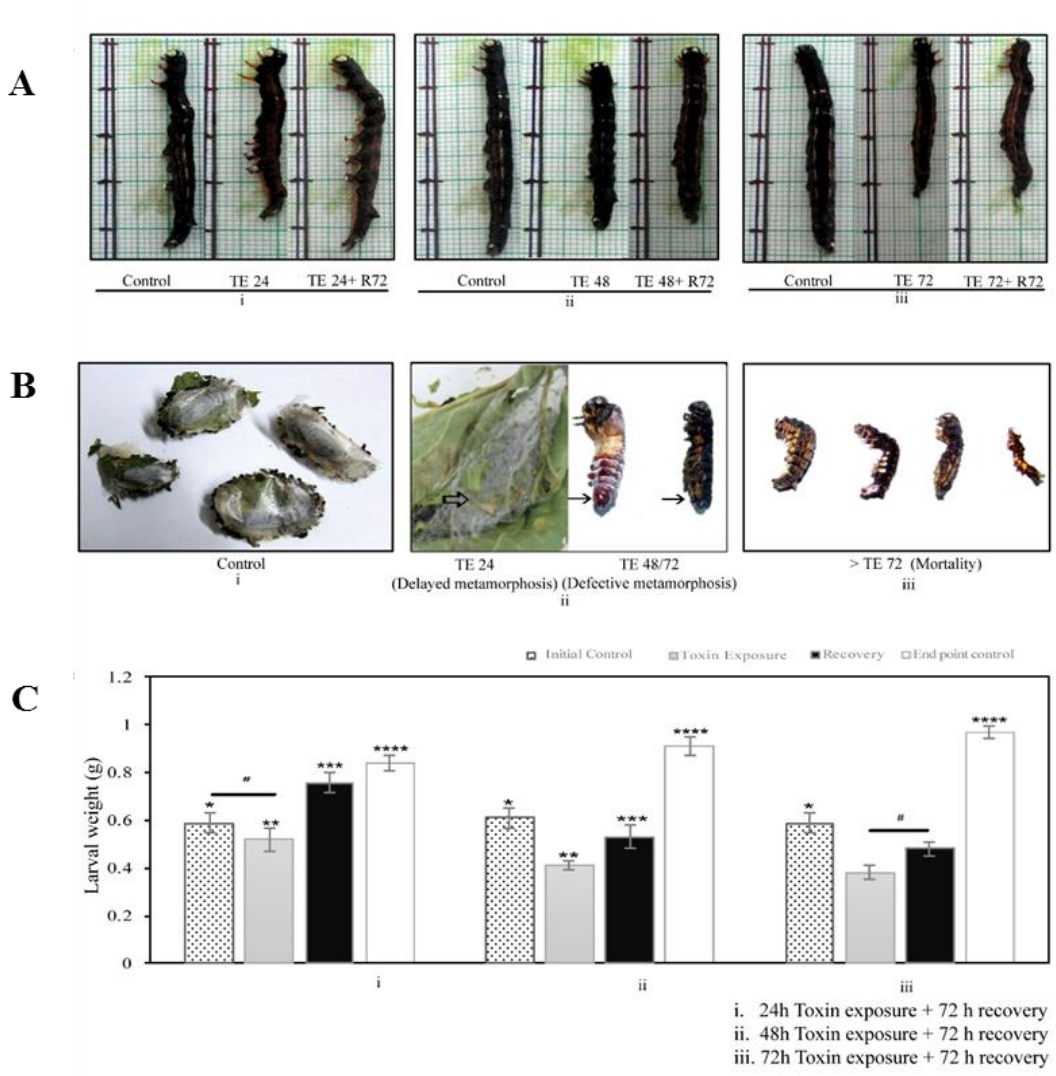


Fig. 11 *A. janata* third instar larvae were maintained either on Cry toxin coated or normal leaves for different time intervals. (A) Growth pattern seen in (i) 24 h, (ii) 48 h and (iii) 72 h after Cry toxin exposure (TE), with respective controls, for recovery toxin exposed larvae were transferred to normal leaves for 72 h (R72). (B) Metamorphosis in toxin exposed larvae (i) control insects with normal metamorphosis **(i**) 24 (⇨), 48 and 72 h (→) toxin exposed larvae with either delayed or defective metamorphosis. (C) Effect of Cry toxin exposure for (i) 24 h, (ii) 48 h and (iii) 72 h on larval weight and after 72 h of recovery. The values presented are the mean ± standard deviation of three independent experiments (n=3), significance between groups was tested by One-Way ANOVA followed by Student-Newman-Keuls (SNK) test using Sigma Plot 12.3 software. Means marked with *$p < 0.05$ are significant with respect to toxin exposed, recovered and age matched end point control larvae,**$p < 0.05$ indicate statistical significance with respect to recovered and age matched end point control, ***$p < 0.05$ indicate statistical significance with respect to initial control and toxin exposed larvae, ****$p < 0.05$ indicate statistical significance with respect to the weight of initial control, toxin exposed and recovered larvae, while # represents difference between compared group that is not statistically significant.

1.3.4 Mid-gut necrosis occurred in toxin-exposed larvae

The viability of mid-gut cells was primarily assessed using PI and Annexin V staining (Vermes *et al.*, 2000). Confocal analysis revealed the presence of PI positive cells in toxin exposed larvae but not in controls (Fig. 12).

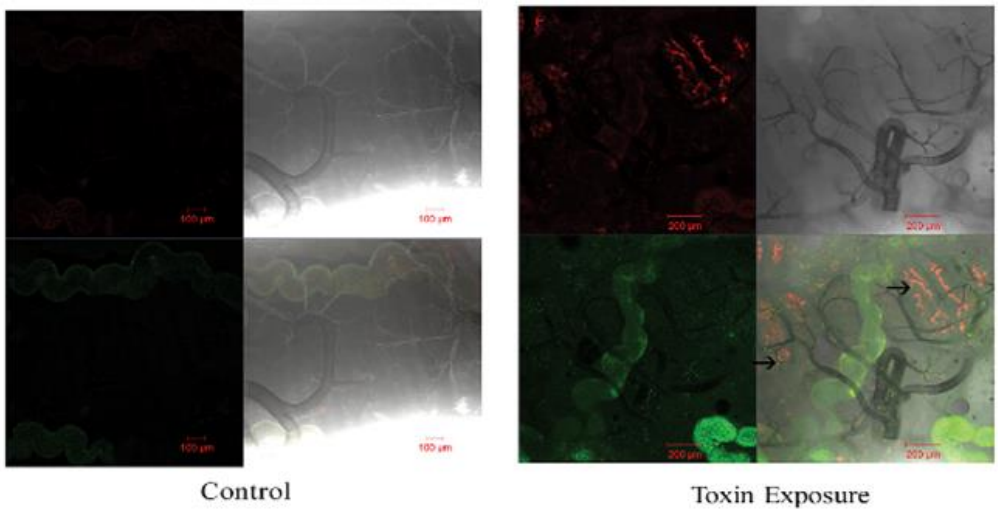


Fig. 12 Cell death through confocal microscopic analysis using Annexin V (AV) and Propidium iodide (PI) stains. Whole mount showing control and Cry toxin exposed larval mid-gut, stained with Annexin V (AV) and Propidium iodide (PI). Note the presence of PI positive cells in the mid-gut epithelium after toxin exposure (→).

The data represented in figure 13 shows the results obtained from FACS analysis. The scatter plot which gives the total cell counts is divided into four quadrants (Q1-Q4), Q1 represents PI^+/AV^- cells (necrotic), Q2 represents PI^+/AV^+ cells (late apoptotic/necrotic), Q3 represents PI^-/AV^- (viable cells) and Q4 represents AV^+/PI^- cells (early apoptotic). Mid-gut cells prepared from toxin exposed insects showed distinct shift into the upper left quadrant (Q1) suggesting that cells were undergoing necrotic type of cell death. Further, their number upon toxin-exposure increased gradually from 12 h to 36 h. However, the results presented here clearly show that the number of necrotic cells declined drastically preparations from the mid-gut of 48 h as well as 72 h toxin-exposed larvae (Fig. 13, inset).

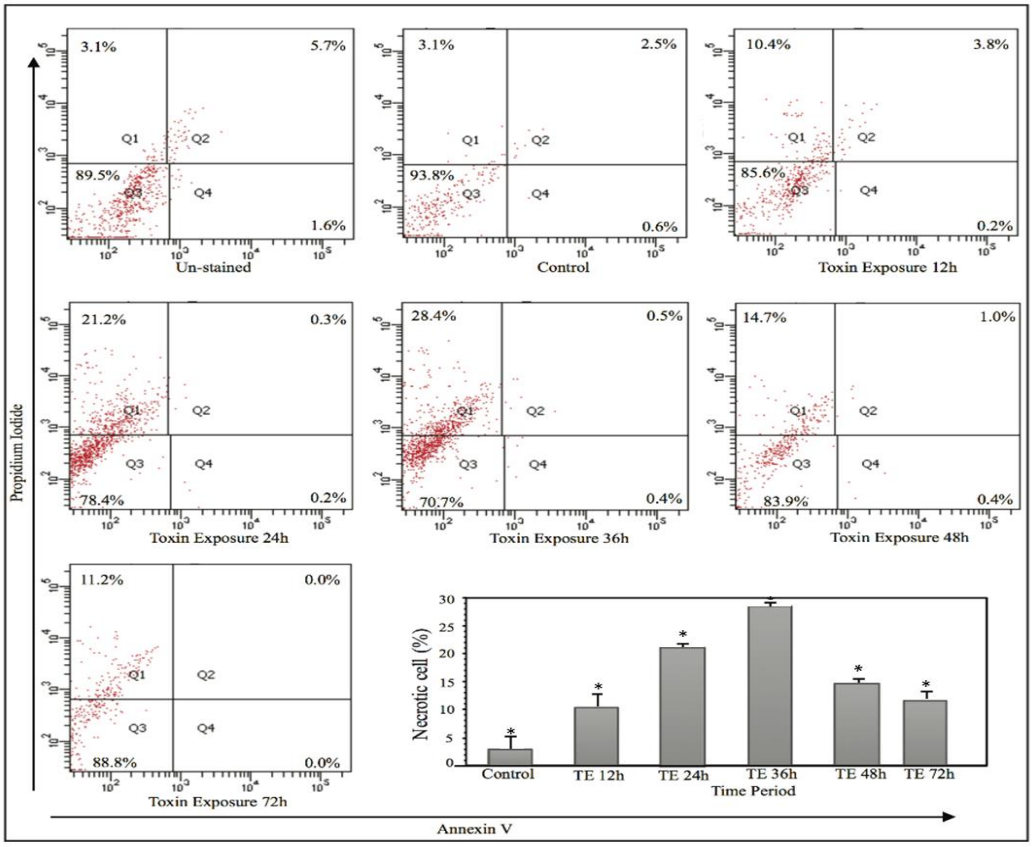


Fig. 13 Scatter plot showing the distribution of Annexin V and PI stained cells suspension prepared from mid-gut of control and Cry toxin exposed larvae. The cells isolated from experimental larvae showed a distinct shift during 24 h and 36 h of exposure into the top left quadrant (Q1). Inset shows percentage of necrotic cells in larval mid-gut upon toxin-exposure during different time points, *p < 0.005 significance between experimental groups.

To further affirm that cell death which occurred in larval mid-gut during early toxin exposure (12-36 h) was due to necrosis and not because of apoptosis, DNA fragmentation assay was carried out using genomic DNA isolated from the tissue. The result revealed that the DNA integrity was preserved in both control and toxin-exposed mid-gut samples and it was comparable with that of control insects (Fig. 14). Histological, FACS and DNA fragmentation analyses carried out in present study indicated that necrosis was the most probable mechanism of cell death upon Cry intoxication.

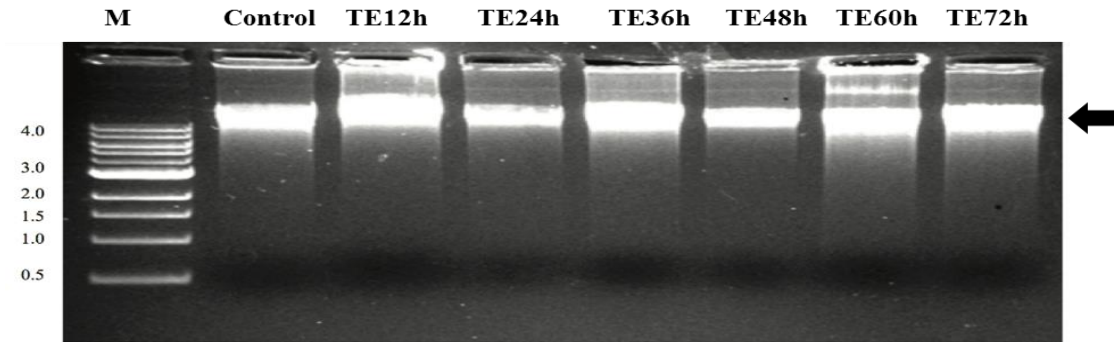


Fig. 14 DNA fragmentation analysis of mid-gut. Ethidium bromide stained agarose gel showing the presence of intact band (⬅) of similar molecular size in both control and toxin exposed larvae after different time points (TE 12- 72 h). Lane M shows the molecular size marker (1kb; #SM0311).

1.3.5 Sub-lethal toxin exposure induced mid-gut cell proliferation

For determination of whether or not cell regeneration occurred in larval mid-gut upon sub-lethal toxin exposure, cell proliferation was monitored using a kit-based BrdU (bromodeoxyuridine) labelling. Mid-gut cross-sections prepared from toxin-exposed larvae were processed for immunocytochemistry using BrdU primary antibody. The cells in the DNA synthesis phase (S phase) were labelled by this method. Few pockets of BrdU-positive cells (brown stained nuclei) were visible in the mid-gut collected from toxin exposed larvae (Fig. 15A). The dividing cells which were primarily seen in the basal region of the gut epithelium, could be ascribed as mid-gut stem cells/ regenerative cells (Fig. 15B, inset).

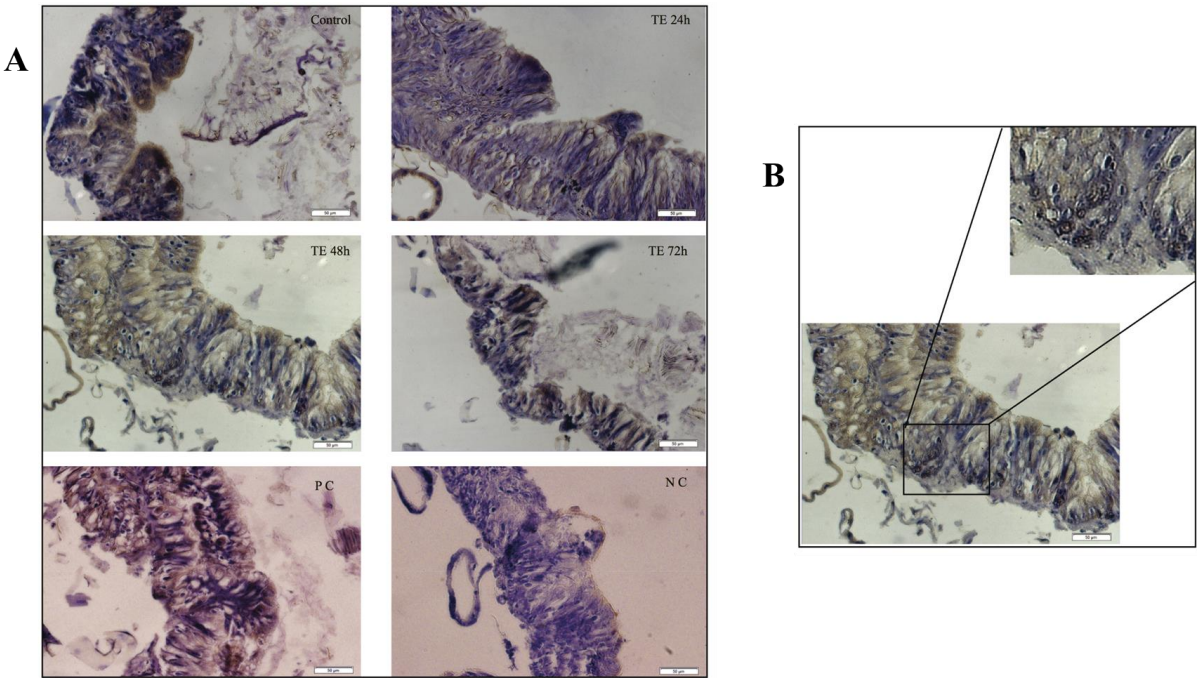


Fig. 15 Mid-gut cell proliferation monitored by BrdU incorporation. (A) T.S. of mid-gut from Cry toxin exposed 24, 48 and 72 h of larvae showing BrdU positive cells. Dextran sulphate sodium injected larvae were used as positive control (PC) and phosphate buffered saline injected larvae were used a negative control (NC). Note the presence of dividing cells with brown stained nucleus. (B) Enlarged portion of mid-gut epithelium from 48 h toxin exposed larvae showing actively dividing cells in the crypt.

1.3.6 Cell death seen upon sub-lethal dose of Cry toxin exposure was associated with epithelial regeneration

To analyse whether or not cell death in mid-gut is associated with regeneration, the toxin exposed larval mid-gut histological sections were stained with either haematoxylin/eosin or toluidine blue. The changes were monitored from 12 h till 72 h of Cry toxin-exposure (Fig. a16).The mid-gut cells showed degeneration with condensed nucleus and fairly low cytoplasmic content. In addition, large number of vacuoles were seen in them. Maximum cell death was observed between 24 h to 36 h (Fig. 16, C-D). However, interesting phenomenon observed was active cell proliferation in toxin exposed larvae. Figure 16 (c-d) clearly showed cell division upon toluidine blue staining. The histological and toluidine blue corroborated and clearly suggested it was not only cell death occurred but cells also divided actively and epithelium regeneration upon Cry intoxication in *A. janata* larvae.

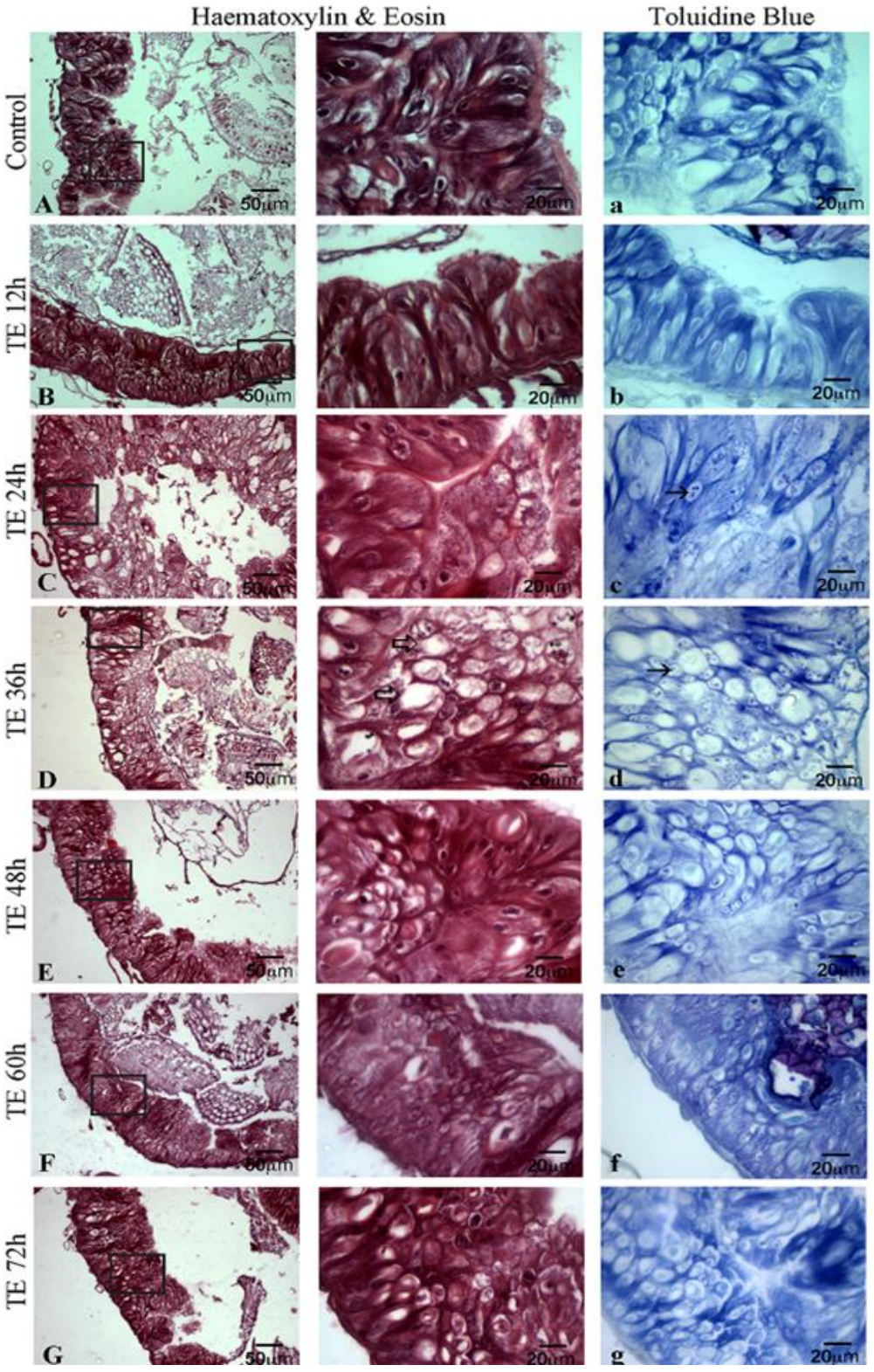


Fig.16 Occurrence of cell death and cell proliferation in mid-gut upon Cry intoxication. Haematoxylin/eosin and toluidine blue staining. T.S. of mid-gut showed extensive damage in epithelium (C) and presence of large number of vacuoles (⇒) (D) after toxin exposure. T.S. of mid-gut stained with toluidine blue (4, a-g), note the presence proliferative cells in the basal region of the epithelium (→) (c-d). (Scale bars 20 µm, 50 µm).

1.3.7 Effects of Cry toxin on other tissue/ organs

Various tissue/organs isolated from sub-lethal toxin exposed/ fed larvae showed the presence of non-viable cells stained reddish pink with PI, which were absent in the tissue dissected from control larvae (Fig. 17B). Once again MTT staining further confirmed the non-viability of fat body cells which appears dark in colour, while the tissue isolated from control larvae stained in light purple in colour (Fig.17D).

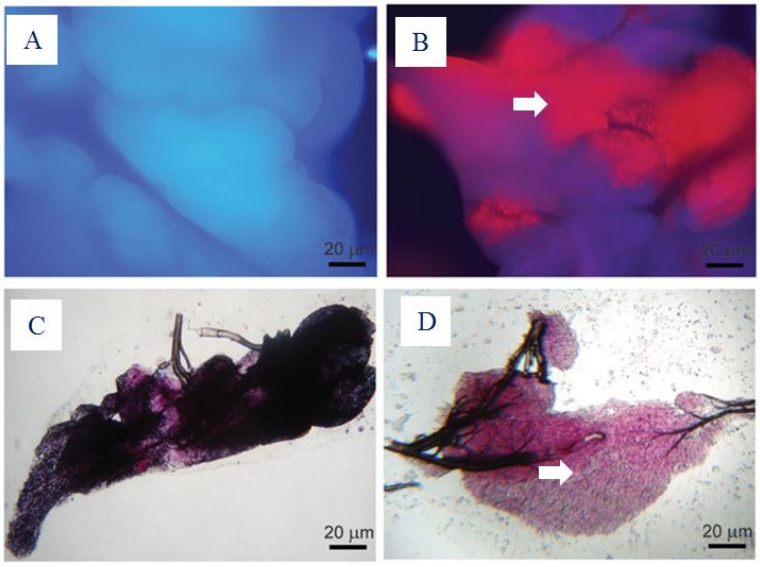


Fig. 17 Effects of Cry toxin on fat body after oral ingestion. Note the presence of non-viable cells which stained reddish pink after PI staining (B). Light purple in MTT assay (D), indicated toxin based cell damage which caused non-viability of tissue upon Cry toxin feeding. A and B are respective control.

Once again the direct interaction of Cry toxin with visceral tissue evaluated by direct haemocoelic delivery of activated Cry toxin in the larvae by micro injection. Mid-gut tissue sections stained with H&E show the presence of irregular arrangement of goblet (g) as well as columnar (c) cells in the gut epithelium (Fig. 18a). In fat body, extensive tissue degeneration was seen (Fig. 18b). Malpighian tubule revealed the presence large number of vacuoles (v) in the cytoplasmic region with chromatin condensation in the nucleus (Fig. 18c). Furthermore salivary gland cells also showed condensation of nuclear chromatin and reduced diameter of the gland with fairly narrow lumen (Fig. 18d)

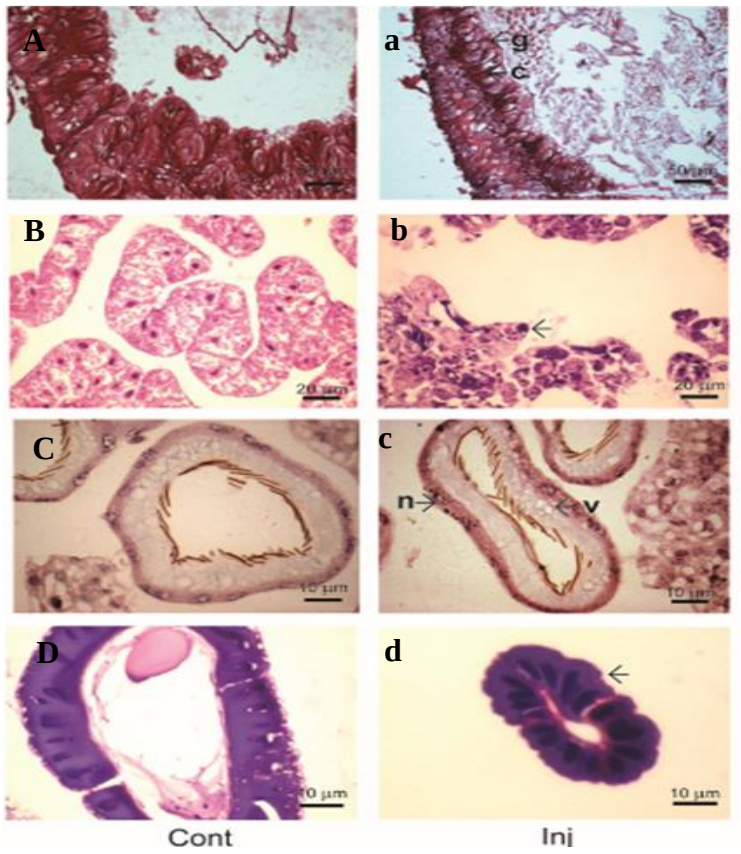


Fig. 18 Histological analysis larval tissue after hemocoelic injection of activated Cry toxin. Tissue sections were prepared from 2 days post-injected larvae and stained with H&E (A) mid-gut, (B) fat body, (C) Malpighian tubule and (D) salivary gland. Note the cytological changes in tissue sections induced by Cry toxin injection (Inj, arrowhead), (g, Goblet cell; c, columnar cell; v, vacuole; n, nucleus.)

All the above observations clearly show that Cry toxin not only caused the damage to mid-gut epithelium but also had deleterious effects to the cells of other visceral tissues of the larvae, which are exposed to hemocoelic toxin upon injection.

1.4 Discussion

In the present work, the effect of sub-lethal dose of Cry toxin was evaluated primarily to assess the larval mid-gut responses in *A.janata*. The formulations used in the present study, DOR *Bt*-1 is a product prepared from a local strain of *Bacillus thuringiensis*, a gift from Dr. P.S.Vimala Devi, of Indian institute of Oilseeds Research, Hyderabad. DOR *Bt*-1 formulations was shown to have high insecticidal activity and was required in very low amount to achieve LC_{50} (4.1 ng/cm^2 of leaf surface) when compared with recombinant Cry toxins obtained from BGSC (Cry 1Aa, Cry1Ab and Cry1Ac) clones. The high toxicity of this strain against *A.janata* larvae was likely associated with presence of multiple *cry* genes, like *cry1* (*cry1Aa*, *cry1Ab* and *cry1Ac*) and *cry2* (*cry2Aa* and *cry2Ab*), which provided broad spectrum potential against Lepidopteran and Dipteran insects (Reddy *et al.*, 2012).

In field, the *Bt* based formulations are generally sprayed when insects are in late 2^{nd} or early 3^{rd} instar larval stages, for effective pest management. The suggested dose for field application for DOR *Bt*-1 formulation is 1g/10L (w/v). The reported LC_{50} for 3^{rd} instar (seven-day old) larvae was reported to be 247.52 µg/ml for ml for seven-day old or 3^{rd} instar larvae (Vimaladevi *et al.*, 2006). In the present study a series of dosages using various dilutions of this formulation were tested and finally $1/10^{th}$ of the LC_{50} i.e 24.75 µg/ml of water (0.170 µg/cm^2 of leaf surface) was chosen as a sub-lethal dose of toxin for the study using for 3^{rd} instar *A. janata* larvae.

As observed in this pest and other reported Lepidopteran models, Cry toxin exposed larvae usually exhibited low digestive activity concomitant with their reduced body weight associated with altered growth and development (Hernandez-Martinez *et al.*, 2010). In the present study one set of larvae (30 no's) was removed after every 24 h of toxin exposure till a period of 72 h, and transferred to uncoated leaves for recovery. The larvae fed on toxin-coated leaves exhibited a time-dependant (24-72 h), reduction in the growth which was reflected in

their body weight. The 24 h toxin-exposed larvae recovered and grow whereas the 48 h and 72 h toxin-fed groups often showed slow as well as poor recovery when transferred to normal leaves. Further, the body weight of the toxin exposed larvae was fairly low i.e. only 50% of that of the age matched control groups after 72 h of recovery, when the larvae were exposed to toxin either for 48h and 72 h. Present study corroborated well the findings of *Helicoverpa armigera*, where upon prolonged sub-lethal exposure to *Btk* HD-1 toxin larvae showed reduced weight and altered behaviour (Mohan *et al.*, 2008).

Histomorphological studies of mid-gut showed that even sub-lethal dose of toxin caused extensive cell death/tissue damage. Further the maximum cell death was observed between 24 h to 36 h, where the mid-gut epithelium showed tissue degeneration. The cells showed the presence of condensed nucleus, fairly low cytoplasmic content and large number of vacuoles in it. Although the pore formation model is most widely accepted for Cry toxin toxicity in Lepidopteran larvae (Bravo *et al.*, 2004), however the exact mechanism of cell death upon Cry toxin intoxication is still not very clear in large number of insects including *A. janata*. Further in this study an attempt was made to identify the type of cell death which occurred upon Cry intoxication. Results obtained from confocal analysis of mid-gut taken from both the control and Cry intoxicated larvae using PI which is a marker for cell death and AV which is also a marker for death associated with apoptosis, clearly showed presence of PI positive cells but not any AV positive cells, suggested that cell death seen in the epithelium upon Cry intoxication was not due to apoptosis but was likely due to other form of cell death (may be necrosis), FACS analysis was carried out by using the both PI and AV stained single cell suspension isolated from both control and toxin exposed larvae. This method primarily allowed the identification of cells which undergo either apoptosis or necrosis (Vermes *et al.*, 2000). In the present analysis maximum number of cells stained with PI and negligible number of cells with Annexin V. Furthermore agarose electrophoresis

carried out with the mid-gut genomic DNA isolated from both control and toxin exposed larvae revealed absence of DNA fragmentation. Histological, FACS and DNA fragmentation assay results of the present study clearly suggested that the cell death induced by Cry toxin was primarily due to necrosis. There is increasing evidence in the field that Cry toxin induced cell death is a fairly complex cellular response. Zhang *et al.*, (2006) using cabbage looper ovarian cell culture demonstrated that Cry1Ab toxin manifested its effect through Mg^{2+}dependent adenylyl cyclase/protein kinase A (PKA) signalling pathway. Recently, using toxin-host interaction system in *Caenorhabditis elegans*, (Zhang *et al.*, 2016) demonstrated that Cry6Aa mediated cell death was promoted through necrotic signalling pathway. However, these reports were either from *in vitro* studies using isolated cells or non-host system and to the best of our knowledge there is no direct evidence reported in insect host model. Present *in vivo* study for the first time clearly showed that Cry toxin promotes necrosis cell death in mid-gut of *A. janata* larvae during Cry intoxication.

One of the notable phenomenon observed in the present study was migration of larvae, after initial feeding on toxin coated leaves for some time, towards the sides of the dishes and reduced feeding to avoid toxin based mid-gut cell damage. Replenishment of injured cells during the mid-gut repair of various Lepidopteran larvae was shown to be facilitated by normal feeding, leading to recovery and metamorphosis (Spies and Spence 1985; Loeb *et al.*, 2001). However, when larvae were exposed to high doses of *Bt* toxin, it lead to mortality and prevented recovery (Tabashnik *et al.*, 2013). Like many other tissues, renewal of epithelium is carried out by regenerative cells localized in the basal crypts which are termed as mid-gut stem cells/ regenerative cells. These were the only population of cells present in the gut capable of division and thus represent the sole source of regenerative cells which play crucial role in repair and growth of damaged epithelium. Continuous replacement of damaged cells by regenerative cells under toxin environment was likely to cause alteration in cellular

properties including changes in the cell surface molecules leading to the development of resistance in insects, against *Bt*/Cry formulations (Martinez-Ramirez *et al.*, 1999; Castagnola and Jurat-Fuentes, 2016).

In the present study we clearly demonstrated not only the cell death and damage of mid-gut epithelium upon Cry intoxication but also proliferation of stem/regenerative cells, which allowed the larvae to repair its gut epithelium and generate new populations of differentiated cells which facilitated the survival of insects. This was further substantiated by the presence of large number of BrdU positive cells in the basal crypts of the gut epithelium, while toluidine blue staining revealed sizable increase in the number of proliferative cells upon Cry intoxication in time dependent manner in *A. janata* larvae. Finally, the present study clearly demonstrated that Cry toxin based damage was associated with cell proliferation in mid-gut, which eventually facilitated the survival of insects upon sub-lethal dosages of Cry intoxication.

Oral ingestion of Cry toxin in *A. janata* not only caused the damage to mid-gut, a well-known organ bearing Cry toxin binding receptor/interacting proteins, but also showed its deleterious effects on visceral tissues. In earlier studies, our group reported the presence Cry toxin binding protein/ receptors among which aminopeptidase N (APN) on the cell membrane of fat body of *A. janata* (Budatha *et al.*, 2007) and also in Malpighian tubules as well as salivary glands (Ningshen *et al.*, 2013). Further, upon feeding the passage of Cry toxin through the mid-gut barrier and its presence in various visceral tissues in larval hemocoel has also been reported (Pérez-Hedo *et al.*, 2012; Zhao *et al.*, 2016). Present study with feeding of sub-lethal dosages of Cry toxin as well as results obtained with hemocoelic delivery of activated Cry toxin, clearly indicated that the receptors/binding proteins present on the surface membrane of various visceral tissues interacted with Cry toxins and promoted cell damage seen in the mid-gut, fat body, Malpighian tubules and salivary glands.

From this part of the study it is concluded that even sub-lethal dose of Cry has deleterious effects on the development and growth of larvae, where mid-gut and fat body cells showed extensive cell death. Further in mid-gut the cell damage was primarily caused due to necrosis, however the cellular damage in epithelium was associated with the cell proliferation, which promoted simultaneous repair of the tissue and facilitated larval survival upon Cry intoxication in *A. janata* larvae.

Chapter 2

Role of endogenous mid-gut arylphorin in cell proliferation/ tissue regeneration

2.1 Introduction

Haemolymph is a circulatory fluid in arthropods including insects which fills the hemocoelic cavity. It basically composed of plasma and cells commonly known as haemocytes. The plasma has high concentrations of variety of macromolecules including proteins, carbohydrates, lipids as well as free amino acids, fatty acids, organic phosphates etc. The haemocytes are of various types, (Gupta, 2009) and play an important role in immune function, storage, synthesis and secretion of variety of molecules including humoral factors (Hopkins and Krmer, 1992; Zhao and Kanost, 1996; Nappi and Christensen, 2005). During the postembryonic development variety of proteins are synthesized by various tissues and released into the haemolymph, which play varied physiological functions.

Hexamerins, are one such group of proteins which are found to be present in very high concentration in the larval haemolymph (40-50% in the total haemolymph) of holometabolous insects including Lepidopteran insects. Their molecular mass ranges around 500 kDa in native form (Telfer and Kunkel, 1991). They consist of six subunits, which are either homo or heteromer. The molecular mass of subunit ranges between 70-86 kDa. Hexamerins are synthesized predominantly by the fat body and released in to larval haemolymph (Telfer and Kunkel, 1991; Burmester, 1999) during active feeding stage. During the non-feeding/ pre-pual stage they get sequestered back through a receptor mediated endocytosis and get accumulated as dense protein granules in the fat body (Bean and Silhacek, 1989; Chrysanthis *et al.*, 1994; Haunerland, 1996; Burmester and Scheller, 1999). Hexamerins share the common features with hemocyanin in term of sequence and structural similarity (Beintema *et al.*, 1994; Burmester and Schellen, 1996; Burmester, 2015), which serve as respiratory proteins in various arthropods as oxygen transport proteins. On the contrary, hexamerins do not bind to oxygen and are known to serve as storage proteins (Burmester, 1999). Although the hexamerins are primarily synthesized during larval/ nymphal stages but their expression has also been reported during

pre-pupal and pupal stages (Martins *et al.*, 2008), and in adults (Martins *et al.*, 2010). However, it needs to be confirmed whether or not they are same genes which get expressed at various developmental stages.

Based on the function and amino acid composition, Lepidopteran larval hexamerins are classified into three or four major categories. Arylphorin, which are rich in aromatic amino acids (tyrosine and phenylalanine) and utilized in cuticle synthesis during larval/pupal/adult molting as well as play variety of other functions (Pan and Telfer, 1996; KiranKumar *et al.*, 1997; Burmester *et al.*, 1998; Nagamanju *et al.*, 2003; Hagner-Holler *et al.*, 2007). They also play role in diapause (Sula *et al.*, 1995; Kludkiewicz *et al.*, 1996; Godlewski *et al.*, 2001; Lewis *et al.*, 2002; Spyliotopoulos *et al.*, 2007). Methionine rich hexamerins present in high concentration in females and facilitate egg production (Wheeler *et al.*, 2000; Pan and Telfer, 2001). The hexamerins, which are neither rich in methionine nor in aromatic amino acids, are sometimes referred as moderately methionine rich hexamerins (Zheng *et al.*, 2000; Spyliotopoulos *et al.*, 2007; Gkouvitsas and Kourti, 2009). A group of hexameins bind to small metabolic molecule riboflavin and are called as riboflavin binding hexamerins (Magee *et al.*, 1994; Rao *et al.*, 2016).

Excising literature survey clearly shows that arylphorins are versatile, multifunctional molecules which help in transport of hormones such as ecdysteroids (20HE) (Enderle *et al.*, 1983; Manohar *et al.*, 2010) and juvenile hormone (JH) (Braun and Wyatt, 1996; Gilbert *et al.*, 2000; Tawfik *et al.*, 2006), and are involved in immune function (Phipps *et al.*, 1994; Beresford *et al.*, 1997; Ma *et al.*, 2005; Poopathi *et al.*, 2014). In last decade or so arylphorins have been shown to promote/stimulate stem cell proliferation *in vitro* (Blackburn *et al.*, 2004; Hakim *et al.*, 2007). However, the exact mechanism by which they facilitate cell proliferation has not been analysed yet.

In this chapter an effort was made to clone and characterize the various hexamerins from larval tissue primarily fat body and mid-gut of *A. janata* with a view to evaluate their role if any in extensive cell/tissue regeneration and repair in Cry toxin based damage caused by sub-lethal exposure of Cry toxin based formulations.

2.2 Materials and Methods

2.2.1 Primers used in the present study

For cloning various larval hexamerins of *A. janata*, following set of primers were designed based on standard protocol (Chen, and Janes, 2002; Dieffenbach and Dveksler, 2003) and synthesized commercially through Oligoanalyzer tool 3.1 (Integrated DNA Technologies, Inc., USA)

S. No.	Primer Name	Primer Sequence (5' – 3')
Degenerate and specific primers for obtaining partial fragment		
1	Aj.DARYL.F	GTC HAG ATH AAT GAC GTG SAA GT
2	Aj.DARYL.R	CTB TGR CBT GGT AGA CYC CTT GG
3	Aj.DMRH.F	GGC ATG TTY STG TAT GCYCT
4	Aj.DMRH.R	CGGATCTTKGGCCARTARCC
5	Aj.DRBX.F	CAR MTN BTN RCH CGY TAY TAY
6	Aj.DRBX R	CAG ATG ATG ATT CCA TAC G
Cloning complete cds		
7	ARYL FF	ATG CCC AAA TAC CAT GAG TTC TCC A
8	ARYL FR	TTA TGA GTG TAG TAT TCG GGA GTG TTC AG
qRT- PCR		
9	ARYL. FRT	GTT AGA GAA GGG CCA GGT GTC TCA TG
10	ARYL. RRT	CTC TGG CTT GGT AGA CTC CTT GGT
11	MRH.FRT	GAC TGG CTG AAG GGA GTC CG
12	MRH.RRT	GCG TAC ATC ATA ACC TCA CCA CGA CG
13	RBX.FRT	CAG GGT TAC GTT GTTAACCACTTAGGC
14	RBX.RRT	GCC GTT GTA GTA CTG ATG TTG ATG CG
Internal control		
15	rS7.FRT	ACG TGG ACG GTT CAC AAC TCA TCA
16	rS7.RRT	TTC GCG GCC TGT TAG CTT CTT GTA

Table 2 Primers used in the cloning, identification, and relative expression analysis of different larval hexamerins. Arylphorin (ARYL), Methionine rich hexamerin (MRH) and Riboflavin binding protein (RBX) of *A. janata*. rS7 gene was used as internal control for expression analysis studies as it was found to be most stable gene for Lepidopteran model (VenkataRao, 2014)

2.2.2 Collection of haemolymph

The prolegs of larvae were cut and bled directly into Eppendorf tubes which was prerinsed with 0.1% of phenylthiourea in order to prevent tyrosinase activity and melanisation of haemolymph. All the haemolymph samples thus obtained were centrifuged at 4000 x g for 2 min at 4℃ to sediment the haemocytes. Peptide/ protein profiling of collected haemolmph at various time interval from both control and toxin exposed larvae was carried by SDS-PAGE (detailed protocol in General Materials and Methods Section).

2.2.3 Protein sequestration analysis

Biolabelling of hexamerin was carried out using [^{35}S]-methionine produced from BRIT, Mumbai. 4th instar *A. janata* larvae were injected with isotope (10 µCi/5 µl) through hemocoelic delivery using Hamilton microsyinge. They were transferred to petriplate with fresh castor leaves and the haemolypmph was collected 14 h after injection. Bio-labelled haemolymph proteins, which were synthesized by the fat body and released into haemolymph were purified through gel-permeation chromatography using Sephadex G-100 (Sigma-Aldrich). 50 µg of purified bio-labelled hexamerins were injected in the hemocoelic cavity of 3rd instar larvae and they were exposed to sub-lethal dose of Cry toxin formulation. Appropriate controls were also maintained. The mid-gut from larvae (n=10) were dissected every 12 h of toxin exposure until 72 h. The gut was homogenized in 1 X PBS (pH. 8.0) and equal quantity of protein (10 µg) was then electrophoresed using 10% SDS-PAGE. Gel was exposed to storage phosphor screen (GE Healthcare, USA) and image was developed using Typhoon image scanner (GE Healthcare, USA).

2.2.4 Arylphorin cRNA probe preparation

A. janata arylphorin gene fragment (160 bp) was positioned as forward locked in pGEM-T Easy Vector (Promega Corporation, USA). The vector was linearized with EcoRI and NdeI restriction enzymes (New England Biolabs®, UK). Sp6 promoter was used for the

synthesis of antisense probe (positive), while T7 promoter was used for the synthesis of sense probe (negative). Further, RNA probe synthesis was carried out using DIG RNA labelling kit using manufactures protocol (Roche Diagnostics, Germany).

2.2.5 *In situ* localization of arylphorin mRNA

Larval mid-gut and fat body were dissected out and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, USA) in 0.1 M PBS (pH 7.4) at 4°C overnight. Post-fixation, tissues were embedded in OCT compound medium (Leica Microsystems, Germany). Tissue sections (5 µm) were obtained using cryostat microtome (Leica CM1850, Leica Microsystems, Germany) and mounted on poly-L-lysine coated slides. The OCT compound was washed using PBST (PBS pH 7.4 containing 0.1% Tween 20) buffer and the sections were permeabilized using proteinase K (1 µg/ml). Once again 4% PFA was used for further tissue fixation and incubated with 200 µl hybridization buffer (50% formamide, 5X SSC, 5 mM EDTA, 0.1% CHAPS, 1% SDS, 50 µg/ml yeast tRNA, 50 µg/ml and heparin) for 1 h at 50°C. A digoxigenin labelled cRNA probe was mixed with 200 µl of hybridization buffer, heat denatured at 80°C for 5 min, draped and covered with parafilm and incubated at 50°C in a sterile incubator overnight. Post-hybridization, the slides were washed four times (5 min each) at 50°C with a wash buffer (SSC, 50% formamide, 0.1% Tween 20) in a series of decreasing SSC concentration. The slides were processed using solution A (0.5 M NaCl, 5 mM EDTA, 10 mM Tris-HCl pH 8.0, containing 0.1% Tween 20,) and incubated at RT for 5 min. This was followed with RNase A (20 µg/ml) incubation for 20 min at RT, finally the slides were washed twice (5 min each) with solution A (0.5 M NaCl, 5 mM EDTA, 10 mM Tris-HCl pH 8.0, containing 0.1% Tween 20).

For the antibody reaction, the slides were initially washed with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 5 min at RT followed by blocking with 10% BSA for 1.5 h at RT. Anti-digoxigenin-ALP (Roche Diagnostics, Germany) was used

for the detection of bound digoxigenin probe (1: 2000). The incubation carried at 4ºC for overnight. These slides were then washed 4 times (15 min each) with DIG wash buffer (Roche Diagnostics, Germany) and incubated with 100 µl BCIP-NBT solution (G-Bioscience, USA) for 10 h at RT to develop the colour. After colour development, the slides were overlaid with detection buffer (Roche Diagnostics, Germany), counterstained with nuclear red (Vector Laboratories Inc., USA) and incubated in TE buffer (Ambion, Life Technologies, USA) for 5 min. Finally after washing with PBST buffer (PBS containing 1% Tween 20), the slides were dehydrated using ascending series of ethanol, cleared in xylene, mounted using DPX mountant and visualized under Olympus IX-81 microscope.

2.2.6 Arylphorin mRNA expression analysis

Total RNA was isolated from the required tissue (100 mg) using TRIzol method (Ambion, Life Technologies, USA). The quantification of the RNA was carried out using NanoDrop spectrophotometer (ND-2000; NanoDrop Technologies, USA). Quality and integrity of RNA was assessed by formaldehyde denaturing gel electrophoresis. cDNA was synthesized using Revert Aid First Stand cDNA synthesis kit (Thermo Scientific, USA). For quantitative PCR analysis, custom-made TaqMan gene expression assay was performed (Applied Biosystems, USA). Arylphorin primers of amplicon size 160 bp (KX462992) were used. Insect rS7 gene of amplicon size of 115 bp (KF984201) was used as internal control. The real-time expression analysis was performed in triplicates. From the exponential phase of PCR amplification Cycle threshold (Ct) values were obtained. Arylphorin expression was normalized against the expression of rS7 rRNA to generate a ΔCt value (Ct of arylphorin– Ct of rS7 rRNA). Comparative Ct method was used to calculate the change in the gene expression (Livak and Schmittgen, 2001).

2.3 Results

2.3.1 Cloning of partial cDNA fragment and confirmation of their identity as hexamerins

Partial fragment for all three hexamerins Arylphorin (ARYL), Methionine rich hehamerin (MRH) and Riboflavin binding protein (RBX) were cloned using degenerate primers based strategy. Based on the sequence similarity obtained with other Lepidopteran insects, they were further cloned using gene specific primers (Table 2) and confirmed by Sanger sequencing. In the present study the partial fragments obtained for Arylprorin was 556 bp, Methionine rich hexamarin was 470 bp and Riboflavin binding hexamerin was 736 bp (Fig19. A, B, C), and their sequence are represented in figure 20. Further sequences were analysed and confirmed through BLAST homology search analysis (BLASTn; https://blast.ncbi.nlm.nih.gov).

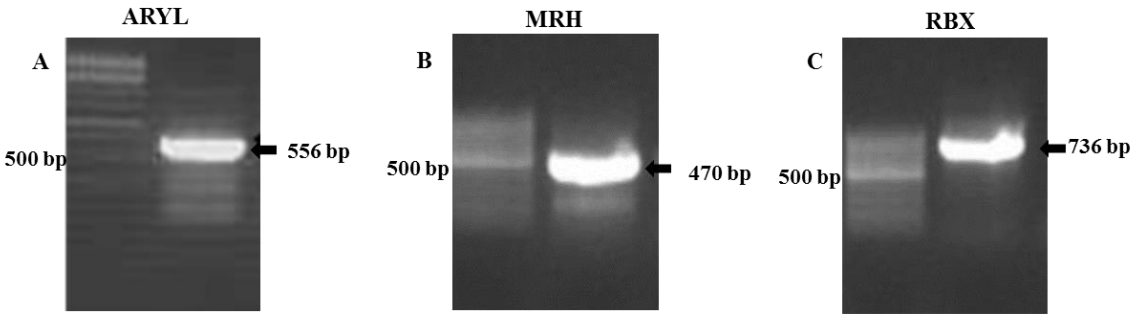


Fig. 19 Amplicon of hexamerins obtained using gene specific primers. (A) Arylphorin (ARYL-556 bp), (B) Methionine rich (MRH-470 bp) and (C) Riboflavin binding (RBX-776 bp).

i) Arylphorin partial cds

CGTCAAGATCAACGACGTGAAAGTCGATGAGCTTGTCACTTACTTCGAATATTATGACTTCAATATTTCAAA
CAGCCTAATGTTCACCAAGAATGTAAAACAGCAACAGCCCGAAAGTTTCGTCATCCGCCAACCCCGTTTGAC
CCACAAGCCGTTCACAGTCAACATCAACCTCAAATCCGATGTAACTTCCCAGGCTGTCTTCAAGATCTTCCT
TGGACCTAAATATGACAACTACGGATATCCTCTGGACCTTGAAAAGGACTGGATGAAGTTCTACGAACTGGA
TTGGTTCGTTCAAAAAATAACTCCTGGTGAAAACAAGGTGGAACGCAAATCTAGTGACTTCATGTTCTTCAA
GGAGGACAGCATGCCGACCAGTGAAATTTGGAAGTGGTTAGAGAAGGGCCAGGTGTCTCATGACATGTCTGT
TGTACCTGACAGCATGCCCAACAGACTGATGCTGCCTAAAGGTACCCACGGTGGATATCCATTCCAGCTGTT
CGTGATGGTATACCCGTACAACGGTGTCACCAAGGAGTCTACCAAGCCAGAG

ii) Methionine rich hexamerin partial cds

GGCATGTTTCTGTATGCCCTCACCGCTGCCGTGTTCCACAGACCCGACTGCAAGGGCATCATCCTCCCCGCC
CCGTACGAGATCTACCCCTACTTCTTCGTCGACAGTCATGTCATTGAAAAGACGATGATGATGAAGATGACC
AAGGCTAAAGTCGACCCCGTTATCAAGGACTACTTCGGCATCAAAGTGACTGACAAGAACCTAGTTGTGATT
GACTGGCGTAAAGGAGTCCGCAACACGATGGCTAAAGAAGACCGTCTCTCATACTTCACCGAGGATGTAGAC
CTGAACACATATATGTACTACCTTCACATGAACTACCCATACTGGATGACTGACGATATGTACACCGTGAAC
AAGGAGCGTCGTGGTGAGGTTATGATGTACGCTAAGATGCAGATGTTGGCAAGGATGAGGCTGGAGCGTCTG
TCTCACGAGATGTGCGACATAAGCCTATGATTGATCCG

iii) Riboflavin binding hexamrin partial cds

CTAATGGCTTTGGTGAAATTCCCGTACTAGGCCAAGATTTTGTAAAGCACGGTTACTCTAGTGGACTTTGTA
CCACAAAGGCATTCCGTTCCCATCCAGGCCTAACAATTACCACCTCGACCAGCCCGAGTTCGTTCAAGCTAT
TACGGCACTCGAAGATTATGAACACCGTATTCGGGAAGCTATTGAACAGGGTTACGTTGTTAACCACTTAGG
CGAACACATCAGCCTGCTGGTTCCCGAAGCTATTGACGTTTTAGGTCGTGTTACTGAGGCTAATGTCGATTC
TCCTAACTGGCACTACTACAAAGACTTTATCACCATTTGGAAACAGCTTTTGGGTAATTCTCAAACTGTTTC
GCATCAACATCAGTACTACAACGGCTTTGCTCCTTTGGTGGTTCCTTCTGTCTTGGAGCACTATCAAACGGC
TCTCCGTGACCCCGCCTTCTACATGATCTGGAAGCGAGTCTTGGGTCTATTCCAGTTGTGGCAAGAGTCACT
ACCTCATTACAAGCGTGAAGAACTGGCTCTCCCTCAAGTGGCTATCCAGAAAGTTGAGGTTGACAAACTAGT
CACCTATTTCGAACATTCATACGTTAACGTTACCAACCATCTTTGGACCCATATGGGCGAAGGTGTGCAGGA
GAAGGTGAGCGTCTTAGTCCAATGGCCGAGGTTGAACCACACAAGTGTTCAAGGTCCGCGTGAACGTCAAGA
GCGCAGTGCGAGACAG

Fig. 20 Partial sequences of hexamerins cloned using gene specific primers (i) Arylphorin amplicon of 556bp, (ii) Methionine rich hexamerin amplicon of 470 bp and (iii) Riboflavin binding hexamerin amplicon of 736 bp.

2.3.2 Expression profile of hexamerin(s)

The expression profiling of all three hexamrins was carried out using Real time primers (Table 2) during the postembryonic larval development from 1st instar to 5th instar. The RNA was isolated from the whole body for 1st and 2nd instar while from 3rd instar onwards it was isolated from the fat body. Results presented in figure 21 revealed that arylphorin expressed from 1st instar, similarly riboflavin binding hexamerin also got expressed from 1st instar, however prominent expression was seen from 3rd instar. However methionine rich hexamerin expression was seen only from 4th instar larval stage.

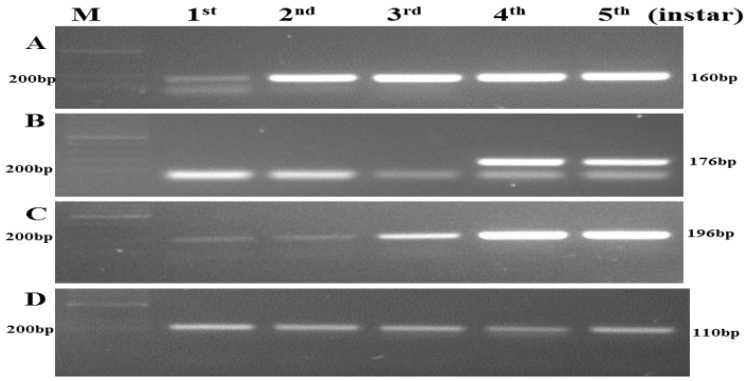


Fig. 21 Expression analysis of hexamerins mRNA during larval development using semi quantitative PCR. Lower expression of aryphorin in 1st and 2nd instar and significant increase observed from 3rd instar onwards (A). Methinone rich hexamerin expression was seen in 4th and 5th instar (B). Riboflavin binding hexamerin expression was observed in 1st instar 5th instar but it was prominent from 3rd instar (C). rS7 was used as internal control (D).

2.3.3 Protein profiling of haemolymph

The protein concentration of haemolymph was determined at various time points in both toxin exposed and normal larvae (7 day old/3rd instar) using Bradford method. Presence of hexamerin in the haemolymph was first confirmed through western blot analysis using Hex2 antibody (Hex2 antibody generated against recombinant arylphorin of *C. cephalonica* which showed 55% protein similarity with *A. janata* arylphorin) (Fig. 22A). An equal amount of haemolymph protein was profiled through 10% SDS-PAGE and the results are presented in figure 22 B and C. A gradual decline in hexamerin protein (70-85 kDa) was noticed in toxin exposed larvae (Fig. 22C). It is interesting to note that in control, at 72 h time period (where insect undergoes 3rd instar to 4th instar molting) there was significant decline in the hexamerin content, while all other protein were present in more or less in the same concentration (Fig. 22B).

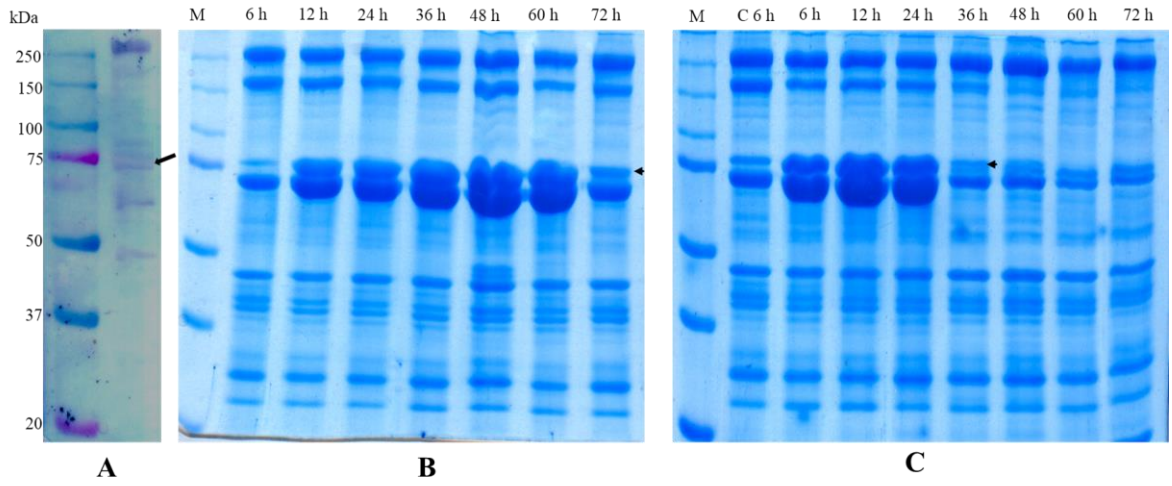


Fig. 22 Haemolymph profile of normal and toxin exposed larvae. (A) Western blot analysis using heterologous Hex2 antibody generated against recombinant araylphorin of *C. cephalonica* (rice moth, stored grain pest) demonstrated it's binding to 70-85 kDa hexamerin proteins from larval haemolymph of *A. janata*. (B) Larval hemolymph protein profile of control insect larvae, (➧) showed the sudden decrease of hexamerin protein during, molting. (C) Larval haemolymph protein profile of toxin exposed larvae, (➧) showed the gradual decrease in the hexamerin protein after 36 h of toxin exposure (➧)

2.3.4 Hexamerin sequestration analysis

Hexamerins are known to be sequestered by various tissue during postembryonic development (KiranKumar *et al.*, 1997; Arif *et al.*, 2008) as well as facilitate cell proliferation. An attempt was made to analyse their sequestration in mid-gut tissue during Cry intoxication and its role if any in promoting cell proliferation seen during tissue regeneration. Bio-labelled larval hexamerins (prepared by injecting [^{35}S] -Methionine) were separated on SDS-PAGE and checked for their integrity (Fig. 23A). The results showed the presence of primarily 80-85 kDa radiolabelled protein in the samples. For uptake studies 50 µg of the radiolabelled protein was injected to 3rd instar larvae of *A. janata* and they were transferred to Cry toxin coated leaves. The insects were exposed for various time periods and the homogenate prepared from mid-gut was analysed for the presence of intact hexamerins (Fig. 23B). Results presented in figure 23B clearly showed that although the intact radiolabelled hexamerin was seen in haemolymph (HL) even after 72 h. None of the mid-gut samples prepared from toxin exposed (12-72 h) showed the intact radiolabelled hexamerin band.

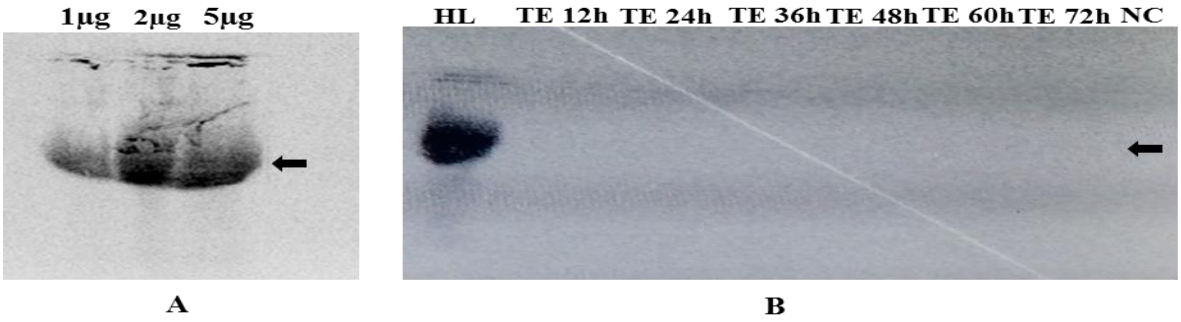


Fig. 23 Autograph to visualize radiolabelled hexamerins. (A) Autoradiograph showed the presence of radiolabeled hexamerins (➡) in the haemolymph of larvae. (B) Autoradiograph showed the presence of the radiolabeled hexamerin in the haemolumph after 72 h injection (➡), while this was not seen in the mid-gut samples prepared from toxin exposed/ fed larvae obtained from various time points (12-72 h).

2.3.5 Expression of arylphorin in mid-gut

Arylphorin gene, is known to be predominantly expressed by the fat body of Lepidopteran insects. qRT- PCR analysis showed detectable but low expression of arylphorin in the larval mid-gut as compared to the fat body (Fig. 24A). For this study 3rd instar larvae were used as the toxin exposure studies were carried out using this stage. Furthermore, *in situ* studies results with arylphorin sense and antisense probes revealed the localization of arylphorin transcript in the gut epithelium primarily in the basal cells (Fig. 24B).

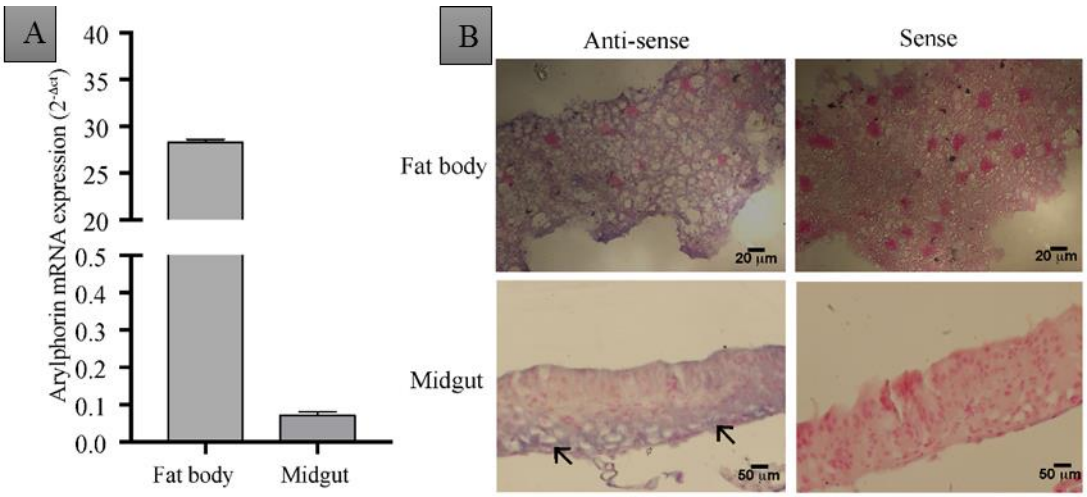


Fig. 24 Analysis of arylphorin gene expression. (A) qPCR analysis showed expression of arylphorin mRNA in the fat body and mid-gut of 3rd instar larvae. (B) *In situ* hybridization demonstrated the synthesis of arylphorin mRNA in the fat body and mid-gut epithelial cells (→) which were primarily localized towards the basal border (Scale bars 20 µm, 50 µm).

2.3.6 Differential expression of arylphorin gene in Cry toxin exposed larval mid-gut

The levels of mid-gut arylphorin transcripts in control and toxin exposed larvae are depicted in figure 25 showed, in control insects, arylphorin transcript level was fairly low throughout the time points monitored, but a steep incline was observed at 72 h (Fig. 25). On the other hand, arylphorin expression in mid-gut gradually increased from 6 h and reached maximum at 36 h in Cry intoxicated larvae, thereafter the mRNA levels declined significantly in 48 h and remained low till 72 h (Fig. 25).

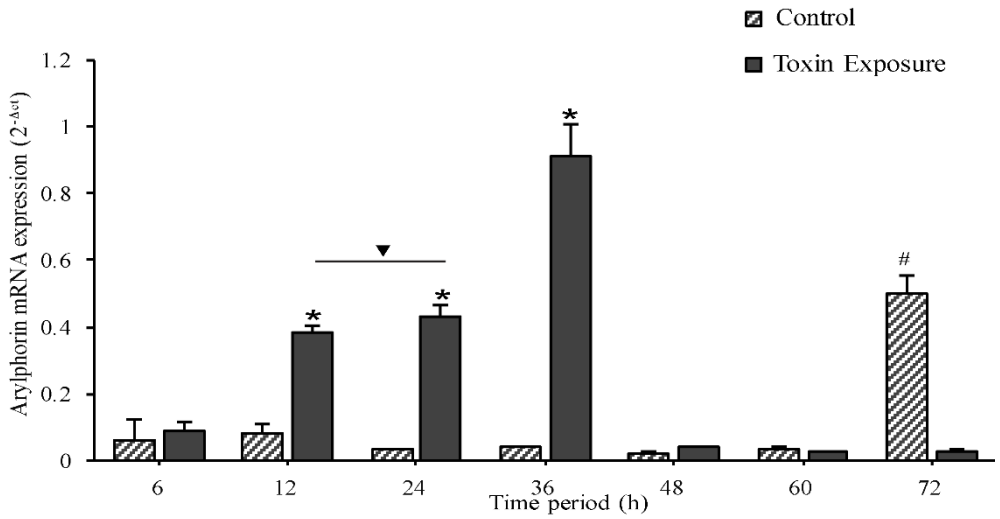


Fig. 25 Expression of mid-gut arylphorin mRNA during toxin exposure: A gradual increase was observed from 6 h to 36 h upon Cry intoxication, which declined later to a basal level. Note the higher expression of arylphorin transcript in 72 h control larvae, a period which coincided with the molting (from 3rd to 4th larval instar). *p < 0.005 significance between experimental groups, #p < 0.005 significant among control groups, ▼ the values are non-significant.

2.4 Discussion

Large number of growth factors and other molecules promoting mid-gut cell proliferation in culture have been reported. They include fetal bovine serum, ecdysteroids (Loeb *et al.*, 2010), mammalian growth-promoting factor (Nishino *et al.*, 1995), alpha-arylphorin, MDFs (Mid-gut differentiation factor) (Blackburn *et al.*, 2004; Hakim *et al.*, 2010) and AlbuMAX II (Castagnola *et al.*, 2011). Hexamerin proteins, particularly exogenous arylphorin synthesised by fat body was shown to promote proliferation of mid-gut cells in culture (Hakim *et al.*, 2007). However, till date there is no direct *in vivo* study which demonstrated the movement of haemolymph hexamerin to mid-gut, and their role in cell proliferation of mid-gut. In the present study haemolymph proteins isolated from both control and toxin exposed larvae were profiled using SDS-PAGE and showed the gradual decline in hexamerin proteins after 36 h of toxin exposure. In addition in control insects a drastic decline at 72 h was noted and at this stage insect larvae prepare themselves to molt from 3rd instar to 4th instar, which was shown to associated with active cell proliferation in *Manduca sexta* (Baldwin and Hakim, 1991). As intact hexamerins were shown to be sequestered by various tissue during postembryonic development (Arif *et al.*, 2008), and play variety of roles. To confirm whether or not the hexamarins were directly involved in mid-gut regeneration. Hexamerins sequestration studies were carried out and results clearly indicated that there was no sequestration of hexamrin protein in toxin exposed gut samples. Furthermore extensive *in vivo* as well as *in vitro* studies earlier demonstrated uptake of hexamerins (sequestration) during non-feeding stage, prepupal stages by the fat body, male accessary gland (MARG), ovary and salivary glands but not in the gut of other Lepidopteran larvae *Chilo partellus* and *Corcyra cephalonica* (Isamail *et al.*, 1993 ; Arif *et al.*, 2008).

Interestingly detailed analysis of transcriptome (unpublished data) generated from the larval mid-gut of *A. janata*, also showed not only the presence but also up regulation of

arylphorin transcript upon Cry toxin intoxication. The same was not seen for the transcripts of methionine rich and riboflavin binding hexamerins. In addition reports from the Xentari™ resistant *Spodoptera exigua* larvae mid-gut transcriptome also reported up-regulation of arylphorin transcript. (Hernández-Martínez *et al.*, 2010). Further, in the present study *in situ* finding provided a direct evidence for the localization of arylphorin transcript in the actively dividing cells of mid-gut, which were primarily localized in the basal region of the epithelium. Further expression analysis using Cry intoxicated larval mid-gut revealed time dependent increase in endogenous arylphorin gene expression which was associated with active cell proliferation, and was not only seen upon Cry intoxication but also during the molting in control larvae. All these further suggested that the arylphorin most likely plays a role in cell proliferation during repair of mid-gut epithelium by facilitating cell proliferation.

Given the versatility of arylphorin, and the results obtained in the present study showing its presence and altered expression in the mid-gut during Cry toxin exposure, we suggests that a low concentration of arylphorin produced endogenously in mid-gut is critical and probably has mitogenic effect during the Cry intoxication. Further characterization of the gut regenerative process will help us in better understanding of the mechanism of the role of this molecule and its probable function in development of resistance.

Chapter 3

Characterization of major receptor aminopeptidase N(s) in larval mid-gut and its expression profile during sub-lethal Cry toxin toxicity

3.1 Introduction

Among various classes of proteases present in animals including insects, aminopeptidase N (APNs) are primarily metallo aminopetidase and have broad specificity. They are known to sequentially/ preferentially cleave the neutral amino acids from the N-terminal end of a peptides/ protein (Terra and Ferreira, 1994). Some of these have been shown to mediate Cry toxin induced toxicity in various insect orders including Lepidoptera (Bravo *et al.*, 2007; Zhao *et al.*, 2016).

Different Cry toxin produced by *Bacillus thuringiensis (Bt)* are known to be target specific for their insecticidal activity. This host specificity was basically due to the presence of different Cry interacting/binding proteins present in the apical brush border membrane of the mid-gut cells of various insects. Existing reports from different insects showed that cadherin like proteins bind to the Cry toxin (Cry1A) in *Manduca sexta* (Vadlamudi *et al.*, 1993), *Bombyx mori* (Nagamatsu *et al.*,1998), *Heliothis virescens* (Gahan *et al.*, 2001), *Helicoverpa armigera* (Xu *et al.*, 2005). In *Anopheles gambiae*, cadherins interacted with Cry4Ba toxins (Hua *et al.*, 2008). GPI-anchored aminopeptidases were recognized as Cry toxin receptor in many Lepidopteran species like *Manduca sexta* (Knight *et al.*, 1995), *Spodoptera litura* (Agrawal *et al.*, 2002), *Heliothis virescens* (Banks *et al.*, 2003), *Helicoverpa armigera* (Rajagopal *et al.*, 2003), *Bombyx mori* (Yaoi *et al.*, 1999), *Lymantria dispar* (Garner *et al.*, 1999), *Plutella xylostella* (Nakanishi *et al.*, 2002), *Achaea janata* (Budatha *et al.*, 2007) and in Dipteran species *Anopheles quadrimaculatus* (Abdullah *et al.*, 2006). GPI-anchored alkaline phosphatase also was shown to interact with Cry toxin in *Manduca sexta* (McNall and Adang, 2003) and *Heliothis virescens* (Jurat-Fuentes and Adang, 2004).

Two distinct and contrasting models have been proposed for the mode of Cry toxin action based on various studies carried out in Lepidopteran insects (Sobern *et al.*, 2009). The pore formation model is a multistep (Bravo *et al.*, 2004) and well accepted model, where series

of events like toxin solubilization, its activation, proteolytic cleavage, binding of toxin with different receptor molecules, oligomerization, and membrane insertion leading to pore formation and epithelial cell damage. However the basic mechanisms by which Cry toxin caused their toxicity is not only poorly understood but also controversial (Knowles, 1994; Pietrantonio et *al.*, 1996; Rajamohan *et al.*, 1998; Schnepf *et al.*, 1998; Soberan *et al.*, 2009).

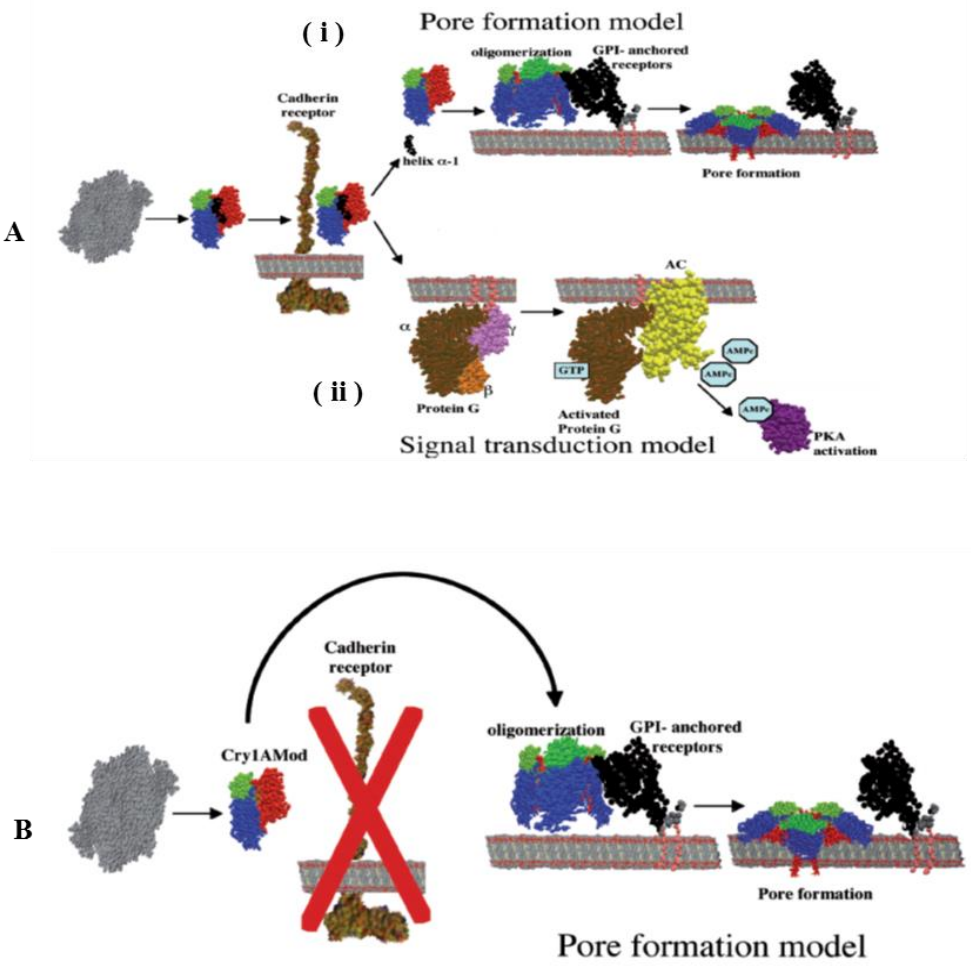


Fig. 26 Mechanism of Cry toxins action. (A) Schematic representation showing (i) Pore formation model where interaction of Cry toxins with cadherin like protein causes cleavage of helix α-1 and its oligomerization and its interaction with the GPI-anchored receptor proteins which facilitate pore formation. (ii) Signal transduction model shows that interaction of the Cry toxin to the cadherin causes the activation of G-protein dependant Protein kinase A (PKA) cellular pathway results in cell death. (B) Pore formation model where interaction of Cry toxin to the cadherin receptor and removal of Cry helix α-1 is not required for toxin oligomerization. (Image adopted from Soberan *et al.*, 2009).

Aminopeptidase N (EC.3.4.11.2) has been identified as Cry toxin receptor in various Lepidopteran insects. These are a class of peptides belonging to Zn^{++} dependent gluzincins family of M1 type metalloproteases (Hooper, 1994; Albiston *et al.*, 2004). They showed the presence of aminopeptidase motif "GAMENWG", Zn^{++} binding motif "$HEXXHX_{18}E$", N-terminal with signal peptides, C- terminal with GPI-anchor signal which helped them in attaching with BBMVs (brush border membrane vesicles) in mid-gut (Fig. 27). *In silico* analysis revealed presence of many O and N- glycosylation sites which was predicted to facilitate Cry toxin binding (Pigott and Ellar, 2007). In Lepidoptera larval mid-gut, enzymes play a vital role in dietary nutrient metabolism which include proteases both luminal and BBMV forms (Terra and Ferreria, 1994; Naseri *et al.*, 2010; Sarate *et al.*, 2012)

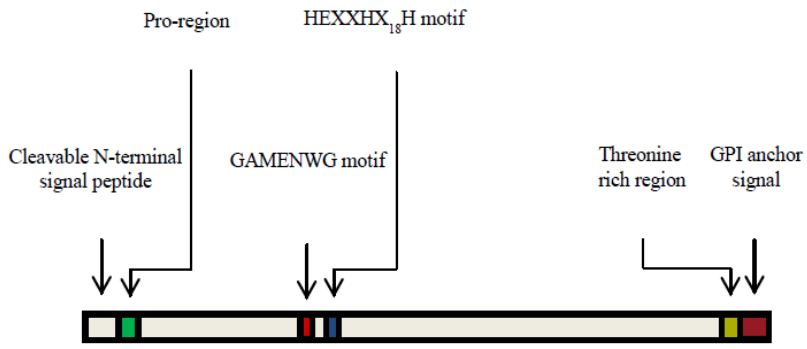


Fig. 27 Schematic representation of Lepidopteran aminopeptidase N.

Further the APNs are classified into eight clusters (APN1-8) based on amino acid identity/similarity in Lepidopteran insects (Crava *et al.*, 2010; Hughes, 2014; Lin, 2014). It was shown that many forms (isoforms) of APN were expressed simultaneously in larval mid-gut of *Helicoverpa armigera* (Angelucci *et al.*, 2008), *Ostrinia nubilalis* (Crava *et al.*, 2010, 2013; Khajuria *et al.*, 2011), *Epiphyas postvittana* (Simpson *et al.*, 2008), *Bombyx mori* (Crava *et al.*, 2010; Nakanishi *et al.*, 2002) and *Trichoplusia ni* (Tiewsiri and Wang, 2011; Wang *et al.*, 2005). All the isoforms found be organized in a single cluster in Lepidopteran genome (Baxter

et al., 2008; Crava *et al.*, 2010; d'Alençon *et al.*, 2010). However, it is still uncertain and not clear as to which isoforms act as Cry toxin receptor.

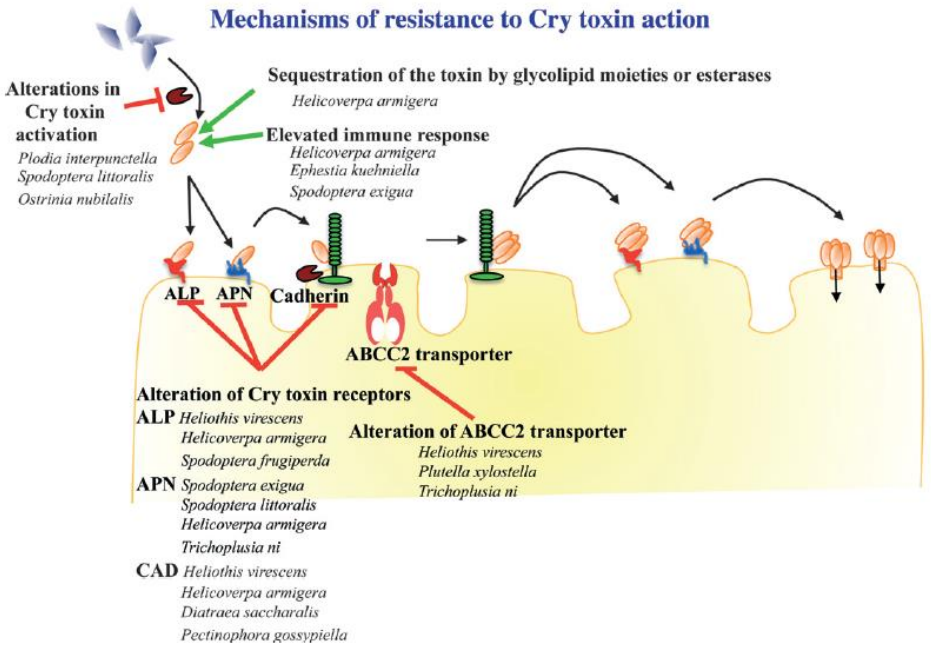


Fig. 28 Schamatic respresentation mechanism(s) of development of resistance. Figure shows possible menchanisms of resistance to Cry toxin which could be due to altration in Cry toxin processing and alteration in Cry toxin binding/ receptors proteins in various Lepidopteran insects (Image adopted from Pardo-Lopez *et al.*, 2012).

Literature survey clearly showed that resistance developed against *Bt*/Cry formulations in selected laboratory populations was primarily due to various mechanisms (Fig. 28). In certain insects it was due to the alteration of in Cry toxin activation process by gut proteases (Keller *et al.*, 1996; Oppert *et al.*, 1997; Li *et al.*, 2004). Further reports suggested that alteration/modification in proteases which acted as toxin receptor, facilitated the development of resistance (Pardo-Lopez *et al.*, 2012). Sequestration of toxin by glycolipids moieties (Ma *et al.*, 2005) and an elevated immune response (Hernandez-Martinez *et al.*, 2010), were also shown to be additional mechanisms for the development of resistance.

Recent studies highlighted that in various insects, toxin resistance was associated with reduced and differntial expression of various APN isoforms (Tiewsiri and Wang, 2011). Reduced interaction of Cry toxin, which was associated with mutation in receptor proteins (Ferre and Van Rie , 2002; Heckel *et al.*, 2007; Zhang *et al.*, 2008; Adang *et al.*, 2014; Guo *et al.*, 2015) and mutation in other molecules like ABCC2 (ATP-binding cassette transportor subfamily C2) (Gahan *et al.*, 2010; Baxter *et al.*, 2011) was also suggested as probable mechanism for resistance in various insect models. They have been characterized as functional Cry toxin receptor (Tanaka *et al.*, 2013; Zhou *et al.*, 2016). Detailed studies with the development of resistance in various insects furthet suggested the Mod1 mechanism, which was primarily due to expression of a mutant cadherin protein (mutation in 12^{th} domain) by the mid-gut cells of Lepidopteran pests (Heckel *et al.*, 2007).

In this chapter we tried to made an attempt to characterize aminopetidase N(s) as Cry toxin (DOR *Bt*-1) receptor, identify various isoforms of APNs and their role in Cry toxin toxicity and/or developmet of toxin tolerance, in toxin tolerated insect populations developed using sub-lethal dose of toxin exposure.

3.2 Materials and methods

3.2.1 Primers used in the present study

Following set of primers were used in this study to cloning the full length of various APN isoforms and also profiling of their expression in toxin exposed larvae of *A. janata*. (Detailed protocol for primer designing explained in General materials and Method Section)

S. No.	Primer Name	Primer Sequence (5' – 3')
Gene specific primers for obtaining full length (ORF)		
1	APN1.FFP	ATG GCG TTG GCA GAA CTG G
2	APN1.FRP	TTA TAC AAT AAC ATTA ACC AAA GC
3	APN2.FFP	ATG TAT TTA CTA ACA CTA TTA GCT
4	APN2.FRP	TAA GAT GGC AAC AAG TGC AG
5	APN4.FFP	ATG CAG CTC CTT ATC ATT CTC
6	APN4.FRP	TTA ACG TAG TAA ATG GGT GAA C
7	APN6.FFP	ATG TCC AAA ATA CTT ATC GCA G
8	APN6.FRP	TAA ACA AAC ATG GAT AGG AGA G
9	APN9. FFP	ATG GCT AGC AAA TTT CAA CCA ACA T
10	APN9. FRP	CTA GGC CCT ATG GTT TGC C
qRT- PCR		
11	APN1.FRT	CAG ACG TCC ACT CTC CTA CTC TAG T
11	APN1.RRT	GCG ATA ACT TCA TGC AGA GCG TTA CC
12	APN2.FRT	CGC TCC ACC ACA GTA CAA TGT TGT C
13	APN2.RRT	CTC TGA AGG GAA TGT TGA AGA TGG GA
14	APN4.FRT	CAC CCG TGA CAT CAA TCT TAG CCC T
15	APN4.RRT	CGT GGG TAA GAT CTT CAG CGA TCG
16	APN6.FRT	CTA TCG AAA CCT GCC GGT TAC ATA GGA
17	APN6. RRT	CGT GGG TAA GAT CTT CAG CGA TCG
18	APN9.FRT	CTG TGG AAA CCT GTT ACA TAT GTG GCT C
19	APN9.RRT	GCT TCC AAC TTC ACC AGA GTC TAT TTG
Internal Control		
20	rS7.FRT	ACG TGG ACG GTT CAC AAC TCA TCA
21	rS7.RRT	TTC GCG GCC TGT TAG CTT CTT GTA

Table 3 List of primers used for cloning and relative expression analysis of different aminopeptidase N isoforms in larval mid-gut of *A. janata.*

3.2.2 Preparation of membrane fraction

i) Tissue isolation: The 3rd instar larvae were narcotized by placing them on ice for 15-20 min. The larval cuticle was incised from the ventral side of abdomen and cut open through the length of the larvae. Mid-gut, fat body, salivary gland and Malpighian tubules were dissected out in ice cold insect Ringer solution (130 mM NaCl, 0.5 mM KCl and 0.1 mM $CaCl_2$). Mid-gut was cleared of food contents and peritrophic membrane prior to usage.

ii) Preparation of mid-gut brush border membrane vesicles factions (BBMVs): This was done by using the protocol of Wolfersberger *et al.*, (1987) with slight modifications. Dissected gut tissue was transferred to centrifuge tube containing ice-cold MET buffer (300 mM mannitol, 17 mM Tris-HCl pH 7.5, 5 mM EGTA and 1 mM PMSF), vigorously vortexed and briefly centrifuged for 5 min at 1000 X g to obtained plant debris free clean tissue. Mid-gut tissue was homogenized on ice in a tightly fitting glass homogenizer in ice-cold MET buffer (10% w/v). The homogenate was diluted with an equal volume of ice-cold 24 mM $MgCl_2$, blended and held on ice for 15 min before centrifugation at 2500 X g for 15 min at 4℃. The supernatant was further centrifuged at 30,000 X g for 1 h at 4℃. The resulting pellet, which contained BBMV fraction was preparation was re-suspended in HBS buffer (10 mM HEPES, pH 7.4, and 150 mM NaCl), flash-frozen in liquid nitrogen and stored at -80^{o}C until use.

iii) Preparation of fat body membrane fractions: The fat body tissue was homogenized on ice in buffer A (5 mM HEPES, pH 8.5 and 0.1 mM $CaCl_2$), and membrane fraction was prepared according to the method described by KiranKumar *et al.*, (1997). The fat body homogenate was centrifuged at 30,000 X g for 30 min at 4^{o}C and 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, 2 mM $CaCl_2$, 10% Sucrose(w/v) and 0.1% Triton X-100). This particular fraction was used as membrane fraction and stored at -80^{o}C until use.

iv) Preparation of Malpighian tubule and salivary gland protein homogenates: The tissues (100 mg) were homogenized in 200 µl of homogenization buffer (10 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 1mM PMSF, 1mM EDTA and 1mM DTT). The homogenates were centrifuged at 1000 X g for 5 min to remove larger debris and the aliquots of the supernatant were used.

3.2.3 *In vitro* Cry toxin binding (ligand blotting) analysis

The gut BBMV's, fat body membrane, Malpighian tubule and salivary gland protein preparations (10 µg each) were separated by 10% SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was blocked using blocking buffer [5% (w/v) skimmed milk powder in 0.01 M Tris buffer saline (TBS, pH 7.4)] for 1 h, followed by incubation in blocking buffer containing 1 µg/ml of Cry toxin (Cry1Ac) for 1 h at RT. Following this blot was incubated with primary Cry 1Ac antibody (antibody was generated in rabbit) overnight then incubated with secondary ALP conjugated anti-rabbit goat antibody (Thermo Fisher Scientific, USA). The blot was developed using BCIP/NBT substrate (G-Biosciences, USA).

3.2.4 Ligand blot of mid-gut BBMVs

The mid- gut BBMV's (10 µg) were separated by 10% SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was blocked using blocking buffer [5% (w/v) skimmed milk powder in 0.01 M Tris buffer saline (TBS, pH 7.4)] for 1 h, followed by incubation in blocking buffer containing 1 µg/ml of activated DOR *Bt*-1 or Cry1Ac toxin for 1 h RT. Following this blot was incubated with polyclonal primary antibody generated against DOR *Bt*-1 toxin and anti-Cry 1Ac toxin antibody (raised in mice) for overnight. The blot was then incubated with secondary HRP conjugated secondary antibody (Thermo Fisher

Scientific, USA). The blot was developed using Western BLoT chemiluminescences Kit (Takara Bio Inc, Japan).

3.2.5 APN and Cry toxin interaction analysis using immunohistochemical colocalization studies

The mid-gut transvers sections were prepared (according to protocol described in General Materials and Methods Section). The slides were deparafinised using xylene and hydrated through decreasing ethanol series. For immunostaining, section were washed with PBS followed by PBST, then blocked using 10% goat serum (Thermo Fisher Scientific, USA). A solution of 1 µg/ml of Cry toxin (Cry1Ac or DOR *Bt*-1) was added on to the slides and inoculated for 2 h RT. Primary antibody generated against aminopeptidase N (1:500 dilutions; raised in rabbit) and anti-Cry toxin(s) antibody (1:500 dilutions; raised in mice) were added and the slides were incubated overnight at 4℃. The slides were washed with PBS solution to remove unbound primary antibodies then respective secondary antibody (anti rabbit goat IgG - FITC conjugated (Green) and anti-mice IgG- TRITC (Red)) was added to the sections and incubated at RT for 2 h. The excess of antibody was washed with PBST and the slides were mountant using gold antifade mountant (Thermo Fisher Scientific, USA) for prolonged storage. The sections were visualized under fluorescence microscope.

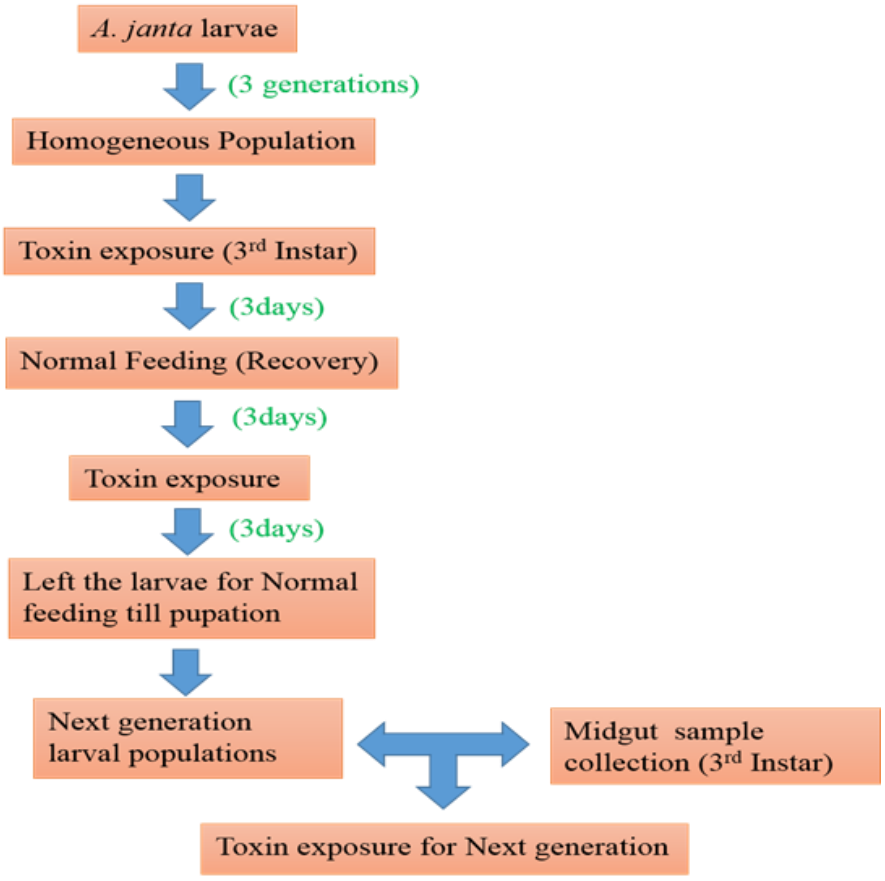
3.2.6 Cloning and characterization of APN isoforms

As the genomic sequence of *A. janta* is not available in database hence an attempt was made to carry out next generation RNA sequencing (*de novo* transcriptome) of larval mid-gut (unpublished data). Based on homology search in NCBI database five different isoforms of APNs were identified. Using standard protocol (mentioned in General materials and Method Section) full length of the APN sequences were cloned using ORF specific primers (Table 3). Detailed *in silico* analysis of these putative APNs was carried using bioinformatics tools. Finally

their expression was analyzed upon either continuous sub-lethal toxin exposure or toxin tolerant insect populations using Real- time primers (Table 3).

3.2.7 Generation of toxin tolerant insect populations

The larvae of *A. janata* were collected from the unexposed *castor* fields of Telangana and Andhra Pradesh which were never exposed to *Bt* formulations and maintained in the laboratory for 3 generations for obtaining homogenous populations. The insects were exposed to sub-lethal dosage of Cry toxin using following strategy (Flow chart 1). The surviving insects were maintained for the next generation. At each generation the required tissue samples were collected and experiments were carried out. Using the above protocol toxin tolerant insects were generated and present study analysis was carried out up to sixth generations. It is being carried out further.



Flow chart 1 Strategy for the development of toxin tolerant insect populations.

3.2.8 *In silico* APN- Cry toxin interaction analysis

Sequence of one of the isoforms of aminopeptidase N i.e APN2 was used for this study. The BLAST program was used for sequence search against known 3D structures available in the Protein Databank (PDB) (http://www.rcsb.org/), in order to select appropriate templates for constructing 3D structure models, using homology modeling methods. The homology model program MODELLER v9.12 was used to generate 3D model of APN. The stereo-chemical quality of the modeled protein structure was checked in Ramachandran plot using PROCHECK. The compatibility of the model with its sequence was measured by Verify-3D graph. 3D coordinates of Cry1Ac protein was downloaded from protein databank (PDB id: 4W8J) and it was used for protein-protein docking studies. Both the proteins were submitted to GRAMM-X protein-protein docking web server. The top ranked result obtained from GRAMM-X web server was identified and further submitted to SPPIDER web server to find out possible interaction between APN and Cry proteins. Binding energy of this interaction was predicted by PRODIIGY. Further 3D structures were viewed, analyzed and figures were generated in PyMOL Molecular Graphics System (1.7).

3.2.9 Two-dimensional electrophoresis

The mid-gut membrane proteins were subjected to clean-up using the 2-D Clean-up Kit (GE Healthcare Life Sciences, USA) as per the manufacturer's instructions. Immobiline Dry strips (4-7 pH 11cm, GE Healthcare, USA) were rehydrated in 350 µl of the protein solution for ~20 h under low viscosity mineral oil (Bangalore Genei, India). The rehydrated IPG strips containing 350 µg proteins were then subjected to isoelectric focusing. After IEF, the strips were equilibrated and second dimensional SDS-PAGE was carried out in duplicate one was for western blotting and a second one for Coomassie brilliant blue R-250 staining.

3.2.10 Two-dimensional electrophoresis and immunoblotting

The mid-gut membrane proteins (BBMVs) separated using 2D SDS-PAGE gel and were electro-transferred to a nitrocellulose membrane. The membrane was blocked using 5% (w/v) skimmed milk powder in 10 mM TBS (pH 7.4) for 1 h. The blot was incubated with primary APN antibody overnight, followed with secondary HRP-conjugated anti-rabbit goat antibody, and developed using Blot chemiluminescenc HRP substrate (Takara Bio.Inc, Japan).

3.2.11 Differential two-dimensional gel electrophoresis (2D-DIGE)

The mid-gut BBMV samples prepared from control and 6th sgeneration toxin tolerant insects, which were exposed to toxin for 24 h. Samples were cleaned up using 2-D Clean-up kit (Amersham Biosciences, USA), the obtained pellet was dissolved in 2-D lysis buffer containing 7 M urea, 2 M thiourea and 4% CHAPS without dithiothreitol (DTT). The protein quantification was done by using Ettan 2-D Quantification kit (Amersham Biosciences, USA). Supplied CyDye (5 nmol) was reconstituted with 99.8% anhydrous dimethylformamide (DMF) from freshly opened source, used as a working solution (400 pmol) for protein labelling. The pH of the protein solution was adjusted to between 8.0-8.5 with 0.5 N NaOH before dye labelling. Minimal labelling of protein samples prepared from control as well as Cry toxin exposed larvae were done with 400 pmol of Cy3 and Cy5 Dyes respectively as per the suppliers instructions. An equal amount of proteins from all the twelve samples (6 control+ 6 treated) were mixed to generate internal standard, which was labelled with Cy2 and equally distributed to all. This helped to generate accurate spot statistics between gels and increased level of confidence. After incubation in the dark for 30 min on ice, the dye quenching was achieved by addition 10 mM L-lysine for 10-20 min in dark. Six biological replicates were used for this experiment, dye swapping was done to minimize the artefacts generated by the dye components.

Each gel consisted of control, treated samples labelled with Cy3 or Cy5 and internal standard labelled with Cy2. Finally, the required volume was made with 2-D rehydration buffer

(40 mM DTT and 0.5% IPG buffer pH 4--7). After rehydration for 12-16 h, IPG strips (pH 4-7, 18 cm) were subjected to isoelectric focusing (IEF) in Ettan IPGPhor 3 system at 36 Volt-hours. Equilibration of IPG strips was done with buffer containing 6 M urea, 30% glycerol, 2% SDS and 50 mM DTT for 20 min followed by alkylation with 100 mM idoacetate (IDA) for 20 min. Finally, IPG strips were equilibrated in SDS-PAGE cathode buffer, before performing the second dimension analysis.

After electrophoresis, gels were scanned at different excitation and emission wavelength for Cy3, Cy5 and Cy2 labelled proteins using Typhoon variable mode scanner (Amersham Biosciences, USA). All the gels were scanned with PMT of 600 and 200 µm/pixel resolution. Images were processed using DeCyder 2-D version 7.0 software through Differential In gel Analysis (DIA) and Biological Variation Analysis (BVA). Finally differentially regulated protein spots were detected with a significant p-value of <0.05 generated by the student t-test and one-way ANOVA.

3.3 Results

3.3.1 Cry toxin interaction with various larval tissues

The profile of proteins/peptides present in tissue homogenates prepared from larval salivary glands (L1) and Malpighian tubules (L2), membrane proteins prepared from fat body (L3) and gut BBMVs (L4) (Fig. 29A). Showed that few of the proteins interact with DOR *Bt* -1 toxin(s) (Fig. 29B). The prominent interaction was seen with proteins of molecular mass of ~110 kDa and ~68 kDa in all the tissues (Lane1- 4) including mid-gut (L4) (Fig. 29B). Thus results obtained indicated that *A. janata* larvae has Cry toxin binding/receptor proteins in almost all the tested tissues.

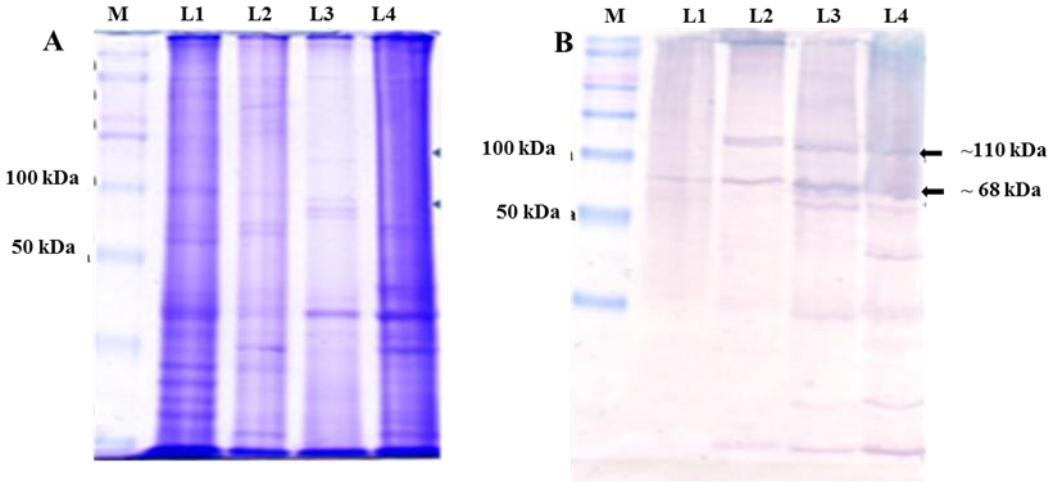


Fig. 29 Cry toxin interaction with larval tissues of *A. janata*. (A) Profiling of membrane and tissues proteins. (B) Ligand blotting with DOR *Bt*-1 toxin(s) and identification of Cry toxin binding proteins/ receptors. Lane M - Protein marker; Lane 1 - Salivary gland protein homogenate; Lane 3 - Malpighian tubule protein homogenate; Lane 3- Fat body membrane fractions and Lane 4 - Gut BBMVs. Note the presence of ~110 kDa and ~68 kDa interacting proteins in all the lanes.

3.3.2 Characterization of aminopeptidase N as mid-gut Cry toxin receptor

SDS-PAGE separated mid-gut BBMV proteins were electrotransfered on nitrocellulose membrane. Ligand binding analysis was carried out using both Cry1Ac and DOR *Bt*-1toxin(s). The results presented in figure 30A clearly showed Cry toxin(s) interaction with 5 to 6 gut membrane proteins. Among them the prominent interaction was seen with 113 kDa protein, with DOR *Bt*-1 toxins (Fig. 30A, L3). Immunoblotting studies using APN antibody generated

against a small fragment of *A. janata* mid-gut APN, revealed that it was an APN (Fig. 30B, L2). Further MALDI-TOF/TOF analysis based on peptide sequence (MS/M), represented that the interacting protein was indeed an aminopeptidase N (APN) (Fig. 31).

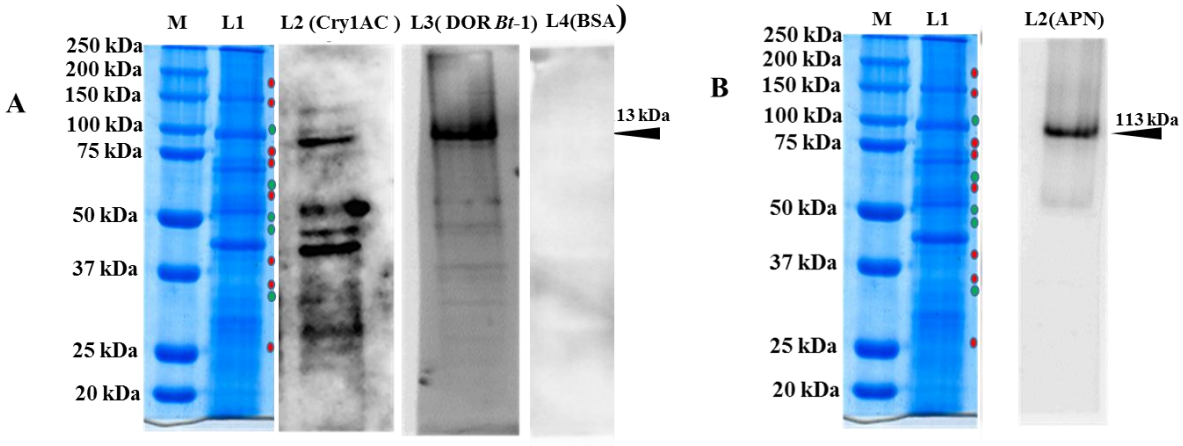


Fig. 30 Identification of Aminopeptidase N as Cry toxin interacting/binding protein. (A) Ligand blot (toxin overlay assay) using active Cry 1Ac (L2) and DOR *Bt*-1(L3) toxins showed the presence of interacting proteins in mid-gut BBMVs. (B) Detection of APN in midgut BBMV's using APN (L2) antibodies generated using recombinant APN protein through immunoblot analysis.

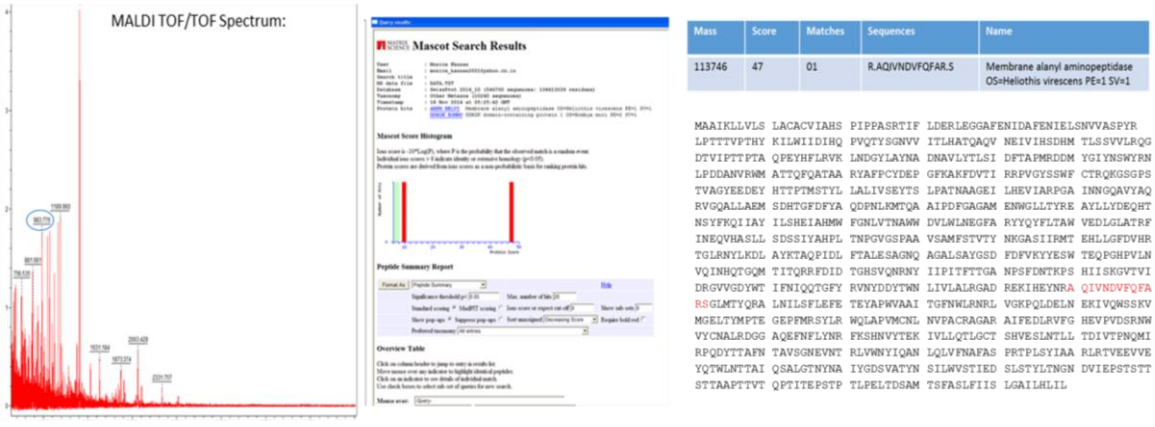


Fig. 31 MALD -TOF/TOF analysis of interacting protein. The peptide peaks obtained analysed through Mascot database search (http://www.matrixscience.com).The peptide (983.776) sequence matched with the reported membrane alanyl aminopeptidase of *Heliothis virescens* with molecular weight of 113 kDa.

3.3.3 APN- Cry toxin interaction analysis through immunohistochemistry

For this interaction analysis the mid-gut cross sections were deparafinized, hydrated and were initially incubated independently with activated DOR *Bt*-1 toxin (test) or Cry1Ac (positive) or BSA (negative) control. Later the sections were incubated simultaneously with Cry toxin antibody (generated in mice) and APN antibody (generated in rabbit). Further interaction were detected using appropriate secondary antibodies, which were tagged with fluorescent dyes. Results obtained showed the presence of overlapping signals in specific regions of mid-gut. i.e inner brush border membrane of epithelial cells (Fig. 32). Further the signal was visible in the Cry1Ac as well as DOR *Bt*-1 toxin but not in BSA incubated sections. This clearly suggested that the interactions were specific and Cry toxins bind to only with the brush border membranes, where APNs were most likely localized.

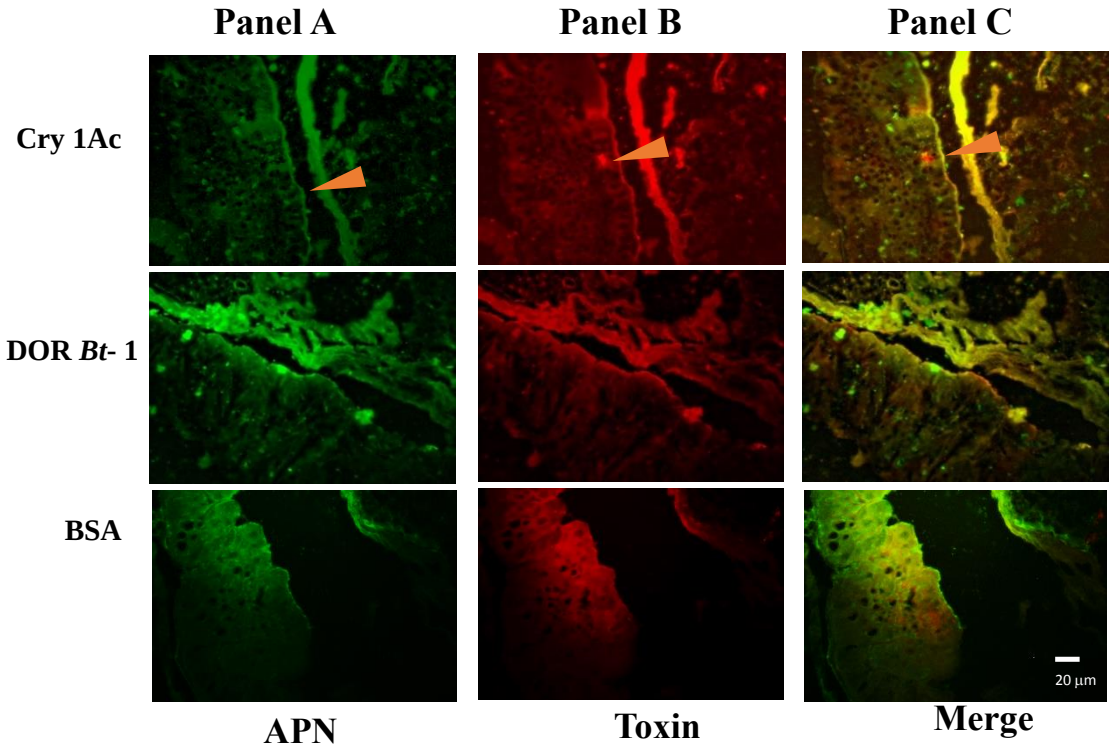


Fig. 32 Aminopeptidase N and Cry toxin interaction analysis. Panel A shows the detection of APN on apical brush border of mid-gut epithelium using APN antibodies. Panel B shows the interaction of Cry toxin with the apical brush border. Panel C shows the colocalization of both APN and Cry interacting proteins. BSA was used as negative control. Please note that merge imaging shows the presence of both the molecules APN and Cry toxin in the same region of the apical membrane of mid-gut ( ). BSA was used as negative control where no signal was detected.

3.3.4 APN isoform analysis

Full length of cDNA for all the five isoforms detected in *A. janata* mid-gut larval trasncriptome were cloned into pTZ57R/T vector (Fig. 33) and Sanger sequenced. The sequences were confirmed and submitted to Gene Bank(https://www.ncbi.nlm.nih.gov/BankIt/) with accession number- APN1 [MF425653], APN 2 [MF425654], APN 4 [DQ872666], APN6 [MF425654] and APN9 [MF425654]. Deduced translated sequences (amino acids) were aligned through Multiple Sequence Alignment (MSA) (http://www.ebi.ac.uk/Tools/msa/) and searched for the presence characteristic aminopeptidase activity motif "GAMENEG" and zinc binding motif "HEXXHX18H" (Fig.34). Similarity between the isoforms was checked using Percent Identity Matrix (Clustal 2.1) and the cloned isoforms did not show more than 37% sequence identity (Fig. 35). Further conserved domain analysis of all the sequences revealed that these proteins belong to "Peptidase M1 Aminopeptidase N" family with Endoplasmic reticulum aminopeptidase 1 (ERAP1) with M1_APN_2 and ERAP1_C domain. Detailed analysis revealed the presence of transmembrane peptide at C-terminal end of APN1, APN2 and APN4 while it was absent in APN6. APN1, APN4 and APN6 possessed signal peptide at N-terminal but it was absent in APN2. Furthermore, APN9 lacked both N and C-terminal signal sequences (Fig. 36).

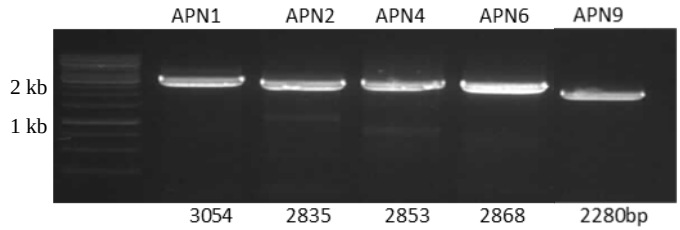


Fig. 33 Agarose gel micrograph showing full length amplicon of APN1 (3054 bp); APN2 (2835 bp); APN4 (2853 bp); APN6 (2868 bp) and APN9 (2280 bp).

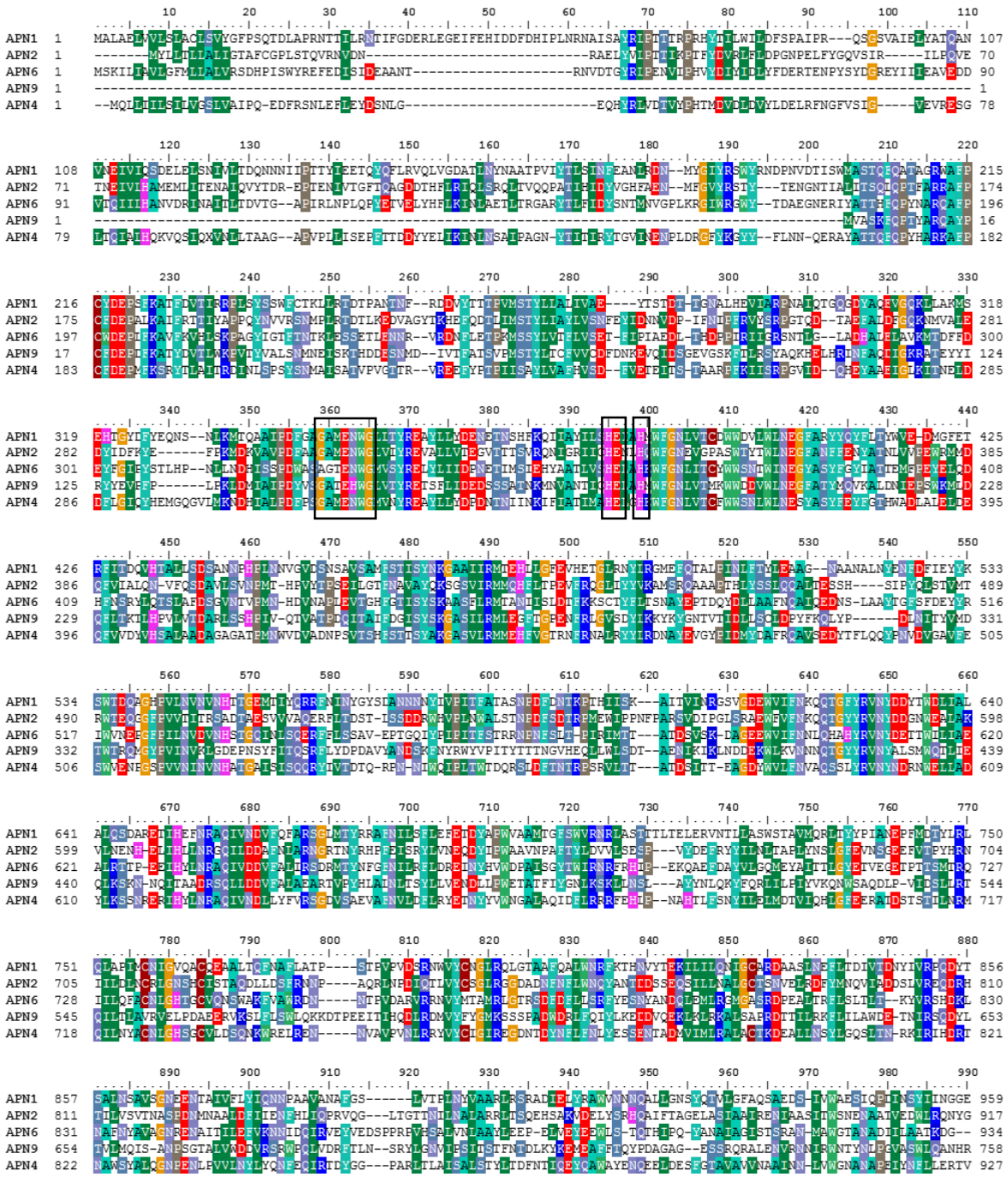


Fig. 34 Multiple sequence alignment of APN's. APN1, APN2, APN3, APN4, APN6 and APN9 from mid-gut of *A. janata* larvae shows the presence of aminopeptidase active motif "GAMENEG" and zinc binding motif "HEXXHX18H". Please note that various isoforms cloned in the present analysis showed sequence identity which was less than 40%.

A

	APN9	APN2	APN1	APN6	APN4
APN9	100.00	27.82	27.50	24.86	26.09
APN2	27.82	100.00	29.15	27.08	29.48
APN1	27.50	29.15	100.00	30.87	31.22
APN6	24.86	27.08	30.87	100.00	37.43
APN4	26.09	29.48	31.22	37.43	100.00

B



Fig. 35 Sequence similarity analysis between different larval mid-gut isoforms of *A. janata*. (A) The results obtained showed the sequence identity match was not more than 37%. (B) Phylogenetic tree showing that APN9 and APN2 fell in one clad, while APN6 and APN4 belonged to same clad, on other hand APN1 fell in a totally different clad.

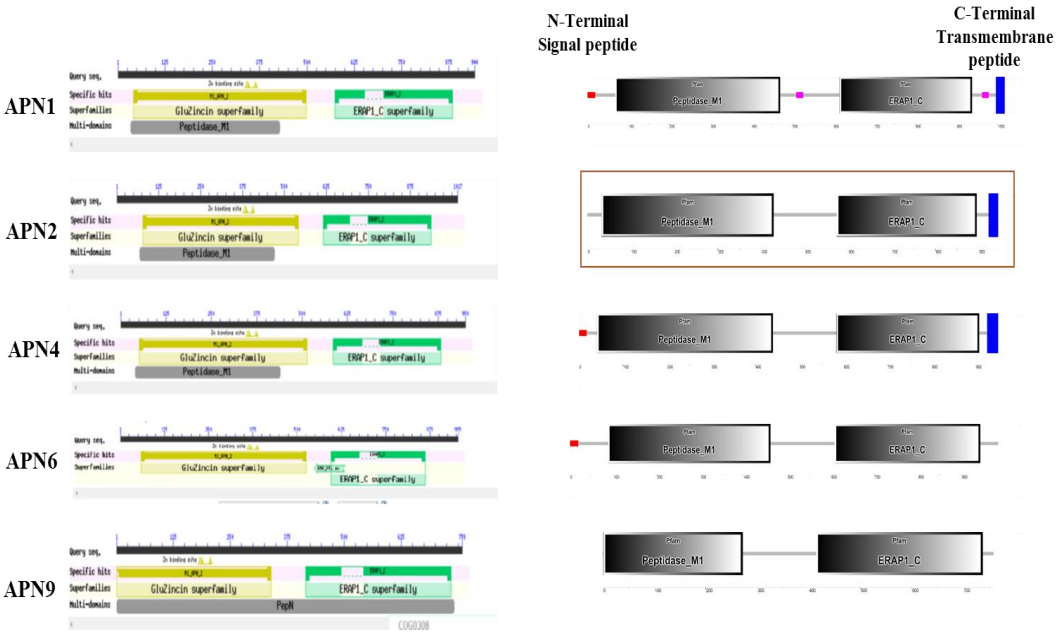


Fig. 36 Conserved domain analysis. The cloned APN proteins in *A. janata* revealed that they belong to Peptidase M1 Aminopeptidase N family having ERAP1 (Endoplasmic reticulum aminopeptidase 1) with M1_APN_2 and ERAP1_C containing domain. APN1, APN4 showed the presence of transmembrane domain and signal peptide further, APN2 had only transmembrane domain, APN6 had N-terminal signal peptide while APN9 lacked both N and C-terminal peptides.

3.3.5 Tissue specific expression APN isoforms

The results presented revealed that all the five APN isoforms cloned from the larval mid-gut in the present study were selectively expressed only in mid-gut tissue (Fig. 37). Further, none of the other tissues tested showed amplification of the cloned APNs.

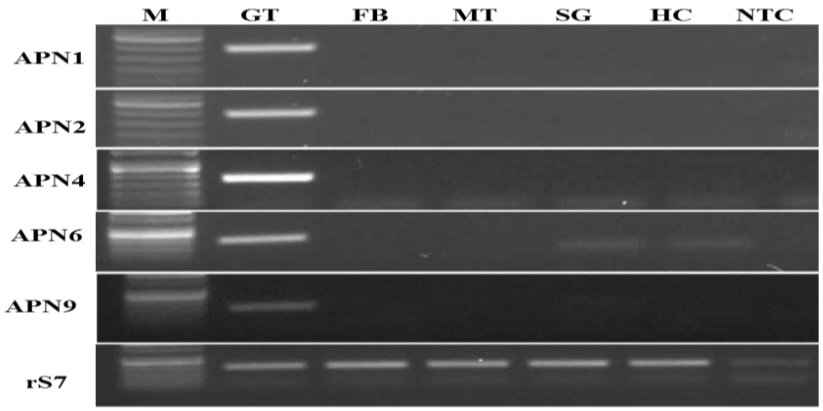


Fig. 37 Tissue distribution of APNs. Semi-quantitative PCR analysis showed the presence of APN transcripts only in gut (GT) but not in other tissues like fat body (FB), Malpighian tubules (MT), salivary gland (SG) and hemocytes (HC).

3.3.6 Expression analysis of APN isoforms

The qRT-PCR analysis revealed the down regulation of all the APN isoforms in time dependent manner upon continuous exposure to sub-lethal dosages of Cry toxins in the larval mid-gut (Fig. 38). Further the isoforms were differentially regulated and it was found that the rate of transcription as well as the total amount of transcript resent for various isoforms differed significantly from one another during Cry toxin expose (Fig. 38).

The generation wise expression analysis revealed that APN1, APN6 and APN9 had similar pattern of expression in each generation. On other hand APN2 showed a decline in expression, while APN4 exhibited inclined in expression (Fig. 39). It is likely that the higher expression seen for APN4 during Cry intoxication might compensate the physiological function of APN2.

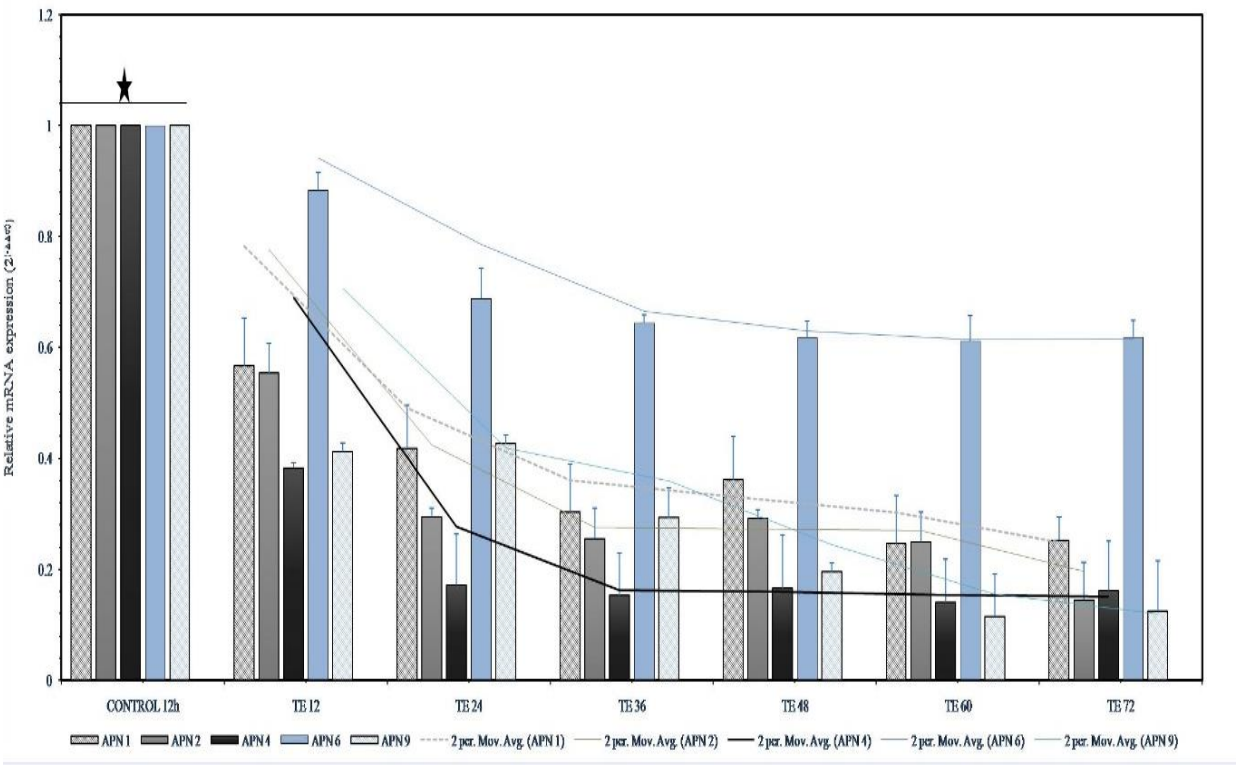
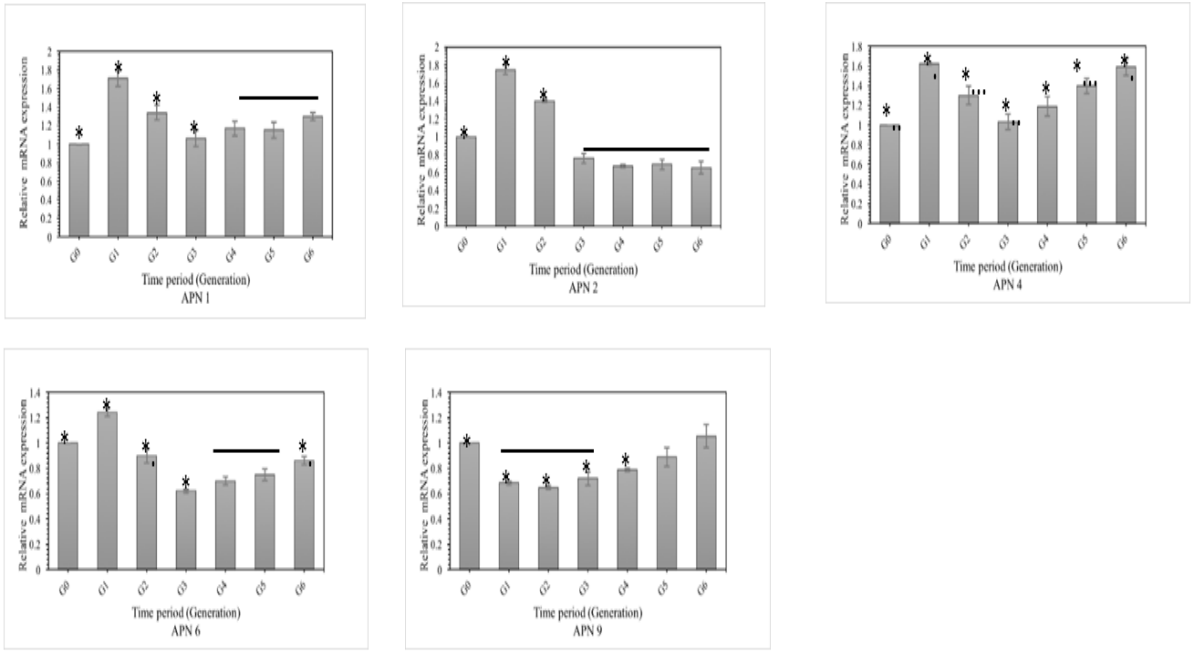


Fig. 38 Expression analysis of mid-gut aminopeptidase N isoforms using qRT-PCR during toxin exposure. A gradual decline in expression of various isoforms from 6 to 72 h of Cry intoxication was seen. Further, each isoform was differentially regulated during various time points. *$p < 0.005$ significance between experimental groups, (n=3).



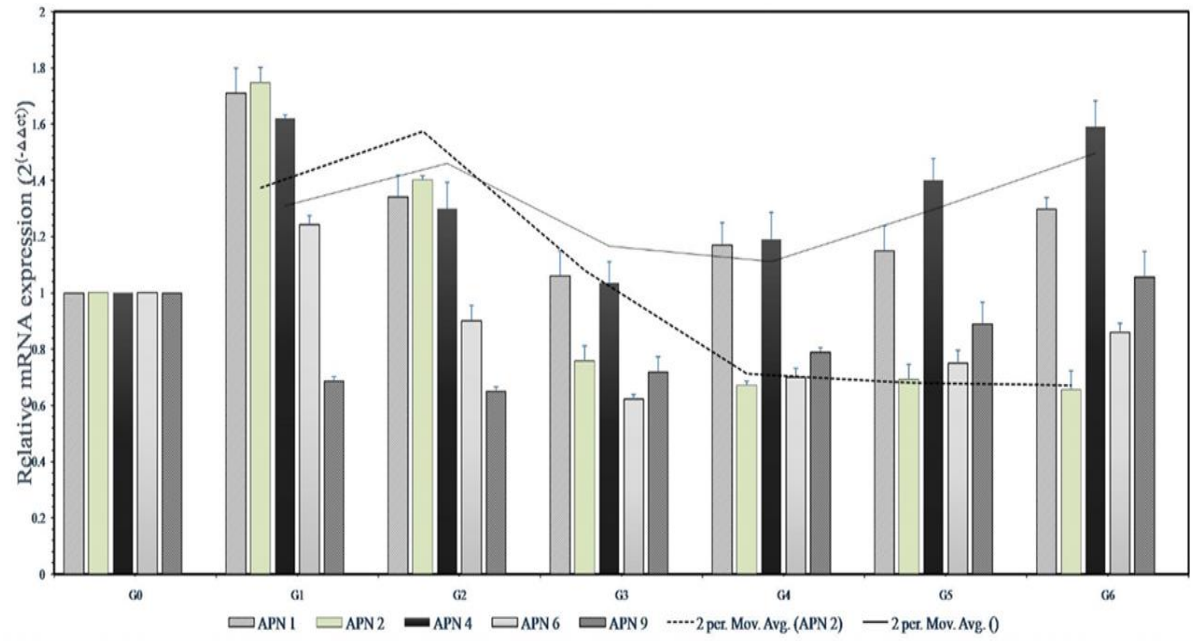


Fig. 39 Expression of various mid-gut aminopeptidase N isoforms transcript during various generations. Generation wise expression analysis of APN isoforms revealed that APN1, APN6 and APN9 had similar expression pattern in all generations, while APN4 was significantly up-regulated and APN2 was down-regulated from 3rd generation onwards *$p < 0.005$ significant between experimental groups (n=3).

3.3.7 *In silico* analysis of Cry-APN interaction

For this analysis APN2 was selected as model protein, while Cry1Ac was used as interacting toxin. The protein-protein interaction analysis revealed that amino acid residues from domains II and III of Cry toxin interact with amino acid residues of peptidase M1 domain of APN2 in *A. janata*. (Figs. 40 and 41). The predicted binding energy complex was found to be is ΔG= -16 (kcal mol-1) and Kd= 1.9 e-12 M.

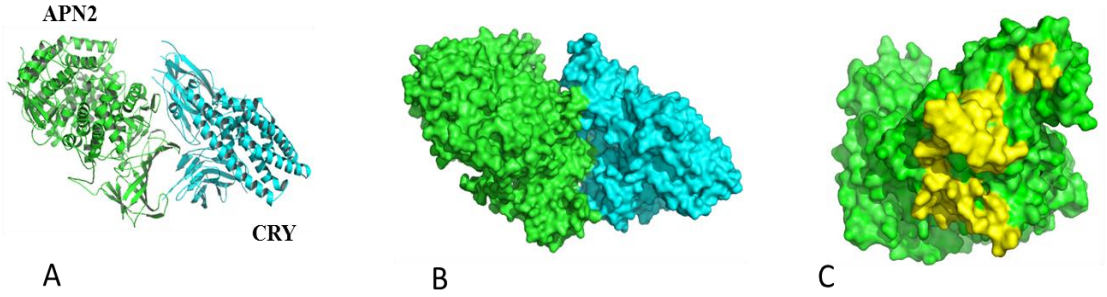


Fig. 40 Protein-protein interactions between APN2 of larval mid-gut of *A. janata* and Cry1Ac toxin. (A) In cartoon representation, (B) In surface representation and (C) Interacting amino acids in APN2 are highlighted in yellow color.

Interacting amino acids in APN2

R46 F48 Q60 A87 Q89 E98 N99 V101 H131 P193 Q194 N196 L204 R205 T206 D207 T208 L209 K210 E211 D212 V213 A214 K218 E220 N238 F239 E240 Y241 I242 D243 N244 N245 V246 D247 P248 I252 P253 R255 Q263 D264 E267

Interacting amino acids in Cry1Ac

P221 D222 R281 G282 S283 Q285 G286 R289 R292 S293 P294 H295 L296 P325 S329 G330 P331 F333 T334 F335 P336 L337 Y338 G339 T340 M341 F436 R437 S438 F440 N480 F481 L482 F483 N484 G485 S486 V487 I488 R500 N502 S503 S504 N507 N510 T537 G594 T595

Fig. 41 List of interacting amino acid residues from aminopeptidase N isoform 2 (APN2) with Cry1Ac toxin identified by *in silico* protein-protein interaction analysis.

3.3.8 Proteomics analysis of APN isoforms

The results obtained from immnunoblot studies with anti-APN antibodies showed the interaction of it with multiple protein spots in two-dimensional gel electrophoresis. This clearly indicated the presence of multiple isoform of aminopeptidases in the larval mid-gut of *A. janata*

(Fig. 42) which might play role in Cry toxin toxicity as well as in development of tolerance/resistance against them upon sub-lethal dose of Cry toxin exposure.

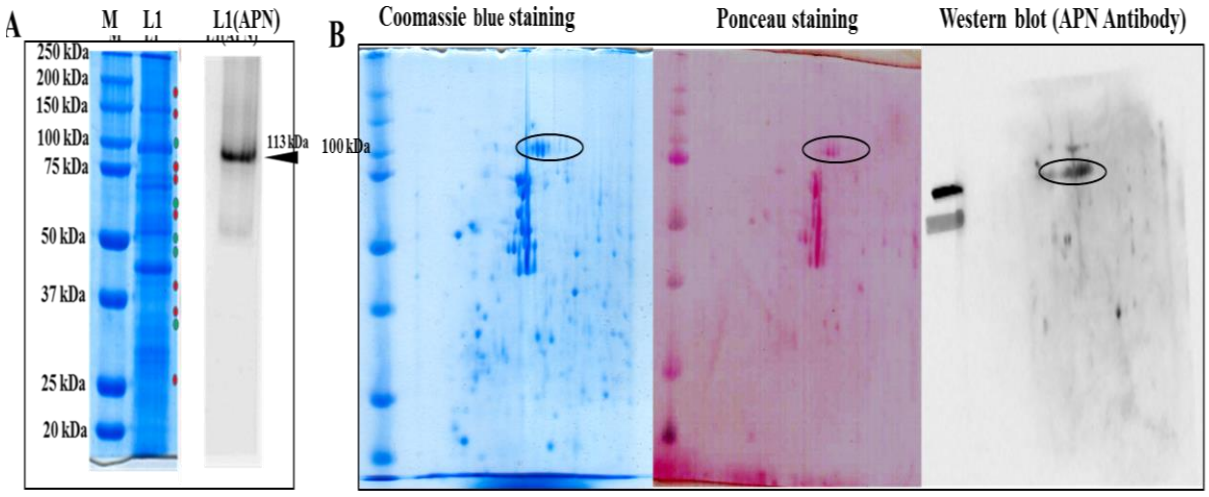


Fig. 42 Identification of membrane bound APN. (A) Detection of APN in membrane proteins with molecular weight 113 kDa (please note that APN membrane proteins in marked (⬭) zone. (B) Two-dimension SDS-PAGE and immunoblotting with APN antibody showed the presence of multiple cross reacting protein isoforms of APNs.

From DIGE analysis mid-gut of both the 6th generation control and toxin exposed insects (24 h), a total of 1552 spots were analysed, and it was found that out of them 225 protein spots were differentially expressed. For statistical analysis we took a cut off of ±1.2 (average ratio). Then student t-test (>0.05) and one way ANOVA (>0.05) was performed. Finally 55 protein spots were identified, which showed significantly differential expression. For the present study we focused on the spots which interacted with APN antibody (Fig. 43). The differential expression analysis showed up-regulation of most of the spots (protein), expect spot no. 4 (Table 4). This down-regulated spot was further analysed through MALDI-TOF/TOF, and identified as aminopeptidase 2 (APN2) by comparing the theoretical values generated through the same parameters used for protein identification through MS/MS analysis using through Mascot (www.matrixscience.com) software (Figs. 44 and 45).

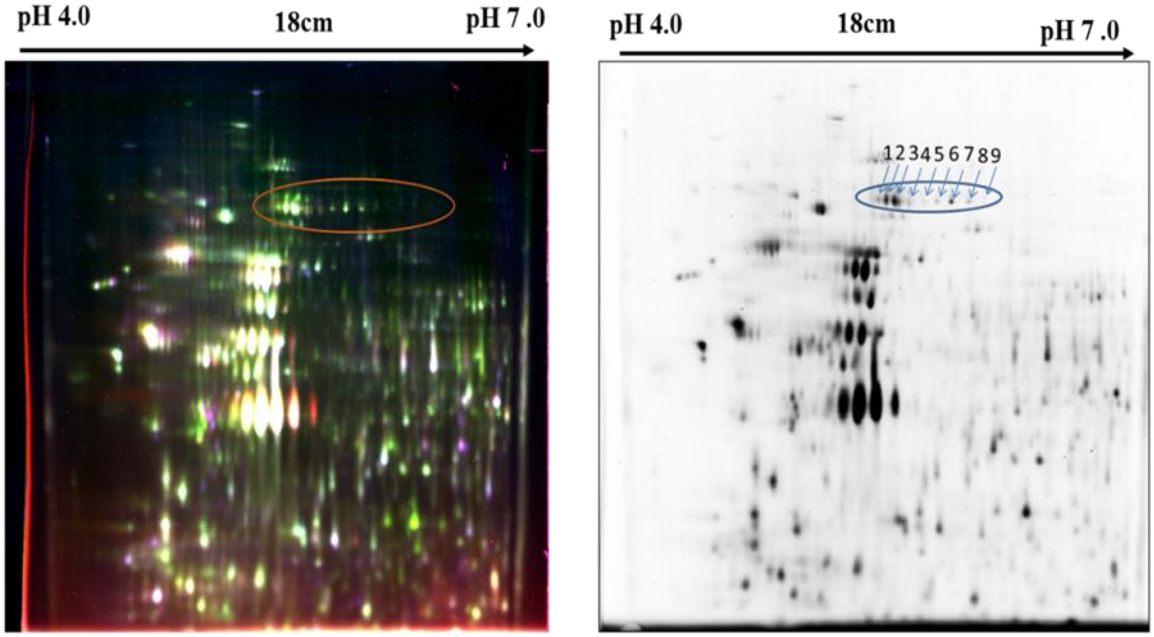
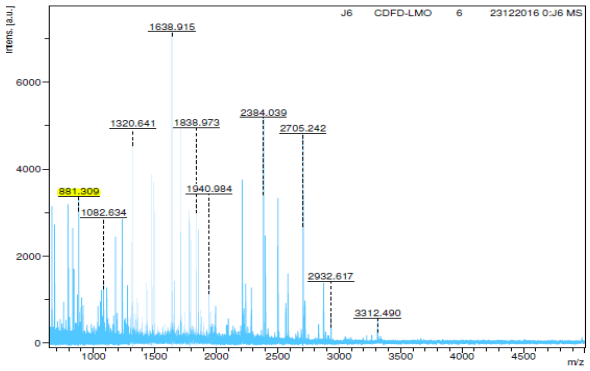


Fig. 43 DIGE expression analysis of membrane bound APN isoforms. BBMVs prepared from the 6th generation control and 24 h toxin exposed (sub-lethal) larval mid-gut was used. Please note the presence of multiple APNs spots (⬭) in the expected zone range.

Spot No	Appearance	T- test	AV.Ratio	One-way ANOVA
1	18/18	0.61	1.21	0.61
2	15/18	0.060	1.66	0.060
3	18/18	0.33	1.41	0.33
4	18/18	0.56	-1.07	0.56
5	18/18	0.58	1.77	0.58
6	18/18	0.41	1.26	0.41
7	18/18	0.017	1.61	0.017
8	18/18	0.031	1.83	0.031
9	18/18	0.39	1.28	0.39

Table 4 DIGE Expression analysis of protein spots which were detected by APN antibody cross reaction. All the protein spots were up- regulated statistically ($p>0.05$) expect spot no.4 which was down regulated.



Search Parameters

Type of search	: Peptide Mass Fingerprint
Enzyme	: Trypsin
Fixed modifications	: Carbamidomethyl (C)
Variable modifications	: Oxidation (M)
Mass values	: Monoisotopic
Protein Mass	: Unrestricted
Peptide Mass Tolerance	: ± 210 ppm
Peptide Charge State	: 1+
Max Missed Cleavages	: 1
Number of queries	: 74

m/z	S/N	Quality Fac.	Res.	Intens.	Area
662.276	19.1	2340	4120	803.64	212
678.334	16.4	4191	4133	686.57	184
683.206	17.9	1635	3707	751.76	225
685.215	6.6	372	3527	279.14	89
704.309	28.3	10517	4541	1180.32	311
706.373	6.4	468	3409	267.71	90
721.335	31.2	26678	4434	1300.83	359
729.250	6.4	197	3313	264.28	96
795.182	8.5	221	2743	350.76	174
797.370	6.7	219	2000	275.34	185
810.433	18.8	8082	4231	782.17	260
828.441	76.9	95225	4311	3197.18	1102
832.338	22.2	7137	3989	927.55	347
842.524	10.9	5966	4249	453.74	163
870.564	8.1	697	3480	332.53	147
881.298	6.6	393	2982	270.45	143
895.520	43.6	51106	4524	1791.87	663
943.489	8.8	1961	3908	361.16	162
954.550	322.3	74127	4568	13175.18	5382
960.524	6.2	3697	5089	254.05	92
966.570	6.1	1095	5241	252.72	88
968.568	12.0	2930	4225	488.99	211
982.590	11.4	3406	4258	464.00	206
1064.648	6.2	247	2694	252.02	197
1109.603	13.1	5692	5373	537.80	237
1133.679	15.9	4607	5406	652.54	296
1139.674	226.8	31245	5022	9300.03	4619
1179.691	9.9	2236	6014	410.87	179
1204.700	30.1	17246	5512	1252.09	616
1232.728	6.9	591	4260	288.71	185
1234.759	18.3	4274	5543	760.52	388
1238.713	28.6	4619	5656	1191.17	605
1307.745	7.7	597	5906	319.00	169
1317.813	15.3	893	5472	626.86	363
1319.734	12.3	353	5108	513.15	312
1371.713	120.5	52680	5950	5083.40	2934
1385.737	8.8	3392	6042	372.10	212
1399.753	10.5	3623	5411	443.39	278
1463.828	11.9	2661	6390	504.14	302

m/z	S/N	Quality Fac.	Res.	Intens.	Area
1475.866	6.1	1715	5871	259.30	167
1483.821	6.9	520	5927	292.33	190
1493.836	14.3	14514	6115	605.65	392
1547.820	11.6	8424	5821	487.65	351
1560.889	6.7	1369	4662	280.14	250
1574.841	147.1	42137	6020	6148.95	4499
1588.879	28.3	20481	6479	1179.06	809
1690.965	32.6	37337	6191	1322.54	1080
1707.890	7.3	1620	5099	295.47	288
1717.012	18.4	10076	6109	741.91	621
1739.963	234.2	60038	6311	9358.47	7986
1754.943	11.0	502	4554	434.88	503
1771.945	7.6	922	4407	300.80	362
1777.011	17.3	2479	4786	684.17	780
1781.961	6.1	522	4918	239.19	264
1785.383	7.7	2146	3123	303.95	554
1824.975	44.6	27819	6773	1712.00	1502
1840.986	22.0	1774	5187	842.34	950
1845.880	37.3	1015	6512	1435.09	1335
1848.108	179.8	14756	5402	6869.59	7690
1918.037	12.4	830	4514	447.55	611
1926.427	21.2	1757	3276	738.99	1432
1947.040	9.0	1025	4944	316.28	401
1967.054	143.6	5690	5953	4969.61	5296
1969.112	43.2	1039	4592	1494.24	2044
1982.018	65.6	11473	6618	2232.47	2266
1996.090	13.5	1379	4571	455.88	641
2090.035	19.3	7809	6358	580.92	636
2147.189	8.3	911	6421	236.28	265
2211.229	8.3	357	5347	220.97	300
2234.203	41.3	8204	7044	1031.43	1109
2384.147	7.4	2229	5793	157.87	243
2501.399	6.9	880	7008	135.53	174
2874.540	13.6	1884	7255	205.35	325
3072.744	20.6	756	9093	276.49	420

Fig. 44 MS/MS analysis of down regulated protein spot using Mascot software. The peptide peaks of M/Z values (highlighted) matched with the theoretical peaks generated with APN2 sequence.

APN2_ACJN Mass: 107367 Score: 8 Expect: 1.2 Matches: 5

Probable Protein APN2 OS=Achaea janata GN=APN2 PE=1 SV=1

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
683.3030	682.2958	682.3326	-54.00	288	292	0	K.YEFPK.M
881.4221	880.4148	880.4290	-16.11	868	874	0	K.VDELYSR.H
943.6217	942.6144	942.5134	107	562	570	0	R.SVDIPGLSR.A
1782.2090	1781.2017	1780.8970	171	172	186	1	R.AFPCFDEPALKAIFR.T
1785.6317	1784.6244	1784.8403	-120.96	791	805	0	R.DFYMNQVIADDSLVR.E

Aj.APN2 sequence

MYLLTLLALI GTAFCGPLST QVRNVDNRAE LYVLPTTIKP TFYDVRLFLD PGNPELFYGQ
VSIRILPQVE TNEIVLHAME MLITENAIQV YTDREPTENI VTGFTQAGDD THFLRIQLSR
QLTVQQPATI HIDYVGHFAE NMFGVYRSTY TENGNTIALI TSQLQPTFAR RAFPCFDEPA
LKAIFRTTIY APPQYNVVRS NMPLRTDTLK EDVAGYTKHE FQDTLIMSTY LIAYLVSNFE
YIDNNVDPIF NIPFRVYSRP GTQDTAEFAL DFGQKNMVAL EDYIDFKYEF PKMDKVAVPD
FAAGAMENWG LVIYREVALL VTEGVTTTSV RQNIGRIICH ENLHQWFGNE VGPASWTYTW
LNEGFANFFE NYATNLVVPE WRMMDQFVIA LQNVFQSDAV LSVNPMTHPV YTPSEILGTF
NAVAYQKSGS VIRMMQHFLT PEVFRQGLIY YVKAMSRQAA APTHLYSSLQ QALTESSHSI
PYQLSTVMTR WTEQGGFPVV TITRSADTAE SVVVAQERFL TDSTLSSDDR WHVPLNWALS
TNPDFSDTRP MEWIPPNFPA RSVDIPGLSR AEWFVFNKQQ TGYYRVNYDD GNWEALAKVL
NENHELIHLL NRGQILDDAF NLARNGRTNY RHPFEISRYL VNEQDYIPWA AVNPAFTYLD
VVLSESPVYD EFRYYILNLT APLYNSLGFE VNSGEEFVTP YHRNIILDLN CRLGNSHCIS
TAQDLLDSFR NNPAQRLNPD IQTLVYCSGL RGGDADNFNF LWNQYANTDD SSEQSILLNA
LGCTSNVELR DFYMNQVIAD DSLVREQDRH TILVSVTNAS PDNMNAALDF IIENFHLIQP
RVQGLTGTTN ILNALARRLT SQEHSAKVDE LYSRHQAIFT AGELASIAAI RENIAASITW
SNENAATVED WLRQNYGDGD GDGAAAMTAS LVLLISALVA IFNY

Fig. 45 Identification of down regulated protein spot. The five peptides obtained from MS/MS analysis marched with the amino acid sequence (highlighted) present in the aminopeptidase isoform 2 (APN2).

.

3.4 Discussion

Mid-gut of Lepidopteran insect larvae is a multifaceted tissue which performs variety of function ranging from digestion, absorption, immune function, transmission of pathogens including baculovirus and interaction with various molecules (Kliot *et al.*, 2014; Wu *et al.*, 2016; Kim *et al.*, 2016). The proteins present in the apical brush border of mid-gut epithelial tissues are digestive proteases, which play important role in digestion and variety of transporters which facilitate transport and absorption. Among the large number of proteins which have been identified in BBMV of mid-gut such as cadherin-like proteins (Nagamatsu *et al.*, 1999; Xu *et al.*, 2005), GPI-anchored aminopeptidase-N (APNs) (Knight *et al.*,1994; Rajagopal *et al.*, 2003; Budatha *et al.*, 2007) and alkaline phosphatases (ALPs) (McNall and Adang,2003; Jurat-Fuentes and Adang, 2004). Glycolipids and glycoconjugates (Valaitis *et al.*, 2001) also bind and/or interact with Cry toxin produced by various strains of *Bt*. Recently ATP-binding cassette transportor subfamily C2 (ABCC2) has also been characterized as Cry toxin functional receptor (Tanaka *et al.*, 2013; Zhou *et al.*, 2016).

Ligand blot analysis (toxin overlay assay) with DOR *Bt*-1 showed the prescence of multiple interacting proteins in the membrne fractions isolated from mid-gut, fat body as well as in tissue homogenates of salivery gland and Malpighian tubules of *A. janata* larvae. Among them two proteins, one with molecular weight around of 100-110 kDa and another one with 60-70 kDa were shown to interact in all four tissues. On the basis of existing literature, the protein with higher molecular weight ranged closure to reported aminopeptidase N (110 kDa) and the one with lower molecular weight matched with alkaline phophatase (68 kDa). Earlier studies from our group have already demostrated them as Cry toxin binding protein/receptor. In *A. janta* the full length APN was cloned and confirmed by lignad blot analysis using Cry 1Aa and DOR 5 toxins, from mid-gut, fat body, Malphighian tubules and salivary glands (Budatha *et al.*, 2007; Ningshen *et al.*, 2013).

In presnt study toxicity of DOR *Bt* -1 strain was analyzed primarily keeping in the view that the Cry toxins produced from this strain have high insecticidal activity towards *A. janata* as well as other Leipidopteran pest (Vimaladevi *et al.*,2006; Reddy *et al.*, 2013). Finding of this study revealed binding of 5-6 membarane protiens with activated toxin obtained from DOR *Bt*-1 and recombinant Cry1Ac (BGSC) in the larval mid-gut of *A. janata.* The prominent interaction was seen primarily with membrane protein of 110 kDa, which was confimed by immunodetection using anti-APN antibody generated against a small frgament of recpmbinant APN from *A. janata.* Furthermore, the sequece obtained through MALDI-TOF/TOF -MS/MS analysis matched with the alanyl aminopeptidase of *Heliothis virescens* with molecular weight 113 kDa (Gill *et al.*, 1995). Colocalization studies were perfromed further to confirm whether or not the Cry toxins really interact with APN presnt in inner apical brush border (BBMVs). Once again it was found that both DOR *Bt*-1 and Cry 1Ac toxins interact with membrane vesicles, where APN was localized using anti APN antibody. The results obtained in present study corroborated well with earlier reports where brush border membrane region was shown to interact and accumulate toixns in intoxicated larvae (Bravo *et al.*, 1992; Yi *et al.*,1996; Valaitis, 2011). The findings presented in chapter 1 clearly showed that Cry intoxication caused a significant changes/ damge in the microvilli mid-gut BBMVs of mid-gut epithelial cells of *A. janata* larvae. Hence, this study cleraly indicateed that mid-gut membane bound protein aminopeptidase N is a major Cry toxin receptor in the larval forms of *A. janata.*

The molecular weight of APNs in various insects ranged between 100-150 kDa. (Crava *et al.*, 2013). In insects they were shown to be produced in multiple forms (isoforms), and same has already been demonstrated in Lepidopteran insects like *Bombyx mori* (Nakanishi *et al.*, 2002); *Trichoplusia ni* (Wang *et al.*, 2005); *Ostrinia nubilalis* (Crava *et al.*, 2013) and *Heliothis virescens* (Perera *et al.*, 2015). As the genome sequences of *A. janata* is not available for finding out the presence of APN isoforms, next generation RNA sequencing (*de novo* trnscriptome) of

larval mid-gut of *A. janata* was carried out (unpublished data). The screening of data revealed the presence of five different isoforms which were further cloned and confirmed by Sanger sequencing. *In silico* analysis of identified sequences revealed the presence of characteristic aminopeptidase activity motif "GAMENEG" and zinc binding motif "HEXXHX18H". The isoforms were named based on the homology found with reported sequences in NCBI data base as APN1 (3054 bp, 1017aa, ~114 kDa, pI 4.73); APN2 (2835 bp, 944 aa, ~107 kDa, pI 4.81); APN4 (2853 bp, 950 aa, ~108 kDa; pI 4.81); APN6 (2868 bp, 955aa, ~109 kDa, pI 4.94) and APN9 (2280 bp; 759 aa, ~88 kDa, pI 5.43). The molecular weight of all the isoforms of APN identified in *A. janata* ranged between 100-120 kDa, except APN9 which was much smaller than others and it was around 88 kDa. A similar low molecular weight aminopeptidase of 90 kDa was also reported in *Bombyx mori* (Nakanishi *et al.*, 2002), further this showed 37% sequence identity with others. Detailed analysis also suggested that these isoforms were paralogs of each other and were expressed by different genes of single clusters. Phylogenetic review also suggested that most of the aminopeptidases arose from gene duplication, from a recent common ancestor of Lepidopteran and Dipteran gene (Hughes, 2014). In addition, conserved domain analysis of the identified sequences revealed that they belonged to peptidase M1 aminopeptidase N family with endoplasmic reticulum aminopeptidase 1 (ERAP1) [M1_APN_2 and ERAP1_C domain-containing protein], which are type 2 integral membrane proteases (Kola *et al.*, 2016). *In silico* analysis showed the presence of transmembrane peptide signal at C-terminal end of APN 1, APN2 and APN4, while APN2 did not have any signal peptides at N-terminal. Further, APN6 showed signal peptides at N-terminal but not the transmembrane peptide at C-terminal. APN9 lacked both the signal sequences.

In *A. janata*, all the above identified isoforms were expressed exclusively in mid-gut tissue and none of the other tissues analysed exhibited their expression, suggesting the tissue

specific expression of these isoforms, and most likely they were important proteases, which play role in physiological function of the mid-gut during larval development.

For the management of this pest (*A. janata*), once pest appears in the field (late 2nd or early 3rd instar) two sprays of *Bt* formulations are applied with a gap of three days. Same strategy was followed in the laboratory to find out the role of aminopeptidase N as Cry toxin receptor. In the present study upon sub-lethal dose of Cry toxin exposure, the process of development of Cry toxin tolerance was monitored in a population of insect using a generation wise analysis. During the continuous exposure to toxin for 72 h the expression of all the APN isoforms gradually declined in the larval mid-gut in time dependent manner. Further, each isoform was differentially regulated. The expression analysis of APN isoforms from generation wise analysis (for 6 continuous generations) revealed no significant difference in APN1, APN6 and APN9 expression, but the expression of APN2 declined while APN4 showed inclined expression. These findings suggest that upon Cry toxin exposure larval mid-gut has ability to modulate the expression of various proteases including APNs (Budatha *et al.*, 2007).

The detailed DIGE expression analysis carried out with BBMVs proteins isolated from 6th generation control as well as 24 h toxin exposed (sub-lethal dose) revealed up regulation of all the proteins spots, which were detected using anti-APN antibody expect the one. A detailed structural analysis of the down regulated protein spot further confirmed it as APN2 using MALD-MS/MS analysis. As it is mentioned in previous section that APN2 lacked signal peptide but showed the presence of transmembrane domain, the reduced expression of APN2 might lower the interaction of Cry toxin with apical membrane of mid-gut epithelial cells and block the cellular damage thus providing toxin tolerance in the larvae. Further, inclined expression seen for APN4 might, compensate the physiological function of down regulated APN2 and facilitate not only survival of the insects but also its growth. In cabbage looper, *Trichoplusia ni* Cry resistant insects were shown to down regulate APN1 (Tiewsiri and Wang, 2011).

Findings of the present study clearly suggests that APN2 most likely acted as a functional Cry toxin receptor in *A. janata* and mediated Cry toxin based damage in susceptible insects. Further it's down regulation upon sub-lethal Cry toxin exposure during generation wise analysis facilitated the tolerance of toxin by the larvae. Finally, this study also suggests that in *A. janata* larvae exploit variety of strategies to avoid the deleterious effects of Cry intoxication, which in long run would make them resistant to *Bt* formulations and/or Cry toxin based transgenics.

Summary and Conclusion

Summary

The lack of homogeneity in field application of *Bt* formulation may often result in ingestion of sub-lethal doses of the biopesticide by a fraction of the pest population and thereby they develop toxin tolerance and resistance in long term. Present laboratory study evaluated various larval mid-gut responses to sub-lethal dose of Cry toxin in Lepidopteran pest *A. janata.*

Prolonged exposure (0-72 h) of sub-lethal dose of DOR *Bt*-1 formulation to seven-day old or 3rd instar larvae showed deleterious effects on larval growth and development. Histomorphological analysis of the toxin exposed mid-gut epithelium showed degeneration of epithelial cells with condensed nucleus and fairly low cytoplasmic content. In addition, large number of vacuoles were also observed within the epithelial cells. Maximum cell death was observed between 24 to 36 h. Further the pattern of cell death was evaluated using AnnexinV and Propidium Iodide staining with confocal microscopy, flow-cytometery and DNA fragmentation analyses and the results obtained indicated that necrosis was the most likely reason for cell death upon Cry intoxication.

Larvae avoided feeding on toxin coated leaves, and made an attempt to overcome toxin mediated damage in gut epithelium by extensive cell proliferation. This was monitored using a toluidine blue staining and kit-based BrdU labelling. Few pockets of BrdU-positive cells were identified from the mid-gut collected after 24 h toxin exposure, and after 48 h, the number of small cells labelled with BrdU increased considerably. These cells were mostly observed in the basal region of the mid-gut epithelium, and they could be ascribed to be mid-gut stem cells/regenerative cells. The events of cell necrosis and regeneration occurred simultaneously during the toxin exposure. Hence, cell proliferation might predictably play a role in compensating the cell death during Cry intoxication.

Arylphorin, a hexamerin protein, is known to be predominantly synthesized by the fat body tissue and released into the haemolymph during active feeding phage of larvae. Detectable but low expression of arylphorin in the mid-gut as compared to the fat body was observed, which was further confirmed through *in situ* hybridization and localization of arylphorin transcript in the gut epithelium primarily in the basal cells. Expression analysis using Cry intoxicated larval mid-gut further showed time dependent increase in endogenous arylphorin gene expression during active cell proliferation, which was not only seen upon Cry intoxication but also during molting in control larvae where regeneration is well documented. Presence of arylphorin transcript and its altered expression in the mid-gut during Cry toxin exposure, indicated a plausible role of arylphorin in rapid gut regeneration, which might facilitate and promote Cry toxin tolerance in the Lepidopteran larvae.

Aminopeptidase N(s) are well known digestive proteases and they cleave the neutral amino acids from N terminal of protein/ peptide. By default they also interact/ bind with Cry toxins and act as interacting/ receptor proteins in Lepidopteran insects including *A. janata*. In the present study five different isoforms (APN isoforms) were identified and named as APN1, APN2, APN4, APN6 and APN9. The expression profile analysis of all the APN isoforms upon continuous sub-lethal dosage of toxin exposure revealed that it gradually declined in time dependent manner and various isoforms were differentially regulated. The field based application strategy for development of toxin tolerant insect populations (6 generations) showed a non-significant difference in APN1, APN6 and APN9 expression but a decline in the expression of APN2 and incline in expression of APN4. Further, DIGE expression analysis of APN isoforms (detected through anti APN antibody) also confirmed the lower expression of APN2 isoform. The present study clearly suggested that APN2 might act as a functional receptor of Cry toxin in *A. janata*, and its binding to the toxin initiated extensive cell damage in mid-gut. Further the analysis revealed that upon long-term generation wise exposure the larval forms

altered the expression profile of various APN isoforms and reduced the expression of APN2 to avoid insecticidal action and develop tolerance against Cry toxin. The upregulation APN4 most likely facilitated and compensated the digestive physiological function of down regulated APN2 for the survival of the larvae.

Conclusion

Present study revealed that prolonged exposure to sub-lethal doses of Cry toxin formulation have deleterious effects on larval growth and development. Cry toxin intoxication promoted necrotic form of cell death. Further larvae reduced toxin feeding to minimize the toxin based mid-gut damage and tried to replenish the injured cells through enhanced proliferation (regeneration) of mid-gut stem cells. The observation that the enhanced mid-gut cell proliferation coincided with the elevated endogenous arylphorin expression suggested a plausible role of arylphorin in rapid gut regeneration, which might facilitate and promote Cry toxin tolerance in the Lepidopteran larvae. Among all the isoforms of aminopeptidase N identified *in A. janata*, APN2 might act as a functional receptor of Cry toxin, hence insect host system tried to reduce its expression in order to survive from toxin based damage and lethality. This strategy might help the insects not only to overcome toxin toxicity but also in development of tolerance against *Bt* biopesticides during long term generation wise field applications.

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Larval Mid-Gut Responses to Sub-Lethal Dose of Cry Toxin in Lepidopteran Pest *Achaea janata*

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The lack of homogeneity in field application of *Bacillus thuringiensis* formulation often results in ingestion of sub-lethal doses of the biopesticide by a fraction of pest population and there by promotes the toxin tolerance and resistance in long term. Gut regeneration seems to be one of the possible mechanism by which this is accomplished. However, the existing information is primarily derived from *in vitro* studies using mid-gut cell cultures. Present study illustrates cellular and molecular changes in mid-gut epithelium of a *Bt*-susceptible polyphagous insect pest castor semilooper, *Achaea janata* in response to a Cry toxin formulation. The present report showed that prolonged exposure to sub-lethal doses of Cry toxin formulation has deleterious effect on larval growth and development. Histological analysis of mid-gut tissue exhibits epithelial cell degeneration, which is due to necrotic form of cell death followed by regeneration through enhanced proliferation of mid-gut stem cells. Cell death is demonstrated by confocal microscopy, flow-cytometry, and DNA fragmentation analysis. Cell proliferation in control vs. toxin-exposed larvae is evaluated by bromodeoxyuridine (BrdU) labeling and toluidine blue staining. Intriguingly, *in situ* mRNA analysis detected the presence of arylphorin transcripts in larval mid-gut epithelial cells. Quantitative PCR analysis further demonstrates altered expression of arylphorin gene in toxin-exposed larvae when compared with the control. The coincidence of enhanced mid-gut cell proliferation coincides with the elevated arylphorin expression upon Cry intoxication suggests that it might play a role in the regeneration of mid-gut epithelial cells.

Keywords: cry toxin, lepidopteran pest, gut regeneration, mid-gut stem cells, arylphorin

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INTRODUCTION

Bacillus thuringiensis (*Bt*) strains synthesize different toxins including crystal (Cry), cytolytic (Cyt), and vegetative insecticidal proteins (Vip). While Cry and Cyt toxins are synthesized during sporulation (Hofte and Whiteley, 1989; Bravo et al., 2007), Vip toxins are produced during the vegetative growth phase (Estruch et al., 1996). Lepidopteran larvae experimentally used to demonstrate the mode of action of Cry toxin, showed sequential event of toxin solubilization, its activation induced by alkaline pH of the gut, proteolytic cleavage, binding of toxin with different receptor molecules, oligomerization, and membrane insertion leading to pore formation

and epithelial cell damage (Bravo et al., 2007). Further, *in vitro* studies suggested that the Cry toxin-induced cell death is primarily of necrotic type (Zhang et al., 2016).

Bt and its insecticidal toxins have been predominantly used for the control of maize and cotton pests (Bravo et al., 2011; Hellmich and Hellmich, 2012). The development of insect resistance to *Bt* is a major concern for its long-term effectiveness. Extensive studies revealed, alteration in mid-gut binding sites leading to reduced interaction of *Bt* toxins to their receptor, as a major mechanism for the development of resistance (Pigott and Ellar, 2007). Since many Cry toxins share common binding sites, the probability of cross-resistance was often found to be high (Ferre and Van Rie, 2002; Tabashnik et al., 2013). In agreement with these reports, insects such as diamond black moth, *Plutella xylostella*; Indian meal moth, *Plodia interpunctella,* and cabbage looper, *Trichoplusia ni* have evolved resistance to commercial *Bt* sprays in the open field and greenhouses (Ferre et al., 1991; Oppert et al., 1997; Janmaat and Myers, 2003). Recently, field-evolved pest resistance to *Bt* corn (maize stem borer, *Busseola fusca*; western corn rootworm, *Diabrotica virgifera virgifera*; and fall armyworm, *Spodoptera frugiperda*), *Bt* cotton (cotton bollworm, *Helicoverpa armigera,* and pink bollworm, *Pectinophora gossypiella*), and *Bt* maize (*Spodoptera frugiperda*) have also been reported (Storer et al., 2010; Dhurua and Gujar, 2011; Kruger et al., 2011; Zhang et al., 2011; Jakka et al., 2015; Pereira et al., 2015).

Additional physiological responses from larvae alike non-receptor mediated resistance mechanisms could interfere with steps common to all Cry toxins vis-à-vis cross-resistance to a wide range of *Bt* toxins. One such process is the enhanced mid-gut regeneration response observed in the resistant larvae. Lepidopteran larvae have been shown to recover from exposure to sub-lethal Cry toxin doses (Dulmage and Martinez, 1973) by mid-gut epithelial regeneration (Spies and Spence, 1985). Further few microscopic studies showed enhanced mid-gut healing response to facilitate Cry toxin resistance in the tobacco budworm, *Heliothis virescens* (Forcada et al., 1999; Martinez-Ramirez et al., 1999). However, there is a dearth of information on the molecular aspects of mid-gut epithelial response to Cry toxin. In addition, most of the information on the regenerative process is derived from *in vitro* studies using primary mid-gut cell cultures (Loeb et al., 2001; Blackburn et al., 2004; Hakim et al., 2010) mainly due to the lack of mid-gut specific biomarkers to allow quantitative detection of lepidopteran mid-gut cells, their proliferation as well as differentiation. The *in vitro* data circumvents specific cell/tissue interactions within the insect system. One of the candidate molecules presumed to be involved in the mid-gut response to *Bt* toxins is arylphorin, which belongs to the hexamerin protein family of insects. Arylphorin was shown to stimulate stem-cell proliferation *in vitro* as well as upon feeding with diet, where it promoted the growth of gut suggesting its mitogenic effect on gut cells (Blackburn et al., 2004; Hakim et al., 2007).

Foliar spray of *Bt* formulation often results in the loss of toxicity from degradation by UV light, wash-off by rain, drying, temperature, and soil acidity as well as its chemistry (Pinnock et al., 1975; Leong et al., 1980; Beckwith and Stelzer, 1987; vanFrankenhuyzen and Nystrom, 1989). Under afore mentioned conditions, there is always a possibility of a population of larva to get exposed to sub-lethal doses of toxin which might exhibit a variable effect and escape mortality. In line with these observations the present study is aimed to evaluate the effect of sub-lethal doses of *Bt* toxin on mid-gut of the larva of *Achaea janata*, an economically important polyphagous pest prevalent in the Indian subcontinent. *In vivo* analysis document the necrotic type of cell death associated with regeneration of mid-gut epithelial cells during Cry intoxication. Additionally, the presence of arylphorin transcript in larval mid-gut cells and its differential expression during Cry intoxication is also demonstrated.

MATERIALS AND METHODS

Insect Rearing and Maintenance

For the present study, larvae of *A. janata* Linn. (Noctuidae: Lepidoptera) were collected from the fields of Indian Institute of Oil Seeds Research, Hyderabad with no prior exposure to any synthetic or *Bt* pesticides, reared in the laboratory conditions (Budatha et al., 2007). They were maintained for three generations as to obtain homogenous population (in a growth chamber). Larvae were fed with sterile castor leaves and maintained at 27 ± 2°, 14:10 h (light: dark) photoperiod and relative humidity of 60–70% till pupation. Pupae were transferred to moist sand container for adult emergence. Adults were transferred to egg laying chamber and were supplemented with 10% honey containing Vitamin E. Adults laid their eggs on the leaves and the neonate larvae hatched were used for continuous culture as well as experiments.

Administration of DOR *Bt*-1 Formulation

DOR *Bt*-1 formulation was obtained from Indian Institute of Oil Seeds Research which exhibited high toxicity against *A. janata* larvae and various other lepidopteran pests. The PCR analysis of DOR *Bt*-1 isolate revealed presence of *cry1* (*cry1Aa*, *cry1Ab,* and *cry1Ac*) and *cry2* (*cry2Aa* and *cry2Ab*) genes, which provide broad-spectrum potential against lepidopteran, and dipteran insects (Reddy et al., 2012). The reported LC_{50} value for the DOR *Bt*-1 formulation was 247.52 μg/ml for seven-day-old or 3rd instar larvae (Vimala Devi and Sudhakar, 2006). One tenth of LC_{50} value was used as a sub-lethal dose (i.e., 24.75 μg/ml of water) for the present study. Toxin coated castor leaves were fed to the larvae. Triplicates were maintained, for each treatment 150 larvae were used. They were divided into two sets of experiments, wherein one batch was continuously exposed to sub-lethal dosage of toxin (0.170 $\mu g/cm^2$) coated castor leaf discs (145 cm^2) till 72 h, and a batch of 30 larvae were intermittently transferred to uncoated leaf discs after every 24 h period of toxin exposure for the recovery. Control larvae were maintained on water coated leaf discs. Furthermore, morphological changes occurring during the development and mortality were also recorded in toxin-exposed as well as recovered insects.

Tissue Isolation

Third instar larvae (a batch of 100) exposed to toxin coated leaves were collected at every 12 h interval till 72 h. These were briefly narcotized on ice and dissected in insect Ringer solution (130 mM NaCl, 0.5 mM KCl, and 0.1 mM $CaCl_2$). An incision was made in the abdominal region and extended through the length of the larvae. The gut content was removed and the mid-gut region was specifically isolated. After removal of the gut, the yellowish-white perivisceral fat body was collected. The isolated tissues were used for histology, *in-situ* hybridization, genomic DNA and total RNA isolation.

Mid-Gut Cell Death Analysis

Cell death/viability of mid-gut cells was primarily assessed using AnnexinV and Propidium Iodide (PI) staining (Vermes et al., 2000). For this experiment mid-gut was dissected out from both control and toxin exposed (24 h) larvae and maintained in Grace insect cell culture media (Invitrogen Corporation, USA) and incubated with AnnexinV and Propidium Iodide staining solutions (BD Biosciences, UK) and visualized under confocal microscope (LSM 880, Carl Zeiss, Jena). Furthermore, gut cells were strained through 70 μm mesh size MACS® smart strainer (Miltenyi Biotec, Singapore) using phosphate buffer saline (PBS) solution (pH 7.4). The cell reservoir was collected in the funnel and centrifuged at 1,500 rpm for 5 min at 4°C. The cell suspension was collected and immediately processed for FACS (fluorescence-activated cell sorting) analysis. An analysis was carried out using Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions (BD Biosciences, UK). In brief, AnnexinV and Propidium Iodide staining solutions were added to the sieved cell suspension and incubated at room temperature in the dark for 15 min. This was followed by addition of binding buffer and then analyzed using a FACS LSR Fortessa™ flow cytometer (BD Biosciences, UK) and BD FACS Diva Software 6.0. For each sample, 10,000 events were acquired. The percentage of live cells vs. cells undergoing death was plotted.

To substantiate that cell death is due to necrosis but not because of apoptosis in larval mid-gut, DNA fragmentation assay was carried out following the protocol of Kasibhatla et al. (2006). Genomic DNA from the mid-gut was isolated using a standard protocol (Lagisz et al., 2010), with minor modifications. The tissue (100 mg) was homogenized using 500 μl of lysis buffer (100 mM Tris-HCl (pH8.0), 50 mM EDTA, 1.5 mM NaCl, 2% CTAB, and 0.1% 2-mercaptoethanol). DNA pellet thus obtained was solubilized in Tris-EDTA buffer (pH 8.0) and analyzed by 0.8% agarose gel electrophoresis.

Histological Analysis of Mid-Gut

The isolated mid-guts were fixed overnight in Bouin's fixative (saturated picric acid: formaldehyde: glacial acetic acid; 15:5:1), processed through a series of increasing ethanol concentrations, cleared in xylene and embedded in molten paraplast (Sigma-Aldrich, USA). The tissues were sectioned (∼5 μm) using a rotatory microtome (Leica Microsystems, Germany) and stained with haematoxylin-eosin or toluidine blue (Sousa et al., 2010), visualized using Olympus IX-81 microscope (Olympus Corporation, Japan), and photographed.

Mid-Gut Cell Proliferation Assay

Streptavidin-biotin based BrdU staining (Invitrogen Corporation, USA) was used for monitoring the cell proliferation in the mid-gut during toxin-induced damage following manufacturer's protocol. Larvae were injected with BrdU labeling reagent (10 μl/g body weight) 4 h prior to isolation of toxin exposed larval mid-gut using a Hamilton syringe. Dissected mid-guts were fixed and processed as mentioned in the previous Section Histological Analysis of Mid-Gut, tissue sections were spread on poly-L-lysine coated slides (Sigma-Aldrich, USA) and incubated overnight at 37°C following which deparaffinization was carried out using xylene and passed through a series of decreasing ethanol concentrations. Peroxidase quenching was performed using 30% H_2O_2 and absolute methanol (1:9). Denaturation and blocking steps were carried out using the kit based reagent prior to antibody incubation. Biotinylated mouse anti-BrdU was overlaid and the slides were incubated at room temperature (RT). After incubation, streptavidin peroxidase (Invitrogen Corporation, USA) was added to detect the incorporation. Color development was monitored after the addition of secondary antibody mixture provided by the manufacturer. Slides were counterstained and mounted using histomount™ (Invitrogen Corporation, USA) and visualized under Olympus IX-81 microscope.

In situ Localization of Arylphorin mRNA

For the preparation of arylphorin cRNA probe, a 160 bp fragment of *A. janata* arylphorin gene was positioned as forward locked in pGEM-T Easy Vector (Promega Corporation, USA). The vector was linearized with EcoRI and NdeI restriction enzymes (New England Biolabs®, UK). Sp6 promoter was used for the synthesis of antisense probe (positive), while T7 promoter was used for the synthesis of sense probe (negative). Further, RNA probe synthesis was carried out using DIG RNA labeling kit (Roche Diagnostics, Germany). Larval mid-gut and fat body were dissected out and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, USA) in 0.1 M PBS at 4°C overnight. Post-fixation, tissues were embedded in OCT compound medium (Leica Microsystems, Germany). Tissue cross sections were obtained using cryostat microtome (Leica CM1850, Leica Microsystems, Germany) and mounted on poly-L-lysine coated slides. The OCT compound was washed using PBST buffer and the sections were permeabilized using proteinase K (1 μg/ml). Once again 4% PFA was used for further tissue fixation and sections were incubated with 200 μl hybridization buffer (50% formamide, 5X SSC, 5 mM EDTA, 0.1% CHAPS, 1% SDS, 50 μg/ml yeast tRNA, 50 μg/ml heparin) for 1 h at 50°C. The digoxigenin labeled cRNA probe was mixed with 200 μl of hybridization buffer, heat denatured at 80°C for 5 min, draped and covered with parafilm and incubated at 50°C in a sterile incubator overnight. Post-hybridization, the slides were washed four times at 50°C (5 min each) with a wash buffer (SSC, 50% formamide, 0.1% Tween 20) in a series of decreasing SSC concentration. The slides were then processed with solution A (0.5 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, 0.1% Tween 20, pH 8.0) and incubated at RT for 5 min followed by incubation with 20 μg/ml of RNase A for 20 min at RT. Later,

the slides were again washed with solution A twice (5 min each) at RT.

For the antibody reaction, the slides were initially washed with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 5 min at RT followed by blocking with 10% BSA for 1.5 h at RT. Anti-digoxigenin-ALP (Roche Diagnostics, Germany) was used for the detection of digoxigenin probe (1:2,000) and incubated overnight at 4°C. These slides were then washed with DIG wash buffer (Roche Diagnostics, Germany) for 4 times (15 min each). Incubation with 100 µl BCIP-NBT solution (G-Bioscience, USA) for 10 h at RT was carried out to develop the color. After color development, the slides were overlaid with detection buffer (Roche Diagnostics, Germany), counterstained with nuclear red (Vector Laboratories Inc., USA), and incubated in TE buffer (Ambion, Life Technologies, USA) for 5 min. After washing with PBST buffer, the slides were processed with increasing series of ethanol, mounted using DPX mountant, and visualized under Olympus IX-81 microscope.

Arylphorin mRNA Expression Analysis

Total RNA was isolated from the tissue (100 mg) using TRIzol method® (Ambion, Life Technologies, USA). The quantification of the RNA was carried out using NanoDrop spectrophotometer (ND-2000; NanoDrop Technologies, USA). Quality and integrity of RNA were assessed by formaldehyde denaturing gel electrophoresis. cDNA was synthesized using Revert Aid First Stand cDNA synthesis kit (Thermo Scientific, USA). For quantitative PCR analysis, a custom-made TaqMan gene expression assay was performed (Applied Biosystems, USA). Arylphorin primers of amplicon size 160 bp (Fwd: 5′-GTT AGA GAA GGG CCA GGT GTC TCA TG-3′; Rev: 5′- CTC TGG CTT GGT AGA CTC CTT GGT-3′) were designed from the arylphorin nucleotide sequence (KX462992). Insect rS7 gene (KF984201) was used as an internal control using primers (Fwd: 5′-ACG TGG ACG GTT CAC AAC TCA TCA-3′; Rev: 5′-TTC GCG GCC TGT TAG CTT CTT GTA-3′) which gave rise amplicon of 115 bp, to normalize the transcript expression levels. The real-time expression analysis was performed in triplicates. From the exponential phase of PCR amplification Cycle threshold (Ct) values were obtained. Arylphorin expression was normalized against the expression of rS7 rRNA to generate a ΔCt value (Ct of Arylphorin—Ct of rS7 rRNA). Comparative Ct method was used to calculate the change in the gene expression (Livak and Schmittgen, 2001).

Statistical Analysis

Presented data was calculated using standard error of the mean (SEM) ($n = 3$) which passed homogeneity and normality tests. Statistical significance was verified using the SigmaPlot v12.3 software (SystatSoftware Inc., USA). Significance between groups was tested using One-Way ANOVA followed by Student-Newman-Keuls (SNK) test for pairwise multiple comparison analysis and the p-value was calculated for each set of experiments.

RESULTS

Cry Toxin Alters Larval Growth and Development

The larvae that fed on toxin-coated leaves exhibited reduced growth in a time-dependent manner (24–72 h; **Figure 1A**). The 24 h toxin-exposed larvae could recover and grow when transferred to normal leaves (**Figure 1B**), whereas the 48 and 72 h toxin-fed groups often showed slow and poor recovery. Further, the body weight of the toxin exposed larvae was fairly low and were only 50% of that of the age matched control groups after 72 h of recovery, when the larvae were exposed to toxin for 48 and 72 h (**Figure 1B**).

Mid-Gut Necrosis Occurs in Toxin-Exposed Larvae

Confocal analysis revealed the presence of PI positive cells in toxin exposed larvae but not in controls (**Figure 2A**). Mid-gut cell suspension prepared from toxin exposed insects showed a distinct shift into the upper left quadrant (Q1) representing necrotic type of cell death (**Figure 2B**). Further, the cells undergoing necrosis have increased gradually from 12 to 36 h upon toxin-exposure. However, it was noteworthy that the number of necrotic cells reduced drastically in 48 and 72 h toxin-exposed samples (**Figure 2B**).

The result from DNA fragmentation assay revealed that the DNA integrity was preserved in both control and toxin-exposed mid-gut samples and the absence of DNA fragmentation in all the samples rules out the possibility of apoptotic form of cell death (**Figure 2C**). Microscopic, FACS and DNA fragmentation analyses indicated that necrosis was the most likely reason for cell death upon Cry intoxication.

Sub-Lethal Toxin Exposure Induces Mid-Gut Cell Proliferation

The results presented in **Figure 3** show few pockets of BrdU-positive cells (brown stained nuclei) were identified in the mid-gut collected from toxin exposed larvae (**Figure 3A**). The dividing cells which were mostly observed in the basal region of the gut epithelium could be ascribed as mid-gut stem cells (**Figure 3B**, inset).

Mid-Gut Cell Death is Associated with Regeneration

Figure 4 shows the histological changes seen in toxin exposed larval mid-gut from 12 to 72 h. The mid-gut cells showed degeneration with a condensed nucleus and fairly low cytoplasmic content. In addition, a large number of vacuoles were seen in them. Maximum cell death was observed between 24 and 36 h (**Figures 4C,D**). However, it was interesting to note that active cell proliferation as depicted by toluidine blue was seen extensively in 36 h Cry toxin exposed larvae (**Figures 4C,D**). BrdU labeling, histological and toluidine blue staining studies in parallel confirmed that not only cell death but also regeneration occurred in larval mid-gut upon Cry intoxication in *A. janata.*

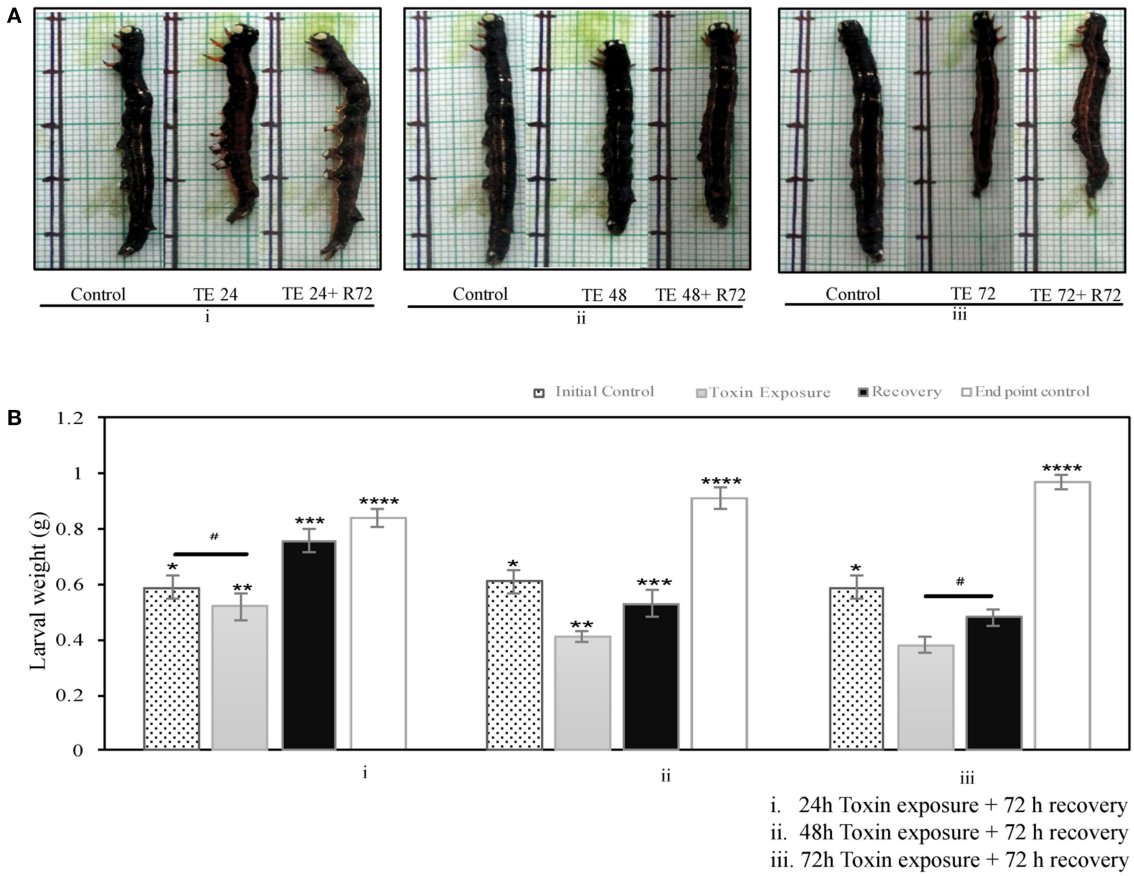


FIGURE 1 | *A. janata,* third instar larvae were maintained either on Cry toxin coated or normal leaves for different time intervals- **(A)** Growth pattern seen in **(i)** 24 h, **(ii)** 48 h, and **(iii)** 72 h after Cry toxin exposure (TE), with respective controls, for recovery toxin exposed larvae were transferred to normal leaves for 72 h (R72), **(B)** Effects of Cry toxin exposure for **(i)** 24 h, **(ii)** 48 h, and **(iii)** 72 h on larval weight, and after 72 h of recovery. The values presented are the mean ± standard deviation of three independent experiments ($n = 3$), significance between groups was tested by One-Way ANOVA followed by Student-Newman-Keuls (SNK) test using SigmaPlot 12.3 software. Means marked with $^{*}p < 0.05$ are significant with respect to toxin exposed, recovered and age matched end point control larvae,$^{**}p < 0.05$ indicates statistical significance with respect to recovered, and age matched end point control, $^{***}p < 0.05$ indicates statistical significance with respect to initial control and toxin exposed larvae, $^{****}p < 0.05$ indicates statistical significance with respect to the weight of initial control, toxin exposed, and recovered larvae, while # represents difference between compared group is not statistically significant.

Expression of Arylphorin in the Mid-Gut

Detectable but low expression of arylphorin in the mid-gut as compared to the fat body was observed using qRT-PCR analysis (**Figure 5A**). In accordance to this, *in situ* hybridization results revealed the localization of arylphorin transcript in the gut epithelium primarily in the basal cells (**Figure 5B**).

Cry Toxin Exposed Larval Mid-Gut Displays Differential Expression of Arylphorin

In control insects, arylphorin transcript level was fairly low throughout the time points monitored, but a steep incline was observed at 72 h (**Figure 6**). On the other hand, arylphorin expression gradually increased from 6 h and reached maximum at 36 h in Cry intoxicated larvae, thereafter the mRNA levels declined significantly in 48 h and remained low till 72 h.

DISCUSSION

In the present study, the effects of a sub-lethal dose of *Bt* toxin was analyzed through the mid-gut responses in *A. janata* larvae. As observed in this pest and other reported lepidopteran models, *Bt* exposed larvae usually have fairly low digestive activity concomitant with their reduced body weight associated with altered growth and development (Hernández-Martinez et al., 2010). DOR *Bt*-1 toxin administration triggered significant changes in the gut epithelium including nuclear condensation, vacuolization, low cytoplasmic content, and loosening of cell followed by necrosis. These observations corroborated well with an earlier study (Budatha et al., 2007) and suggested that even in *A. janata*, the binding of Cry to mid-gut cell receptors promote cell death. However, there is increasing evidence that Cry toxin induced cell death is a fairly complex cellular response. Zhang et al. (2006) using cabbage looper ovarian cell culture

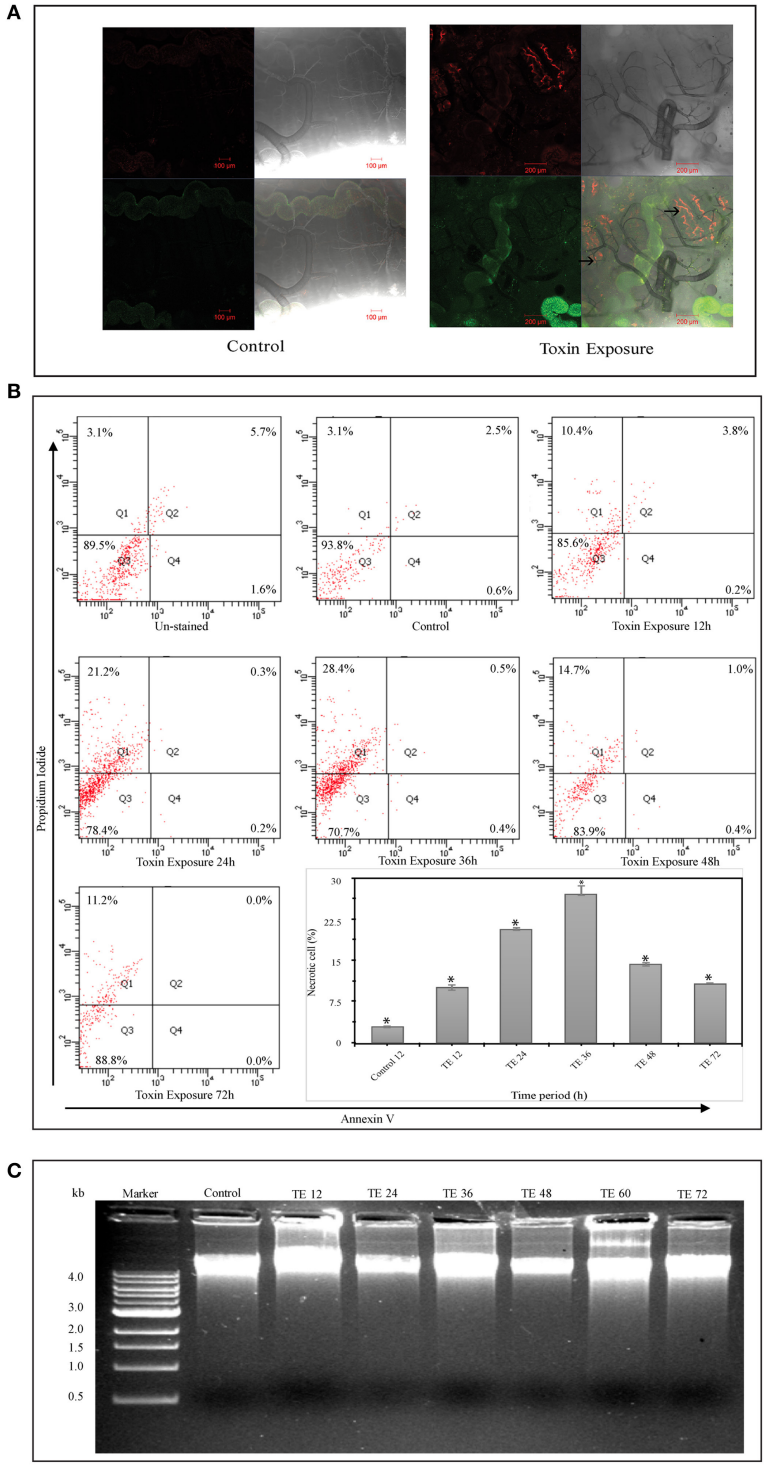


FIGURE 2 | Cell death analysis using Annexin V and Propidium iodide (PI) staining and DNA fragmentation assay—**(A)** Whole mount of control and Cry toxin exposed larval mid-gut, stained with Annexin V and Propidium iodide (PI). Note the presence of PI^+ positive cells in the mid-gut epithelium after toxin exposure (→). **(B)** FACS analysis, where scatter plot is divided into four quadrants (Q1–Q4), which give the total cell counts. Q1 represents PI^+/Annexin V^- cells (necrotic), Q2 represents PI^+/Annexin V^+ cells (late apoptotic/necrotic), Q3 represents the viable cells and Q4 represents Annexin V^+/PI^- cells (early apoptotic). Scatter plots showing the distribution of Annexin V and PI stained cells in mid-gut cell suspension prepared from control and Cry toxin exposed larvae. The cell suspension prepared from experimental larvae shows a distinct shift during 24 and 36 h of exposure into the top left quadrant (Q1). Inset shows the percentage of necrotic cells in mid-gut of toxin-exposed larvae at different points, $^*p < 0.005$ significance between experimental groups, **(C)** DNA fragmentation analysis of mid-gut shows the presence of intact band in agarose gel in both control and toxin exposed larvae after different time points.

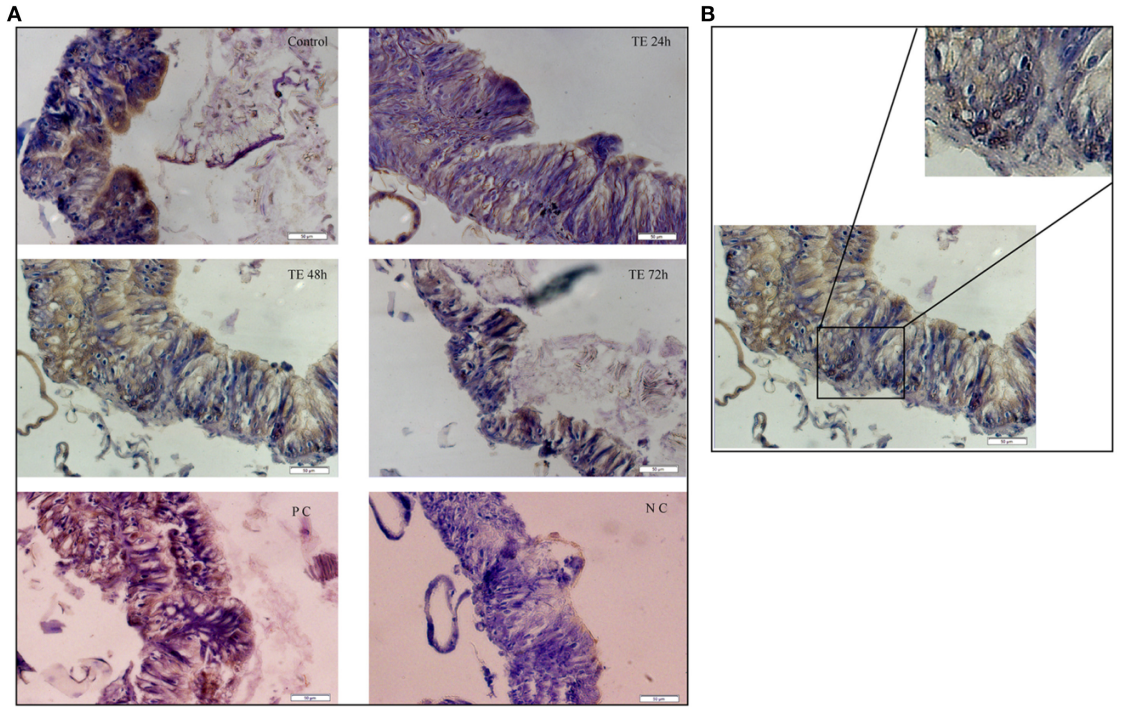


FIGURE 3 | Mid-gut cell proliferation monitored by bromodeoxyuridine (BrdU) incorporation—**(A)** T.S. of mid-gut from Cry toxin exposed 24, 48, and 72 h of larvae showing BrdU positive cells. Dextran sulfate sodium injected larvae were used as positive control (PC) and phosphate buffered saline injected larvae were used a negative control (NC). Note the presence of dividing cells with the brown stained nucleus. **(B)** Enlarged portion of mid-gut epithelium from 48 h toxin exposed larvae showing actively dividing cells in the crypt.

demonstrated that Cry1Ab toxin manifests its effect through Mg^{2+}dependent adenylyl cyclase/protein kinase A signaling pathway. Present microscopic and FACS analyses in *A. janata* showed Cry toxin induced necrotic type of cell death in larval mid-gut under *in-vivo* condition without DNA fragmentation (a hallmark of apoptosis). Recently, using toxin-host interaction system in *Caenorhabditis elegans*, Cry6Aa was shown to promote cell death through necrotic signaling pathway (Zhang et al., 2016). In addition, many bacterial toxins such as β-toxin from *Clostridium perfringens*, delta-toxin from *Clostridium septicum*, and VacA from *Helicobacter pylori* were shown to cause cell necrosis (Knapp et al., 2010; Radin et al., 2011; Sridharan and Upton, 2014; Seike et al., 2016). Taken together, the results obtained in the present study confirm that exposure to sub-lethal doses of Cry toxin triggers cell death in larval mid-gut of *A. janata* through necrosis.

Inspite of mid-gut cell death, larva did survive the exposure to sub-lethal doses of *Bt* toxin. Replenishment of injured cells is facilitated by normal feeding leading to recovery and metamorphosis (Spies and Spence, 1985; Loeb et al., 2001), while high doses of *Bt* toxin are known to kill insect pests (Tabashnik et al., 2013). Like many other tissues, renewal of epithelium is carried out by regenerative cells localized in the basal crypts, termed as mid-gut stem cells. These are the only cell present in gut capable of division and thus represent the sole source of new cells during tissue repair and growth. Continuous replacement of damaged cells by regenerative cells under toxin environment is likely to cause an alteration in cellular properties, leading to the development of resistance in insects against Cry formulations (Martinez-Ramirez et al., 1999; Castagnola and Jurat-Fuentes, 2016). Majority of cases of resistance against Cry toxins, reported reduction in binding of *Bt* toxins to their specific mid-gut receptors, i.e., cadherins, aminopeptidases, and alkaline phosphatases, which resulted in cross-resistance to Cry toxins sharing common altered receptor site (Morin et al., 2003; Pigott and Ellar, 2007; Zhang et al., 2009). However, Cry toxin resistance involving alterations in toxin processing by mid-gut secretion was also reported (Oppert et al., 1997; Caccia et al., 2012).

A number of growth factors promoting mid-gut cell culture regeneration have been reported, which include fetal bovine serum, ecdysteroids (Loeb, 2010), mammalian growth-promoting factor (Nishino and Mitsuhashi, 1995), alpha-arylphorin, MDFs (Blackburn et al., 2004; Hakim et al., 2010), and AlbuMAX II (Castagnola et al., 2011). The proliferation and differentiation of mid-gut epithelial cell seen upon Cry intoxication in *A. janata* might have been triggered and modulated by similar factors. Hexamerin proteins, particularly exogenous arylphorin (fat body synthesized arylphorin) were shown to promote proliferation of mid-gut cells in culture (Hakim et al., 2007). Mid-gut transcriptome profile showed up-regulation of arylphorin transcripts in the Xentari™ resistant *Spodoptera exigua* larvae (Hernández-Martinez et al., 2010).

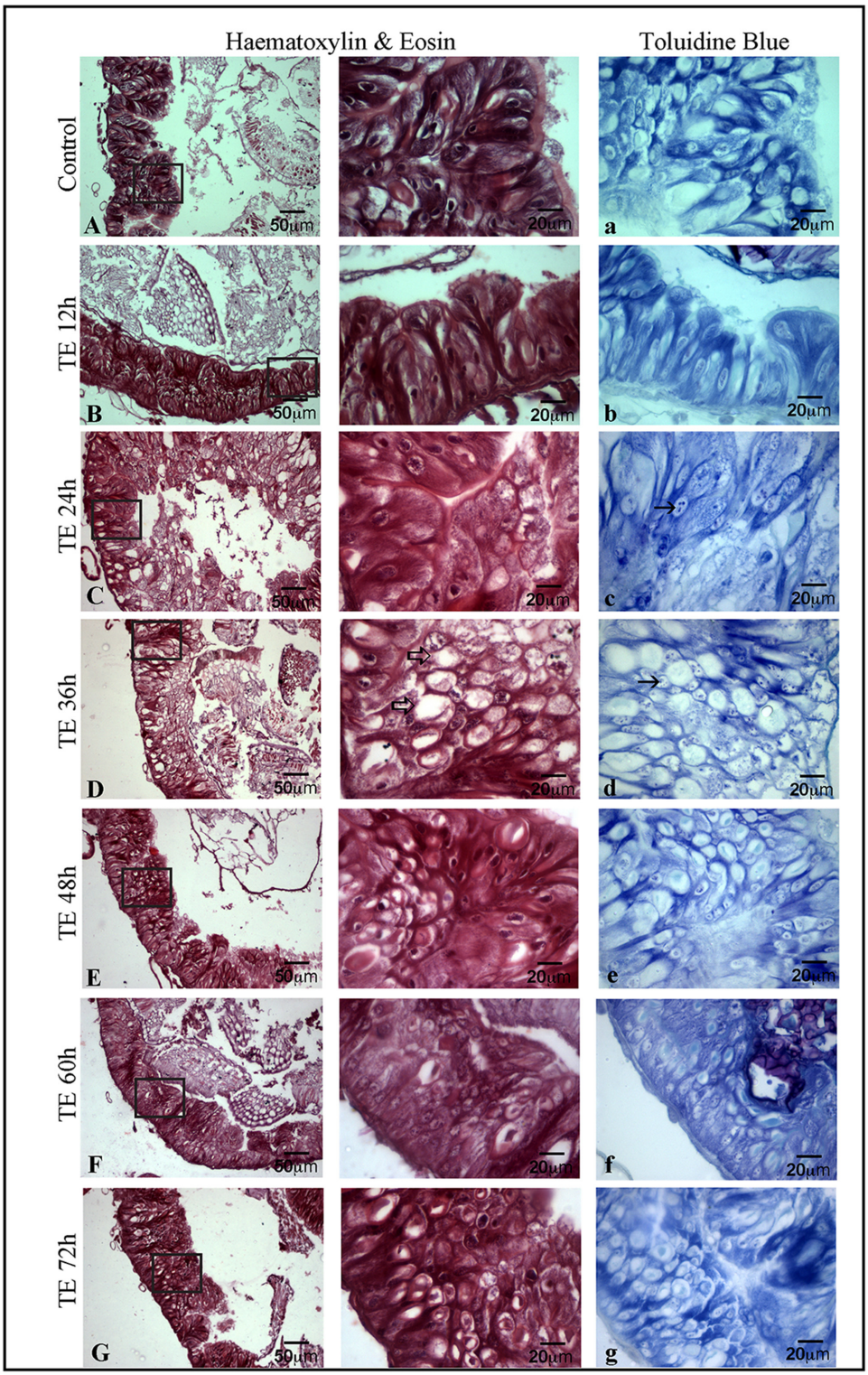


FIGURE 4 | Occurrence of cell death and proliferation in mid-gut demonstrated by haematoxylin-eosin (H & E) and toluidine blue staining, T.S. of mid-gut shows extensive damage in epithelium **(C)** and the presence of a large number of vacuoles (⟹) **(D)** after toxin exposure. T.S. of mid-gut stained with toluidine blue **(A–G)** showing the proliferative cells (→) **(C–D)**. (Scale bars 20, 50 μm).

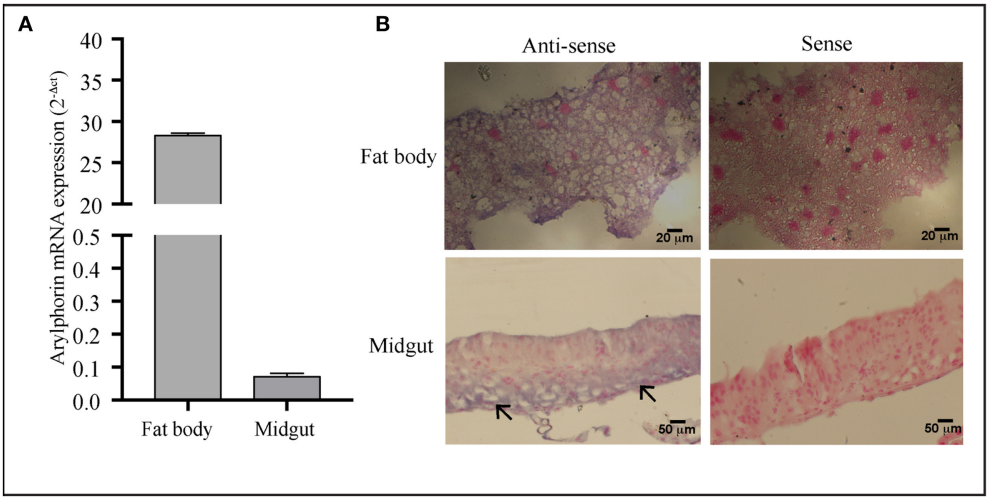


FIGURE 5 | Arylphorin gene expression—**(A)** qPCR analysis showing expression of arylphorin mRNA in the fat body and mid-gut of 3rd instar larvae, **(B)** *In situ* hybridization demonstrated the localization of arylphorin mRNA in the fat body and mid-gut epithelial cells (→) which are primarily observed toward the basal border (Scale bars 20, 50 µm).

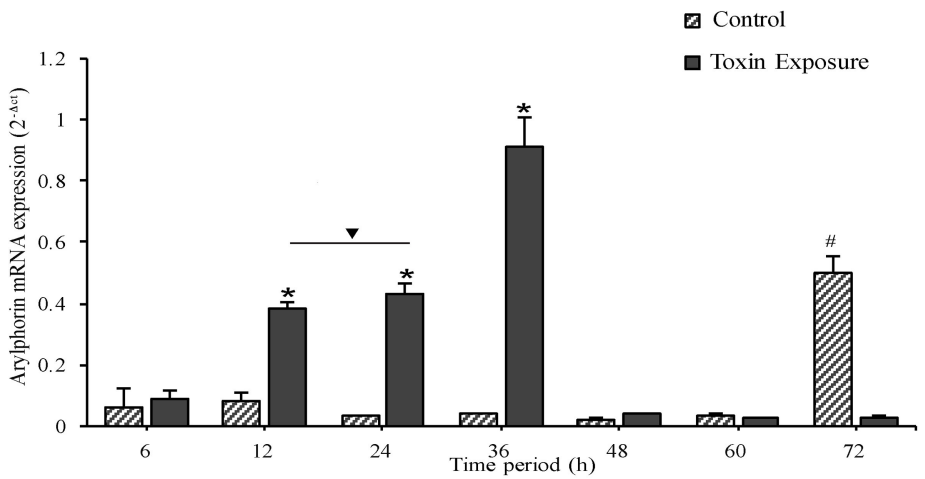


FIGURE 6 | Expression of mid-gut arylphorin mRNA during toxin exposure, A gradual increase was observed from 6 to 36 h of Cry intoxication exposure which declines later to a basal level. Note the higher expression of arylphorin transcript in 72 h control larvae, a period which coincides with molting (from 3rd to 4th larval instar). *$p < 0.005$ indicates significance between experimental groups, #$p < 0.005$ significant among control groups, ∇ the values are non-significant.

The transcriptome analysis with *A. janata* larval mid-gut also revealed up-regulation of arylphorin upon Cry intoxication (Unpublished data). In addition, in the present study *in situ* hybridization provided direct evidence for the localization of arylphorin transcripts in the actively dividing cells of mid-gut, which were primarily confined in the basal region of the epithelium. Expression analysis using Cry intoxicated larva mid-gut further showed time dependent increase in endogenous arylphorin gene expression during active cell proliferation, which was not only seen upon Cry intoxication but also during molting in control larvae where regeneration has been well-documented (Webb and Riddiford, 1988; Kawaguchi et al., 1993).

The present study, not only demonstrated cell death and damage of mid-gut epithelium upon Cry intoxication but also proliferation of stem/regenerative cells that allow the larvae to repair its gut epithelium and generate differentiated cells to facilitate their survival. This is further substantiated by the presence of a large number of BrdU positive cells in the basal crypts of the gut epithelium, while toludine blue staining reveals a sizable increase in the number of proliferative cell upon Cry intoxication in time dependent manner. Given the versatility of arylphorin, its presence and altered expression in the mid-gut during Cry toxin exposure, it is possible that a low concentration of arylphorin

produced endogenously in mid-gut is critical and probably inculcates mitogenic effect during the Cry intoxication. Further characterization of the gut regenerative process in the future will help in obtaining a better understanding of the resistance mechanism.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AD, VC, and ND; Performance of the experiments: VC and ND; Analysis of the data: AD, VC, ND, and RC; Suggestion and help for *in-situ* analysis: BS; Manuscript writing: VC and ND; Manuscript editing: AD, BS, and RC.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Insecticidal Effects of Hemocoelic Delivery of *Bacillus thuringiensis* Cry Toxins in *Achaea janata* Larvae

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Insecticidal effects of *Bacillus thuringiensis* Cry toxins in hemocoel of larvae have not been properly evaluated. In the present study, hemocoelic injection of four representative Cry toxins i.e., Cry1Aa, Cry1Ab, Cry1Ac, and DOR5 to an economically important lepidopteran insect pest *Achaea janata*, induced larval mortality, reduced larval growth rate and gave rise to smaller pupae, all in a dose-dependent manner. We observed extensive degeneration as well as the disintegration of larval tissues, most notably, fat body, and the possible involvement of lysosomal enzymes in tissue histolysis. The resultant "hypoproteinemia" and most relevantly, the drastic reduction of 80–85 kDa hexamerin proteins levels of hemolymph could be attributed to the pathological state of the fat body induced by Cry toxin injection. Formation of non-viable larval-pupal intermediates and emergence of defective adults also indicate toxicity effects of Cry toxins during metamorphosis. Thus, findings from our study suggest Cry toxins in larval hemocoel are also toxic to *A. janata* larval survival and subsequent development.

Keywords: ***Achaea janata*, *Bacillus thuringiensis*, cry toxins, fat body, hemocoelic injection**

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INTRODUCTION

Insecticidal proteins called crystal (Cry) toxins, produced as crystal inclusions by gram-positive bacteria *Bacillus thuringiensis* (Bt), are toxic to a wide range of insects including those belonging to the order Lepidoptera (Bravo et al., 2011). When susceptible insects ingest the Cry toxins, they are solubilized in the alkaline medium of the gut, processed by the gut proteases to yield the active toxins which then bind to specific receptors located on the brush-border membranes of the larval midgut epithelial cells, followed by insertion into the cell membrane, leading to pore formation, then cell lysis, and eventual death of the insects (Ferré and Van Rie, 2002; Bravo et al., 2007). Development of Bt-based sprayable products and Bt transgenic crops have provided valuable alternatives to chemical insecticides as they are more insect species-specific and least harmful to the natural enemies (James, 2009; Bravo et al., 2011). However, as a consequence of the uncontrolled widespread use of Bt-based biopesticides in agriculture, a number of insect species including those belonging to Lepidoptera have developed resistance to Bt Cry toxins (Ferré and Van Rie, 2002; Tabashnik et al., 2005, 2013). Further, laboratory-selected resistance experiments have indicated the potential of many species to still evolve resistance against Cry toxins (Bravo et al., 2011). As an

Abbreviations: 5E, early fifth instar; 5L, late fifth instar; ACP, acid phosphatase; ALP, alkaline phosphatase; APN, aminopeptidase N; BCIP, 5-bromo-4-chloro-3′-indolyl phosphate; BSA, bovine serum albumin; Bt, *Bacillus thuringiensis*; GPI, glycosylphosphatidyl-inositol; IgG, immunoglobulin G; NBT, nitro blue tetrazolium chloride; PP, pre-pupa; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

effort to tackle this problem, many workers have been continuously working to understand various aspects of modes of action and mechanisms of pest's resistance to Cry toxins after oral ingestion (Bravo and Soberón, 2008; Pardo-López et al., 2009), but no effective control strategy has been developed to date.

Earlier studies have suggested a possible mechanism where Cry toxins ingested along with the feed can also penetrate from gut through the gut wall to hemolymph in the insect hemocoel, which accumulate in the insect body and suppress the reproductive potential (Arpaia et al., 2000; Hussein et al., 2006). The fat body of lepidopteran larvae is the principal metabolic center, synthesizes, and releases a large number of haemolymph proteins including hexamerins, which play an important role during the pupal-adult transformation (Kanost et al., 1990). A recent report in *Helicoverpa armigera* further reveals accumulation of toxin in fat body and hemolymph of larvae fed with artificial diet containing high dosage of Cry1Ac (Zhao et al., 2016). Hemocoelic injection of Cry1 toxins into *Lymantria dispar* (Lepidoptera) and *Neobellieria bullata* (Diptera) larvae was found to illicit various toxic responses (Cerstians et al., 2001). However, proper evaluation of insecticidal effects of Cry toxins in hemocoelic visceral organs of insects has been lacking. Hence, the present study was undertaken to generate useful information on various aspects of toxicity induced by Cry toxins in the larvae of an economically important lepidopteran pest *Achaea janata*. Among various insect pests of castor, *A. janata* is a major pest in Indian subcontinent, whose larvae feed on castor foliage leading to serious crop loss. Hemocoelic injection of four representative Cry toxins i.e., Cry1Aa, Cry1Ab, Cry1Ac, and DOR5 to *A. janata* larvae, induced mortality, reduced growth rate and gave rise to smaller pupae, all in a dose-dependent manner. We also observed defective metamorphosis during larval-pupal and pupal-adult transformation. As we earlier reported the presence of Cry toxin binding protein (Aminopeptidase-N) in larval fat body of *A. janata* (Budatha et al., 2007), an attempt was also made to evaluate the effect of Cry toxin on the haemolymph protein profile. The study suggests Cry toxins in hemocoel are insecticidal to larval forms of *A. janata*, have deleterious effects on larval survival, growth, and subsequent development.

MATERIALS AND METHODS

Insect Culture

Achaea janata egg masses collected from castor fields of Indian Institute of Oilseeds Research, Hyderabad were allowed to hatch into neonate larvae which were then reared on fresh castor leaves (*Ricinus communis*) as diet, at 27 ± 2°C in the insect culture facility under a photoperiod of 14:10 h (light:dark) and 60–70% relative humidity. The larval development proceeds through five instars and the whole life cycle is completed in about 35–40 days. Each instar from first to fourth lasts for about 2 days while the final or fifth instar lasts 4–5 days. The fifth instar is further classified into early (5E) and late (5L) fifth instar which lasts 2 and 2–3 days, respectively, then followed by non-feeding pre-pupal (PP) stage.

Feeding Bioassay

Feeding bioassay was performed on castor leave discs. Both sides of the leave discs were painted with Cry toxins, shade dried and placed onto moist cotton in petriplates. Seven treatment concentrations i.e., 2, 4, 6, 8, 10, 12, and 14 ng/cm^2 and one control [carrier solvent coated; insect Ringer containing 1 mM of Tris-HCl (pH 7.0)] were selected. Triplicates were maintained for each treatment and twenty 2 days old *A. janata* larvae were released on each leaf disc. Mortality was recorded every 24 h interval for 5 days and LC_{50} was calculated with 2 days post-treatment data using probit analysis with the help of Finney's table (Finney, 1952).

Collection of Larval Tissues

Larvae were narcotized on ice and tissues were dissected out in ice-cold insect Ringer solution (130 mM NaCl, 0.5 mM KCl, and 0.1 mM $CaCl_2$) and used immediately. A cut was made in the ventral region through full length of larvae, along with midgut, perivisceral fat body, Malpighian tubule, and salivary gland were dissected out. Further, experiments were carried out using these tissue.

Preparation of Activated Cry Toxins

The DOR5 Bt spores and crystals were obtained using the method described by Aronson et al. (1971). Spores and crystals were separated by differential ultracentrifugation on a discontinuous sucrose density gradient in a Beckman L8–80M ultracentrifuge with a SW 50.1 rotor operating at 42,000 rpm for 4 h at 4°C (Thomas and Ellar, 1983). On the other hand, Cry1Aa, Cry1Ab, and Cry1Ac protoxins were obtained from recombinant *Escherichia coli* JM103 strains ECE52, ECE53, and ECE54 harboring *cry1Aa*, *cry1Ab*, and *cry1Ac* genes respectively, which were supplied by *Bacillus* Genetic Stock Center (Ohio State University, USA). The activated toxins were prepared according to the method of Lee et al. (1992) and purified by gel filtration on Sephadex G-100 column.

Hemocoelic Cry Toxin Injection Assay

Third instar larvae were narcotized on ice for 15 min and were then injected individually with activated Cry1Aa, Cry1Ab, Cry1Ac, and DOR5 (Directorate of Oilseeds Research Strain 5) Cry toxin doses of 10, 50, 100, 150, and 200 ng/0.2 g body weight through the mid-dorsal region using a Hamilton microsyringe. Control larvae received the same volume of the carrier solvent. Immediately after injection, the wound was sealed with bee wax and the larvae were again placed on ice for 15 min before transferring them back to the rearing chamber provided with fresh castor leaves. A minimum of 20 larvae were/was used for each dose and the experiment was repeated three times independently. The body weight of larva was examined every 24 h for 5 days. Larval mortality was recorded 2 days post-injection (dpi). The weights of resultant pupae from toxin injected larvae and control were also recorded.

Western Blotting

Hemolymph from required larval stages were collected in centrifuge tubes precoated with 0.025% phenylthiourea in insect

Ringer solution by cutting the proleg and were then centrifuged at 1000 × g for 3 min at 4°C to sediment the hemocytes. The supernatant was diluted with 10 mM Tris-HCl (pH 7.8) and used immediately. Total protein content was estimated by Bradford's method (1976) using BSA as standard. The hemolymph proteins were resolved by 10% SDS-PAGE and electro-blotted onto a nitrocellulose membrane using Trans-Blot apparatus (Bio-Rad) according to the procedure of Towbin et al. (1979). Non-specific binding sites were blocked with 5% skimmed milk (w/v) in Tris-buffered saline (TBS) and then incubated with *A. janata* hexamerin polyclonal antibody (1:5,000 dilutions) (Budatha et al., 2011). Subsequently, the blots were incubated with ALP-conjugated goat anti-rabbit IgG and finally detected with NBT-BCIP substrate (Sigma-Aldrich).

Hematoxylin-Eosin (HE) Staining

Two days post-injection, various larval tissues were dissected out in ice-cold Ringer solution and then fixed in 4% paraformaldehyde. After washing with 0.1 M phosphate buffered saline (PBS, pH 7.4), the tissues were dehydrated in ethanol series and embedded in paraffin. Sections of 5 μm thickness were prepared using rotary microtome (Leica). The sections were deparafinized in xylene, rehydrated in ethanol series, followed by staining with hematoxylin (nuclear stain) and eosin (cytoplasmic stain). The sections were then dehydrated in ethanol series, cleared in xylene and mounted using DPX mountant.

Acid Phosphatase (ACP) Assay

Acid phosphatase assay is widely used as the lysosomal marker for the demonstration of tissue degeneration and remodeling in various insect models. The assay was carried out according to the method of Henrikson and Clever (1972) with minor modifications. The reaction mixture containing 100 mM sodium acetate buffer (pH 5.0) and protein preparation (100 μg) was incubated at 37°C for 10 min to exclude glucose-6-phosphatase activity. The reaction was initiated with the addition of 8 mM *p*-nitrophenyl bi-sodium phosphate followed by incubation for 1 h at 37°C. The reaction was terminated by adding 0.5 ml of 0.1 N NaOH. The yellow color thus developed was measured at 410 nm against a substrate blank. The *p*-nitrophenol (PNP) was used for the preparation of a standard curve. The activity of the enzyme was expressed as nanomoles of PNP released/h/μg protein. The assay was performed with 2 days post-injected larval tissues.

Phenotypic Analysis

This was primarily carried out to analyze the effect of Cry toxin on the gross morphology of the insect. Photographs were taken with a FinePix S9600 digital camera (Nikon). The wings of the insects were placed on aluminum stubs and coated with gold in a FullamEMS-76 m evaporator for 15 min and the wing scale morphology was examined using Jeol scanning electron microscope.

Statistical Analysis

Data are expressed as mean ± standard deviation of three independent experiments ($n = 3$). Differences between groups were analyzed for statistical significance by One-Way ANOVA followed by Students-Newman-Keuls (SNK) test using SigmaPlot 11.0 software. A probability of $p < 0.05$ is considered statistically significant.

RESULTS

Toxicity Effects of Cry Toxin Injection in *A. janata*

Cry1Aa, Cry1Ab, Cry1Ac, and DOR5 Cry protoxins and their subsequent activated forms were prepared and then purified by gel filtration using Sephadex G-100 column (**Supplementary Figure 1**). Lepidopteran-specific insecticidal gene profiling of DOR5 Bt isolate revealed the presence *cry1Aa*, *cry1Ab*, *cry1C*, *cry2A*, *cry2B*, and *vip* (vegetative insecticidal proteins) genes (unpublished data).

Feeding bioassay with 2 days old *A. janata* larvae showed that recombinant Cry toxins i.e., Cry1Aa, Cry1Ab, and Cry1Ac feeding causes larval mortality, However, DOR5 Cry toxins showed much higher insecticidal activity with fairly low LC_{50} (4.9 ng/cm^2 of leaf surface) (**Table 1**). Hemocoelic injection with doses of 200 ng/0.2 g body weight of Cry1Aa (**Figure 1A**), 150 ng/0.2 g body weight each of Cry1Ab (**Figure 1B**) and Cry1Ac (**Figure 1C**), and 100 ng/0.2 g body weight of DOR5 (**Figure 1D**) toxins resulted in 50–70% larval mortality. Further, increase in DOR 5 toxin dose to 150 ng/0.2 g body weight, caused nearly 100% larval mortality (**Figure 1D**). All the doses of Cry1Aa, Cry1Ab, Cry1Ac, and DOR5 Cry toxins reduced food consumption. The toxins inhibited larval growth in a dose-dependent manner. Results presented in **Figure 2** clearly show that Cry1Aa (**Figure 2A**), Cry1Ab (**Figure 2B**), and Cry1Ac (**Figure 2C**) at doses ≥100 ng/0.2 g body weight reduced larval growth and the mean body weight was fairly low after 5 days after injection. Further, DOR5 Cry toxin was found to be more effective than the recombinant toxins (**Figure 2D**). The control larvae reached a maximum body weight of around 0.8 g during fifth instar, while the toxin injected larvae weighed around 0.3 g or less, even at 5 days post-injection (number of days after which the larvae should be in fifth instar). Furthermore, injection of ≥100 ng/0.2 g body weight of Cry1Aa (**Figure 3A**), Cry1Ab (**Figure 3B**), and Cry1Ac (**Figure 3C**) and ≥50 ng/0.2 g body weight of DOR5 (**Figure 3D**) toxins caused the significant reduction in the weight of resultant pupae ($p < 0.05$). The weight of the control pupa reached 0.55 ± 0.013 g while the pupae obtained after injection weighed only around 0.38 ± 0.04 g (**Figure 3D**).

TABLE 1 | Evaluation of insecticidal activity of Cry toxins against *A. janata* larvae after oral ingestion [Details of experiment are provided in Section Cross-Compression Entropy (CCE)].

Cry toxin	Feeding bioassay LC_{50}(ng/cm^2 of leaf surface)
Cry 1Aa	107.56
Cry1Ab	47.83
Cry1Ac	35.87
DOR5	4.908

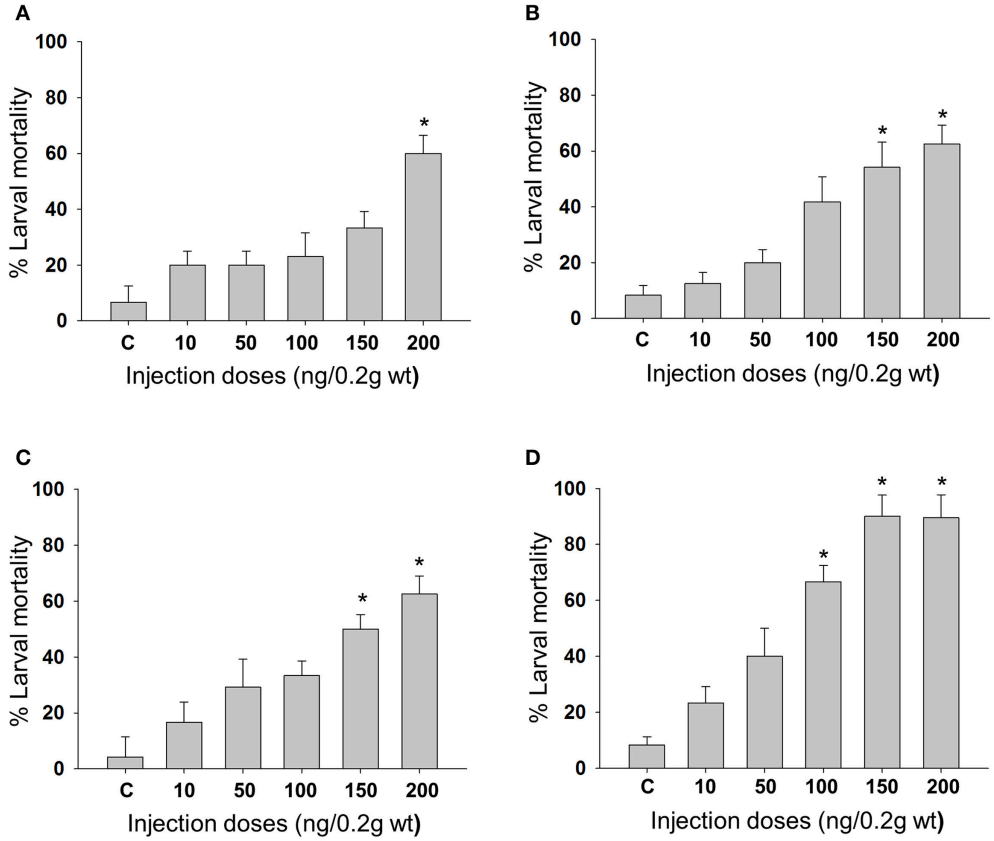


FIGURE 1 | Mortality of *A. janata* larvae upon Cry toxin injection. Third instar larvae were injected with Cry toxin doses of 10, 50, 100, 150, and 200 ng/0.2 g body weight. Percentage of larval mortality for each dose was calculated 2 days post-injection. **(A)** Cry1Aa; **(B)** Cry1Ab; **(C)** Cry1Ac; **(D)** DOR5. Values presented are the mean ± standard deviation of three independent experiments ($n = 3$). *represent ≥ 50% mortality.

Pathological Effects of Cry Toxin Injection

Observation of vital tissues of 2 days post-injected larvae revealed thin and delicately developed fat body tissue (**Figure 4**). The midgut of the toxin injected larvae had comparatively narrower lumen with lower food contents and the Malpighian tubules appeared thin and opaque (**Figure 4**). The size of salivary glands of the toxin injected insects was much smaller (**Figure 4**).

Hematoxylin and eosin (HE) staining of midgut tissue sections of Cry toxin injected larvae revealed the presence of irregular arrangement of Goblet (g) as well as columnar (c) cells in the gut epithelium (**Figure 5A**; Inj). In fat body, extensive tissue degeneration was seen (**Figure 5B**; Inj), viability/non-viability of fat body tissue from control and toxin injected larvae was confirmed using propidium iodide and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] staining (**Supplementary Figure 2**). We observed vacuoles (v) in the cytoplasmic regions of the single cell layered epithelium of Malpighian tubule with chromatin condensation in the nucleus (n) of these cells (**Figure 5C**; Inj). The salivary gland of the Cry toxin injected larvae did not show major structural differences but condensation of nuclear chromatin and reduced diameter of the gland with much narrow lumen was observed (**Figure 5D**; Inj). HE staining revealed fat body to be most affected by haemocoelic Cry toxin injection. Estimation of ACP activity in tissues of Cry toxin injected larvae showed a significant ($p < 0.05$) increase in the level of enzyme activity only in fat body and not in other tissues (**Figure 6**).

Effect of Cry Toxin Injection on Larval Hemolymph Hexamerin Profile

Upon injection of Cry toxins into third instar larvae, the increase in larval hemolymph total protein content (seen during normal larval development) was significantly inhibited ($p < 0.001$; **Table 2**). The total protein content of the 2 and 4 days post-injected control larvae reached 2.12 ± 0.06 and 2.3 ± 0.07 mg/mL hemolymph respectively, while the Cry toxin injected larvae of same chronological age showed only 0.3 ± 0.05 and 0.34 ± 0.04 mg/mL hemolymph respectively (**Table 2**). SDS-PAGE (**Figure 7A**) and western blot (**Figure 7B**) analyses revealed drastic reduction in 80–85 kDa hexamerin proteins level in hemolymph of the 2 (**Figures 7A,B**, Lane: 4) as well as 4 days post-injected (**Figures 7A,B**, Lane: 6) larvae. However, the hemolymph of 2 and 4 days post-injected control larvae showed fairly high amount of the 80–85 kDa hexamerin proteins (**Figures 7A,B**, Lanes: 3 and 5).

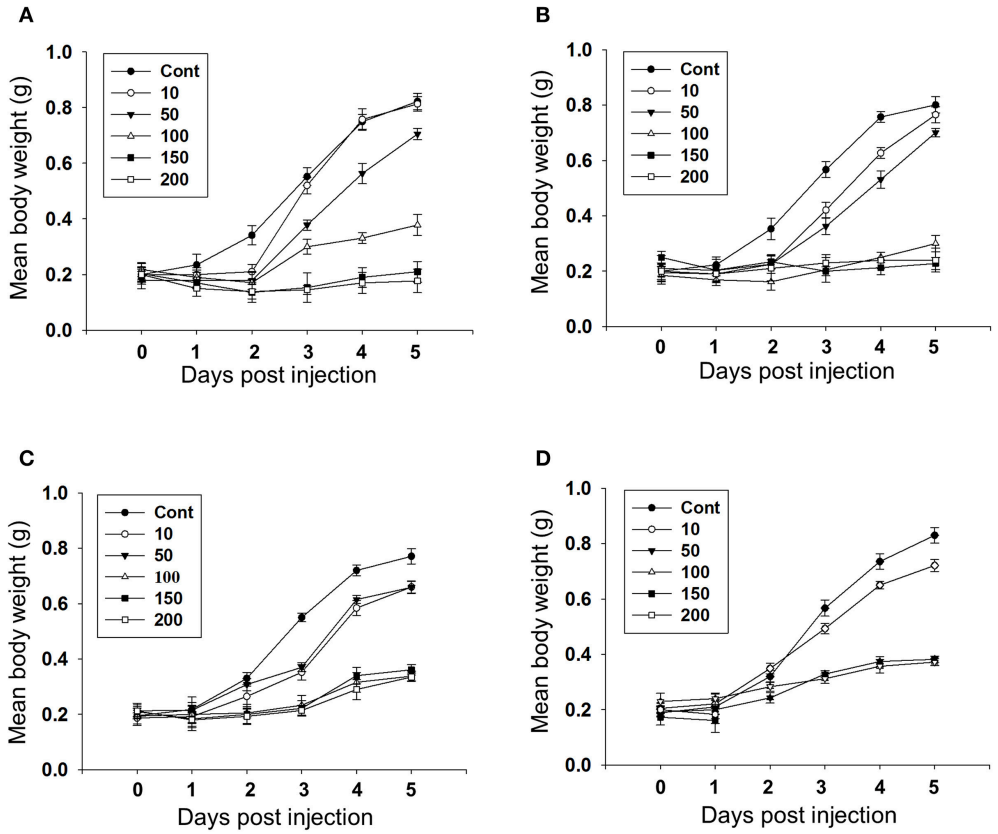


FIGURE 2 | Effect of Cry toxin injection on larval growth. Third instar larvae were injected with Cry toxin doses of 10, 50, 100, 150, and 200 ng/0.2 g body weight and the weight of larvae was measured every 24 h for 5 days. **(A)** Cry1Aa; **(B)** Cry1Ab; **(C)** Cry1Ac; **(D)** DOR5. Values presented are the mean ± standard deviation of three independent experiments ($n = 3$).

Effect of Cry Toxin Injection on Larval-Pupal and Pupal-Adult Transformation

Injection of Cry toxins to third instar larvae resulted in formation of 20% non-viable larval-pupal intermediate which died and could not molt into pupae (**Figure 8A**), while 9% of them emerged into defective but viable adults and 5% developed into phenotypically normal adults. The non-viable larval-pupal intermediates exhibited an "incomplete-ecdysis" phenotype. The defective adults that emerged from the toxin injected insects had fairly large but improperly folded and crumbled wings and as a result, the adults could not fly or even mate (**Figure 8B**, panel 1). On the other hand, only 5% of the control larvae died due to injury during the larval stages while 95% of the control larvae molted normally throughout the larval stages and emerged as normal adults (**Figure 8C**, panel 1). Scanning electron microscopic analysis of the wing surface morphology of the deformed adult revealed the presence of numerous piliform (hair-like) minute scales in addition to the usual lamellar (blade-like) form of wing scales and most of these lamellar form of scales showed irregular transverse and longitudinal ridges (**Figure 8B**, panels 2 and 3). On the other hand, the control insects showed the presence of only lamellar scales with smooth inferior lamella and proper transverse and longitudinal ridges (**Figure 8C**, panels 2 and 3).

DISCUSSION

Bt-based biopesticides, perhaps the most well-known and widely used biopesticides are now less effective as the number of agricultural insect pests have already developed resistance (Tabashnik et al., 2005; Bravo et al., 2011). Hence an approach that could promise more efficient and eco-friendly control of insect pests in agriculture has become more urgent. No organ or cell other than the midgut epithelial cell has been established as target organ for Cry toxin action in insect pests. However, reports do suggest interaction of Cry toxins with other tissues in the insect body cavity (Arpaia et al., 2000; Cerstians et al., 2001; Hussein et al., 2006). In the larval hemocoel, the major tissues or organs present include the fat body, Malpighian tubule, and salivary gland, where they all play important functions during post-embryonic development. In the present study, hemocoelic injection of four representative Cry toxins i.e., Cry1Aa, Cry1Ab, Cry1Ac, and DOR5 individually to third instar larvae of *A. janata* induced larval mortality, reduced larval growth and gave rise to smaller pupae in a dose-dependent manner, indicating

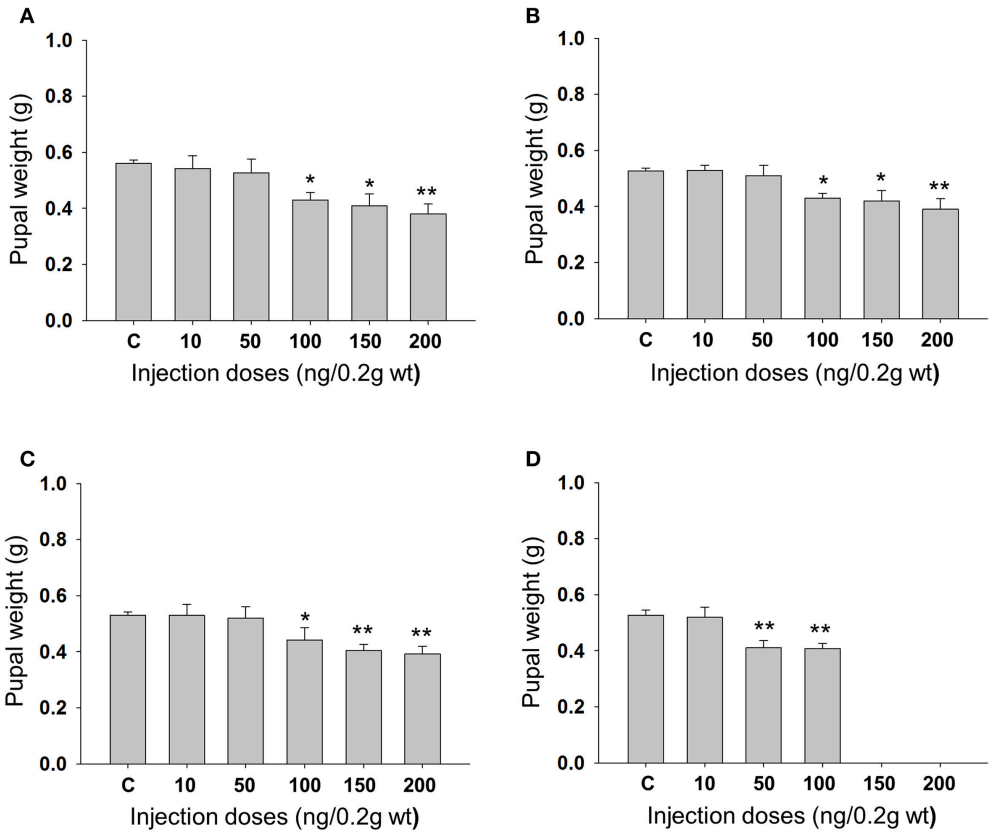


FIGURE 3 | Effect of larval Cry toxin injection on the pupal weight. Third instar larvae were injected with Cry toxin doses of 10, 50, 100, 150, and 200 ng/0.2 g body weight. **(A)** Cry1Aa; **(B)** Cry1Ab; **(C)** Cry1Ac; **(D)** DOR5, they were allowed to feed, grow, and molt into pupae. Values presented are mean ± standard deviation of three independent experiments ($n = 3$). Significance between groups was tested by One-Way ANOVA followed by Student-Newman-Keuls (SNK) test using SigmaPlot 11.0 software. Means marked with $^{*}p < 0.05$, $^{**}p < 0.001$ indicate statistical significance.

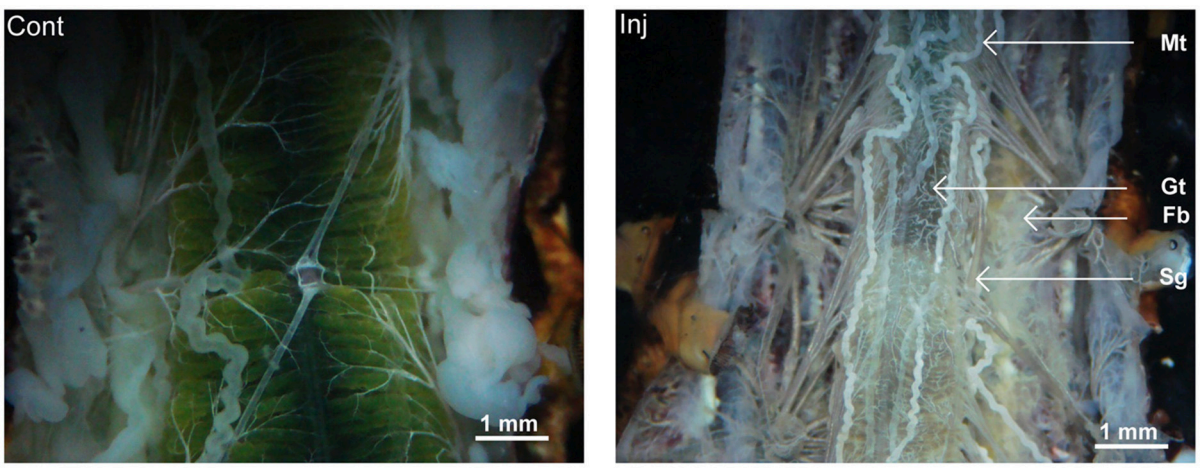


FIGURE 4 | Pathological condition of Cry toxin injected *A. janata* larvae. Two days post-injected larvae were dissected and morphological features of various visceral tissue was viewed. Note the differences between the morphology of the organs in control and Cry toxin injected (Inj, arrow), Gt, gut; Fb, fat body; Mt, Malpighian tubule; and Sg, salivary gland.

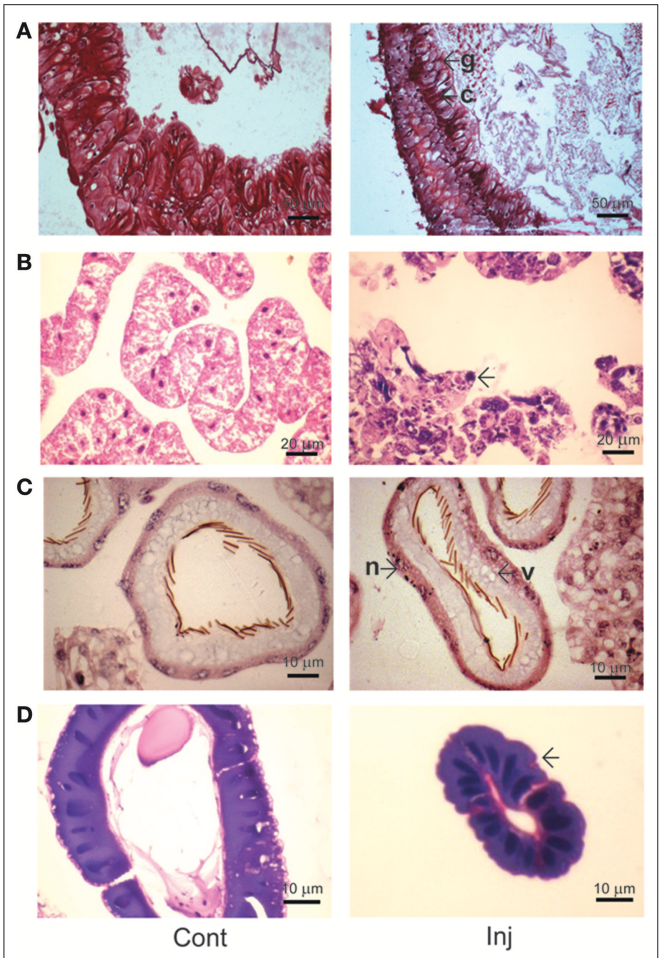


FIGURE 5 | Histological analysis of Cry toxin injected *A. janata* larval tissues. Tissue sections were prepared from 2 days post-injected larvae and stained with hematoxylin-eosin (HE). **(A)** midgut; **(B)** fat body; **(C)** Malpighian tubule; **(D)** salivary gland. Note the cytological changes in tissue sections induced by Cry toxin injection (Inj, arrowhead), g, Goblet cell; c, columnar cell; v, vacuole; n, nucleus.

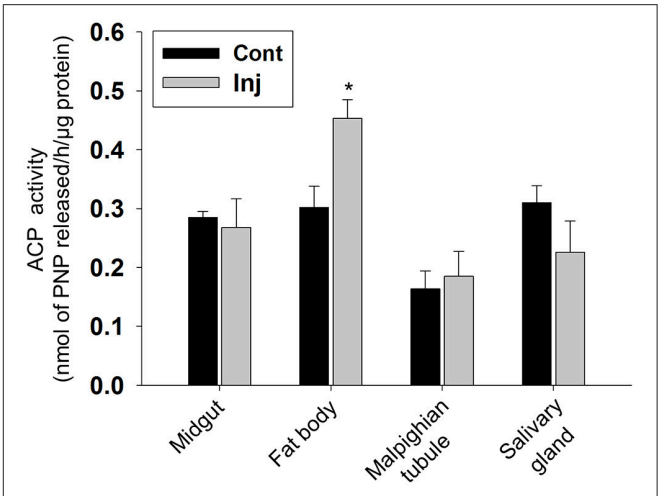


FIGURE 6 | Analysis of ACP activity in Cry toxin injected larval tissues. Assay was performed with 2 days post-injected larval tissues. The enzyme activity is expressed as nmol of PNP released/h/mg protein. Values are the mean ± standard deviation of three independent experiments ($n = 3$). *$p <$ 0.050 (One-Way ANOVA followed by SNK-test).

TABLE 2 | Effect of hemocoelic Cry toxin injection on larval hemolymph total protein content.

	2 dpi (4th instar) (±*SD*)	4 dpi (5th instar) (±*SD*)
Control	2.12 ± 0.06	2.3 ± 0.07
Injected	0.3 ± 0.05**	0.34 ± 0.04**

Total protein content expressed as mg/mL hemolymph.
***$p < 0.01$. Total protein content was analyzed 2 and 4 dpi of third instar larvae with Cry toxins. The results shown in the table represent DOR5 Cry toxin dose 100 ng/0.2 g body weight.*

a fairly high insecticidal activity. A dose-dependent increase in larval mortality rate as compared to the control larvae (carrier solvent-injected) clearly suggests that the mortality was the result of toxicity induced by Cry toxins rather than the effects of injury. Since larval mortality was analyzed just 2 days post-injection, we are also convinced that the larvae did not die of starvation, as larvae of this age can normally survive for up to 4–5 days without feeding. Hemocoelic injection to larvae younger than third instar was not possible as they are too small and delicate. DOR5 isolate, which contained *cry1Aa*, *cry1Ab*, *cry1C*, *cry2A*, *cry2B*, and *vip* genes obviously was more potent than individual recombinant Cry1Aa, Cry1Ab, and Cry1Ac toxins.

The presence of large cytoplasmic cavities in fat body tissue sections suggest degeneration and disintegration of the tissue. Till date, a majority of studies show cytotoxic activity of Cry toxins only against midgut cells of insects. With this study, we report the induction of cell death in fat body tissue of a lepidopteran insect by Cry toxins. In addition, the significant increase in the level of ACP activity primarily in the fat body and not in other tissues indicates involvement of lysosomes in Cry toxin-induced cell death of fat body. During post-embryonic development of holometabolous insects, programmed cell death of fat body follows two major pathways viz. apoptotic and autophagic cell death (Sumithra et al., 2009). During metamorphosis, acidic autophagic vacuoles accumulate in fat body cells and the activity of several lysosomal enzymes including ACP increase and cause the histolysis of larval organs (Ashok and Dutta-Gupta, 1988, 1991; Lee and Baehriecke, 2001). Therefore, it could be that cell death in fat body follows both the pathways during normal development as well as during Cry toxin intoxication, though it may be prematurely induced in the latter case.

The absence of significant change in the ACP activity level in midgut, Malpighian tubule, and salivary gland clearly rules out the involvement of apoptotic and autophagic modes of cell death in these tissues upon Cry toxin injection. However, disorganization of epithelial cells indicates loss of gut tissue structure and damage. This would affect the food storage and digestive roles of the gut and hence disturb the overall

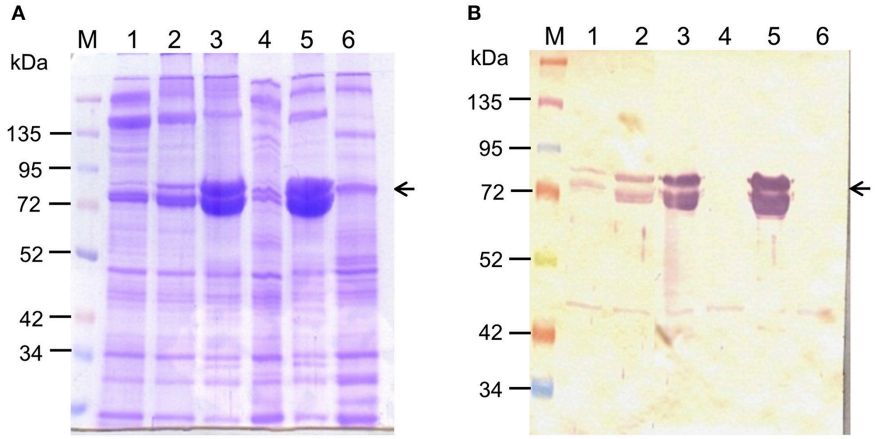


FIGURE 7 | Effect of Cry toxin injection on hemolymph hexamerin protein profile. Hemolymph hexamerin proteins profile was analyzed 2 and 4 days post-injection. **(A)** 10% SDS-PAGE analysis of larval hemolymph proteins profile. **(B)** Western blot analysis of hexamerin protein profile. Note the disappearance of 80–85 kDa hexamerin protein from the hemolymph of 2 and 4 days post-injected larvae (arrowhead). lane M: protein ladder, lane 1: second instar (4 days old), lane 2: third instar (6 days old), lane 3: fourth instar control (2 dpi), lane 4: fourth instar Cry toxin injected (2 dpi), lane 5: fifth instar control (4 dpi), lane 6: fifth instar Cry toxin injected (4 days post-injection).

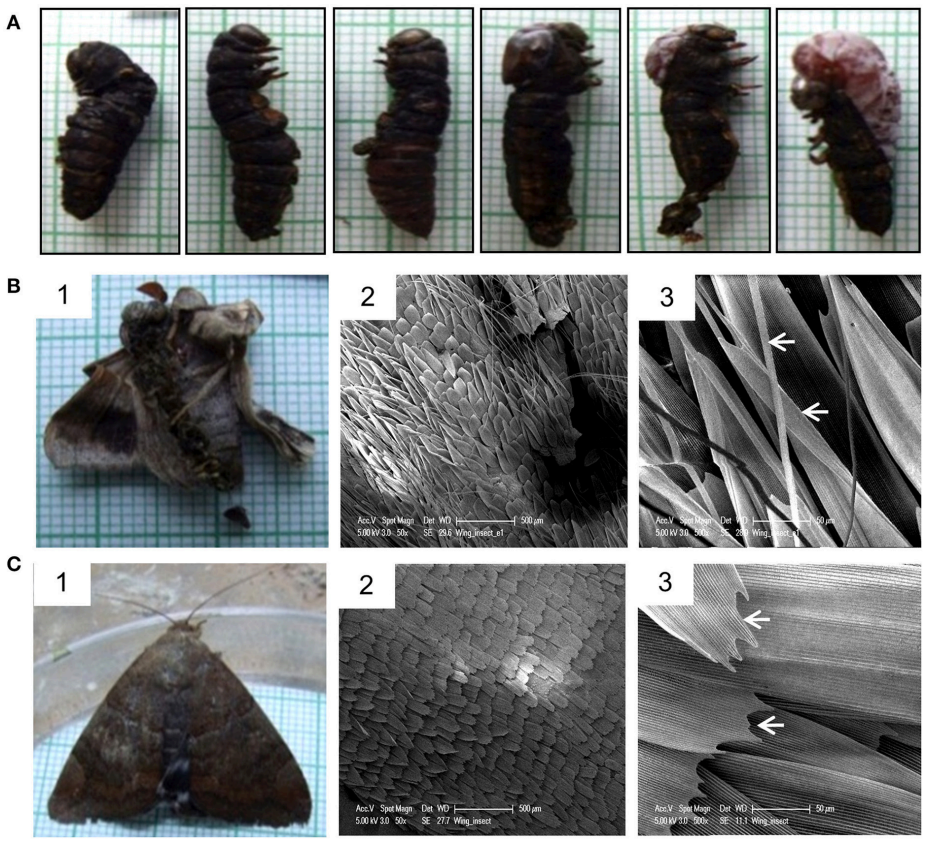


FIGURE 8 | Effect of Cry toxin injection on larval-pupal and pupal-adult transformation. (A) Non-viable larval-pupal intermediates showing "incomplete ecdysis" phenotype. Note the presence of both larval and pupal structures. **(B)** Defective adult emerged from the larvae injected with Cry toxin and SEM micrographs of its wing scale morphology. Note the presence of numerous hair-like and under-developed blade-like scales (arrowhead). **(C)** Normal adult emerged from the control larvae and SEM micrographs of normal wing morphology. Note the presence of only lamellar scales with smooth proper transverse and longitudinal ridges (arrowhead).

supply of nutrients. The chromatin condensation in the nucleus of epithelial cells of Malpighian tubule and a tremendous reduction in the size of salivary gland of the Cry toxin injected larvae are pieces of evidence, suggesting that these tissues are also seriously affected. In a lepidopteran insect like *A. janata*, salivary glands attain maximum size during the last larval instar and pre-pupal stage, and are involved in silk secretion during these stages, which constitute an important physiological event that allows puparia formation for successful larval-pupal transformation. Thus, the decrease in the gland size of the Cry toxin injected larvae and the subsequent insufficient silk secretion during larval-pupal transformation might contribute to abnormal development and metamorphosis seen in the present study. Different larval tissues appeared to respond to Cry toxin injection differently. The fat body being the primary metabolic organ in the larval forms (Price, 1973; Haunerland and Shirk, 1995; Burmester and Scheller, 1999), any damage to this tissue could derail all the ongoing metabolic activities and significantly affect normal development and metamorphosis.

In holometabolous insects, storage proteins, mainly hexamerins are synthesized primarily by fat body of the actively feeding larvae, released into the hemolymph, and later during the non-feeding stage are sequestered back into the fat body again and stored as protein granules, which then serve as the main source of nitrogen and amino acids during pupal-adult metamorphosis and reproduction (Kanost et al., 1990). Contrary to the hemolymph hyperproteinemia reported in the Cry toxin ingested larvae of *S. litura* (Tripathi and Singh, 2002), a significant hypoproteinemia was observed in *A. janata* when Cry toxins were injected into the hemocoel. The significant decline in total protein content of hemolymph associated with a drastic reduction of 80–85 kDa hexamerin proteins to an undetectable level in hemolypmh could indicate inhibition of its synthesis and release, which clearly corroborates with the damage and pathological condition of the fat body seen in toxin injected larvae. Thus, the larval mortality, formation of non-viable larval-pupal intermediates and the emergence of defective adults clearly indicate abnormal development and incomplete metamorphosis which could be the consequences of degeneration of vital larval tissues induced by the toxicity of Cry toxins in the larval hemocoel. There are evidences in literature that when noctuid lepidopteran larvae are fed upon Cry toxin, the toxins are processed in midgut and activated. Further, a small quantity of active toxin was shown to pass through midgut and appear in hemocoel (Pérez-Hedo et al., 2012, 2013; Zhao et al., 2016). The mode of action of Cry toxins in the larval hemocoel is not known but could be different from the one that occurs after oral ingestion. However, binding and pore forming ability of Cry toxin on *in vitro* cultured fat body cells was demonstrated by Cheon et al. (1997), and presence of aminopeptidase in fat body membrane and other non-gut hemocoelic tissue of *A. janata* (Budatha et al., 2007; Ningshen et al., 2013) indicate the presence of Cry toxin binding proteins/receptors which might induce toxicity. In conclusion, the toxicity effects induced by Cry toxins in hemocoel indicate the presence of a specific yet unknown mode of action that possibly targets the tissues and cells present in the insect body cavity. Cry toxin delivery system (e.g., nanoparticle-based) that targets multiple tissues through both oral ingestion and body diffusion/absorption modes predictably would offer a much more effective approach in controlling many economically important pests like *A. janata*.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AD; Performance of the experiments: TN, VC, and ND; Analysis of the data: AD and TN; Manuscript writing: TN, VC, and ND; Manuscript editing: AD.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00289/full#supplementary-material

Supplementary Figure1 | Purification of activated Cry toxins for hemocoelic injection. The activated Cry toxins were purified by gel filtration on Sephadex G-100 column. (**A**) Cry1Aa; Lane M: protein ladder, Lane 1: trypsinized Cry1Aa toxin mixture, remaining lanes: purified activated Cry1Aa toxins. (**B**) Cry1Ab; Lane M: protein ladder, Lane 1: trypsinized Cry1Ab toxin mixture, remaining lanes: purified Cry1Ab toxins. (**C**) Cry1Ac; Lane M: protein ladder; Lane 1: trypsinized Cry1Ac toxin mixture, remaining lanes: purified activated Cry1Ac. (**D**) DOR5; Lane M: protein ladder, Lane 1: discontinuous sucrose density ultracentrifugation prepared DOR5 protoxins, Lane 2: trypsinized DOR5 Cry toxin mixture, remaining lanes: purified activated DOR5 Cry toxins.

Supplementary Figure2 | Propidium iodide and MTT staining: Fat body tissue was dissected out from toxin injected and control larvae. The tissue was placed in 500 μl of TC-100 insect culture medium (Sigma-Aldrich, USA) containing trace amounts of streptomycin sulfate. For PI (**A**) staining tissue were washed twice in PBS (pH 7.4) and incubated with 100 μl of 1X PI staining solution (Cat. No. 556463, BD Biosciences, USA) for 15 min. in dark at room temperature. After this 400 μl of 1X binding buffer were added and analyzed under microcope. While for MTT (**B**) 100 μl of staining solution (5 ng/ml) was added and incubated at 25°C with gentle shaking for 30 min in dark. The tissue was then washed twice in PBS (pH 7.4) and mounted on slide and visualized under microcope. The tissue isolated from toxin injected larvae showed the presence of non-viable cells stained reddish pink with PI, which is absent in viable tissue from control. With MTT staining the non-viable fat body cells from toxin injected didn't shows the dark color, while viable tissue from control larvae stained dark purple in color.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Nucleotide Sequences submitted to NCBI Gene bank

(https://www.ncbi.nlm.nih.gov/BankIt/)

Sl. No	Accession. No	Gene Name
1	MF425653	*Achaea janata* aminopeptidase N (APN1)
2	MF425654	*Achaea janata* aminopeptidase N (APN2)
3	DQ872666	*Achaea janata* aminopeptidase N (APN4)
4	MF425655	*Achaea janata* aminopeptidase N (APN6)
5	MF425656	*Achaea janata* glutamine aminopeptidase-like protein (APN 9)
6	KX462992	*Achaea janata* arylporin

Anti-plagiarism Certificate

Larval mid-gut responses against sub-lethal dosages of Cry toxins in Lepidopteran pest, Achaea janata

by Vinod Kumar Chauhan

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Larval mid-gut responses against sub-lethal dosages of Cry toxins in Lepidopteran pest, *Achaea janata*

VINOD KUMAR CHAUHAN

Farming and pest management

Worldwide nearly 1.8 billion people are engaged in agriculture practice, while in India around 60-70% of its population practices agriculture. Variety of practices are being used to protect the agricultural fields from various pests. They range from traditional practices to pesticides. Pesticides which are categorized as herbicides (weed killer), fungicides (kill fungi and their spores), insecticides (kill insect pests) and others including rodenticides (kill rodents). In India most of the pesticides used are of chemical origin, and it is only less than 5% are of biological origin. When compared to world's per capita usage, Indian use of pesticides is much lower (0.6 Kg/Ha) when compared to other countries (Fig. 1).

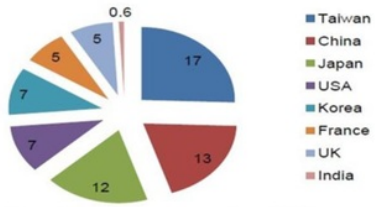


Fig. 2 Per capita usages of pesticide by country (Kg/Ha)

Fig. 1 Per capita usage of pesticides by various countries (Kg/Ha). (Image adopted from www.ficci.com/)

Consumptions pesticides

India uses more than 80% of insecticides as compared to total world usage (De *et al.*, 2014). This is primarily due to the tropical climate conditions and the diversity of insects which is more (Fig. 2).

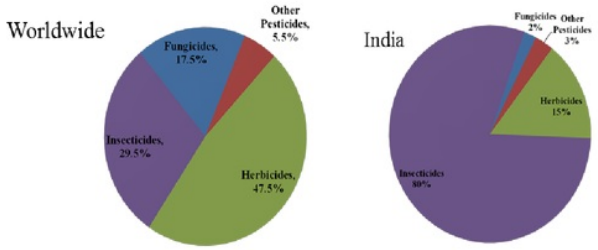


Fig. 2 Consumption of pesticides in world as well as in India. (Adapted from De *et al.*, 2014).

There is always an attempt to reduce the usage of deleterious chemical pesticides by implementing organic agricultural practices and incorporating alternate approaches including usage of biopesticides. Although the biopesticide market presently makes a very small component of total world market, i.e. $3 billion in 2016, but expected is expected to rise to ~$5.0 billion by 2024 (Fig. 3)

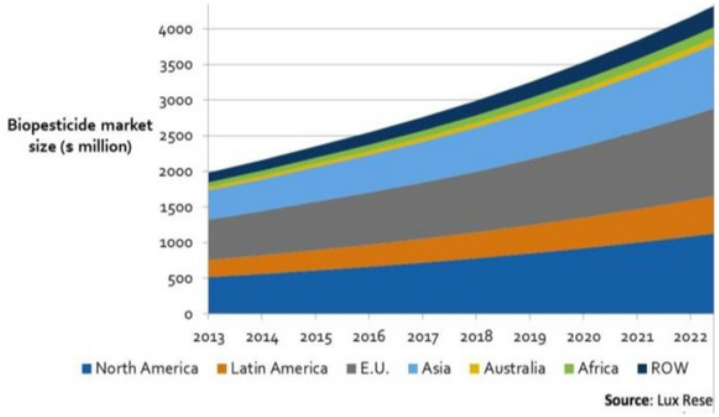


Fig. 3 Forecast of the global market for biopesticides. (Adopted from http://www.luxresearchinc.com/)

Biopesticieds

Biopesticides, can be defined as the group of control agents derived from natural sources. These are the common alternatives to some of the most problematic chemical pesticides currently in use. Biopesticides are primarily classified into: (i) Phytochemicals and biochemical substances commonly present in plants are derived from animals that control pests by various mechanisms, (ii) Microbial pesticides consisting of microorganisms such as bacteria (*Bacillus thuringiensis*), fungi (*Beauveria sp, Nomeria sp*), virus (Nucleopolyhydrous virus) as well as their active ingredients, which are target specific. For example, there are fungi that control certain weeds (*Colletotrichum, Phoma,* and *Sclerotinia sp)* and other fungi that kill specific insects (*Beauveria, Isaria Hirsutella, Metarhizium* and *Nomaria sp)*. The most widely used microbial pesticide for the management of insects pests are subspecies and strains of *Bacillus*

thuringiensis (Bt) either in the form of formulations or *Bt* transgenic plants with their toxin genes.

Bacillus thuringiensis

Bacillus thuringiensis (Bt), a gram positive spore forming bacteria, produces vegetative insecticidal proteins (Vip's) during early growth phase and Crystal (Cry) inclusions as well as Cytolytic (Cyt) proteins during sporulation (Fig. 4) These inclusions are composed of δ-endotoxins (protoxins). The *Bt* strains produce Cry toxins in combinations which are product of various *cry* genes and are effective against Lepidopteran (moths), Coleopteran (beetles and weevils), Dipteran (flies and mosquitoes) and Hynopteran (wasps and bees) order of insects. The advantage associated with *Bt* based biopesticies include target specificity (entomopathogenic), their ecofriendly nature which have very little or no side effects on mammals and non-target organisms and their high effectively as they are normally required in low dosage to show insecticidal activity. The use of *Bt* toxins in the form of *Bt* based spray formulations (mixtures of spores and crystals) as well as *Bt* transgenic plants where these effective protein(s) (*cry*) genes are introduced into economically important crops like *Bt* cotton, *Bt* corn, *Bt* maize, *Bt* brinzal etc. are widely being used for the effective management of insect pests.

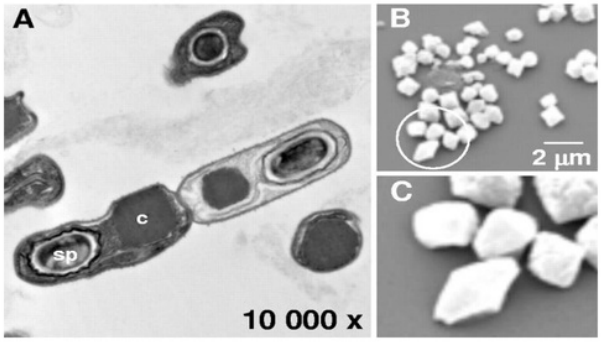


Fig. 4 Morphology of insecticidal crystals *B. thuringiensis* subsp. *thuringiensis*. (A) Transmission electron micrograph of sporulated cells, sp, spore; c, crystal (B) Scanning electron micrograph of purified crystals(circled in panel B). (C) Higher magnification crystals (Image was adopted from Swiecicka *et al.*, 1997)

Cry toxin and its binding/ receptors proteins

The crystal inclusions upon ingestion are solubilized and activated in the host digestive system, in larval mid-gut the active toxins bind to specific binding/ receptor proteins like glycosylphosphotidyl- inositol (GPI)-anchored aminopeptidase-N (APN) (Knight *et al.,* 1995), transmembrane cadherin-like proteins (Nagamastsu *et al.,* 1999), GPI-anchored alkaline phosphatase (ALP) (Jurat-Fuentes and Adang, 2004), or to a high molecular weight glycoconjugate (Valaitis *et al.,* 2001). In general all these aforesaid proteins play important physiological role in digestive function, however by default they also bind to the Cry toxins, and facilitate pore formation in gut cells and cause septicemia leading to death of the larvae.

i) Cadherins: The cadherin family proteins are diverse in nature and perform variety of functions like cell adhesion, migration, cytoskeletal organization and morphogenesis (Angst *et al.,* 2001). These are primarily transmembrane proteins, which possess a cytoplasmic domain as well as extracellular ectodomain with several repeats (Nagamastu *et al.,* 1999). Further they are located in the microvilli of larval mid-gut epithelial cells. Various studies have demonstrated that Cry toxins primarily interact with these proteins (Vadlamudi *et al.,* 1993), which promotes oligomerization. The oligomeric toxins later interact/bind to secondary receptors/binding proteins like aminopeptidase N and alkaline phosphatase present in the brush border membrane of mid-gut cells.

ii) Aminopeptidase N (APNs): The APN's are digestive enzymes that cleave the neutral amino acids from the N-terminal end of polypeptides. These proteins belong to the Zn^{++} binding metalloprotease family of peptides called gluzincins (Hopper, 1994) and have highly conserved aminopeptidase activity motif "GAMENWG" and ZN^{++} binding motif " $HEXXXHX_{18}H$" (Pigott and Elllar, 2007). Mid-gut APNs have been identified as Cry toxin receptor/binding proteins in various lepidopteran insect pests (Soberon *et al.,* 2009).

iii) Alkaline phosphatase (ALP): These enzymes are mainly involved in removing phosphate groups from organic molecules. ALPs are shown to play many biological functions in insects (Funk, 2001). They bind to Cry1Ac toxin, which points to the fact they might play a role in Cry toxin toxicity (Jurat-Fuentes and Adang, 2004, 2007).

iv) Glycolipids: They are known to act as putative Cry toxin receptor. It is hypothesized that both glycolipids and Cry toxin receptors may play role simultaneously or sequentially in the toxin insertion into the membrane bilayer of mid-gut cells. However, the exact function of these molecules in Cry toxicity is yet to be validated. However, an alteration in glycolipids levels in *Plutella xylostella* is believed to play role in evolution of resistance to Cry1Ac (Kumaraswami *et al.*, 2001).

Cry toxin-Mode of action

Two distinct and contrasting models have been proposed for the mode of Cry toxin action based on various studies carried out in lepidopteran insects (*Soberón et al.*, 2009) (Fig. 5).

i) Pore formation model: This is a multistep (Bravo *et al.*, 2004) and well accepted model to describe the mechanism of Cry toxin toxicity (Fig. 5). Upon ingestion of Cry toxin present in crystal inclusions as δ-endotoxins (130 kDa protoxin), is solubilized and released in mid-gut lumen. The alkaline medium and reducing condition of the gut facilitates protoxins cleavage by gut proteases. They are converted into active form toxins which are 64-66 kDa proteins with three domain structure (Schnepf *et al.*, 1998). Similarly from the *Bt* transgenic plants which express the protoxin, upon feeding it is released in mid-gut lumen of larvae and gets activated. (Gasser and Fraley 1989, Fischhoff 1996). This active toxin binds to the receptor/binding protein present in inner apical brush border membrane vesicles (BBMV's) primarily with cadherin like proteins (Vadlamudi *et al.*, 1993; Gomez *et al.*, 2001, 2002), where one α helix

from domain 1 gets cleaved (Gomez *et al.*, 2002) . They interact among themselves and give rise to highly effective oligomeric forms of Cry proteins (Peng *et al.*, 2010), which then further bind either to GPI-anchored receptors like aminopeptidase N (APNs) (eg. *Manduca sexta* (*Pacheco et al.*, 2009) or alkaline phosphatase (ALPs) (eg. *Heliothis virescences*) (Bravo *et al.*, 2004; Jurat-Fuentes and Adang, 2004). Binding of the oligomers to secondary receptors (APNs/ALPs) is followed by its insertion into the microdomains, which causes pore formation in the apical membrane epithelial cells, leading to septicemia and death of the larvae (Schenepf *et al.*, 1998; de Maagd *et al.*, 2003; Bravo *et al.*, 2004).

ii) Signal transduction model: A recent model proposes that toxicity of Cry toxin is through Mg^{++} - dependent signal cascade pathway, where the interaction of activated toxin with primary receptor (cadherin like proteins) activates a guanine nucleotide binding protein (G-protein), which in turn activates adenylyl cyclase and promotes increased production of intracellular cAMP. This causes protein kinase A (PKA) activation which promotes intracellular downstream pathway leading to cell death (Fig. 5). This model is derived entirely through *in vitro* studies carried out using Cry toxin receptor transfected ovarian cells (H5- High five) from cabbage looper *Trichoplusia ni* induced by Cry1Ab toxin (Zhang *et al.*, 2006).

For effective toxicity of Cry toxin, it is not necessary that all the proteins reported as Cry toxin binding/receptor proteins are involved at a given time and in different insect model. The mechanism varies from toxin to toxin and type of receptors involved in it differ significantly. However, at present aforesaid pore formation model is well accepted model for Cry toxin toxicity.

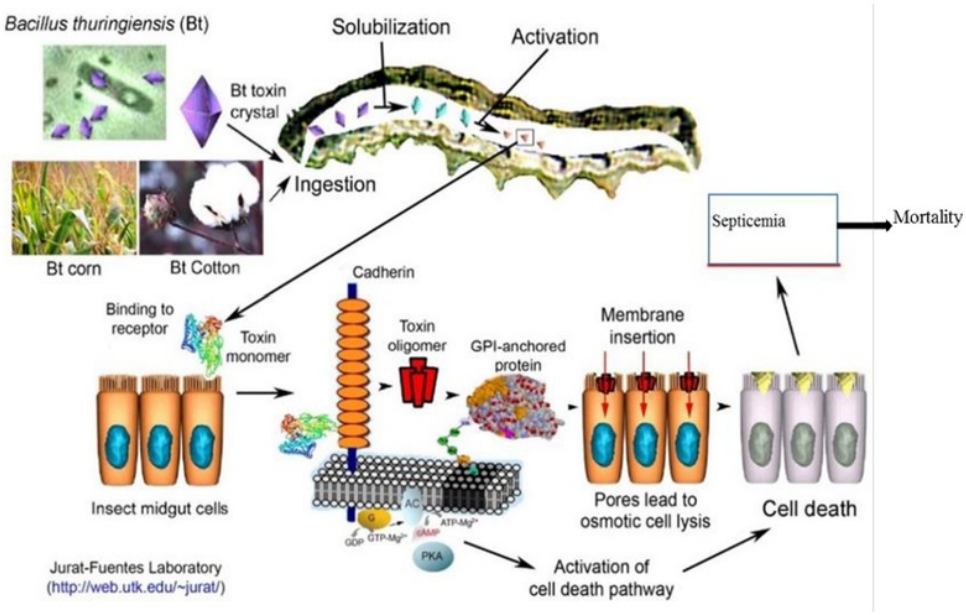


Fig. 5 Mode of action. This multi-step toxicity process of mode of Cry toxin action includes ingestion of the Cry protein, solubilisation, activation, interaction primarily with cadherin like proteins, oligomerization, binding to the secondary APN/ALP receptors, toxin insertion, pore formation, septicaemia and death of the larvae (Image was adopted and modified from Jurat-Fuentes laboratory, http://web.utk.edu/~jurat/).

Development of tolerance and resistance in lepidopteran pests

Bt- based biopesticides are currently being used as an alternative to chemical insecticides for the effective management of economically important Lepidopteran pests in the form of either *Bt* based formulations or *Bt* transgenic plants. However, uncontrolled widespread use of *Bt* sprays and *Bt* crops in last two decades is a topic of concern because of its adverse effects on environment and human health (Shelton *et al.*, 2002). Further, development of resistance against Cry toxin based control strategies in insect populations is a serious matter that needs a careful

evaluation (Tabashnik *et al.*, 2013). Various reports from different insects such as diamond black moth; *Plutella xylostella*, Indian meal moth ; *Plodia interpunctella*, and cabbage looper; *Trichoplusia ni* are the evidence for the evolved resistance to commercial *Bt* sprays, when used in open field and greenhouses (Ferre and Van-Rie 1991; Oppert et al., 1997; Janmaat and Myers, 2003). Recently, field-evolved pest resistance to variety of *Bt* transgenic crops such as *Bt* corn (maize stem borer- *Busseola fusca*, western corn rootworm- *Diabrotica virgifera virgifera*, and fall armyworm- *Spodoptera frugiperda*), *Bt* cotton (cotton bollworm- *Helicoverpa armigera* and pink bollworm- *Pectinophora gossypiella*) and *Bt* maize (Fall armyworm-*Spodptera frugiperda*) have also been reported (Storer *et al.*, 2010; Dhurua and Gujar 2011; Zhang *et al.*, 2012; Pereira *et al.*, 2015; Jakka *et al.*, 2015)

The insect were shown to develop resistance due to alteration in mid-gut binding sites leading to reduced interaction of *Bt* toxins to their receptor (Pigott and Ellar, 2007) and also mutation in toxin binding/interacting protein genes (Guo *et al.*, 2015). In addition variety of physiological responses which are primarily non-receptor mediated mechanisms could also interfere with steps common to more than one Cry toxin leads to resistant against a wide range of *Bt* toxins. One of the process was shown to be enhanced mid-gut regeneration response observed in resistant larvae (Martinez-Ramirez *et al.*, 1999; Castagnola and Jurat-Fuentes 2016). Dulmage *et. al.*, (1978) demonstrated that Lepidopteran larvae recovered from exposure to sub-lethal doses of Cry toxins and it was primarily due to mid-gut epithelial regeneration (Spies and Spence, 1985).

Experimental Model

Achaea janata

Achaea janata is a holometabolus insect. It is commonly known as castor semilooper, belongs to super family of Noctudidae order of Lepidoptera. It is a widely spread agricultural pest of the tropical and sub-tropical region of the world. Although it is a polyphagous insect but primarily feeds on castor plant (*Ricinus communis)* and in absence of it, occasionally feeds on other plants belonging to *Euphorbiaceae, Brassica* and *Ficus* species.

Economic importance

Castor is an important non-edible oil seed crop grown in India, China and Brazil (Lakshminarayana and Raoof, 2005) and it provides completely biodegradable industrial oil (Gaur, 2013). India is the world leader in castor production (Shah and Anish, 2017). Major insect pests of this crop belongs to Lepidopteran species like *Spodoptera litura*, *Amascata albistriga* and *Achaea janata.* Among this *A.janata* is a serious castor pest which leads to 50-70% loss in India during the period of July to September (Gaikwad and Bilapte, 1992).

Life cycle

A. janata is a holometabolus insect pest. It completes its life cycle in 48 -50 days under laboratory conditions. It follows four different life stages consisting of egg, larval, pupal and adults (Karmawati, and Tobing, 1988) (Fig. 6). There are five instars during the larval development which are voracious feeders. The total larval period lasts between 15-20 days, the duration of first to fourth larval stages is of nearly three days, while the last instar takes 4-5 days. The full grown larvae are about 7 cm long. Pupation takes place either in soil or between the fallen leaves. The pupae are normally enclosed in loose silk cocoon. The pupal period extends between 10-15 days and gets prolonged to few months under unfavourable conditions (diapause).The duration of pupal stage is directly influenced by the temperature and humidity.

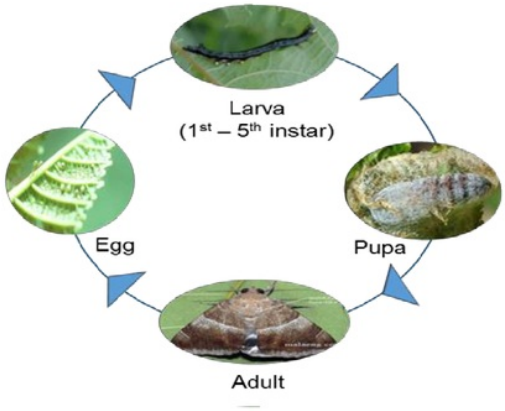


Fig. 6 The life cycle of *Achaea janata*. A holometabolous life cycle consisting of four distinct stages- egg, larva, pupa and adult.

Organs of *A .janata* larvae (used for the present study)

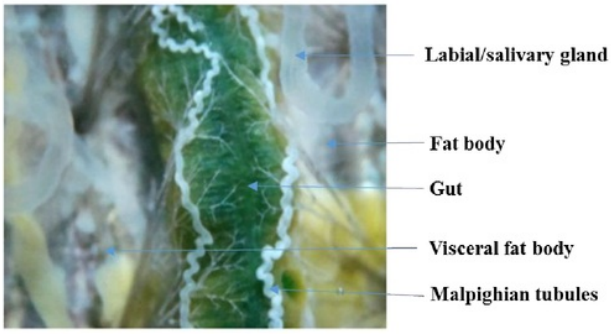


Fig. 7 Organs of *A. janata*. Abdominal cavity of 5th instar larvae showing gut, fat body, Malpighian tubules and Salivary gland (Labial gland).

i) Gut: This is primary organ which is involved in digestion and absorption. Larval gut is further divided into fore-gut, mid-gut and hind-gut. The mid-gut of lepidopteran larva is the major region of digestive tract, which is not only involved in digestion but also in other physiological functions such as metabolism, immune response etc. It is composed of columnar epithelial cells with microvillar apical brush border and Goblet cells with large cavity, which connect the apical surface by inter digitated processes (Cavalcante and Cruz-Landim, 1999; Pinheiro *et al.*, 2008).

Presence of microvilli in mid-gut increases the surface area, which allows to exchange of molecules/ nutrients (Dow, 1987).

ii) Fat body: It is the major organ of metabolism, and nutrient storage in insects (Law and Wells, 1989), Primarily composed of variety of cells like adipocytes, oenocytes, urocytes and bacteriocytes (Arrese and Soulages, 2010). It is known to be a multifunctional organ which co-ordinates the hormonal and nutritional signals and regulates insect development, metamorphosis and reproduction (Colombani *et al.*, 2005).

iii) Malpighian tubules: They are primary excretory and osmoregulatory organ of insects. These tubules spread throughout the surface of gut and visceral cavity. At the junction of mid-gut and hind-gut, they are connected with the gut lumen. The metabolic waste from the surrounding haemolymph as well as gut is excreted and released outside, by them to the exterior through hind-gut and/or rectum. They are composed of a single layer of epithelial cells.

iv) Larval salivary glands: These are a pair of tubular structure, which get specialized in Lepidopteran larvae and produce silk. Constitutive expression of silk protein genes is transient during the larval development. However, the amount of silk protein produced during prepual stage is copious and plays important role in cocoon formation.

v) Haemolymph: It is a circulatory fluid constitute of the plasma and cells, which are commonly known as haemocytes. The plasma of haemolymph has high concentrations of a variety of macromolecules including proteins, carbohydrates, lipids, free amino acids, fatty acids, organic phosphates etc. The larval haemolymph, shows the presence of large number of proteins and among them storage proteins (hexamerins) constitute 40-50% of them. These hexamerins are synthesised by fat body during feeding stage and released into the circulatory fluid (Telfer and Kunkel, 1991; Burmester, 1999). They are then sequestered back into the fat body during non-

feeding (pre-pupal) stage, and are utilized for pupal-adult transformation as well as in reproduction (Pan and Telfer, 1996).

Felid application of *Bt* based formulations

As *Bt* formulations are proteinaceous in nature, hence their efficacy/ activity gets affected by varied field conditions. Foliar spray of *Bt* formulation can result in the loss of toxicity from degradation through different ways such as UV light, wash-off by rain, drying, temperature and chemistry as well as soil acidity (Leong *et al.*, 1980; Beckwith *et al.*, 1987; Van Frankenhuyzen and Nystrom 1989). Uneven sprays of formulation further leaves the unexposed area of the plants, thus giving insects the chance to move from exposed to unexposed part of plants, even unexposed neighbouring fields. Under such conditions, there is always a possibility for the larvae to get exposed to sub-lethal doses of toxin, and exhibit a variable effect leading to survival of insects. Continuous exposure of Cry toxin(s) in sub-lethal dosage either in single generation or in the continuous generations is likely to promote the development tolerance towards this toxin which in turn might promotes insect pests resistance to *Bt* formulations.

On the basis above concept as well as preliminary analysis carried out in laboratory, the present study was designed with the following objectives to evaluate the effect of sub-lethal dosages of Cry toxins in Lepidopteran pest, *Achaea janata*.

i) Evaluation of physiological responses of *A. janata* larvae to sub-lethal dosages of Cry toxin exposure.

ii) Role of endogenous mid-gut aryphorin in cell proliferation/ tissue regeneration.

iii) Characterization of major receptor aminopeptidase N(s) in larval mid-gut and its expression profile during sub-lethal Cry toxin toxicity.

Insect culture maintenance

Egg masses were collected from the agricultural fields of Telangana region where the crops were never sprayed with *Bt*- based biopesticides. Immediately after hatching, the neonates were transferred onto fresh sterile castor leaves. The larvae were maintained in insect culture facility under of 14:10 h (light: dark period), 70 ± 5% RH and 27 ± 2ºC temperature until pupation. The glass troughs containing sterile moist sand was used for maintaining the pupae which were allowed to emerge into adults. The adult moths (male/ female) were collected and transferred to breeding cages where they were fed with 10% honey (Pavani *et al.*, 2015). Adult female moths laid eggs on fresh castor leaves surface, which hatched in 4-5 days. Neonate larvae were used for maintaining generation wise cultures. In laboratory three continuous generations were maintained to get homogenous populations. Later based on the experimental requirement the larvae were exposed to various treatments and the cultures were parallely maintained throughout the study.

Total RNA isolation

Total RNA isolation from collected tissue was carried out using TRI reagent TM (Sigma Aldrich, USA) following the manufacturer's instructions. Tissue and Tri reagent were mixed gently (1 ml TRI reagent for 100 mg of tissue). After homogenization, chloroform was added (0.2 ml for 1 ml sample) and left for 10 min at RT followed by centrifugation at 15000 rpm at 4°C for 15 min. Two layers were separated after centrifugation. The aqueous layer was carefully transferred in a fresh Eppendorf tube to which two volumes of 2-propanol was added, and kept at -20°C for 60 min. The precipitated RNA was then pelleted down at 15000 rpm at 4°C for 15 min and pellet was washed three times with 70% alcohol (DEPC treated) followed by centrifugation. The obtained pellet was air dried and dissolved in DEPC treated nuclease free water. Quantity and quality of isolated RNA was assessed using Nanodrop 1000 (Thermo Scientific, USA). A260/A280 ratio of ~2.0 was considered as a good quality of RNA.

RNA gel electrophoresis

Integrity of RNA was assessed by performing denaturing electrophoresis on a 1% formaldehyde agarose gel (Sambrook *et al.*, 1989). The total RNA (1-2 µg) was mixed with RNA denaturing buffer 25 µl (4 µl of formaldehyde (37%), 13.4 µl formamide (deionized), 3.6 µl of 10X MOPS gel buffer (0.2 M MOPS pH 7.0; 50 mM sodium acetate, pH 6.0; 10 mM EDTA, pH 8.0) 4 µl of DEPC treated water). It was boiled at 65°C for 5 min, and then cooled on ice. Finally 2.5 µl of gel loading dye containing bromophenol blue and Xylene cyanol was added (Pavani *et al.*, 2015). 1% agarose gel was prepared (1gm of agarose, 1X MOPS buffer, 12.3 M formaldehyde and 4 µl of ethidium bromide (10 mg/ml) and RNA samples were loaded on to it. Electrophoresis was carried out using 1X MOPS buffer until dye reached 3/4th portion, at a voltage of 5V/cm^2. Electrophoresed RNA was visualized using UV transilluminator.

DNase 1 treatment

DNase treatment was carried out using reaction mixture containing 1 µl of 10X DNase1 reaction buffer, 1 µl of DNase1 enzyme (Invitrogen, USA) and 2 µg of total RNA and total volume was made up to 10 µl using DEPC treated water. The reaction mix was incubated at 37°C for 20 min. After incubation, DNase I enzymatic activity was inactivated by adding 1 µl of 25 mM EDTA at 65°C for 10 min. This DNA free RNA was used for first strand cDNA synthesis.

First strand cDNA synthesis

First stand cDNA synthesis was carried out according to manufacturer's protocol (Invitrogen, Life Technologies, USA). The reaction mixture was set up by adding 1 µg of RNA, 1 µl of oligo dT primers (50 µM) and 2 µl of dNTP mix (10 mM). The volume was made upto 14 µl using nuclease free water in a PCR tube and incubated at 65°C for 5 min. To this 4 µl of reaction buffer (5X), 1 µl of RT enhancer and 1 µl of reverse transcriptase (200U/µl) were added

to carry out first strand cDNA synthesis at 42ºC for 60 min. The reaction was terminated at 85°C using thermocycler. The synthesized cDNA was stored at -20°C until further use.

Primer (short oligos) designing

As the whole organism genome sequence database for *A. janata* is not available, hence the gene sequence analysis was carried out using degenerate primer based strategy. Primers were designed based on the sequences similarity, reported for other related lepidopteran species in NCBI data base (https://www.ncbi.nlm.nih.gov/). The retrieved sequences were aligned using multiple sequence alignment tool (http://www.ebi.ac.uk). The highly conserved region of the sequence in MulTalin was used for designing the degenerate primers. Primers were designed based on standard protocol (Chen, and Janes, 2002; Dieffenbach and Dveksler, 2003). The parameters considered were primer length (15-30 bp), primer annealing temperature, percentage of GC content (at least 50%), chances of hairpin as well as homodimer formation etc. All the designed primers used in the present study were analysed for the above basic parameters using Oligoanalyzer tool 3.1 (Integrated DNA Technologies Inc., USA).

Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out using thermocycler (Eppendorf, USA). The 10 µl reaction was setup using 5 µl of PCR master mix (2X) (Emerald Amp GT PCR master mix- Clonetech, USA) , 4.5 µl nuclease free water, 0.5 µl forward primer (10 pmol), 0.5 µl reverse primer (10 pmol) and 0.5 µl cDNA template (dilutions of 1:10). Polymerase chain reaction was carried out for 94°C for 2min (initial denaturation); 94°C for 30 sec (denaturation); *54°C for 30 sec (annealing temperature * differ for various primers) and 35 cycles of amplification at 72°C and final extension at 72°C for 10 min. The amplification time for the cycles depended on the size of fragment (30 sec for below 500 bp; 1min for 1kb; 1.5 min for 1.5 kb etc.)

DNA gel electrophoresis

Agarose gel electrophoresis was used for the separation of DNA molecules (genomic DNA, plasmid DNA and PCR amplified DNA product) according to charge and size. Agarose gel was prepared using 1X TAE buffer diluted from 50X stock solution (24.2 g - tris base, 5.7 mL - acetic acid and 10 ml 0.5 M EDTA made upto 100 ml with distilled water). For 1% agarose gel preparation 1g of agarose (Lonza, USA) was suspended in 1X 100 ml of TAE buffer and boiled using microwave. The solution was allowed to cool and 5 µl of ethidium bromide (10 mg/ml) was added and poured into gel casting boat with comb slotted for the loading of DNA samples. The DNA samples were mixed with 6X DNA loading dye (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cynol) and loaded in the wells. 4 µl of respective DNA marker (100 bp - SM0241, 1 Kb - SM0312, Thermo Scientific, USA) was loaded along with the samples (amplified DNA or Plasmid DNA or genomic DNA) was electrophored using 1X TAE buffer at 100 V for 30-40 min, and visualized and documented using gel documentation system (Bio-Rad, USA).

Elution of PCR amplified product

The desired amplified nucleic acid band was sliced using sterile blade and DNA extraction was carried out using commercial gel extraction kit (Qiagen, Germany) according to manufacturer's protocol. For this 100 mg of gel was suspended in three volumes of solubilisation buffer followed by incubation at 50°C for 10 min (till it gets dispersed completely), to this one volume of isopropanol was added and mixed. The solution was loaded onto the silica gel column. It was centrifugation at 13,000 rpm for 1 min, then the column was washed with buffer provided in the kit. The bound DNA molecules were eluted using elution buffer (10 mM Tris-HCl, pH 8.5) in a fresh Eppendorf tube.

Construction of recombinant vector

The desired gene of interest (amplified DNA product) was inserted in pTZ57R/T (cloning vector; Thermo Fisher Scientific, USA) or pET-28a$^{(+)}$ (expression vector; Novagen, USA) using commercial kit (Thermo Fisher Scientific, USA). Insert/vector ratio (3:1 or 5:1) was calculated according to manufacturer's protocol. Ligation reaction (20 µl) was set up by adding vector pTZ57R/T (55 ng)/ pET-28a$^{(+)}$ 2 µl, 5X reaction buffer 4 µl, PCR product (variable), nuclease free water (up to 13 µl) and T4 DNA ligase (1 µl). The reaction mixture was vortexed, briefly centrifuged and was allowed to undergo ligation either at room temperature (22°C) for 1 h or at 4°C for overnight.

Preparation of competent cells and transformation

Glycerol stock of DH5α or BL21 *E.coli* cells was streaked on to LB agar plates and incubated overnight at 37°C. A single colony from plate was inoculated in 5 ml of LB broth (Himedia, India) incubated overnight at 37°C with 180 rpm shaking. The overnight primary culture was further inoculated, 1% in 10 ml of fresh LB broth and allowed to grow for 2 to 3 h till it reached 0.5-0.6 OD at 600 nm. This actively growing culture cells (0.5 OD) were further incubated on ice for 30 min and centrifuged at 6000 rpm for 10 min at 4°C. The pellet was resuspended in 10 ml of 0.1M $CaCl_2$ and incubated on ice for 1 h followed by centrifugation at 6000 rpm at 4°C. The prepared competent cells were aliquoted (100 µl) and used. For the long term storage the pellet was resuspended in 1ml cold 0.1 M $CaCl_2$ containing 15% (v/v) of glycerol and snap frozen in liquid nitrogen and stored at – 80°C.

For transformation 10 µl of ligation reaction containing 50-100 ng of plasmid was added to 100 µl DH5α or BL21 *E.coli* competent cells and kept on ice for 30 min and subjected to heat shock at 42°C for 90 sec followed by immediate incubation on ice for 5 min. 1ml of LB broth was added to transformed cells and incubated for 1 h at 37°C with continuous shaking at 180 rpm. The actively grown cells were centrifuged at 3000 rpm for 5 min, 800 µl of the supernatant

was discarded and the pellet was resuspended in remaining 200 µl of cell culture media. To this 10 µl of IPTG (1M) and 20 µl of X-gal (for blue white screening) was added and the cells were plated on LB plate containing desired antibiotics (for the growth and selection of recombinant vector containing cells) and plates were incubated at 37°C overnight/ maximum of 14 h.

Plasmid isolation

Plasmid was isolated from 10 ml overnight culture using Miniprep plasmid isolation kit (Qiagen, Germany) according to manufacturer's protocol. In brief, from the overnight culture cells were harvested at 8000 rpm for 3 min at RT. The pellet was resuspended in 250 µl of cold resuspension buffer (P1) containing RNase followed by addition 250 µl cell lysis buffer (P2) and mixed thoroughly by inverting the tube 4-6 times. To this 350 µl of neutralization buffer (N3) was added and immediately mixed by inverting tube 4-6 times, and centrifuged at 13000 rpm for 10 min. The supernatant was loaded onto QiAprep spin column and centrifuged at 13000 rpm for 1 min. The flow through was discarded and the column was washed twice with 750 µl wash buffer (PE). The bound plasmid was eluted using 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5). The concentration of plasmid was quantified using Nano drop and stored at -20°C. The same plasmid preparation was used for Sanger sequencing.

Gene expression analysis

For quantitative PCR analysis, a 20 µl reaction was carried out using a custom-made TaqMan gene expression assay (Applied Biosystems, USA). For this both forward and reverse primers of amplicon size (120-150 bp) were designed for various genes. Insect rS7 (KF984201) was used as an internal reference to normalize the transcript expression levels. The real-time expression analysis was performed in triplicates. Relative expression of various genes were calculated using $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).

Heterologous expression and purification of recombinant proteins

i) Construction of recombinant expression vector: The full length cDNA sequence (ORF) of the protein to be expressed was screened to identify the restriction for zero cutters (no cleavage site for this restriction enzyme was present in the ORF sequence but it was present in the expression vector pET28a+) (http://www.labtools.us/nebcutter-v2-0/). Based on the analysis, expression primers were designed with restriction site sequence at the 5'end. The same restriction enzyme was used for the digestion of both the amplified product (insert) and expression vector (pET28a+) to create restriction site overhangs with sticky ends. This was followed by ligation reaction in which insert and vector (5:1 or 3:1) were ligated using T4 DNA ligase. Ligated recombinant vector mixture was used for transformation of BL21 *E.coli* expression host cells.

ii) Recombinant protein expression: Transformed single positive colony (BL21 *E. coli)* containing recombinant expression vector was picked up and inoculated in 10 ml of LB broth with desired antibiotics (Kanamycin (50 µg/ml)) and incubated at 37°C. This overnight culture was used for inoculating (1%) 100 ml LB broth with of kanamycin (50µg/ml) and allowed to grow at 37° C till it reached an OD around 0.4-0.6 at 600 nm. This actively grown culture was induced with 0.5 mM IPTG and allowed to grow for 3 h at 37°C. It was centrifuged at 6000 rpm for 10 min. Pellet thus obtained was washed twice with PBS (137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 and 2.7 mM KCl, pH 7.4).

iii) Purification of recombinant protein using Ni-NTA column (His-tag proteins): The induced bacterial cell pellet obtained was resuspended in equilibration buffer (50 mM Tris, 250 mM NaCl and 10 mM imidazole, pH 7.4). Cell lysis was allowed by addling lysozyme (10 mg/ml), followed by sonication for 10 min on ice (31% amplitude, 10 sec on and 30 sec off). The lysate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was then applied to equilibrated Ni-NTA column (Qiagen, Gemany) and allowed to pass through for binding.

The column was further washed with 20 bed volumes of wash buffer (50 mM Tris, 250 mM NaCl and 40 mM imidazole, pH 7.4) and the bound histidine tagged protein was eluted using elution buffer (50 mM Tris, 250 mM NaCl and 250 mM imidazole, pH 7.4) in 1 ml fractions. Purity of the eluted protein fractions was assessed by SDS-PAGE.

Protein estimation

Protein concentration in various samples was estimated using colorimetric assay i.e, Bradford method (1976). For this procedure bovine serum albumin (BSA 10 mg/ml) was used for preparation of standard sample. An aliquot was taken and volume was adjusted to 100 µl with 10 mM Tris-HCl (pH 7.4). To this 900 µl 1X Bradford reagent (Bio-Rad, USA) was added and incubated for 10 min in the dark. Absorbance was then measured at 595 nm against blank using spectrophotometer (Shimadzu, Japan). The protein concentration of the sample was calculated using a BSA standard curve.

Profiling of proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): It was carried out using protocol of Laemmli (1970). For this 1/4th of 5% stacking gel (2 ml 30% acrylamide mix, 3 ml 0.5 M Tris-HCl pH 6.8, 6.76 ml distilled water, 120 µl 10% SDS, 50 µl 10% of APS and 6 µl of TEMED) and 3/4th of 10 % resolving gel (3.3ml 30% acrylamide mix, 2.5 ml 1.5 M Tris-HCl pH 8.8, 4.1 ml distilled water, 120 µl 10% of SDS, 150 µl 10% APS and 10 µl TEMED) was polymerised. The comb of desired volume size was inserted into stacking gel and allowed to polymerize. Samples were prepared by mixing known concentrations of protein and loading buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue). Proteins were electrophoresed at 60 V in stacking gel and at 100 V for resolving gel. The tracking dye was allowed to run into the tank buffer, and the gel was stained.

Visualization of separated protein/ peptides

i) Coomassie staining: The gel staining was carried out using protocol of Wilson *et al.,* (1983). Protein/polypeptides separated on gel were stained with staining solution (0.025% Brilliant blue- R 250 in 40% methanol and 7% acetic acid) for overnight by using a rocker. It was then destained with destaining solution (40% methanol and 10% glacial acetic acid) to clear the background leaving stained protein bands and molecular weight markers (Bia-Rad, USA). For storage the stained gel were kept in 5% glacial acetic acid solution.

ii) Silver staining: This is a more sensitive method when compared with coomassie. As per protocol of Blum *et al.,* (1987), the gel with separated protein/polypeptides was incubated in a fixative solution (50% methanol, 12% acetic acid and 0.05% formaldehyde) for 3 h and washed thrice in 50% ethanol. Sensitization was carried out using hypo solution (sodium thiosulphate - 20 mg/100 ml) for 30 -60 sec followed by rinsing three times with distilled water (1 min each). The gel was then incubated in silver nitrate solution (0.2% silver nitrate containing 0.076% of formaldehyde) with gentle agitation for 30 min. After incubation, it was rinsed three times with distilled water (1 min each). The gel was finally developed using developing solution (6% sodium carbonate containing 0.05% formaldehyde) till the clear protein bands were visible. Finally the reaction development was stopped by adding 12% acetic acid.

Generation of polyclonal antibodies

The polyclonal antibodies against DOR *Bt*-1and Cry1Ac proteins were raised in 6 weeks old mice (BALB/c) while APN antibodies were generated using 3 months old male rabbit (New Zealand variety) using standard protocol which was approved by Institutional Animal Ethics Committee. For antibody generation in mice, 100 µg of protein and in rabbit 200 µg of protein was emulsified with 500 µl of complete Freund's adjuvant and injected at multiple sites subcutaneously. Prior to injection, the lateral ear vein of rabbit and tail vein of mice was bled to collect pre immune serum. After14 days, first booster dose was given, which was followed

by a second booster dose (14 days after first injection), where 50 µg of protein was emulsified with incomplete Freund's adjuvant. Blood was collected after one week of second booster dose and left overnight at 4°C for clotting. The serum was collected by centrifugation at 5000 X g for 20 min.

Antibody purification

The IgG fraction was purified by affinity chromatography using protein A-agarose column (Amersham, Germany) according to manufacturer's protocol. The serum protein was diluted (1:1) binding/wash buffer (20 mM sodium phosphate pH 7.4 with 150 mM NaCl) and was loaded on to the column preequilibrated with binding/wash buffer. This was followed with the washing of columns using the same buffer until the absorbance of elutes at 280 nm approaches the background level. The bound IgG fraction was eluted with 4 column volume of elution buffer (100 mM glycine-HCl, pH 3.0) in tubes containing 100 µl of neutralization buffer (1 M Tris-HCl, pH 9.0) for rapid neutralization of the eluted purified IgG fraction. The purified IgG fraction was aliquoted, lyophilized and stored at -20°C till use.

Western blotting and immune detection

The proteins/ peptides from electrophoresed gel were transblotted to nitrocellulose membrane (Amersham, Germany) (Towbin *et al.*, 1979). The membrane as well as gel were immersed for 10 min, in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol). The gel and membrane along with filter paper were stacked into a cassette without air bubble and placed in transfer unit containing Towbin buffer. The transfer of proteins/peptides to the membrane was carried out at 25 V with 250 mA current limit at least for 14 h. Finally transfer of proteins to membrane was checked by Ponceau S (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) staining. It was followed with extensive washing with TBS (Tris buffered saline, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl). For immunoblotting above membrane was soaked in 5% blocking solution (5% skim milk powder (w/v) in TBS) or 3% BSA for 3 h to block nonspecific

regions on blot. Blot was washed three times (15 min each) with TBST (0.1% Tween- 20 in TBS (Sigma, USA)). Incubation of blot was carried out with desired primary antibody (1:1000 dilution) overnight at 4°C followed by secondary antibody (1:4000 dilutions). Either alkaline phosphatase (ALP) or Horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit IgG were diluted in 5% skim milk powder (w/v) in TBS and the blot was soaked for 2 h. The blot was washed to remove unbound antibodies and processed for blot development with BCIP/NBT (G-Biosciences, USA) for ALP or chemiluminiscence (Takara Bio Inc, Japan) for HRP conjugated antibodies. Finally blots were imaged with Kodak Photo Imager.

Histological procedures

i) Tissue fixation: The isolated tissues mid-gut, fat body, Malpighian tubules and salivary glands were fixed overnight in Bouin's fixative (saturated picric acid: formaldehyde: glacial acetic acid 15:5:1) at RT for a minimum of 12 h but not more than 36 h. The tissue was washed 3- 4 times with 70% ethanol and stored in same. It is recommended that the tissue should be used in a short period and the storage may not be more than one month for further processing.

ii) Paraffin embedding and sectioning: The fixed tissue was processed through a series of increasing alcohol concentrations (70%, 90% and 100%) for dehydration followed by clearance in xylene (5-10 min). The tissue was penetrated with molten paraplast (Sigma-Aldrich, USA) and finally embedded in the same as blocks. The blocks were sectioned (~5 µm) using a rotator microtome (Leica Microsystems, Germany). Wax strips containing tissue sections were placed on the Mayer's albumin coated glass slides containing some water on it. Stretching of the ribbon with sections was carried out by placing the slides hot plate (40°C) for 10-20 min. Water was drained and the slides were left overnight at 37°C.

iii) Haematoxylin and eosin staining: The slides containing sections were placed in coupling jars and processed through two changes of xylene (each of 5 min) and then rehydrated in series

of ethanol changes (2 changes in 100% for each of 10 min each; 90% for 5 min; 70% for 5 min; 50% for 5 min; 30% for 5 min) then transferred to distilled water for 5 min. The slides were stained with haematoxylin ($C_{16}H_{14}O_6$. xH_2O) for 20 min, followed by 10 min wash with distilled water. The slides were transferred to acid water to remove excess of staining and finally eosin ($C_{20}H_6Br_4Na_2O_5$) staining was carried out for 3-5 min. The stained sections were further dehydrated in increasing ethanol concentration (30%; 50%; 70% 90% and 100%) and cleared through xylene (3 changes; 5 min each). The slides were then mounted with DPX mountant (Sigma-Aldrich, USA), visualized using Olympus IX-81 microscope (Olympus Corporation, Japan) and photographed.

Statistical analysis

Presented data was validated using standard error of mean (SEM) (n=3), which passed homogeneity and normality tests. Statistical significance was verified using the Sigma Plot v12.3 software (SystatSoftware Inc., USA). Significance between groups was tested using One-Way ANOVA followed by Student-Newman-Keuls (SNK) test for pair wise multiple comparison analysis and p value was calculated for each set of experiments.

1 Introduction

The toxins produced by *Bacillus thuringiensis (Bt),* are classified into three types (i) vegetative insecticidal toxins(Vips) (Warren *et al.,* 1996), (ii) Cytolytic (Cyt) toxins and (iii) Crystalline (Cry) toxins. Further the parasporal crystals produced during sporulation generally contain more than one proteins (Cyt and Cry toxins) (Schnepf *et al.,* 1998). Both Cyt and Cry toxin are known as pore forming toxins (PFT), they share common structural and functional features which facilitate their penetration in the cell membranes and formation of pores, which eventually cause cell lysis followed by insect death (González *et al.,* 2000, 2008 ; Feil *et al.,* 2010). Cry inclusion proteins are also referred as delta-endotoxins (Heimpel, 1967), which are primarily protoxin, activated in the mid-gut of target insects by proteolytic cleavage. Cry toxins have been shown toxic to the larval forms of Lepidopteran, Coleopteran, Hymenopteran and Dipteran order insects as well as towards soil nematodes (Hofte, and Whiteley, 1989; Zhong *et al.,* 2000; Crickmore 2005; Likitvivatanavong *et al.,* 2011).

The nomenclature committee for *Bt* delta-endotoxin was set up in 1993 and proposed nomenclature of various toxins based on amino acid identity which allows closely related toxins to be ranked together. More than 700 *cry* genes have been classified and their products have been grouped into 73 different Cry proteins (Cry1-Cry73) (Crickmore *et al.,* 2016). Further based on the host specificity Cry toxins have been grouped into: Group 1- Lepidopteran (Cry1, Cry9 and Cry15); group 2- Lepidopteran and Dipteran (Cry2); group 3- Coleopteran (Cry3, Cry7 and Cry8); group 4- Dipteran (Cry4, Cry10, Cry11, Cry16, Cry17, Cry19 and Cry20); group 5- Lepidopteran and Coleopteran (Cry1I); and group 6-nematodes specific (Cry6). Further Cry2, Cry3, Cry10 and Cry11 toxins (73–82 kDa) are unique because they appear to be natural product of the larger Cry1 and Cry4 proteins (130–140 kDa). (de Maagd *et al.,* 2001; Pardo-Lopez *et al.,* 2012).

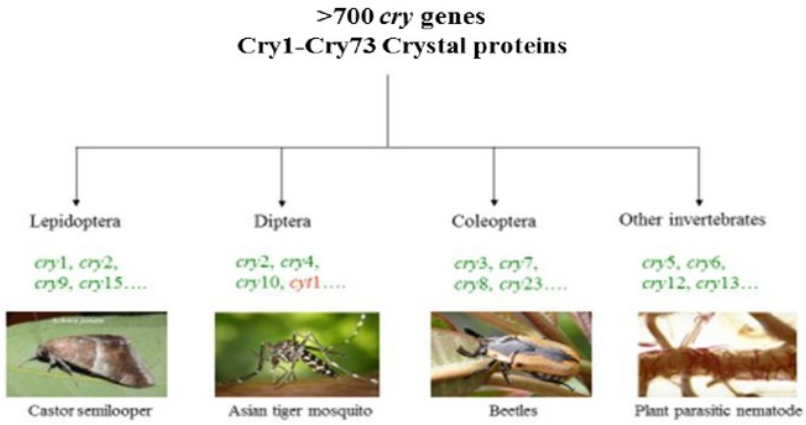


Fig. 8 Summarised spectrum of delta-endotoxin (Cry). Represents the different groups of toxin show its effect on different orders of phylum Arthropoda and other invertebrates including nematodes (data adopted from http://www.btnomenclature.info/ and modified)

Cry toxins present in the parasporal crystal range between 120-130 kDa, are activated in the host digestive system by an array of proteases and form 64-67 kDa active toxins. X-ray crystallographic analysis of activated toxin revealed it three dimensional (3D) structure (Li *et al.*, 1991) which consisted of three domains. Domain I, a seven α helical structure, resembled to pore forming domain of colicin and was designated as pore-forming determinant and membrane insertion domain. Domain II which possessed to three anti-parallel β sheets, was suggested to help in receptor binding. Domain III consisted of two anti-parallel β sheets, played a role in receptor recognition and membrane insertion. Furthermore domains II and III lie at the same side while domain I is located on opposite side (Grochulski *et al.*, 1995). Both domain I and III have been demonstrated to play role in specificity and interactions with different gut receptors (Bravo *et al.*, 2007).

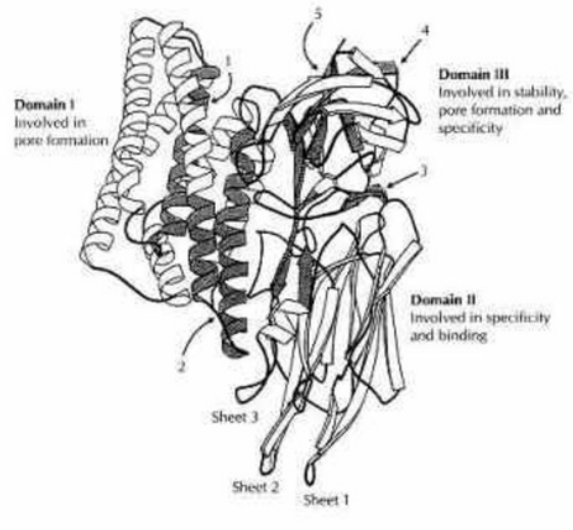


Fig. 9 Schematic representation of activated Cry1A toxin. Consists of three domains; domain I (seven α helices), domain II (three anti-parallel β sheets) and domain III (two anti-parallel β sheets). (image adopted from http://www.mrclmb.cam.ac.uk/genomes/madanm/articles/dnashuff.htm)

For the mode of action of Cry *(Bt)* toxins two different models have been proposed. (i) The pore formation model is widely accepted one for the Cry toxin toxicity in *M. sexta* (Bravo *et al.,* 2004). In this model, several sequential steps like protoxin activation by gut proteases, oligomerization through cadherin like proteins, binding to the receptor proteins like APN/ALP, which lead to membrane insertion and pore formation. (ii) The other model is signal transduction model which is primarily based on *in vitro* transfection of cadherin receptor gene of *M. sexta* in High Five (H5) cells derived from ovarian cells of cabbage looper; *Trichoplusia ni.* Cry1Ab interaction activated Mg^{+2} dependant cellular signalling cascade which involved protein kinase A (PKA) pathway, and caused the cell death (Zhang *et al.,* 2006). This model further suggested that cleavage of α helix and oligomerization were not necessarily required for the pore formation (Soberaon *et al.,* 2009).

As the *Bacillus thuringiensis (Bt)* have potential insecticidal activity, they are formulated into various forms (powder/granules or liquid) for application to the agricultural/ horticultural fields as biological control agents, for the management of insect pests. More than 400 *Bt* based formulations are available in the market (Ahmedani *et al.*, 2008; Sanahuja, *et al.*, 2011) and applied in the form of sprays (Ali *et al.*, 2010). Majority of the sprayable formulations are based on natural stains of *Bt* like *B. thuringiensis var. kurstaki (Btk)* HD1 (Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa proteins), HD73 (Cry1Ac), HD137 (Cry1Aa, Cry1Ba, Cry1Ca and Cry1Da) and *B. thuringiensis var. tenebrionis* (Cry3Aa). *Btk* (HD1) was shown to produce Lepidopteran specific insecticidal proteins (Soberaon *et al.*, 2009). As the Cry toxins were found to be have potential insecticidal activity, plant biotechnologist took lead on to generating *Bt* based transgenic plants which expressed toxin encoding genes (Barton *et al.*, 1987; Walter *et al.*, 2010) and were resistant to variety of insect pests.

Continuous usage of *Bt* based formulations and *Bt* transgenic plants had always been a threat to the development of resistance in insect pests to these Cry toxins. Field evolved resistance against *Bt* was demonstrated in diamondback moth, *Plutella xylostella* (Sayyed *et al.*, 2004) and the same was shown in laboratory reared Europian corn borer, *Ostrinia nubilalis* (Pereira *et al.*, 2008) and pink bollworm, *Pectinophora gossypiella* (Fabrick *et al.*, 2009). Further studies revealed that some pests not only developed resistance to a particular toxin, but also exhibited resistance to a newer toxin(s) to which they were not exposed earlier. This kind of cross resistance was observed in *Plutella xylostella* (Gong *et al.*, 2010) and Asian corn borer, *Ostrinia nubilalis* (Xu *et al.*, 2010). Development of resistance against *Bt* toxin has been attributed to various reasons such as (i) reduced solubilisation of protoxin (ii) alteration of proteolytic activation of toxin (iii) precipitation in gut environment and toxin degradation (Bruce *et al.*, 2007) and (iv) reduced in binding of toxin to the receptors (Tiewsiri and Wang, 2011; Guo *et al.*, 2015)

Additional physiological responses from larvae like non-receptor mediated resistance mechanisms could also interfere with steps which are common to all Cry toxins and thereby result in cross-resistance to a wide range of *Bt* toxins. One such process is the enhanced mid-gut regeneration response observed in resistant larvae. It is documented that the Lepidopteran larvae could recover from exposure to sub-lethal Cry toxin doses (Spies *et al.*, 1985) by mid-gut epithelial regeneration (Forcada *et al.*, 1999). Cry toxin resistance involving an enhanced mid-gut healing response has only been reported in the tobacco budworm; *H. virescens* so far (Martinez-Ramirez and Ferre, 1999; Blanco *et al.*, 2009).

In this chapter a detailed study has been carried out decipher the probable mechanisms which facilitate repair of mid-gut epithelium upon Cry intoxication in *A. janata* larvae.

2 Materials and Methods

2.1 Bacterial strains

DOR *Bt*-1 (a *Bacillus thuringiensis thuringiensis* strain) was obtained from Indian Institute of Oilseeds Research, Hyderabad which exhibited high toxicity against larval forms of *A. janata* and as well as various other Lepidopteran pests. Recombinant Cry toxin (Cry1 Aa, Cry1Ab and Cry1Ac) containing JM103 *E .coli* strains were obtained from Bacillus Genetic Stock Centre (BGSC), Ohio State University, USA. The Cry toxins obtained from both local isolate and recombinant strains were used for the present studies.

2.2 Collection and purification of *Bt* crystal Spores

For preparation of *Bt* crystal spores (δ- endotoxin), the local DOR *Bt*-1 isolate was grown in Nutrient broth (Himedia, India) for 72 h with agitation (180 rpm/min) at 28°C until sporulation and cell lysis was complete. The parasporal crystal and spores were harvested from the above culture by centrifugation at 1100 X g for 10 min at 4°C followed by 3 times washing with distilled water. Pure crystals thus obtained were stored at 4°C till use. Crystal incisions obtained from DOR *Bt*-1 were suspended in water and smeared on microscopic stub. The samples were air dried, gold coated and examined under Jeol scanning electron microscope (Jeol Inc, USA).

For preparation of recombinant toxins, a standard protocol was followed (Lee *et al.*, 1992), where JM103 cells were grown and harvested (10000 X g, 10 min) and the pellet was suspended in buffer (50 mM Tris-HCl, 50 mM EDTA, 15% Sucrose, pH 8.0). Cell suspension was sonicated on ice (10 min, 10 sec on and 30 sec off) and centrifuged at 15000 X g for 10 min. The pellet thus obtained was washed three times with ice cold 0.5 M NaCl containing 2% Triton X-100, five times with 0.5 M NaCl and finally two times with distilled water.

2.3 Solubilisation and activation of protoxin

The crystal pellet was solubilized in 50 mM sodium carbonate/bicarbonate buffer (pH 9.5) containing 10 mM DTT and incubated at 37°C in water bath for 2 h. After solubilization the fraction was dialyzed to remove DTT. The solubilized protoxins were digested with trypsin in a trypsin/protoxin ratio of 1:50 (by mass) at 37°C for 2 h. To this 1mM PMSF was added, to stop the proteolytic reaction. The activated toxins were purified using Sephadex G-100 (Sigma-Aldrich, USA) through gel permeation chromatography and profile of the digested toxin was assessed by SDS-PAGE. Presence of active Cry protein was confirmed by immunoblotting using antibody generated against recombinant Cry1Ac toxin.

2.4 *Bt* toxin activity evaluation

Feeding bioassay was performed where specific area of the upper leaf surface was coated with various concentrations of activated Cry toxin(s) (2, 4, 6, 10, 12, and 14 ng/cm2). Control were maintained on buffer coated leaves. Two days old (neonates) *A. janata* larvae (20 no's) were allowed to feed on both toxin coated and normal (buffer coated) leaves. Triplicates were maintained for each treatment and larval mortality was recorded at every 24 h interval for 5 days. LC_{50} dose (toxin concentration at which 50% larval lethality/mortality obtained) was calculated, using Probit analysis with the help of Finney'S table (Finney, 1978).

2.5 Feeding/ exposure of 3rd instar larvae with (DOR *Bt*-1) formulation

The reported LC_{50} value of DOR *Bt*-1 formulation was 247.52 µg/ml for seven-day old or 3rd instar larvae (Vimaladevi *et al.*, 2006). For the present study various dosage of toxin formulation was coated on fresh castor leaves. Laboratory standardized sub-lethal dosage ($1/10^{th}$ of the LC_{50}) of DOR1 *Bt-1* formulations coated leaves were air dried and used for feeding to a fairly large number of 3rd instar larvae (150 no's) in a restricted environment. For each feeding assay triplicates were maintained. The feeding was carried out for various time periods, the insects (30 no's) were removed after 24 h , 48 h and 72 h of toxin exposure

and were transferred to normal uncoated castor leaves and allowed to recover. Rest of the insects (60 no's) were continuously maintained on toxin coated leaves. For each time period age matched control larvae were maintained on normal (buffer coated) leaves. Furthermore the data was collected on mortality and morphological changes during development were recorded in toxin-exposed, recovered insects, and control insects for the comparative study.

2.6 Tissue isolation

Third instar larvae exposed to toxin coated leaves were collected at every 12 h interval till 72 h, along with appropriate age matched control insects. The larvae were briefly narcotized on ice and dissected in insect Ringer solution (130 mM NaCl, 0.5 mM KCl and 0.1 mM $CaCl_2$). An incision was made in the abdominal region and extended through the length of the larvae. The gut content was removed and the mid-gut region was isolated. After removal of the gut from the insect, the yellowish-white perivisceral fat body was collected. The isolated tissues were used for histology, *in-situ* hybridization, genomic DNA as well as total RNA isolation and protein analysis.

2.7 Histological analysis of mid-gut

The detailed protocol have been described in the General Materials and Method Section. The isolated mid-guts were fixed overnight in Bouin's fixative, processed through a series of increasing alcohol concentration, cleared in xylene and embedded in molten paraplast (Sigma-Aldrich, USA). The tissue was sectioned (5 µm) using a rotator microtome (Leica Microsystems, Germany) and stained with hematoxylin-eosin or toluidine blue (Sousa *et al.*, 2010), visualized and photographed using Olympus IX-81 microscope (Olympus Corporation, Japan).

2.8 Confocal analysis

The larval mid-gut tissue from both control and toxin exposed larvae were dissected out and placed in Grace's insect culture medium (Thermo Fisher Scientific, USA). They were incubated in presence of both Propidium iodide and Annexin V staining solutions (AnnexinV-FITC Apoptosis Detection kit, Abcam pk, UK) for 10 min in dark and analysed using confocal microscope LSM 880 (Carl Zeiss,Germany).

2.9 FACS (Fluorescence-activated cell sorting) analysis

Single cell suspension prepared from mid-gut of both normal and toxin exposed larvae using 70 µm mesh size MACS® smart strainer (MiltenyiBiotec, Singapore) and phosphate buffer saline (PBS) solution (pH 7.4). Cell suspension collected through funnel was centrifuged at 1500 rpm for 5 min at 4ºC, and the cells were immediately processed for FACS analysis. It was carried out using Apoptosis Detection Kit according to the manufacturer's instruction (BD Biosciences, UK). AnnexinV and Propidium Iodide (PI) staining solutions were added to cell suspension, which was incubated at room temperature in the dark for 15 min. This was followed by addition of binding buffer and then analyzed using a FACS LSR Fortessa^{TM} flow cytometer (BD Biosciences, UK) and BD FACS Diva Software 6.0. For each sample, 10,000 events were acquired. The percentage of live cells versus cells undergoing death was plotted and for analysis data was represented in four quadrant (Q1: non-viable, PI^+ (necrotic); Q2: both PI^+ (necrotic)/ AV^+ (apoptosis); Q3: PI^-/ AV^- (viable cells) and Q4: AV^+ (apoptotic) cells.

2.10 Genomic DNA isolation and DNA fragmentation analysis

Genomic DNA from mid-gut of both control and toxin exposed insect larvae was isolated using standard protocol (Lagisz *et. al.*, 2010) with slight modifications. Homogenization of mid-gut tissue was carried out in 500 µl of lysis buffer (100 mM Tris-HCl. (pH8.0), 50 mM EDTA (pH 8.0), 1.5 mM NaCl, 2% CTAB and 0.1% Mercaptoethanol).

The sample was incubated at 65°C for 45 min with intermittent mixing. The phenol: chloroform: isoamyl alcohol (25:24:1) mix was added with vigorous mixing for 5 min, followed by centrifugation at 15000 rpm for 15 min. The supernatant was transferred to a fresh Eppendroff tube and two volumes of pre chilled 2-propanol was added along with 5 µl RNase A (10 mg/ml) and centrifuged at 10000 rpm for 10 min. The obtained pellet was washed thrice with 70% ethanol. The pellet was dried and dissolved in TE buffer. Quality of genomic DNA was analyzed using Nanodrop spectrophotometer. DNA fragmentation analysed using 0.8% of agarose gel electrophoresis.

2.11 BrdU labelling and detection

Streptavidin-biotin based BrdU staining (Invitrogen Corporation, USA) was used for monitoring the cell proliferation in the mid-gut during toxin-induced damage following manufacturer's protocol. Larvae were injected with BrdU labelling reagent (10 µl/g body weight) 4 h prior to isolation of toxin exposed larval mid-gut using a Hamilton syringe. Tissue was fixed in Bouin's fixative and processed (as mentioned in the General Materials and Methods Section).Tissue sections were adhered on poly-L-lysine coated slides (Sigma-Aldrich, USA) and incubated overnight at 37°C, following which deparaffinization was carried out using xylene and passed through a series of decreasing ethanol concentrations. Peroxidase quenching was performed using 30% H_2O_2 and absolute methanol (1:9). Denaturation and blocking steps were carried out using the kit based reagents prior to incubation with antibody. Biotinylated mouse anti-BrdU antibody was overlaid and the slides were incubated at room temperature. After incubation, streptavidin peroxidase (Invitrogen Corporation, USA) was added to detect the incorporation. Colour development was monitored after the addition of secondary antibody mixture provided by the manufacturer. Slides were counterstained and mounted using histomount TM (Invitrogen Corporation, USA) and visualized under Olympus IX-81 microscope.

2.12 Propidium Iodide (PI) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide staining (MTT) of fat body tissue

Fat body was collected from the sub-lethal dosage toxin exposed insects and analysed for the effects of Cry toxin using PI and MTT staining. The tissue was placed in 500 µl of TC-100 insect culture medium (Sigma-Aldrich, USA) containing trace amounts of streptomycin sulphate. For PI staining tissue was washed twice in PBS (pH 7.4) and incubated with 100 µl of 1X PI staining solution (BD Biosciences, USA) for 15 min, in dark at room temperature. After this 400 µl of 1X binding buffer was added and tissue was visualized under microscope. While for MTT 100 µl of staining solution (5 ng/ml) was added and incubated at 25°C with gentle shaking for 30 min in dark. The tissue was then washed twice in PBS (pH 7.4) and mounted on slide and visualized under microscope.

2.13 Hemocoelic injection of Cry toxin

Third instar larvae were narcotized on ice for 15 min and were then injected with activated Cry toxins i.e. 200 ng/0.2 g body weight through the mid-dorsal region using a Hamilton microsyringe. Control larvae received the same volume of the carrier solvent. Immediately after injection, the wound was sealed with bees wax and the larvae were placed on ice for 15 min before transferring them back to the rearing dishes/troughs provided with fresh castor leaves. Pathological effects of Cry toxin on various larval tissues were analysed using histological sections stained with haematoxylin-eosin staining.

3 Results

3.1 Preparation and purification of Cry toxins from local isolate

The scanning electron microscopic analysis clearly showed the presence of crystal proteins of various shapes in DOR *Bt* -1 preparation, which is suggestive of the expression of more than one *cry* gene in this local isolate (Fig. 10A). The *Bt* crystal spores solubilized in sodium carbonate buffer (pH 9.5) and activated by trypsinization showed the presence of both protoxin (120 kDa) and active toxin (64 kDa) bands (Fig. 10B), which is further purified with gel permeation chromatography using (Sephadex G-100) beads (Fig. 10C). Presence of toxin was confirmed by using Cry1Ac antibody generated against recombinant Cry1Ac protein purified from BGSC clones (Fig. 10D).

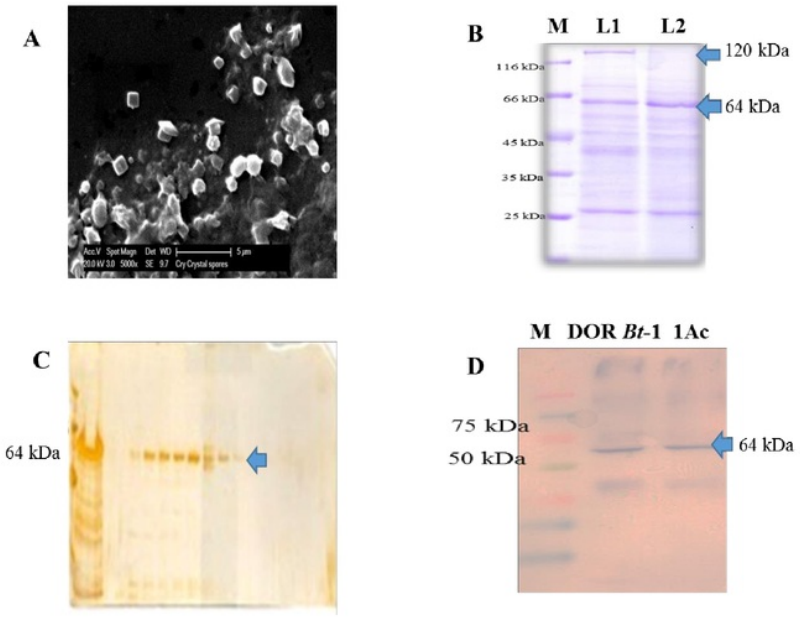


Fig. 10 Purification of Cry toxins from local isolate (DOR *Bt*-1). (A) Scanning electron micrograph of DOR *Bt*-1 crystal spores. (B) SDS-PAGE profiling of solubilized protoxin (⇨120 kDa) and active toxin(s) (⇨64 kDa). (C) Purification of active Cry toxin through gel permeation chromatography (Sephadex G-100). (D) Confirmation of purified activated Cry toxin(s) (⇨64 kDa) with recombinant Cry1Ac antibody.

3.2 Toxicity effects of Cry toxin

Feeding bioassay with 2 days old *A. janata* larvae showed that feeding of local isolate (DOR *Bt*-1) and recombinant Cry toxin(s) i.e. Cry1Aa, Cry1Ab, and Cry1Ac caused larval mortality. However, DOR *Bt*-1 Cry toxins showed much higher insecticidal activity with fairly low LC_{50} (4.1 ng/cm^2 of leaf surface) (Table 1). Furthermore DOR *Bt*-1 were reported to be free from any exotoxin activity (Vimaladevi *et al.*, 2006).

Toxin	LC_{50} (ng/cm^2 of leaf surface)
Cry1Aa	107.56
Cry1Ab	47.83
Cry1Ac	35.87
DOR *Bt*-1	4.010

Table 1 Evaluation of insecticidal activity of Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac and DOR *Bt*-1) against *A. janta* larvae after oral ingestion. Feeding assay was performed using neonate larvae (2 days old). The specific area of castor leaf was coated with various concentrations of activated proteins (2, 4, 6, 8, 10, 12 and 14 ng/cm^2). The experiment was performed in triplicates. Larval mortality was recorded at 24 h intervals for 5 days. LC_{50} was calculated based on area of feeding and mortality rate of insects and analysed using probit analysis using Finney's table (Finney, 1952).

3.3 Cry toxin altered larval growth and development

The larvae which were fed on toxin-coated leaves exhibited reduced growth in a time-dependent manner (24-72 h). The 24 h toxin-exposed larvae could recover and grow when transferred to normal leaves, whereas the 48 h and 72 h toxin-fed groups often showed slow growth as well as poor recovery (Fig. 11A).Eventually also resulting in defective metamorphosis and premature death (Fig. 11B). The 24 h toxin-exposed larvae could recover and normally develop when transferred onto uncoated leaves, whereas the 48 and 72h toxin-fed groups could not recover from the effect of *Bt* and exhibited defective molting. Further,

the body weight of the toxin exposed larvae was fairly low and was only 50% of that of the age matched control groups after 72 h of recovery, when the larvae were exposed to toxin for 48 h or 72h (Fig. 11C).

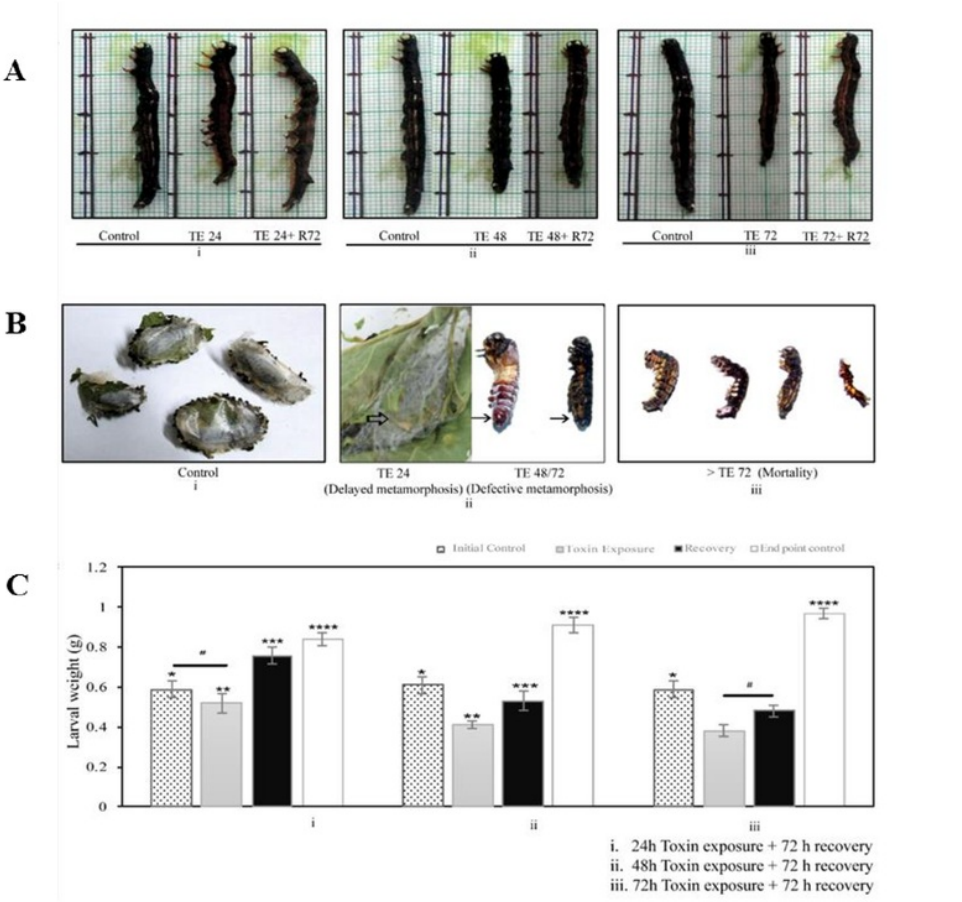


Fig. 11 ***A. janata*** **third instar larvae were maintained either on Cry toxin coated or normal leaves for different time intervals.** (A) Growth pattern seen in (i) 24 h, (ii) 48 h and (iii) 72 h after Cry toxin exposure (TE), with respective controls, for recovery toxin exposed larvae were transferred to normal leaves for 72 h (R72). (B) Metamorphosis in toxin exposed larvae (i) control insects with normal metamorphosis (i)24 h (⇨), 48 and 72 h (→) toxin exposed larvae with either delayed or defective metamorphosis. (C) Effect of Cry toxin exposure for (i) 24 h, (ii) 48 h and (iii) 72 h on larval weight and after 72 h of recovery. The values presented are the mean ± standard deviation of three independent experiments (n=3), significance between groups was tested by One-Way ANOVA followed by Student-Newman-Keuls (SNK) test using Sigma Plot 12.3 software. Means marked with *$p < 0.05$ are significant with respect to toxin exposed, recovered and age matched end point control larvae,**$p < 0.05$ indicate statistical significance with respect to recovered and age matched end point control, ***$p < 0.05$ indicate statistical significance with respect to initial control and toxin exposed larvae, ****$p < 0.05$ indicate statistical significance with respect to the weight of initial control, toxin exposed and recovered larvae, while # represents difference between compared group that is not statistically significant.

3.4 Mid-gut necrosis occurred in toxin-exposed larvae

The viability of mid-gut cells was primarily assessed using PI and Annexin V staining (Vermes *et al.*, 2000). Confocal analysis revealed the presence of PI positive cells in toxin exposed larvae but not in controls (Fig. 12).

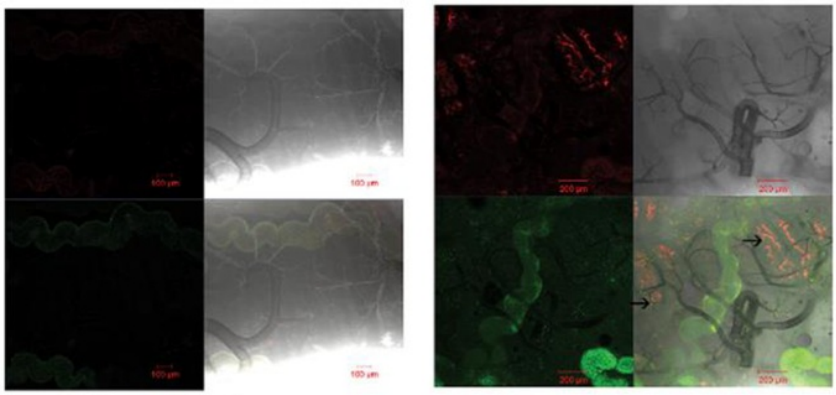


Fig. 12 Cell death through confocal microscopic analysis using Annexin V (AV) and Propidium iodide (PI) stains. Whole mount showing control and Cry toxin exposed larval mid-gut, stained with Annexin V (AV) and Propidium iodide (PI). Note the presence of PI positive cells in the mid-gut epithelium after toxin exposure (→)

The data represented in figure 13 shows the results obtained from FACS analysis. The scatter plot which gives the total cell counts is divided into four quadrants (Q1-Q4), Q1 represents PI^{+}/AV^{-} cells (necrotic), Q2 represents PI^{+}/AV^{+} cells (late apoptotic/necrotic), Q3 represents PI^{-}/AV^{-} (viable cells) and Q4 represents AV^{+}/PI^{-} cells (early apoptotic). Mid-gut cells prepared from toxin exposed insects showed distinct shift into the upper left quadrant (Q1) suggesting that cells were undergoing necrotic type of cell death. Further, their number upon toxin-exposure increased gradually from 12 h to 36 h. However, the results presented here clearly show that the number of necrotic cells declined drastically preparations from the mid-gut of 48 h as well as 72 h toxin-exposed larvae (Fig. 13, inset).

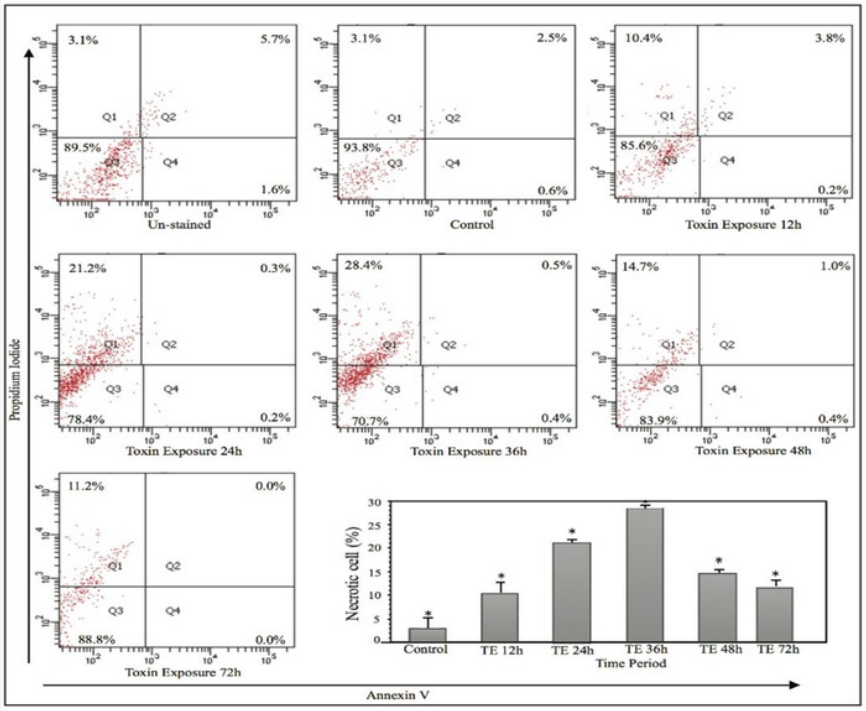


Fig. 13 Scatter plot showing the distribution of Annexin V and PI stained cells suspension prepared from mid-gut of control and Cry toxin exposed larvae. The cells isolated from experimental larvae showed a distinct shift during 24 h and 36 h of exposure into the top left quadrant (Q1). Inset shows percentage of necrotic cells in larval mid-gut upon toxin-exposure during different time points, *p < 0.005 significance between experimental groups.

To further affirm that cell death which occurred in larval mid-gut during early toxin exposure (12-36 h) was due to necrosis and not because of apoptosis, DNA fragmentation assay was carried out using genomic DNA isolated from the tissue. The result revealed that the DNA integrity was preserved in both control and toxin-exposed mid-gut samples and it was comparable with that of control insects (Fig. 14). Histological, FACS and DNA fragmentation analyses carried out in present study indicated that necrosis was the most probable mechanism of cell death upon Cry intoxication.

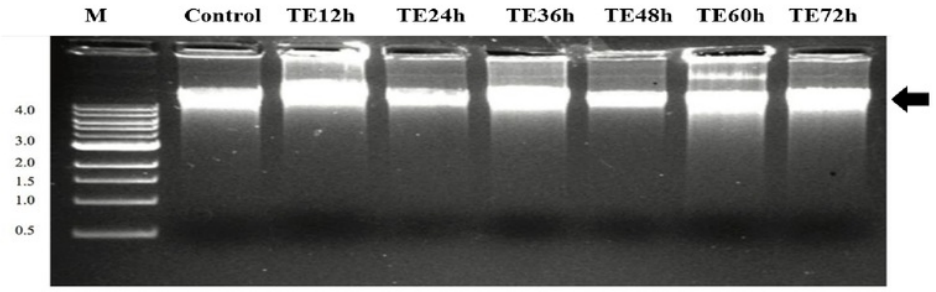


Fig 14 DNA fragmentation analysis of mid-gut. Ethidium bromide stained agarose gel showing the presence of intact band (⬅) of similar molecular size in both control and toxin exposed larvae after different time points (TE 12- 72 h). Lane M shows the molecular size marker (1kb; #SM0311).

3.5 Sub-lethal toxin exposure induced mid-gut cell proliferation

For determination of whether or not cell regeneration occured in larval mid-gut upon sub-lethal toxin exposure, cell proliferation was monitored using a kit-based BrdU (bromodeoxyuridine) labelling. Mid-gut cross-sections prepared from toxin-exposed larvae were processed for immunocytochemistry using BrdU primary antibody. The cells in the DNA synthesis phase (S phase) were labelled by this method. Few pockets of BrdU-positive cells (brown stained nuclei) were visible in the mid-gut collected from toxin exposed larvae (Fig. 15A). The dividing cells which were primarily seen in the basal region of the gut epithelium, could be ascribed as mid-gut stem cells/ regenerative cells (Fig. 15B, inset).

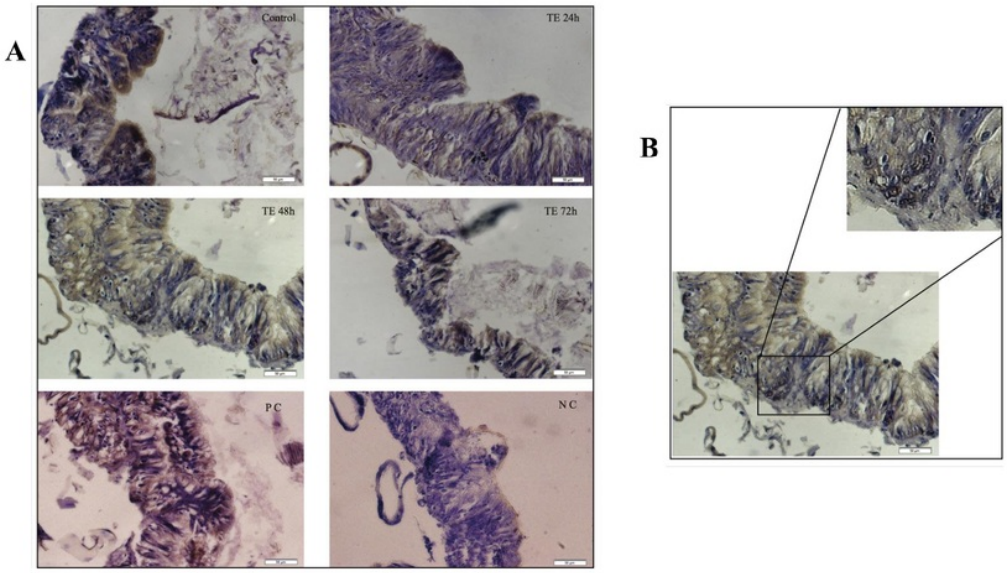


Fig 15 Mid-gut cell proliferation monitored by BrdU incorporation. (A) T.S. of mid-gut from Cry toxin exposed 24, 48 and 72 h of larvae showing BrdU positive cells. Dextran sulphate sodium injected larvae were used as positive control (PC) and phosphate buffered saline injected larvae were used a negative control (NC). Note the presence of dividing cells with brown stained nucleus. (B) Enlarged portion of mid-gut epithelium from 48 h toxin exposed larvae showing actively dividing cells in the crypt.

3.6 Cell death seen upon sub-lethal dose of Cry toxin exposure was associated with epithelial regeneration

To analyse whether or not cell death in mid-gut is associated with regeneration, the toxin exposed larval mid-gut histological sections were stained with either haematoxylin/eosin or toluidine blue. The changes were monitored from 12 h till 72 h of Cry toxin-exposure (Fig. 16).The mid-gut cells showed degeneration with condensed nucleus and fairly low cytoplasmic content. In addition, large number of vacuoles were seen in them. Maximum cell death was observed between 24 h to 36 h (Fig. 16, C-D). However, interesting phenomenon observed was active cell proliferation in toxin exposed larvae. Figure 16 (c-d) clearly showed cell division upon toluidine blue staining. The histological and toluidine blue corroborated and clearly suggested it was not only cell death occurred but cells also divided actively and epithelium regeneration upon Cry intoxication in *A. janata* larvae.

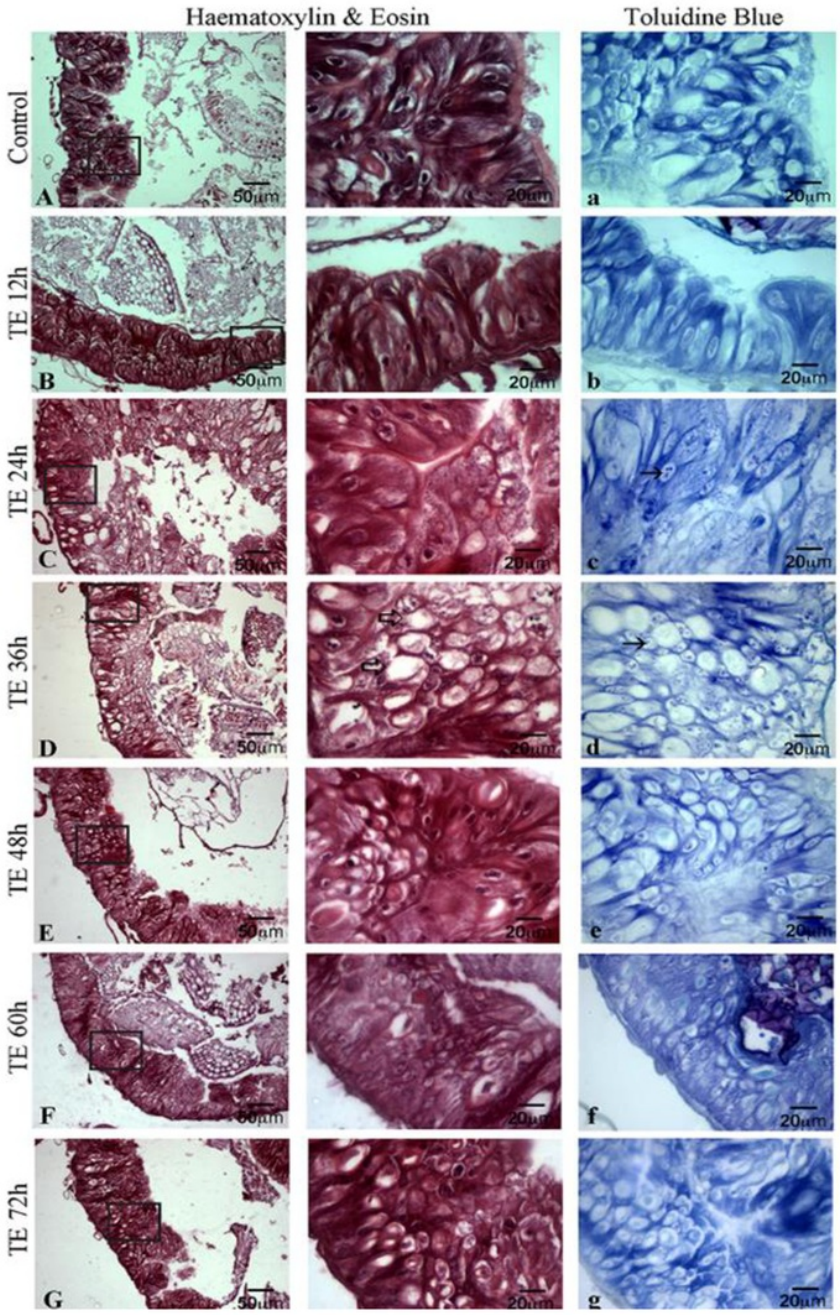


Fig.16 Occurrence of cell death and cell proliferation in mid-gut upon Cry intoxication. Haematoxylin/eosin and toluidine blue staining. T.S. of mid-gut showed extensive damage in epithelium (C) and presence of large number of vacuoles (⇒) (D) after toxin exposure. T.S. of mid-gut stained with toluidine blue (4, a-g), note the presence proliferative cells in the basal region of the epithelium (→) (c-d). (Scale bars 20 µm, 50 µm).

3.7 Effects of Cry toxin on other tissue/ organs

Various tissue/organs isolated from sub-lethal toxin exposed/ fed larvae showed the presence of non-viable cells stained reddish pink with PI, which were absent in the tissue dissected from control larvae (Fig. 17B). Once again MTT staining further confirmed the non-viability of fat body cells which appears dark in colour, while the tissue isolated from control larvae stained in light purple in colour (Fig.17D).

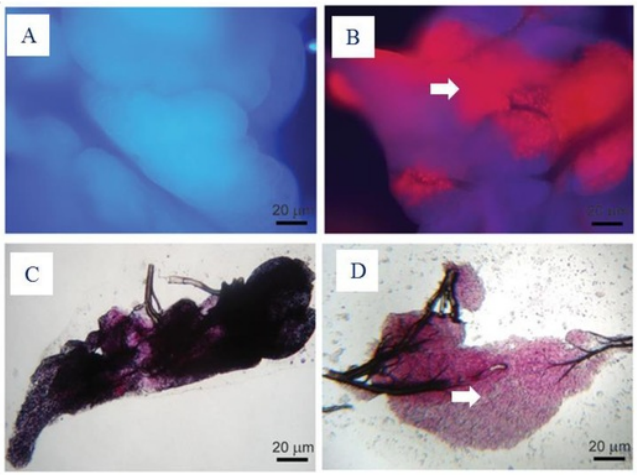


Fig. 17 Effects of Cry toxin on fat body after oral ingestion. Note the presence of non-viable cells which stained reddish pink after PI staining (B). Light purple in MTT assay (D), indicated toxin based cell damage which caused non-viability of tissue upon Cry toxin feeding. A and B are respective control.

Once again the direct interaction of Cry toxin with visceral tissue evaluated by direct haemocoelic delivery of activated Cry toxin in the larvae by micro injection. Mid-gut tissue sections stained with H&E show the presence of irregular arrangement of goblet (g) as well as columnar (c) cells in the gut epithelium (Fig. 18a). In fat body, extensive tissue degeneration was seen (Fig. 18b). Malpighian tubule revealed the presence large number of vacuoles (v) in the cytoplasmic region with chromatin condensation in the nucleus (Fig. 18c). Furthermore salivary gland cells also showed condensation of nuclear chromatin and reduced diameter of the gland with fairly narrow lumen (Fig. 18d)

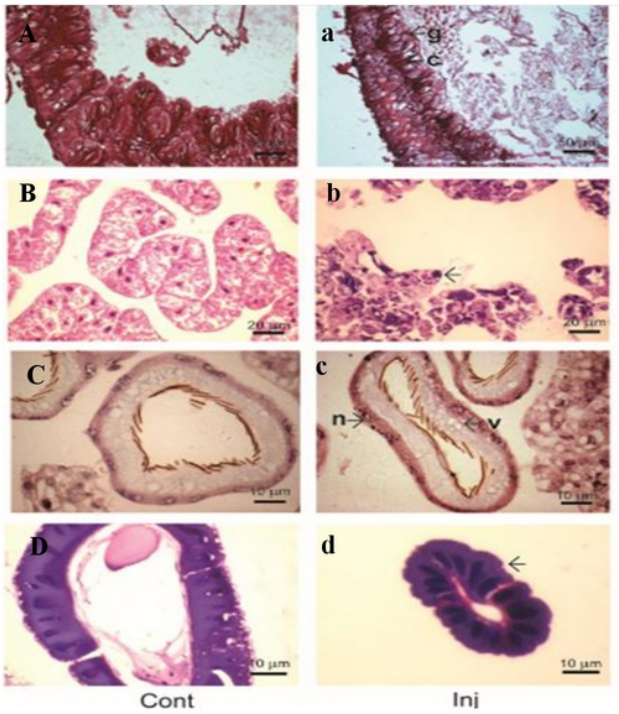


Fig. 18 Histological analysis larval tissue after hemocoelic injection of activated Cry toxin. Tissue sections were prepared from 2 days post-injected larvae and stained with H&E (A) mid-gut, (B) fat body, (C) Malpighian tubule and (D) salivary gland. Note the cytological changes in tissue sections induced by Cry toxin injection (Inj, arrowhead), (g, Goblet cell; c, columnar cell; v, vacuole; n, nucleus.)

All the above observations clearly show that Cry toxin not only caused the damage to mid-gut epithelium but also had deleterious effects to the cells of other visceral tissues of the larvae, which are exposed to hemocoelic toxin upon injection.

4. Discussion

In the present work, the effect of sub-lethal dose of Cry toxin was evaluated primarily to assess the larval mid-gut responses in *A.janata*. The formulations used in the present study, DOR *Bt*-1 is a product prepared from a local strain of *Bacillus thuringiensis*, a gift from Dr. P.S.Vimala Devi, of Indian institute of Oilseeds Research, Hyderabad. DOR *Bt*-1 formulations was shown to have high insecticidal activity and was required in very low amount to achieve LC_{50} (4.1 ng/cm^2 of leaf surface) when compared with recombinant Cry toxins obtained from BGSC (Cry 1Aa, Cry1Ab and Cry1Ac) clones. The high toxicity of this strain against *A.janata* larvae was likely associated with presence of multiple *cry* genes, like *cry1* (*cry1Aa*, *cry1Ab* and *cry1Ac*) and *cry2* (*cry2Aa* and *cry2Ab*), which provided broad spectrum potential against Lepidopteran and Dipteran insects (Reddy *et al.*, 2012).

In field, the *Bt* based formulations are generally sprayed when insects are in late 2nd or early 3rd instar larval stages, for effective pest management. The suggested dose for field application for DOR *Bt*-1 formulation is 1g/10L (w/v). The reported LC_{50} for 3rd instar (seven-day old) larvae was reported to be 247.52 µg/ml for ml for seven-day old or 3rd instar larvae (Vimaladevi *et al.*, 2006). In the present study a series of dosages using various dilutions of this formulation were tested and finally 1/10th of the LC_{50} i.e 24.75 µg/ml of water (0.170 µg/cm^2 of leaf surface) was chosen as a sub-lethal dose of toxin for the study using for 3rd instar *A. janata* larvae.

As observed in this pest and other reported Lepidopteran models, Cry toxin exposed larvae usually exhibited low digestive activity concomitant with their reduced body weight associated with altered growth and development (Hernandez-Martinez *et al.*, 2010). In the present study one set of larvae (30 no's) was removed after every 24 h of toxin exposure till a period of 72 h, and transferred to uncoated leaves for recovery. The larvae fed on toxin-coated leaves exhibited a time-dependant (24-72 h), reduction in the growth which was reflected in

their body weight. The 24 h toxin-exposed larvae recovered and grow whereas the 48 h and 72 h toxin-fed groups often showed slow as well as poor recovery when transferred to normal leaves. Further, the body weight of the toxin exposed larvae was fairly low i.e. only 50% of that of the age matched control groups after 72 h of recovery, when the larvae were exposed to toxin either for 48h and 72 h. Present study corroborated well the findings of *Helicoverpa armigera*, where upon prolonged sub-lethal exposure to *Btk* HD-1 toxin larvae showed reduced weight and altered behaviour (Mohan *et al.*, 2008).

Histomorphological studies of mid-gut showed that even sub-lethal dose of toxin caused extensive cell death/tissue damage. Further the maximum cell death was observed between 24 h to 36 h, where the mid-gut epithelium showed tissue degeneration. The cells showed the presence of condensed nucleus, fairly low cytoplasmic content and large number of vacuoles in it. Although the pore formation model is most widely accepted for Cry toxin toxicity in Lepidopteran larvae (Baravo *et al.*, 2004), however the exact mechanism of cell death upon Cry toxin intoxication is still not very clear in large number of insects including *A. janata*. Further in this study an attempt was made to identify the type of cell death which occurred upon Cry intoxication. Results obtained from confocal analysis of mid-gut taken from both the control and Cry intoxicated larvae using PI which is a marker for cell death and AV which is also a marker for death associated with apoptosis, clearly showed presence of PI positive cells but not any AV positive cells, suggested that cell death seen in the epithelium upon Cry intoxication was not due to apoptosis but was likely due to other form of cell death (may be necrosis), FACS analysis was carried out by using the both PI and AV stained single cell suspension isolated from both control and toxin exposed larvae. This method primarily allowed the identification of cells which undergo either apoptosis or necrosis (Vermes *et al.*, 2000). In the present analysis maximum number of cells stained with PI and negligible number of cells with Annexin V. Furthermore agarose electrophoresis

carried out with the mid-gut genomic DNA isolated from both control and toxin exposed larvae revealed absence of DNA fragmentation. Histological, FACS and DNA fragmentation assay results of the present study clearly suggested that the cell death induced by Cry toxin was primarily due to necrosis. There is increasing evidence in the field that Cry toxin induced cell death is a fairly complex cellular response. Zhang *et al.,* (2006) using cabbage looper ovarian cell culture demonstrated that Cry1Ab toxin manifested its effect through Mg^{2+}dependent adenylyl cyclase/protein kinase A (PKA) signalling pathway. Recently, using toxin-host interaction system in *Caenorhabditis elegans*, (Zhang *et al.,* 2016) demonstrated that Cry6Aa mediated cell death was promoted through necrotic signalling pathway. However, these reports were either from *in vitro* studies using isolated cells or non-host system and to the best of our knowledge there is no direct evidence reported in insect host model. Present *in vivo* study for the first time clearly showed that Cry toxin promotes necrosis cell death in mid-gut of *A. janata* larvae during Cry intoxication.

One of the notable phenomenon observed in the present study was migration of larvae, after initial feeding on toxin coated leaves for some time, towards the sides of the dishes and reduced feeding to avoid toxin based mid-gut cell damage. Replenishment of injured cells during the mid-gut repair of various Lepidopteran larvae was shown to be facilitated by normal feeding, leading to recovery and metamorphosis (Spies and Spence 1985; Loeb *et al.,* 2001). However, when larvae were exposed to high doses of *Bt* toxin, it lead to mortality and prevented recovery (Tabashnik *et al.,* 2013). Like many other tissues, renewal of epithelium is carried out by regenerative cells localized in the basal crypts which are termed as mid-gut stem cells/ regenerative cells. These were the only population of cells present in the gut capable of division and thus represent the sole source of regenerative cells which play crucial role in repair and growth of damaged epithelium. Continuous replacement of damaged cells by regenerative cells under toxin environment was likely to cause alteration in cellular

properties including changes in the cell surface molecules leading to the development of resistance in insects, against *Bt*/Cry formulations (Martinez-Ramirez *et al.*, 1999; Castagnola and Jurat-Fuentes, 2016).

In the present study we clearly demonstrated not only the cell death and damage of mid-gut epithelium upon Cry intoxication but also proliferation of stem/regenerative cells, which allowed the larvae to repair its gut epithelium and generate new populations of differentiated cells which facilitated the survival of insects. This was further substantiated by the presence of large number of BrdU positive cells in the basal crypts of the gut epithelium, while toluidine blue staining revealed sizable increase in the number of proliferative cells upon Cry intoxication in time dependent manner in *A. janta* larvae. Finally, the present study clearly demonstrated that Cry toxin based damage was associated with cell proliferation in mid-gut, which eventually facilitated the survival of insects upon sub-lethal dosages of Cry intoxication.

Oral ingestion of Cry toxin in *A. janata* not only caused the damage to mid-gut, a well-known organ bearing Cry toxin binding receptor/interacting proteins, but also showed its deleterious effects on visceral tissues. In earlier studies, our group reported the presence Cry toxin binding protein/ receptors among which aminopeptidase N (APN) on the cell membrane of fat body of *A. janata* (Budatha *et al.*, 2007) and also in Malpighian tubules as well as salivary glands (Ningshen *et al.*, 2013). Further, upon feeding the passage of Cry toxin through the mid-gut barrier and its presence in various visceral tissues in larval hemocoel has also been reported (Pérez-Hedo *et al.*, 2012; Zhao *et al.*, 2016). Present study with feeding of sub-lethal dosages of Cry toxin as well as results obtained with hemocoelic delivery of activated Cry toxin, clearly indicated that the receptors/binding proteins present on the surface membrane of various visceral tissues interacted with Cry toxins and promoted cell damage seen in the mid-gut, fat body, Malpighian tubules and salivary glands.

From this part of the study it is concluded that even sub-lethal dose of Cry has deleterious effects on the development and growth of larvae, where mid-gut and fat body cells showed extensive cell death. Further in mid-gut the cell damage was primarily caused due to necrosis, however the cellular damage in epithelium was associated with the cell proliferation, which promoted simultaneous repair of the tissue and facilitated larval survival upon Cry intoxication in *A. janata* larvae.

1 Introduction

Haemolymph is a circulatory fluid in arthropods including insects which fills the hemocoelic cavity. It basically composed of plasma and cells commonly known as haemocytes. The plasma has high concentrations of variety of macromolecules including proteins, carbohydrates, lipids as well as free amino acids, fatty acids, organic phosphates etc. The haemocytes are of various types, (Gupta, 2009) and play an important role in immune function, storage, synthesis and secretion of variety of molecules including humoral factors (Hopkins and Krmer, 1992; Zhao and Kanost, 1996; Nappi and Christensen, 2005). During the postembryonic development variety of proteins are synthesized by various tissues and released into the haemolymph, which play varied physiological functions.

Hexamerins, are one such group of proteins which are found to be present in very high concentration in the larval haemolymph (40-50% in the total haemolymph) of holometablous insects including Lepidopteran insects. Their molecular mass ranges around 500 kDa in native form (Tefler and Kunkel, 1991). They consist of six subunits, which are either homo or heteromer. The molecular mass of subunit ranges between 70-86 kDa. Hexamerins are synthesized predominantly by the fat body and released in to larval haemolymph (Telfer and Kunkel, 1991; Burmester, 1999) during active feeding stage. During the non-feeding/ pre-pual stage they get sequestered back through a receptor mediated endocytosis and get accumulated as dense protein granules in the fat body (Bean and Silhacek, 1989; Chrysanthis *et al.*, 1994; Haunerland, 1996; Burmester and Scheller, 1999). Hexamerins share the common features with hemocyanin in term of sequence and structural similarity (Beintema *et al.*, 1994; Burmester and Schellen, 1996; Burmester, 2015) which serve as respiratory proteins in various arthropods as oxygen transport proteins. On the contrary, hexamerins do not bind to oxygen and are known to serve as storage proteins (Burmester, 1999). Although the hexamerins are primarily synthesized during larval/ nymphal stages but their expression has also been reported during

pre-pupal and pupal stages (Martins *et al.*, 2008), and in adults (Martins *et al.*, 2010). However, it needs to be confirmed whether or not they are same genes which get expressed at various developmental stages.

Based on the function and amino acid composition, Lepidopteran larval hexamerins are classified into three or four major categories. Arylphorin, which are rich in aromatic amino acids (tyrosine and phenylalanine) and utilized in cuticle synthesis during larval/pupal/adult molting as well as play variety of other functions (Pan and Telfer, 1996; KiranKumar *et al.*, 1997; Burmester *et al.*, 1998; Nagamanju *et al.*, 2003; Hagner-Holler *et al.*, 2007). They also play role in diapause (Sula *et al.*, 1995; Kludkiewicz *et al.*, 1996; Godlewski *et al.*, 2001; Lewis *et al.*, 2002; Spyliotopoulos *et al.*, 2007). Methionine rich hexamerins present in high concentration in females and facilitate egg production (Wheeler *et al.*, 2000; Pan and Telfer, 2001). The hexamerins, which are neither rich in methionine nor in aromatic amino acids, are sometimes referred as moderately methionine rich hexamerins (Zheng *et al.*, 2000; Spyliotopoulos *et al.*, 2007; Gkouvitsas and Kourti, 2009). A group of hexameins bind to small metabolic molecule riboflavin and are called as riboflavin binding hexamerins (Magee *et al.*, 1994; Rao *et al.*, 2016).

Excising literature survey clearly shows that arylphorins are versatile, multifunctional molecules which help in transport of hormones such as ecdysteroids (20HE) (Enderle *et al.*, 1983; Manohar *et al.*, 2010) and juvenile hormone (JH) (Braun and Wyatt, 1996; Gilbert *et al.*, 2000; Tawfik *et al.*, 2006), and are involved in immune function (Phipps *et al.*, 1994; Beresford *et al.*, 1997; Ma *et al.*, 2005; Poopathi *et al.*, 2014). In last decade or so arylphorins have been shown to promote/stimulate stem cell proliferation *in vitro* (Blackburn *et al.*, 2004; Hakim *et al.*, 2007). However, the exact mechanism by which they facilitate cell proliferation has not been analysed yet.

In this chapter an effort was made to clone and characterize the various hexamerins from larval tissue primarily fat body and mid-gut of *A. janata* with a view to evaluate their role if any in extensive cell/tissue regeneration and repair in Cry toxin based damage caused by sub-lethal exposure of Cry toxin based formulations.

2 Materials and Methods

2.1 Primers used in the present study

For cloning various larval hexamerins of *A. janata*, following set of primers were designed based on standard protocol (Chen, and Janes, 2002; Dieffenbach and Dveksler, 2003) and synthesized commercially through Oligoanalyzer tool 3.1 (Integrated DNA Technologies, Inc., USA)

S. No.	Primer Name	Primer Sequence (5' – 3')
Degenerate and specific primers for obtaining partial fragment		
1	Aj.DARYL.F	GTC HAG ATH AAT GAC GTG SAA GT
2	Aj.DARYL.R	CTB TGR CBT GGT AGA CYC CTT GG
3	Aj.DMRH.F	GGC ATG TTY STG TAT GCYCT
4	Aj.DMRH.R	CGGATCTTKGGCCARTARCC
5	Aj.DRBX.F	CAR MTN BTN RCH CGY TAY TAY
6	Aj.DRBX R	CAG ATG ATG ATT CCA TAC G
Cloning complete cds		
7	ARYL FF	ATG CCC AAA TAC CAT GAG TTC TCC A
8	ARYL FR	TTA TGA GTG TAG TAT TCG GGA GTG TTC AG
qRT- PCR		
9	ARYL. FRT	GTT AGA GAA GGG CCA GGT GTC TCA TG
10	ARYL. RRT	CTC TGG CTT GGT AGA CTC CTT GGT
11	MRH.FRT	GAC TGG CTG AAG GGA GTC CG
12	MRH.RRT	GCG TAC ATC ATA ACC TCA CCA CGA CG
13	RBX.FRT	CAG GGT TAC GTT GTTAACCACTTAGGC
14	RBX.RRT	GCC GTT GTA GTA CTG ATG TTG ATG CG
Internal Control		
15	rS7.FRT	ACG TGG ACG GTT CAC AAC TCA TCA
16	rS7.RRT	TTC GCG GCC TGT TAG CTT CTT GTA

Table 2 Primers used in the cloning, identification, and relative expression analysis of different larval hexamerins. Arylphorin (ARYL), Methionine rich hexamerin (MRH) and Riboflavin binding protein (RBX) of *A. janata*. rS7 gene was used as internal control for expression analysis studies as it was found to be most stable gene for Lepidopteran model (VenkataRao, 2014)

2.2 Collection of haemolymph

The prolegs of larvae were cut and bled directly into Eppendorf tubes which was prerinsed with 0.1% of phenylthiourea in order to prevent tyrosinase activity and melanisation of haemolymph. All the haemolymph samples thus obtained were centrifuged at 4000 x g for 2 min at 4ºC to sediment the haemocytes. Peptide/ protein profiling of collected haemolmph at various time interval from both control and toxin exposed larvae was carried by SDS-PAGE (detailed protocol in General Materials and Methods Section).

2.3 Protein sequestration analysis

Biolabelling of hexamerin was carried out using [^{35}S]-methionine produced from BRIT, Mumbai. 4th instar *A. janata* larvae were injected with isotope (10 µCi/5 µl) through hemocoelic delivery using Hamilton microsyinge. They were transferred to petriplate with fresh castor leaves and the haemolypmph was collected 14 h after injection. Bio-labelled haemolymph proteins, which were synthesized by the fat body and released into haemolymph were purified through gel-permeation chromatography using Sephadex G-100 (Sigma-Aldrich). 50 µg of purified bio-labelled hexamerins were injected in the hemocoelic cavity of 3rd instar larvae and they were exposed to sub-lethal dose of Cry toxin formulation. Appropriate controls were also maintained. The mid-gut from larvae (n=10) were dissected every 12 h of toxin exposure until 72 h. The gut was homogenized in 1 X PBS (pH. 8.0) and equal quantity of protein (10 µg) was then electrophoresed using 10% SDS-PAGE. Gel was exposed to storage phosphor screen (GE Healthcare, USA) and image was developed using Typhoon image scanner (GE Healthcare, USA).

2.4 Arylphorin cRNA probe preparation

A. janata arylphorin gene fragment (160 bp) was positioned as forward locked in pGEM-T Easy Vector (Promega Corporation, USA). The vector was linearized with EcoRI and NdeI restriction enzymes (New England Biolabs®, UK). Sp6 promoter was used for the

synthesis of antisense probe (positive), while T7 promoter was used for the synthesis of sense probe (negative). Further, RNA probe synthesis was carried out using DIG RNA labelling kit using manufactures protocol (Roche Diagnostics, Germany).

2.5 In situ localization of arylphorin mRNA

Larval mid-gut and fat body were dissected out and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, USA) in 0.1 M PBS (pH 7.4) at 4°C overnight. Post-fixation, tissues were embedded in OCT compound medium (Leica Microsystems, Germany). Tissue sections (5 µm) were obtained using cryostat microtome (Leica CM1850, Leica Microsystems, Germany) and mounted on poly-L-lysine coated slides. The OCT compound was washed using PBST (PBS pH 7.4 containing 0.1% Tween 20) buffer and the sections were permeabilized using proteinase K (1 µg/ml). Once again 4% PFA was used for further tissue fixation and incubated with 200 µl hybridization buffer (50% formamide, 5X SSC, 5 mM EDTA, 0.1% CHAPS, 1% SDS, 50 µg/ml yeast tRNA, 50 µg/ml and heparin) for 1 h at 50°C. A digoxigenin labelled cRNA probe was mixed with 200 µl of hybridization buffer, heat denatured at 80°C for 5 min, draped and covered with parafilm and incubated at 50°C in a sterile incubator overnight. Post-hybridization, the slides were washed four times (5 min each) at 50°C with a wash buffer (SSC, 50% formamide, 0.1% Tween 20) in a series of decreasing SSC concentration. The slides were processed using solution A (0.5 M NaCl, 5 mM EDTA, 10 mM Tris-HCl pH 8.0, containing 0.1% Tween 20,) and incubated at RT for 5 min. This was followed with RNase A (20 µg/ml) incubation for 20 min at RT, finally the slides were washed twice (5 min each) with solution A (0.5 M NaCl, 5 mM EDTA, 10 mM Tris-HCl pH 8.0, containing 0.1% Tween 20).

For the antibody reaction, the slides were initially washed with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 5 min at RT followed by blocking with 10% BSA for 1.5 h at RT. Anti-digoxigenin-ALP (Roche Diagnostics, Germany) was used

for the detection of bound digoxigenin probe (1: 2000). The incubation carried at 4°C for overnight. These slides were then washed 4 times (15 min each) with DIG wash buffer (Roche Diagnostics, Germany) and incubated with 100 µl BCIP-NBT solution (G-Bioscience, USA) for 10 h at RT to develop the colour. After colour development, the slides were overlaid with detection buffer (Roche Diagnostics, Germany), counterstained with nuclear red (Vector Laboratories Inc., USA) and incubated in TE buffer (Ambion, Life Technologies, USA) for 5 min. Finally after washing with PBST buffer (PBS containing 1% Tween 20), the slides were dehydrated using ascending series of ethanol, cleared in xylene, mounted using DPX mountant and visualized under Olympus IX-81 microscope.

2.6 Arylphorin mRNA expression analysis

Total RNA was isolated from the required tissue (100 mg) using TRIzol method (Ambion, Life Technologies, USA). The quantification of the RNA was carried out using NanoDrop spectrophotometer (ND-2000; NanoDrop Technologies, USA). Quality and integrity of RNA was assessed by formaldehyde denaturing gel electrophoresis. cDNA was synthesized using Revert Aid First Stand cDNA synthesis kit (Thermo Scientific, USA). For quantitative PCR analysis, custom-made TaqMan gene expression assay was performed (Applied Biosystems, USA). Arylphorin primers of amplicon size 160 bp (KX462992) were used. Insect rS7 gene of amplicon size of 115 bp (KF984201) was used as internal control. The real-time expression analysis was performed in triplicates. From the exponential phase of PCR amplification Cycle threshold (Ct) values were obtained. Arylphorin expression was normalized against the expression of rS7 rRNA to generate a ΔCt value (Ct of Arylphorin– Ct of rS7 rRNA). Comparative Ct method was used to calculate the change in the gene expression (Livak and Schmittgen, 2001).

3 Results

3.1 Cloning of partial cDNA fragment and confirmation of their identity as hexamerins

Partial fragment for all three hexamerins Arylphorin (ARYL), Methionine rich hehamerin (MRH) and Riboflavin binding protein (RBX) were cloned using degenerate primers based strategy. Based on the sequence similarity obtained with other Lepidopteran insects, they were further cloned using gene specific primers (Table 2) and confirmed by Sanger sequencing. In the present study the partial fragments obtained for Arylprorin was 556 bp, Methionine rich hexamarin was 470 bp and Riboflavin binding hexamerin was 736 bp (Fig19. A, B, C), and their sequence are represented in figure 20. Further sequences were analysed and confirmed through BLAST homology search analysis (BLASTn; https://blast.ncbi.nlm.nih.gov).

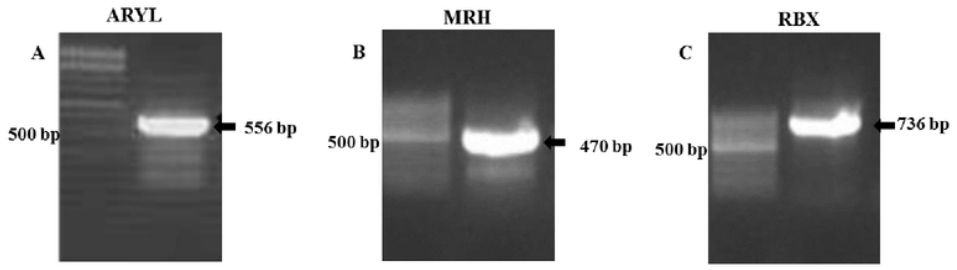


Fig. 19 Amplicon of hexamerins obtained using gene specific primers. (A) Arylphorin (ARYL-556 bp), (B) Methionine rich (MRH-470 bp) and (C) Riboflavin binding (RBX-776 bp).

i) Arylphorin mRNA partial cds

CGTCAAGATCAACGACGTGAAAGTCGATGAGCTTGTCACTTACTTCGAATATTATGACTTCAATATTTCAAA
CAGCCTAATGTTCACCAAGAATGTAAAACAGCAACAGCCCGAAAGTTTCGTCATCCGCCAACCCCGTTTGAC
CCACAAGCCGTTCACAGTCAACATCAACCTCAAATCCGATGTAACTTCCCAGGCTGTCTTCAAGATCTTCCT
TGGACCTAAATATGACAACTACGGATATCCTCTGGACCTTGAAAAGGACTGGATGAAGTTCTACGAACTGGA
TTGGTTCGTTCAAAAAATAACTCCTGGTGAAAACAAGGTGGAACGCAAATCTAGTGACTTCATGTTCTTCAA
GGAGGACAGCATGCCGACCAGTGAAATTTGGAAGTGGTTAGAGAAGGGCCAGGTGTCTCATGACATGTCTGT
TGTACCTGACAGCATGCCCAACAGACTGATGCTGCCTAAAGGTACCCACGGTGGATATCCATTCCAGCTGTT
CGTGATGGTATACCCGTACAACGGTGTCACCAAGGAGTCTACCAAGCCAGAG

ii) Methionine rich hexamerins partial cds

GGCATGTTTCTGTATGCCCTCACCGCTGCCGTGTTCCACAGACCCGACTGCAAGGGCATCATCCTCCCCGCC
CCGTACGAGATCTACCCCTACTTCTTCGTCGACAGTCATGTCATTGAAAAGACGATGATGATGAAGATGACC
AAGGCTAAAGTCGACCCCGTTATCAAGGACTACTTCGGCATCAAAGTGACTGACAAGAACCTAGTTGTGATT
GACTGGCGTAAAGGAGTCCGCAACACGATGGCTAAAGAAGACCGTCTCTCATACTTCACCGAGGATGTAGAC
CTGAACACATATATGTACTACCTTCACATGAACTACCCATACTGGATGACTGACGATATGTACACCGTGAAC
AAGGAGCGTCGTGGTGAGGTTATGATGTACGCTAAGATGCAGATGTTGGCAAGGATGAGGCTGGAGCGTCTG
TCTCACGAGATGTGCGACATAAGCCTATGATTGATCCG

iii) Riboflavin binding hexamrins partial cds

CTAATGGCTTTGGTGAAATTCCCGTACTAGGCCAAGATTTTGTAAAGCACGGTTACTCTAGTGGACTTTGTA
CCACAAAGGCATTCCGTTCCCATCCAGGCCTAACAATTACCACCTCGACCAGCCCGAGTTCGTTCAAGCTAT
TACGGCACTCGAAGATTATGAACACCGTATTCGGGAAGCTATTGAACAGGGTTACGTTGTTAACCACTTAGG
CGAACACATCAGCCTGCTGGTTCCCGAAGCTATTGACGTTTTAGGTCGTGTTACTGAGGCTAATGTCGATTC
TCCTAACTGGCACTACTACAAAGACTTTATCACCATTTGGAAACAGCTTTTGGGTAATTCTCAAACTGTTTC
GCATCAACATCAGTACTACAACGGCTTTGCTCCTTTGGTGGTTCCTTCTGTCTTGGAGCACTATCAAACGGC
TCTCCGTGACCCCGCCTTCTACATGATCTGGAAGCGAGTCTTGGGTCTATTCCAGTTGTGGCAAGAGTCACT
ACCTCATTACAAGCGTGAAGAACTGGCTCTCCCTCAAGTGGCTATCCAGAAAGTTGAGGTTGACAAACTAGT
CACCTATTTCGAACATTCATACGTTAACGTTACCAACCATCTTTGGACCCATATGGGCGAAGGTGTGCAGGA
GAAGGTGAGCGTCTTAGTCCAATGGCCGAGGTTGAACCACACAAGTGTTCAAGGTCCGCGTGAACGTCAAGA
GCGCAGTGCGAGACAG

Fig. 20 Partial sequences of hexamerins cloned using gene specific primers (i) Arylphorin amplicon of 556bp, (ii) Methionine rich hexamerin amplicon of 470 bp and (iii) Riboflavin binding hexamerin amplicon of 736 bp.

3.2 Expression profile of hexamerin(s)

The expression profiling of all three hexamrins was carried out using Real time primers (Table 2) during the postembryonic larval development from 1st instar to 5th instar. The RNA was isolated from the whole body for 1st and 2nd instar while from 3rd instar onwards it was isolated from the fat body. Results presented in figure 21 revealed that arylphorin expressed was from 1st instar, similarly riboflavin binding hexamerin also got expressed from 1st instar, however prominent expression was seen from 3rd instar. However methionine rich hexamerin expression was seen only from 4th instar larval stage.

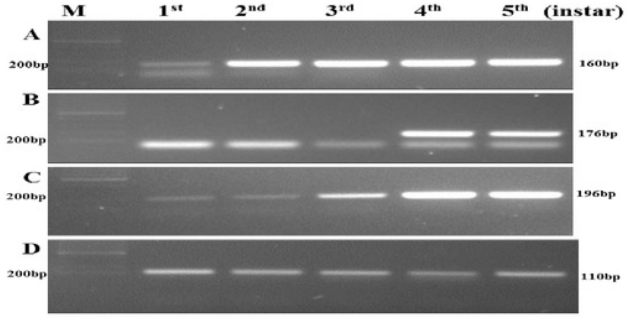


Fig. 21 Expression analysis of hexamerins mRNA during larval development using semi quantitative PCR. Lower expression of aryphorin in 1st and 2nd instar and significant increase observed from 3rd instar onwards (A). Methinone rich hexamerin expression was seen in 4th and 5th instar (B). Riboflavin binding hexamerin expression was observed in 1st instar 5th instar but it was prominent from 3rd instar (C). rS7 was used as internal control (D).

3.3 Protein profiling of haemolymph

The protein concentration of haemolymph was determined at various time points in both toxin exposed and normal larvae (7 day old/3rd instar) using Bradford method. Presence of hexamerin in the haemolymph was first confirmed through western blot analysis using Hex2 antibody (Hex2 antibody generated against recombinant arylphorin of *C. cephalonica* which showed 55% protein similarity with *A. janata* arylphorin) (Fig. 22A). An equal amount of haemolymph protein was profiled through 10% SDS-PAGE and the results are presented in figure 22 B and C. A gradual decline in hexamerin protein (70-85 kDa) was noticed in toxin exposed larvae (Fig. 22C). It is interesting to note that in control, at 72 h time period (where insect undergoes 3rd instar to 4th instar molting) there was significant decline in the hexamerin content, while all other protein were present in more or less in the same concentration (Fig. 22B).

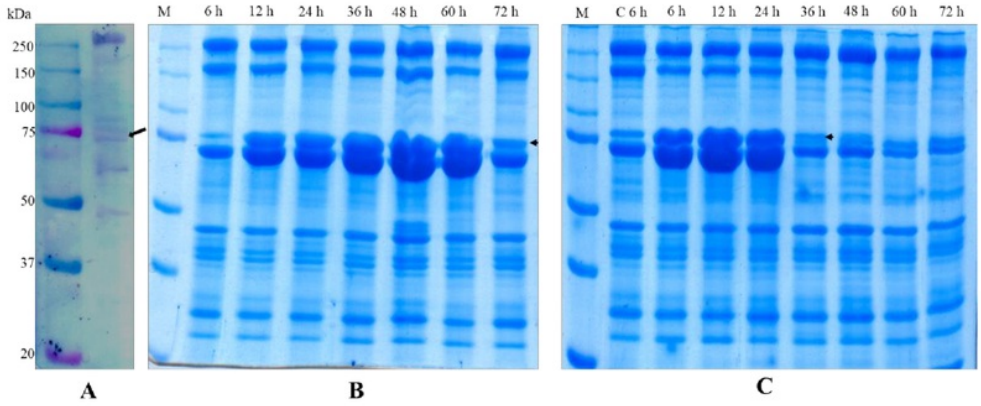


Fig. 22 Haemolymph profile of normal and toxin exposed larvae. (A) Western blot analysis using heterologous Hex2 antibody generated against recombinant araylphorin of *C. cephalonica* (rice moth, stored grain pest) demonstrated it's binding to 70-85 kDa hexamerin proteins from larval haemolymph of *A. janata*. (B) Larval hemolymph protein profile of control insect larvae, (➧) showed the sudden decrease of hexamerin protein during, molting. (C) Larval haemolymph protein profile of toxin exposed larvae, (➧) showed the gradual decrease in the hexamerin protein after 36 h of toxin exposure (➧)

3.4 Hexamerin sequestration analysis

Hexamerins are known to be sequestered by various tissue during postembryonic development (KiranKumar *et al.*, 1997; Arif *et al.*, 2008) as well as facilitate cell proliferation. An attempt was made to analyse their sequestration in mid-gut tissue during Cry intoxication and its role if any in promoting cell proliferation seen during tissue regeneration. Bio-labelled larval hexamerins (prepared by injecting [^{35}S] -Methionine) were separated on SDS-PAGE and checked for their integrity (Fig. 23A). The results showed the presence of primarily 80-85 kDa radiolabelled protein in the samples. For uptake studies 50 µg of the radiolabelled protein was injected to 3rd instar larvae of *A. janata* and they were transferred to Cry toxin coated leaves. The insects were exposed for various time periods and the homogenate prepared from mid-gut was analysed for the presence of intact hexamerins (Fig. 23B). Results presented in figure 23B clearly showed that although the intact radiolabelled hexamerin was seen in haemolymph (HL) even after 72 h. None of the mid-gut samples prepared from toxin exposed (12-72 h) showed the intact radiolabelled hexamerin band.

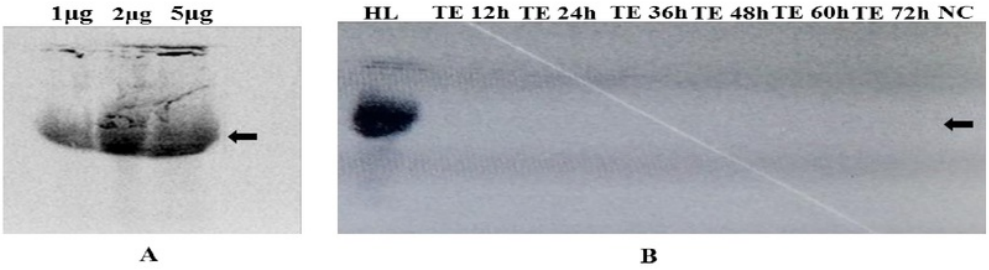


Fig. 23 Autograph to visualize radiolabelled hexamerins. (A) Autoradiograph showed the presence of radiolabeled hexamerins (➡) in the haemolymph of larvae. (B) Autoradiograph showed the presence of the radiolabeled hexamerin in the haemolumph after 72 h injection (➡), while this was not seen in the mid-gut samples prepared from toxin exposed/ fed larvae obtained from various time points (12-72 h).

3.5 Expression of arylphorin in mid-gut

Arylphorin gene, is known to be predominantly expressed by the fat body of Lepidopteran insects. qRT- PCR analysis showed detectable but low expression of arylphorin in the larval mid-gut as compared to the fat body (Fig. 24A). For this study 3rd instar larvae were used as the toxin exposure studies were carried out using this stage. Furthermore, *in situ* studies results with arylphorin sense and antisense probes revealed the localization of arylphorin transcript in the gut epithelium primarily in the basal cells (Fig. 24B).

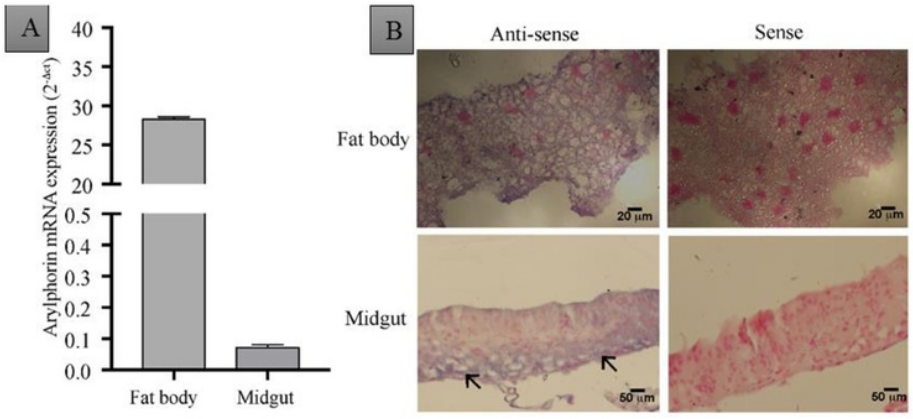


Fig. 24 Analysis of arylphorin gene expression. (A) qPCR analysis showed expression of arylphorin mRNA in the fat body and mid-gut of 3rd instar larvae. (B) *In situ* hybridization demonstrated the synthesis of arylphorin mRNA in the fat body and mid-gut epithelial cells (→) which were primarily localized towards the basal border (Scale bars 20 µm, 50 µm).

3.6 Differential expression of arylphorin gene in Cry toxin exposed larval mid-gut

The levels of mid-gut arylphorin transcripts in control and toxin exposed larvae are depicted in figure 25 showed, in control insects, arylphorin transcript level was fairly low throughout the time points monitored, but a steep incline was observed at 72 h (Fig. 25). On the other hand, arylphorin expression in mid-gut gradually increased from 6 h and reached maximum at 36 h in Cry intoxicated larvae, thereafter the mRNA levels declined significantly in 48 h and remained low till 72 h (Fig. 25).

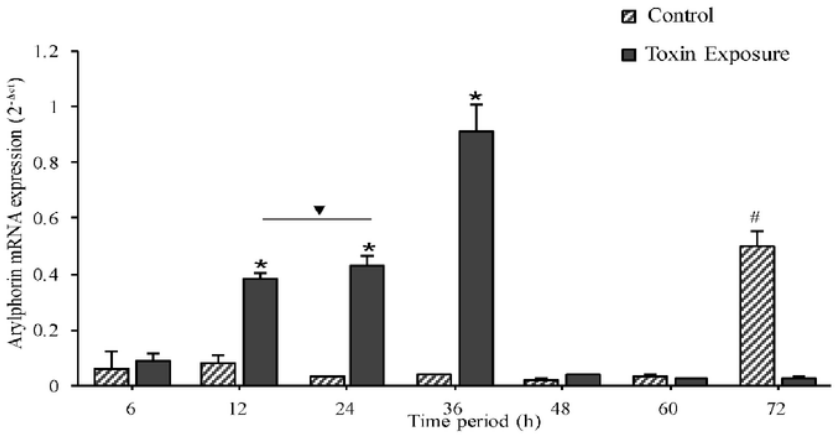


Fig. 25 Expression of mid-gut arylphorin mRNA during toxin exposure: A gradual increase was observed from 6 h to 36 h upon Cry intoxication, which declined later to a basal level. Note the higher expression of arylphorin transcript in 72 h control larvae, a period which coincided with the molting (from 3rd to 4th larval instar). *p < 0.005 significance between experimental groups, #p < 0.005 significant among control groups, ▼ the values are non-significant.

4. Discussions

Large number of growth factors and other molecules promoting mid-gut cell proliferation in culture have been reported. They include fetal bovine serum, ecdysteroids (Loeb *et al.*, 2010), mammalian growth-promoting factor (Nishino *et al.*, 1995), alpha-arylphorin, MDFs (Mid-gut differentiation factor) (Blackburn *et al.*, 2004; Hakim *et al.*, 2010) and AlbuMAX II (Castagnola *et al.*, 2011). Hexamerin proteins, particularly exogenous arylphorin synthesised by fat body was shown to promote proliferation of mid-gut cells in culture (Hakim *et al.*, 2007). However, till date there is no direct *in vivo* study which demonstrated the movement of haemolymph hexamerin to mid-gut, and their role in cell proliferation of mid-gut. In the present study haemolymph proteins isolated from both control and toxin exposed larvae were profiled using SDS-PAGE and showed the gradual decline in hexamerin proteins after 36 h of toxin exposure. In addition in control insects a drastic decline at 72 h was noted and at this stage insect larvae prepare themselves to molt from 3rd instar to 4th instar, which was shown to associated with active cell proliferation in *Manduca sexta* (Baldwin and Hakim, 1991). As intact hexamerins were shown to be sequestered by various tissue during postembryonic development (Arif *et al.*, 2008), and play variety of roles. To confirm whether or not the hexamarins were directly involved in mid-gut regeneration. Hexamerins sequestration studies were carried out and results clearly indicated that there was no sequestration of hexamrin protein in toxin exposed gut samples. Furthermore extensive *in vivo* as well as *in vitro* studies earlier demonstrated uptake of hexamerins (sequestration) during non-feeding stage, prepupal stages by the fat body, male accessary gland (MARG), ovary and salivary glands but not in the gut of other Lepidopteran larvae *Chilo partellus* and *Corcyra cephalonica* (Isamail *et al.*, 1993 ; Arif *et al.*, 2008).

Interestingly detailed analysis of transcriptome (unpublished data) generated from the larval mid-gut of *A. janata*, also showed not only the presence but also up regulation of

arylphorin transcript upon Cry toxin intoxication. The same was not seen for the transcripts of methionine rich and riboflavin binding hexamerins. In addition reports from the Xentari™ resistant *Spodoptera exigua* larvae mid-gut transcriptome also reported up-regulation of arylphorin transcript. (Hernández-Martínez *et al.*, 2010). Further, in the present study *in situ* finding provided a direct evidence for the localization of arylphorin transcript in the actively dividing cells of mid-gut, which were primarily localized in the basal region of the epithelium. Further expression analysis using Cry intoxicated larval mid-gut revealed time dependent increase in endogenous arylphorin gene expression which was associated with active cell proliferation, and was not only seen upon Cry intoxication but also during the molting in control larvae. All these further suggested that the arylphorin most likely plays a role in cell proliferation during repair of mid-gut epithelium by facilitating cell proliferation.

Given the versatility of arylphorin, and the results obtained in the present study showing its presence and altered expression in the mid-gut during Cry toxin exposure, we suggests that a low concentration of arylphorin produced endogenously in mid-gut is critical and probably has mitogenic effect during the Cry intoxication. Further characterization of the gut regenerative process will help us in better understanding of the mechanism of the role of this molecule and its probable function in development of resistance.

1 Introduction

Among various classes of proteases present in animals including insects, aminopeptidase N (APNs) are primarily metallo aminopetidase and have broad specificity. They are known to sequentially/ preferentially cleave the neutral amino acids from the N-terminal end of a peptides/ protein (Terra and Ferreira, 1994). Some of these have been shown to mediate Cry toxin induced toxicity in various insect orders including Lepidoptera (Bravo *et al.*, 2007; Zhao *et al.*, 2016).

Different Cry toxin produced by *Bacillus thuringiensis (Bt)* are known to be target specific for their insecticidal activity. This host specificity was basically due to the presence of different Cry interacting/binding proteins present in the apical brush border membrane of the mid-gut cells of various insects. Existing reports from different insects showed that cadherin like proteins bind to the Cry toxin (Cry1A) in *Manduca sexta* (Vadlamudi *et al.*, 1993), *Bombyx mori* (Nagamatsu *et al.*, 1998), *Heliothis virescens* (Gahan *et al.*, 2001), *Helicoverpa armigera* (Xu *et al.*, 2005). In *Anopheles gambiae,* cadherins interacted with Cry4Ba toxins (Hua *et al.*, 2008). GPI-anchored aminopeptidases were recognized as Cry toxin receptor in many Lepidopteran species like *Manduca sexta* (Knight *et al.*, 1995), *Spodoptera litura* (Agrawal *et al.*, 2002), *Heliothis virescens* (Banks *et al.*, 2003), *Helicoverpa armigera* (Rajagopal *et al.*, 2003), *Bombyx mori* (Yaoi *et al.*, 1999), *Lymantria dispar* (Garner *et al.*, 1999), *Plutella xylostella* (Nakanishi *et al.*, 2002), *Achaea janata* (Budatha *et al.*, 2007) and in Dipteran species *Anopheles quadrimaculatus* (Abdullah *et al.*, 2006). GPI-anchored alkaline phosphatase also was shown to interact with Cry toxin in *Manduca sexta* (McNall and Adang, 2003) and *Heliothis virescens* (Jurat-Fuentes and Adang, 2004).

Two distinct and contrasting models have been proposed for the mode of Cry toxin action based on various studies carried out in Lepidopteran insects (Sobern *et al.*, 2009). The pore formation model is a multistep (Bravo *et al.*, 2004) and well accepted model, where series

of events like toxin solubilization, its activation , proteolytic cleavage, binding of toxin with different receptor molecules, oligomerization, and membrane insertion leading to pore formation and epithelial cell damage. However the basic mechanisms by which Cry toxin caused their toxicity is not only poorly understood but also controversial (Knowles, 1994; Pietrantonio et *al.*, 1996; Rajamohan *et al.*, 1998; Schnepf *et al.*, 1998; Soberan *et al.*, 2009).

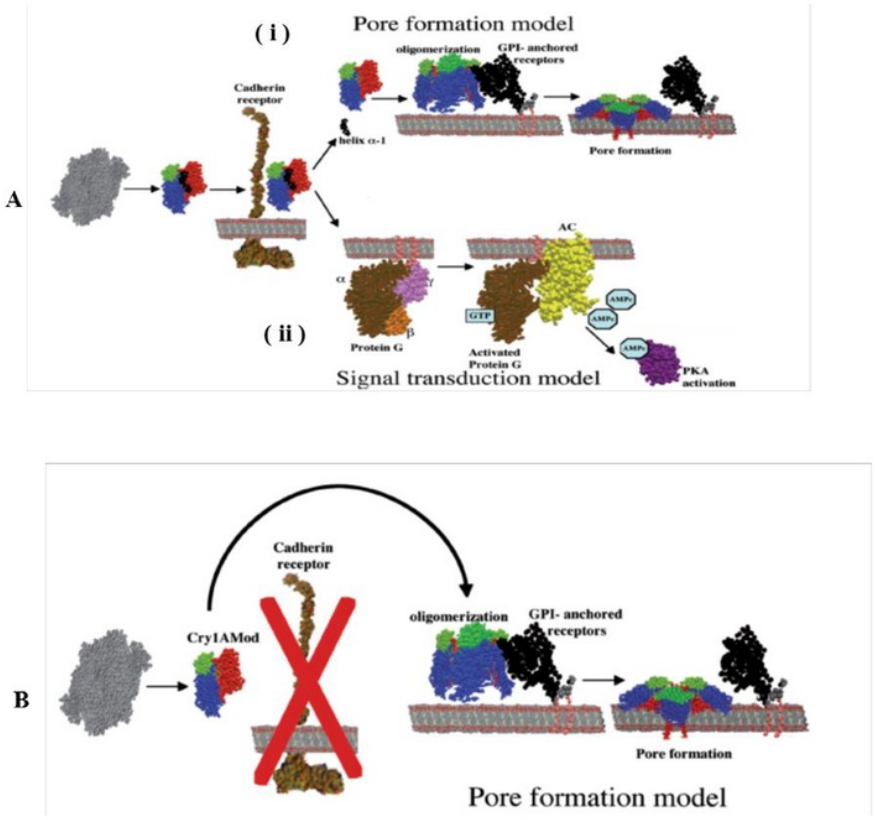


Fig. 26 Mechanism of Cry toxins action. (A) Schematic representation showing (i) Pore formation model where interaction of Cry toxins with cadherin like protein causes cleavage of helix α-1 and its oligomerization and its interaction with the GPI-anchored receptor proteins which facilitate pore formation. (ii) Signal transduction model shows that interaction of the Cry toxin to the cadherin causes the activation of G-protein dependant Protein kinase A (PKA) cellular pathway results in cell death. (B) Pore formation model where interaction of Cry toxin to the cadherin receptor and removal of Cry helix α-1 is not required for toxin oligomerization. (Image adopted from Soberan *et al.*, 2009).

Aminopeptidase N (EC.3.4.11.2) has been identified as Cry toxin receptor in various Lepidopteran insects. These are a class of peptides belonging to Zn^{++} dependent gluzincins family of M1 type metalloproteases (Hooper, 1994; Albiston *et al.*, 2004). They showed the presence of aminopeptidase motif "GAMENWG", Zn^{++} binding motif "$HEXXHX_{18}E$", N-terminal with signal peptides, C- terminal with GPI-anchor signal which helped them in attaching with BBMVs (brush border membrane vesicles) in mid-gut (Fig. 27). *In silico* analysis revealed presence of many O and N- glycosylation sites which was predicted to facilitate Cry toxin binding (Pigott and Ellar, 2007). In Lepidoptera larval mid-gut, enzymes play a vital role in dietary nutrient metabolism which include proteases both luminal and BBMV forms (Terra and Ferreria, 1994; Naseri *et al.*, 2010; Sarate *et al.*, 2012)

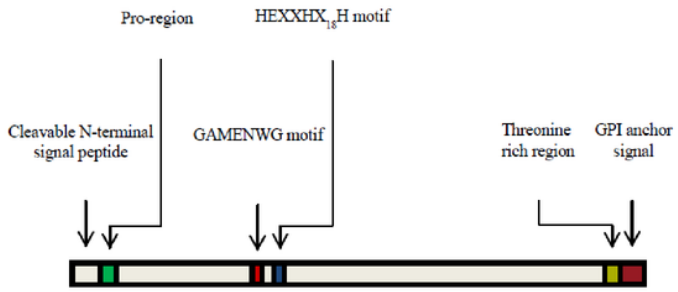


Fig. 27 Schematic representation of Lepidopteran aminopeptidase N.

Further the APNs are classified into eight clusters (APN1-8) based on amino acid identity/similarity in Lepidopteran insects (Crava *et al.*, 2010; Hughes, 2014; Lin, 2014). It was shown that many forms (isoforms) of APN were expressed simultaneously in larval mid-gut of *Helicoverpa armigera* (Angelucci *et al.*, 2008), *Ostrinia nubilalis* (Crava et al., 2010, 2013; Khajuria *et al.*, 2011), *Epiphyas postvittana* (Simpson *et al.*, 2008), *Bombyx mori* (Crava *et al.*, 2010; Nakanishi *et al.*, 2002) and *Trichoplusia ni* (Tiewsiri and Wang, 2011; Wang *et al.*, 2005). All the isoforms found be organized in a single cluster in Lepidopteran genome (Baxter

et al., 2008; Crava *et al.*, 2010; d'Alençon *et al.*, 2010). However, it is still uncertain and not clear as to which isoforms act as Cry toxin receptor.

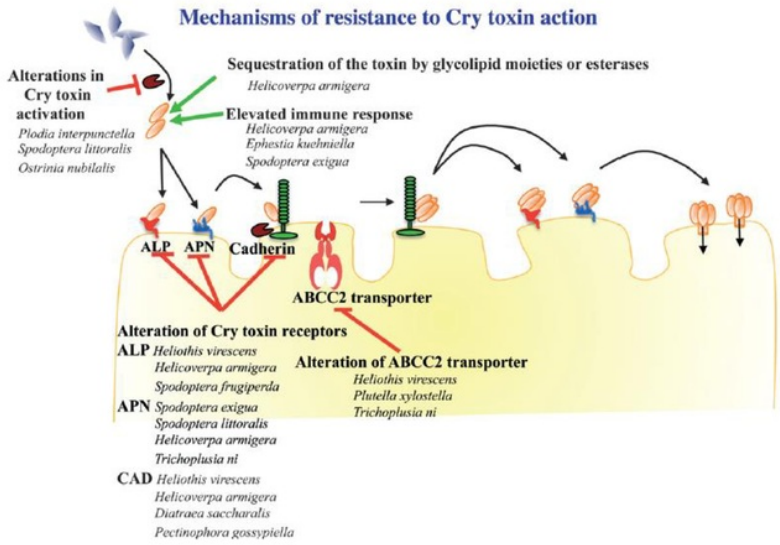


Fig. 28 Schamatic respresentation mechanism(s) of development of resistance. Figure shows possible menchanisms of resistance to Cry toxin which could be due to altration in Cry toxin processing and alteration in Cry toxin binding/ receptors proteins in various Lepidopteran insects (Figure adopted from Pardo-Lopez *et al.*, 2012).

Literature survey clearly showed that resistance developed against *Bt*/Cry formulations in selected laboratory populations was primarily due to various mechanisms (Fig. 28). In certain insects it was due to the alteration of in Cry toxin activation process by gut proteases (Keller *et al.*, 1996; Oppert *et al.*, 1997; Li *et al.*, 2004). Further reports suggested that alteration/modification in proteases which acted as toxin receptor, facilitated the development of resistance (Pardo-Lopez *et al.*, 2012). Sequestration of toxin by glycolipids moieties (Ma *et al.*, 2005) and an elevated immune response (Hernandez-Martinez *et al.*, 2010), were also shown to be additional mechanisms for the development of resistance.

Recent studies highlighted that in various insects, toxin resistance was associated with reduced and differntial expression of various APN isoforms (Tiewsiri *et al.*, 2011). Reduced interaction of Cry toxin, which was associated with mutation in receptor proteins (Ferre and Van Rie , 2002; Hackel *et al.*, 2007; Zhang *et al.*, 2008; Adang *et al.*, 2014; Guo *et al.*, 2015) and mutation in other molecules like ABCC2 (ATP-binding cassette transportor subfamily C2) (Gahan *et al.*, 2010; Baxter *et al.*, 2011) was also suggested as probable mechanism for resistance in various insect models. They have been characterized as functional Cry toxin receptor (Tanaka *et al.*, 2013; Zhou *et al.*, 2016). Detailed studies with the development of resistance in various insects furthet suggested the Mod1 mechanism, which was primarily due to expression of a mutant cadherin protein (mutation in 12^{th} domain) by the mid-gut cells of Lepidopteran pests (Heckel *et al.*, 2007).

In this chapter we tried to made an attempt to characterize aminopetidase N as Cry toxin (DOR *Bt*-1) repotor, identify various isoforms of APNs and their role in Cry toxin toxicity and/or developmet of toxin tolerance, in toxin tolerated insect populations developed using sub-lethal dose of toxin exposure.

2 Materials and methods

2.1 Primers used in the present study

Following set of primers were used in this study to cloning the full length of various APN isoforms and also profiling of their expression in toxin exposed larvae of *A. janata*. (Detailed protocol for primer designing explained in General materials and Method Section)

S. No.	Primer Name	Primer Sequence (5' – 3')
Gene specific primers for obtaining full length (ORF)		
1	APN1.FFP	ATG GCG TTG GCA GAA CTG G
2	APN1.FRP	TTA TAC AAT AAC ATTA ACC AAA GC
3	APN2.FFP	ATG TAT TTA CTA ACA CTA TTA GCT
4	APN2.FRP	TAA GAT GGC AAC AAG TGC AG
5	APN4.FFP	ATG CAG CTC CTT ATC ATT CTC
6	APN4.FRP	TTA ACG TAG TAA ATG GGT GAA C
7	APN6.FFP	ATG TCC AAA ATA CTT ATC GCA G
8	APN6.FRP	TAA ACA AAC ATG GAT AGG AGA G
9	APN9. FFP	ATG GCT AGC AAA TTT CAA CCA ACA T
10	APN9. FRP	CTA GGC CCT ATG GTT TGC C
qRT- PCR		
11	APN1.FRT	CAG ACG TCC ACT CTC CTA CTC TAG T
11	APN1.RRT	GCG ATA ACT TCA TGC AGA GCG TTA CC
12	APN2.FRT	CGC TCC ACC ACA GTA CAA TGT TGT C
13	APN2.RRT	CTC TGA AGG GAA TGT TGA AGA TGG GA
14	APN4.FRT	CAC CCG TGA CAT CAA TCT TAG CCC T
15	APN4.RRT	CGT GGG TAA GAT CTT CAG CGA TCG
16	APN6.FRT	CTA TCG AAA CCT GCC GGT TAC ATA GGA
17	APN6. RRT	CGT GGG TAA GAT CTT CAG CGA TCG
18	APN9.FRT	CTG TGG AAA CCT GTT ACA TAT GTG GCT C
19	APN9.RRT	GCT TCC AAC TTC ACC AGA GTC TAT TTG
Internal Control		
20	rS7.FRT	ACG TGG ACG GTT CAC AAC TCA TCA
21	rS7.RRT	TTC GCG GCC TGT TAG CTT CTT GTA

Table 3 List of primers used for cloning and relative expression analysis of different aminopeptidase N isoforms in larval mid-gut of *A. janata*.

2.2 Preparation of membrane fraction

i) Tissue isolation: The 3rd instar larvae were narcotized by placing them on ice for 15-20 min. The larval cuticle was incised from the ventral side of abdomen and cut open through the length of the larvae. Mid-gut, fat body, salivary gland and Malpighian tubules were dissected out in ice cold insect Ringer solution (130 mM NaCl, 0.5 mM KCl and 0.1 mM $CaCl_2$). Mid-gut was cleared of food contents and peritrophic membrane prior to usage.

ii) Preparation of mid-gut brush border membrane vesicles factions (BBMVs): This was done by using the protocol of Wolfersberger *et al.*, (1987) with slight modifications. Dissected gut tissue was transferred to centrifuge tube containing ice-cold MET buffer (300 mM mannitol, 17 mM Tris-HCl pH 7.5, 5 mM EGTA and 1 mM PMSF), vigorously vortexed and briefly centrifuged for 5 min at 1000 X g to obtained plant debris free clean tissue. Mid-gut tissue was homogenized on ice in a tightly fitting glass homogenizer in ice-cold MET buffer (10% w/v). The homogenate was diluted with an equal volume of ice-cold 24 mM $MgCl_2$, blended and held on ice for 15 min before centrifugation at 2500 X g for 15 min at 4ºC. The supernatant was further centrifuged at 30,000 X g for 1 h at 4ºC. The resulting pellet, which contained BBMV fraction was preparation was re-suspended in HBS buffer (10 mM HEPES, pH 7.4, and 150 mM NaCl), flash-frozen in liquid nitrogen and stored at -80ºC until use.

iii) Preparation of fat body membrane fractions: The fat body tissue was homogenized on ice in buffer A (5 mM HEPES, pH 8.5 and 0.1 mM $CaCl_2$), and membrane fraction was prepared according to the method described by Kirankumar *et al.*, (1997). The fat body homogenate was centrifuged at 30,000 X g for 30 min at 4ºC and 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, 2 mM $CaCl_2$, 10% Sucrose(w/v) and 0.1% Triton X-100). This particular fraction was used as membrane fraction and stored at -80ºC until use.

iv) Preparation of Malpighian tubule and salivary gland protein homogenates: The tissues (100 mg) were homogenized in 200 µl of homogenization buffer (10 mM Tris –HCl, pH 7.4, 0.1% Triton X-100, 1mM PMSF, 1mM EDTA and 1mM DTT). The homogenates were centrifuged at 1000 X g for 5 min to remove larger debris and the aliquots of the supernatant were used.

2.3 *In vitro* Cry toxin binding (ligand blotting) analysis

The gut BBMV's, fat body membrane, Malpighian tubule and salivary gland protein preparations (10 µg each) were separated by 10% SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was blocked using blocking buffer [5% (w/v) skimmed milk powder in 0.01 M Tris buffer saline (TBS, pH 7.4)] for 1 h, followed by incubation in blocking buffer containing 1 µg/ml of Cry toxin (Cry1Ac) for 1 h at RT. Following this blot was incubated with primary Cry 1Ac antibody (antibody was generated in rabbit) overnight then incubated with secondary ALP conjugated anti-rabbit goat antibody (Thermo Fisher Scientific, USA). The blot was developed using BCIP/NBT substrate (G-Biosciences, USA).

2.4 Ligand blot of mid-gut BBMVs

The mid- gut BBMV's (10 µg) were separated by 10% SDS-PAGE and electro-transferred to a nitrocellulose membrane. The membrane was blocked using blocking buffer [5% (w/v) skimmed milk powder in 0.01 M Tris buffer saline (TBS, pH 7.4)] for 1 h, followed by incubation in blocking buffer containing 1 µg/ml of activated DOR *Bt*-1 or Cry1Ac toxin for 1 h RT. Following this blot was incubated with polyclonal primary antibody generated against DOR *Bt*-1 toxin and anti-Cry 1Ac toxin antibody (raised in mice) for overnight. The blot was then incubated with secondary HRP conjugated secondary antibody (Thermo Fisher

Scientific, USA). The blot was developed using Western BLoT chemiluminescences Kit (Takara Bio Inc, Japan).

2.5 APN and Cry toxin interaction analysis using immunohistochemical colocalization studies

The mid-gut transvers sections were prepared (according to protocol described in General Materials and Method Section). The slides were deparafinised using xylene and hydrated through decreasing ethanol series. For immunostaining, section were washed with PBS followed by PBST, then blocked using 10% goat serum (Thermo Fisher Scientific, USA). A solution of 1 µg/ml of Cry toxin (Cry1Ac or DOR *Bt*-1) was added on to the slides and inoculated for 2 h RT. Primary antibody generated against aminopeptidase N (1:500 dilutions; raised in rabbit) and anti-Cry toxin(s) antibody (1:500 dilutions; raised in mice) were added and the slides were incubated overnight at 4°C. The slides were washed with PBS solution to remove unbound primary antibodies then respective secondary antibody (anti rabbit goat IgG - FITC conjugated (Green) and anti-mice IgG- TRITC (Red)) was added to the sections and incubated at RT for 2 h. The excess of antibody was washed with PBST and the slides were mountant using gold antifade mountant (Thermo Fisher Scientific, USA) for prolonged storage. The sections were visualized under fluorescence microscope.

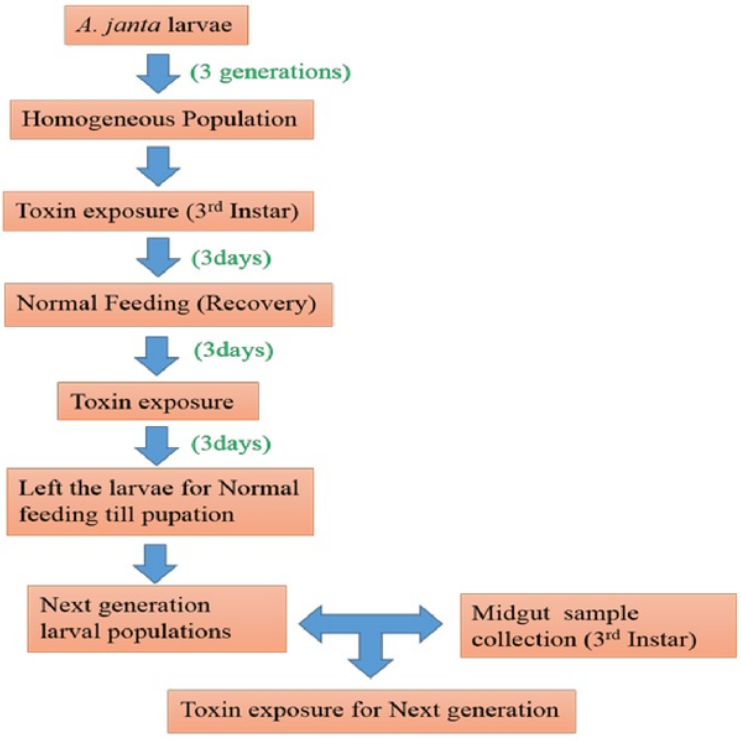
2.6 Cloning and characterization of APN isoforms

As the genomic sequence of *A. janta* is not available in database hence an attempt was made to carry out next generation RNA sequencing (*de novo* transcriptome) of larval mid-gut (unpublished data). Based on homology search in NCBI database five different isoforms of APNs were identified. Using standard protocol (mentioned in General materials and Method Section) full length of the APN sequences were cloned using ORF specific primers (Table 3). Detailed *in silico* analysis of these putative APNs was carried using bioinformatics tools. Finally

their expression was analyzed upon either continuous sub-lethal toxin exposure or toxin tolerant insect populations using Real- time primers (Table 3).

2.7 Generation of toxin tolerant insect populations

The larvae of *A. janata* were collected from the unexposed *castor* fields of Telangana and Andhra Pradesh which were never exposed to *Bt* formulations and maintained in the laboratory for 3 generations for obtaining homogenous populations. The insects were exposed to sub-lethal dosage of Cry toxin using following strategy (Flow chart 1). The surviving insects were maintained for the next generation. At each generation the required tissue samples were collected and experiments were carried out. Using the above protocol toxin tolerant insects were generated and present study analysis was carried out up to sixth generations. It is being carried out further.



Flow chart 1 Strategy for the development of toxin tolerant insect populations.

2.8 *In-silico* APN- Cry toxin interaction analysis

Sequence of one of the isoforms of aminopeptidase N i.e APN2 was used for this study. The BLAST program was used for sequence search against known 3D structures available in the Protein Databank (PDB) (http://www.rcsb.org/), in order to select appropriate templates for constructing 3D structure models, using homology modeling methods. The homology model program MODELLER v9.12 was used to generate 3D model of APN. The stereo-chemical quality of the modeled protein structure was checked in Ramachandran plot using PROCHECK. The compatibility of the model with its sequence was measured by Verify-3D graph. 3D coordinates of Cry1Ac protein was downloaded from protein databank (PDB id: 4W8J) and it was used for protein-protein docking studies. Both the proteins were submitted to GRAMM-X protein-protein docking web server. The top ranked result obtained from GRAMM-X web server was identified and further submitted to SPPIDER web server to find out possible interaction between APN and Cry proteins. Binding energy of this interaction was predicted by PRODIIGY. Further 3D structures were viewed, analyzed and figures were generated in PyMOL Molecular Graphics System (1.7).

2.9 Two-dimensional electrophoresis

The mid-gut membrane proteins were subjected to clean-up using the 2-D Clean-up Kit (GE Healthcare Life Sciences, USA) as per the manufacturer's instructions. Immobiline Dry strips (4-7 pH 11cm, GE Healthcare, USA) were rehydrated in 350 µl of the protein solution for ~20 h under low viscosity mineral oil (Bangalore Genei, India). The rehydrated IPG strips containing 350 µg proteins were then subjected to isoelectric focusing. After IEF, the strips were equilibrated and second dimensional SDS-PAGE was carried out in duplicate one was for western blotting and a second one for Coomassie brilliant blue R-250 staining.

2.10 Two-dimensional electrophoresis and immunoblotting

The mid-gut membrane proteins (BBMVs) separated using 2D SDS-PAGE gel and were electro-transferred to a nitrocellulose membrane. The membrane was blocked using 5% (w/v) skimmed milk powder in 10 mM TBS (pH 7.4) for 1 h. The blot was incubated with primary APN antibody overnight, followed with secondary HRP-conjugated anti-rabbit goat antibody, and developed using Blot chemiluminescenc HRP substrate (Takara Bio.Inc, Japan).

2.11 Differential two-dimensional gel electrophoresis (2D-DIGE)

The mid-gut BBMV samples prepared from control and 6th sgeneration toxin tolerant insects, which were exposed to toxin for 24 h. Samples were cleaned up using 2-D Clean-up kit (Amersham Biosciences, USA), the obtained pellet was dissolved in 2-D lysis buffer containing 7 M urea, 2 M thiourea and 4% CHAPS without dithiothreitol (DTT). The protein quantification was done by using Ettan 2-D Quantification kit (Amersham Biosciences, USA). Supplied CyDye (5 nmol) was reconstituted with 99.8% anhydrous dimethylformamide (DMF) from freshly opened source, used as a working solution (400 pmol) for protein labelling. The pH of the protein solution was adjusted to between 8.0-8.5 with 0.5 N NaOH before dye labelling. Minimal labelling of protein samples prepared from control as well as Cry toxin exposed larvae were done with 400 pmol of Cy3 and Cy5 Dyes respectively as per the suppliers instructions. An equal amount of proteins from all the twelve samples (6 control+ 6 treated) were mixed to generate internal standard, which was labelled with Cy2 and equally distributed to all. This helped to generate accurate spot statistics between gels and increased level of confidence. After incubation in the dark for 30 min on ice, the dye quenching was achieved by addition 10 mM L-lysine for 10-20 min in dark. Six biological replicates were used for this experiment, dye swapping was done to minimize the artefacts generated by the dye components.

Each gel consisted of control, treated samples labelled with Cy3 or Cy5 and internal standard labelled with Cy2. Finally, the required volume was made with 2-D rehydration buffer

(40 mM DTT and 0.5% IPG buffer pH 4--7). After rehydration for 12-16 h, IPG strips (pH 4-7, 18 cm) were subjected to isoelectric focusing (IEF) in Ettan IPGPhor 3 system at 36 Volt-hours. Equilibration of IPG strips was done with buffer containing 6 M urea, 30% glycerol, 2% SDS and 50 mM DTT for 20 min followed by alkylation with 100 mM idoacetate (IDA) for 20 min. Finally, IPG strips were equilibrated in SDS-PAGE cathode buffer, before performing the second dimension analysis.

After electrophoresis, gels were scanned at different excitation and emission wavelength for Cy3, Cy5 and Cy2 labelled proteins using Typhoon variable mode scanner (Amersham Biosciences, USA). All the gels were scanned with PMT of 600 and 200 µm/pixel resolution. Images were processed using DeCyder 2-D version 7.0 software through Differential In gel Analysis (DIA) and Biological Variation Analysis (BVA). Finally differentially regulated protein spots were detected with a significant p-value of <0.05 generated by the student t-test and one-way ANOVA.

3 Results

3.1 Cry toxin interaction with various larval tissues

The profile of proteins/peptides present in tissue homogenates prepared from larval salivary glands (L1) and Malpighian tubules (L2), membrane proteins prepared from fat body (L3) and gut BBMVs (L4) (Fig. 29A). Showed that few of the proteins interact with DOR *Bt* - 1 toxin(s) (Fig. 29B). The prominent interaction was seen with proteins of molecular mass of ~110 kDa and ~68 kDa in all the tissues (Lane1- 4) including mid-gut (L4) (Fig. 29B). Thus results obtained indicated that *A.janata* larvae has Cry toxin binding/receptor proteins in almost all the tested tissues.

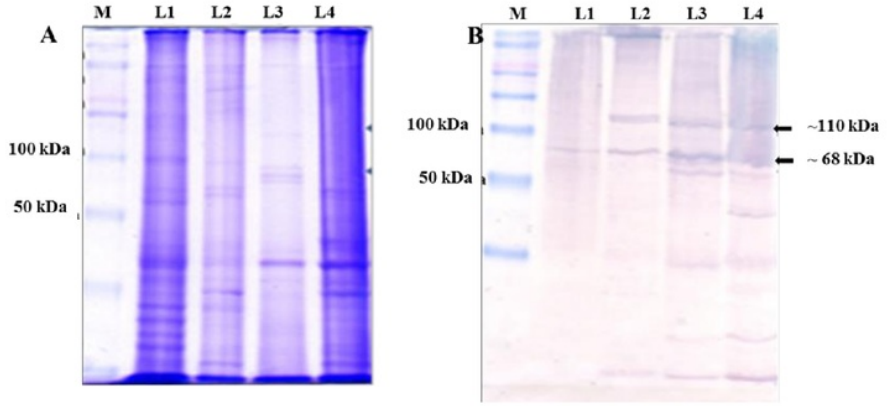


Fig. 29 Cry toxin interaction with larval tissues of *A. janata*. (A) Profiling of membrane and tissues proteins. (B) Ligand blotting with DOR *Bt*-1 toxin(s) and identification of Cry toxin binding proteins/ receptors. Lane M - Protein marker; Lane 1 - Salivary gland protein homogenate; Lane 3 - Malpighian tubule protein homogenate; Lane 3- Fat body membrane fractions and Lane 4 - Gut BBMVs. Note the presence of ~110 kDa and ~68 kDa interacting proteins in all the lanes.

3.2 Characterization of aminopeptidase N as mid-gut Cry toxin receptor

SDS-PAGE separated mid-gut BBMV proteins were electrotransfered on nitrocellulose membrane. Ligand binding analysis was carried out using both Cry1Ac and DOR *Bt*-1toxin(s). The results presented in figure 30A clearly showed Cry toxin(s) interaction with 5 to 6 gut membrane proteins. Among them the prominent interaction was seen with 113 kDa protein, with DOR *Bt*-1 toxins (Fig. 30A, L3). Immunoblotting studies using APN antibody generated

against a small fragment of *A. janata* mid-gut APN, revealed that it was an APN (Fig. 30B, L2). Further MALDI-TOF/TOF analysis based on peptide sequence (MS/M), represented that the interacting protein was indeed an aminopeptidase N (APN) (Fig. 31).

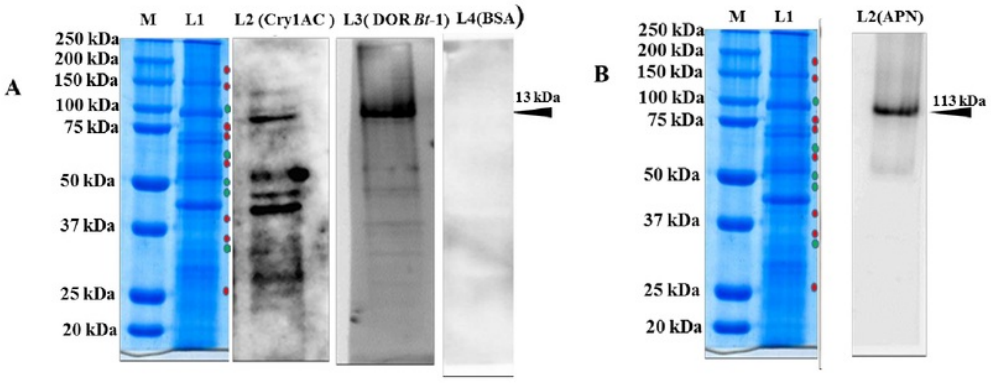


Fig. 30 Identification of Aminopeptidase N as Cry toxin interacting/binding protein.(A) Ligand blot (toxin overlay assay) using active Cry 1Ac (L2) and DOR *Bt*-1(L3) toxins showed the presence of interacting proteins in mid-gut BBMVs. (B) Detection of APN in midgut BBMV's using APN (L2) antibodies generated using recombinant APN protein through immunoblot analysis.

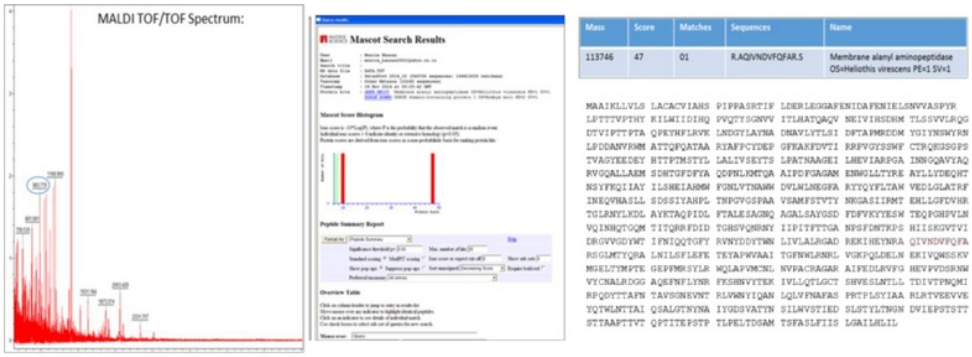


Fig. 31 MALD -TOF/TOF analysis of interacting protein. The peptide peaks obtained analysed through Mascot database search (http://www.matrixscience.com).The peptide (983.776) sequence matched with the reported membrane alanyl aminopeptidase of *Heliothis virescens* with molecular weight of 113 kDa.

3.3 APN- Cry toxin interaction analysis through immunohistochemistry

For this interaction analysis the mid-gut cross sections were deparafinized, hydrated and were initially incubated independently with activated DOR *Bt*-1 toxin (test) or Cry1Ac (positive) or BSA (negative) control. Later the sections were incubated simultaneously with Cry toxin antibody (generated in mice) and APN antibody (generated in rabbit). Further interaction were detected using appropriate secondary antibodies, which were tagged with fluorescent dyes. Results obtained showed the presence of overlapping signals in specific regions of mid-gut. i.e inner brush border membrane of epithelial cells.(Fig. 32). Further the signal was visible in the Cry1Ac as well as DOR *Bt*-1 toxin but not in BSA incubated sections. This clearly suggested that the interactions were specific and Cry toxins bind to only with the brush border membranes, where APNs were most likely localized.

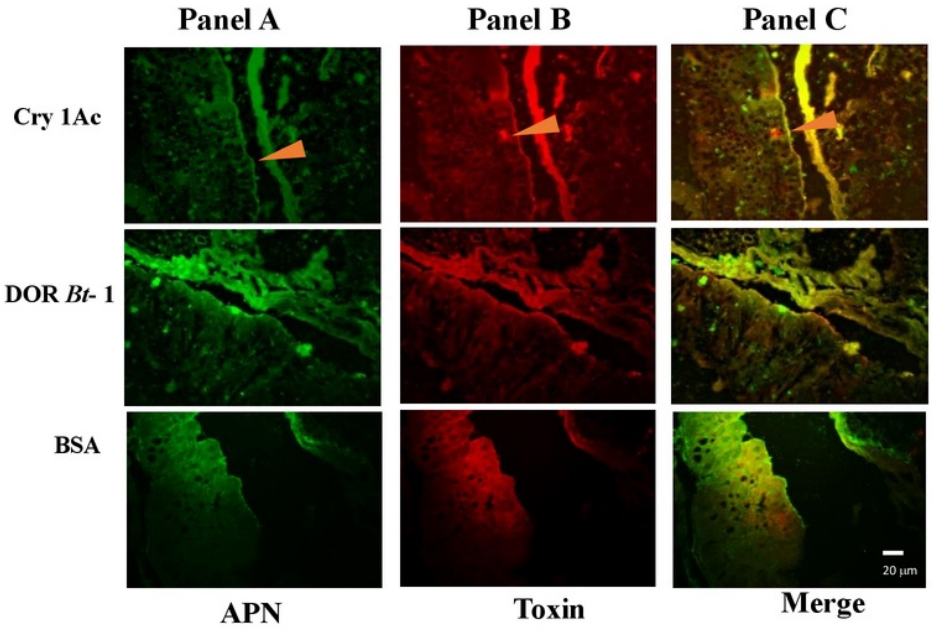


Fig. 32 Aminopeptidase N and Cry toxin interaction analysis. Panel A shows the detection of APN on apical brush border of mid-gut epithelium using APN antibodies. Panel B shows the interaction of Cry toxin with the apical brush border. Panel C shows the colocalization of both APN and Cry interacting proteins. BSA was used as negative control. Please note that merge imaging shows the presence of both the molecules APN and Cry toxin in the same region of the apical membrane of mid-gut ( ). BSA was used as negative control where no signal was detected.

3.4 APN isoform analysis

Full length of cDNA for all the five isoforms detected in *A. janata* mid-gut larval trasncriptome were cloned into pTZ57R/T vector (Fig. 33) and Sanger sequenced. The sequences were confirmed and submitted to Gene Bank(https://www.ncbi.nlm.nih.gov/BankIt/) with accession number- APN1 [MF425653], APN 2 [MF425654], APN 4 [DQ872666], APN6 [MF425654] and APN9 [MF425654]. Deduced translated sequences (amino acids) were aligned through Multiple Sequence Alignment (MSA) (http://www.ebi.ac.uk/Tools/msa/) and searched for the presence characteristic aminopeptidase activity motif "GAMENEG" and zinc binding motif "HEXXHX18H" (Fig.34). Similarity between the isoforms was checked using Percent Identity Matrix (Clustal 2.1) and the cloned isoforms did not show more than 37% sequence identity (Fig. 35). Further conserved domain analysis of all the sequences revealed that these proteins belong to "Peptidase M1 Aminopeptidase N" family with Endoplasmic reticulum aminopeptidase 1 (ERAP1) with M1_APN_2 and ERAP1_C domain. Detailed analysis revealed the presence of transmembrane peptide at C-terminal end of APN1, APN2 and APN4 while it was absent in APN6. APN1, APN4 and APN6 possessed signal peptide at N-terminal but it was absent in APN2. Furthermore, APN9 lacked both N and C-terminal signal sequences (Fig. 36).

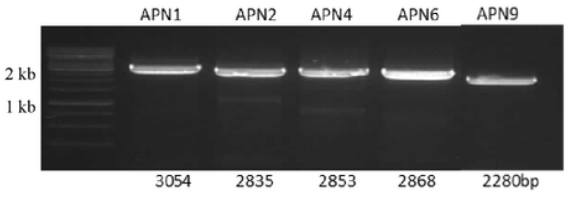


Fig. 33 Agarose gel micrograph showing full length amplicon of APN1 (3054 bp); APN2 (2835 bp); APN4 (2853 bp); APN6 (2868 bp) and APN9 (2280 bp).

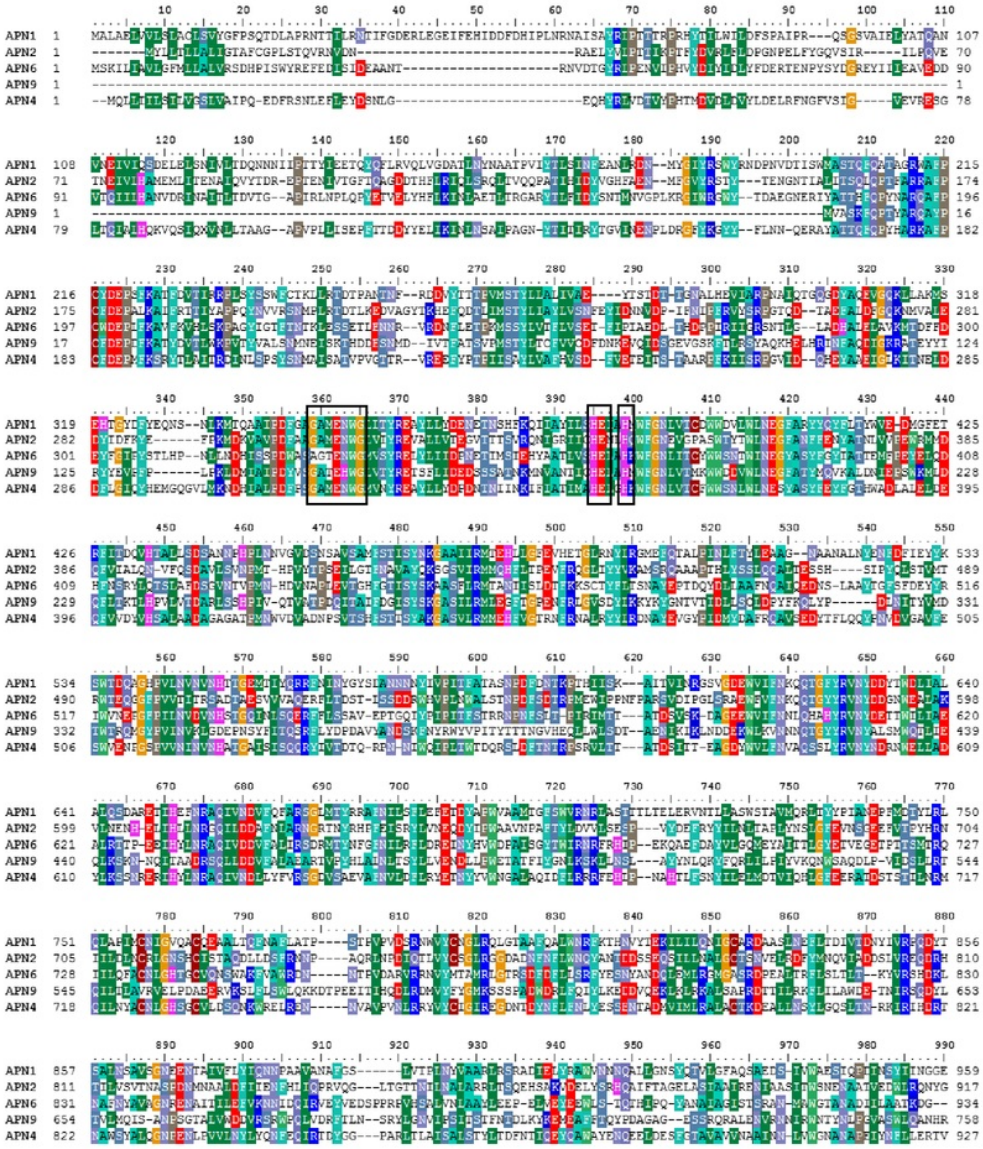


Fig. 34 Multiple sequence alignment of APN's. APN1, APN2, APN3, APN4, APN6 and APN9 from mid-gut of *A. janata* larvae shows the presence of *A. janata* shows the presence of aminopeptidase active motif "GAMENEG" and zinc binding motif "HEXXHX18H". Please note that various isoforms cloned in the present analysis showed sequence identity which was less than 40%.

A

	APN9	APN2	APN1	APN6	APN4
APN9	100.00	27.82	27.50	24.86	26.09
APN2	27.82	100.00	29.15	27.08	29.48
APN1	27.50	29.15	100.00	30.87	31.22
APN6	24.86	27.08	30.87	100.00	37.43
APN4	26.09	29.48	31.22	37.43	100.00

B



Fig. 35 Sequence similarity analysis between different larval mid-gut isoforms of *A. janata*. (A) The results obtained showed the sequence identity match was not more than 37%. (B) Phylogenetic tree showing that APN9 and APN2 fell in one clad, while APN6 and APN4 belonged to same clad, on other hand APN1 fell in a totally different clad.

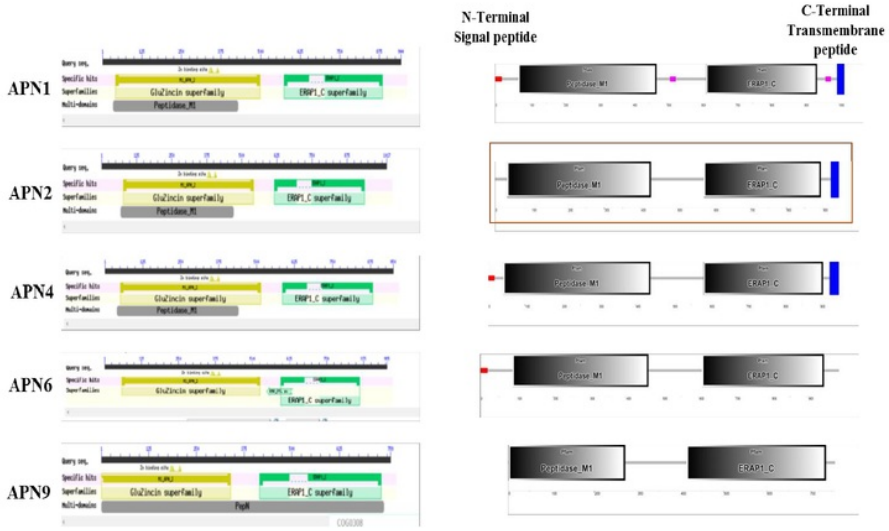


Fig. 36 Conserved domain analysis. The cloned APN proteins in *A. janta* revealed that they belong to Peptidase M1 Aminopeptidase N family having ERAP1 (Endoplasmic reticulum aminopeptidase 1) with M1_APN_2 and ERAP1_C containing domain. APN1, APN4 showed the presence of transmembrane domain and signal peptide further, APN2 had only transmembrane domain, APN6 had N-terminal signal peptide while APN9 lacked both N and C-terminal peptides.

3.5 Tissue specific expression APN isoforms

The results presented revealed that all the five APN isoforms cloned from the larval mid-gut in the present study were selectively expressed only in mid-gut tissue (Fig. 37). Further, none of the other tissues tested showed amplification of the cloned APNs.

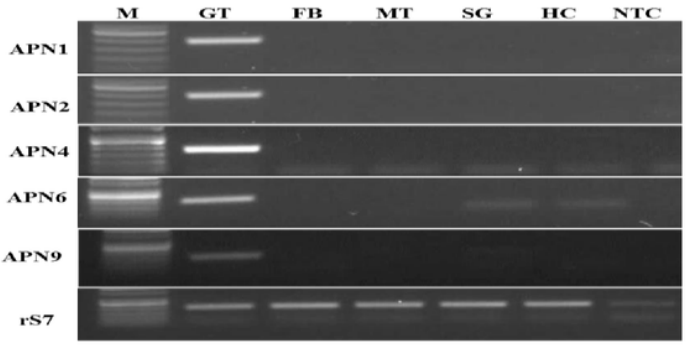


Fig. 37 Tissue distribution of APNs. Semi- quantitative PCR analysis showed the presence of APN transcripts only in gut (GT) but not in other tissues like fat body (FB), Malpighian tubules (MT), salivary gland (SG) and hemocytes (HC).

3.6 Expression analysis of APN isoforms

The qRT-PCR analysis revealed the down regulation of all the APN isoforms in time dependent manner upon continuous exposure to sub-lethal dosages of Cry toxins in the larval mid-gut (Fig. 38). Further the isoforms were differentially regulated and it was found that the rate of transcription as well as the total amount of transcript resent for various isoforms differed significantly from one another during Cry toxin expose (Fig. 38).

The generation wise expression analysis revealed that APN1, APN6 and APN9 had similar pattern of expression in each generation. On other hand APN2 showed a decline in expression, while APN4 exhibited inclined in expression (Fig. 39). It is likely that the higher expression seen for APN4 during Cry intoxication might compensate the physiological function of APN2.

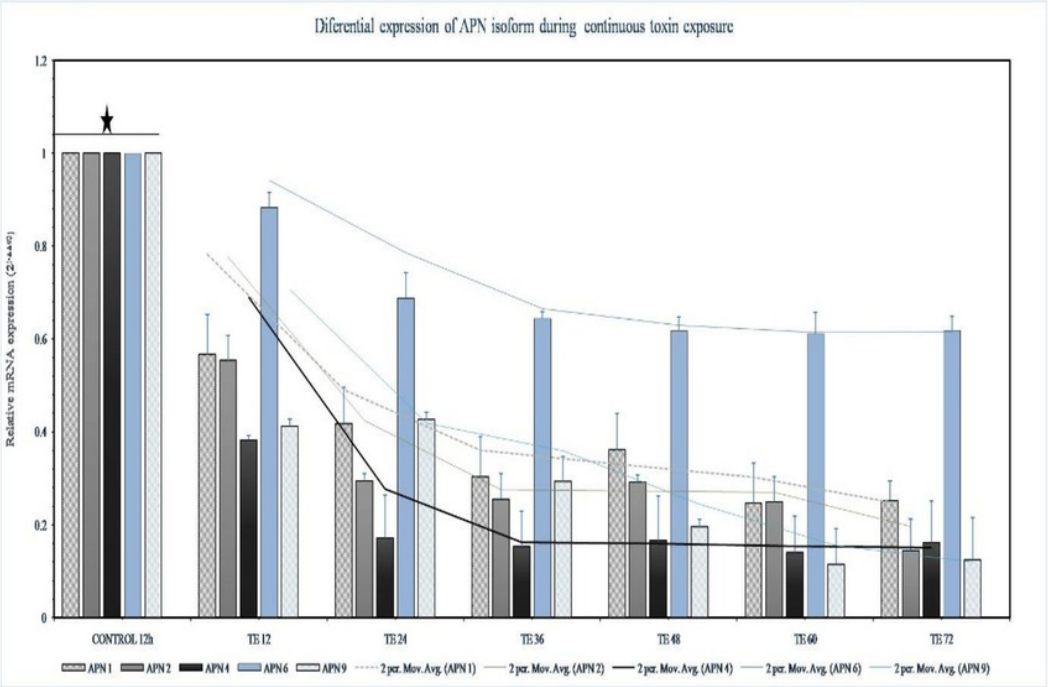
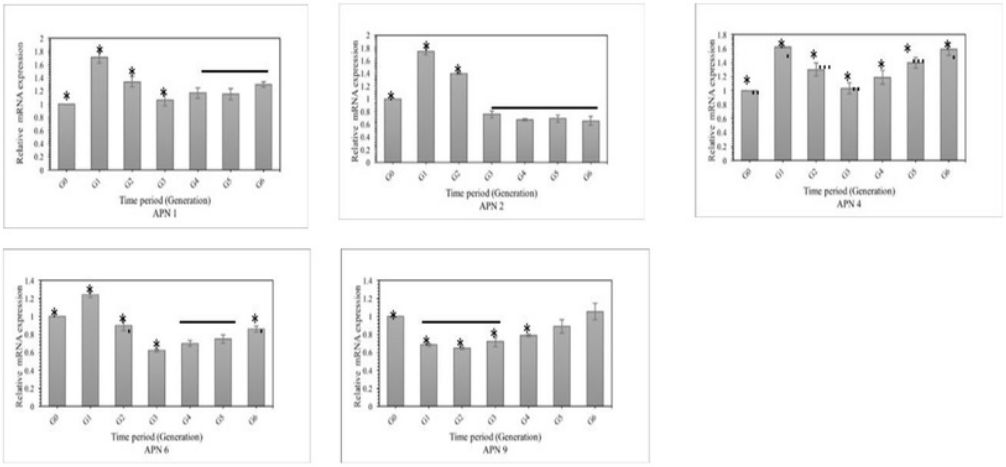


Fig. 38 Expression analysis of mid-gut aminopeptidase N isoforms using qRT-PCR during toxin exposure. A gradual decline in expression of various isoforms from 1 to 72 h of Cry intoxication was seen. Further, each isoforms was differentially regulated during various time points. *p < 0.005 significance between experimental groups, (n=3).



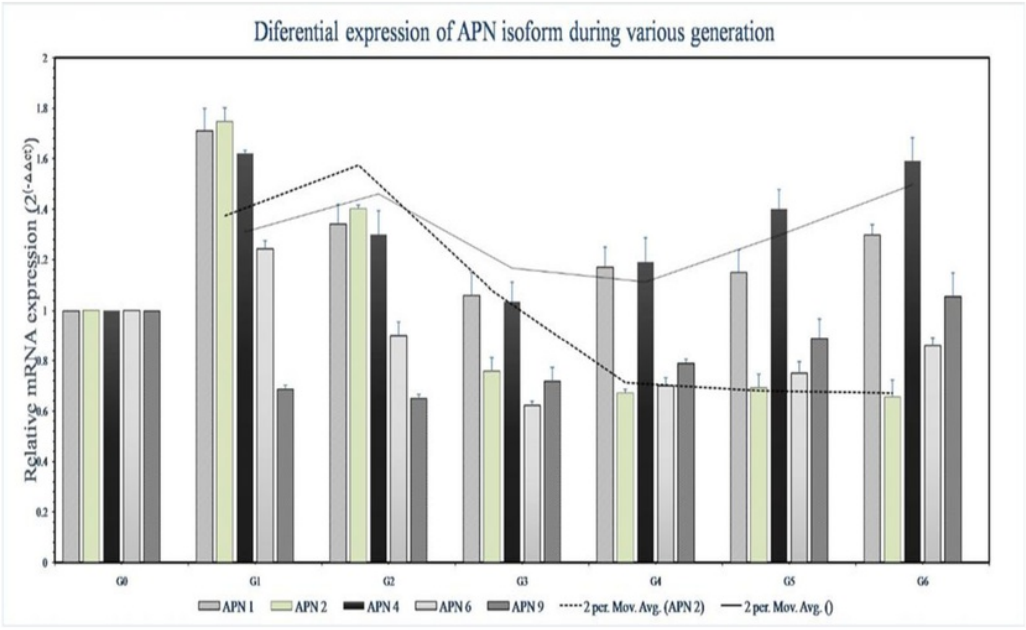


Fig. 39 Expression of various mid-gut aminopeptidase N isoforms transcript during various generations. Generation wise expression analysis of APN isoforms revealed that APN1, APN6 and APN9 had similar expression pattern in all generations, while APN4 was significantly up-regulated and APN2 was down-regulated from 3rd generation onwards *$p < 0.005$ significance between experimental groups, (n=3).

3.7 *In silico* analysis of Cry-APN interaction

For this analysis APN2 was selected as model protein, while Cry1Ac was used as interacting toxin. The protein-protein interaction analysis revealed that amino acid residues from domains II and III of Cry toxin interact with amino acid residues of peptidase M1 domain of APN2 in *A. janata.* (Figs. 40 and 41). The predicted binding energy complex was found to be is ΔG= -16 (kcal mol-1) and Kd= 1.9 e-12 M.

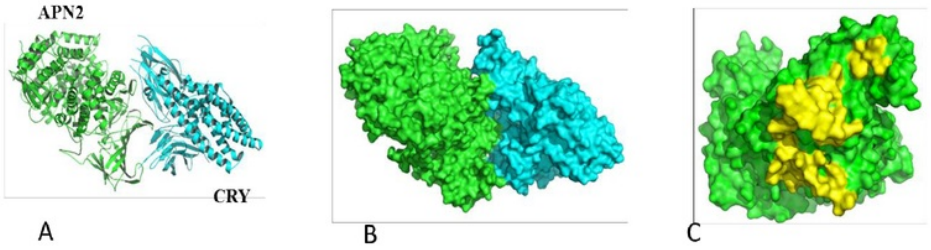


Fig. 40 Protein-protein interactions between APN2 of larval mid-gut of *A. janata* and Cry1Ac toxin (A) In cartoon representation, (B) In surface representation, (C) Interacting amino acids in APN2 are highlighted in yellow color.

Interacting amino acids in APN2
R46 F48 Q60 A87 Q89 E98 N99 V101 H131 P193 Q194 N196 L204 R205 T206 D207 T208 L209 K210 E211 D212 V213 A214 K218 E220 N238 F239 E240 Y241 I242 D243 N244 N245 V246 D247 P248 I252 P253 R255 Q263 D264 E267

Interacting amino acids in Cry1Ac
P221 D222 R281 G282 S283 Q285 G286 R289 R292 S293 P294 H295 L296 P325 S329 G330 P331 F333 T334 F335 P336 L337 Y338 G339 T340 M341 F436 R437 S438 F440 N480 F481 L482 F483 N484 G485 S486 V487 I488 R500 N502 S503 S504 N507 N510 T537 G594 T595

Fig. 41 List of interacting amino acid residues from aminopeptidase N isoform 2 (APN2) with Cry1Ac toxin identified by *in silico* protein-protein interaction analysis.

3.8 Proteomics analysis of APN isoforms

The results obtained from immnunoblot studies with anti-APN antibodies showed the interaction of it with multiple protein spots in two-dimensional gel electrophoresis. This clearly indicated the presence of multiple isoform of aminopeptidases in the larval mid-gut of *A. janata*

(Fig. 42) which might play role in Cry toxin toxicity as well as in development of tolerance/resistance against them upon sub-lethal dose of Cry toxin exposure.

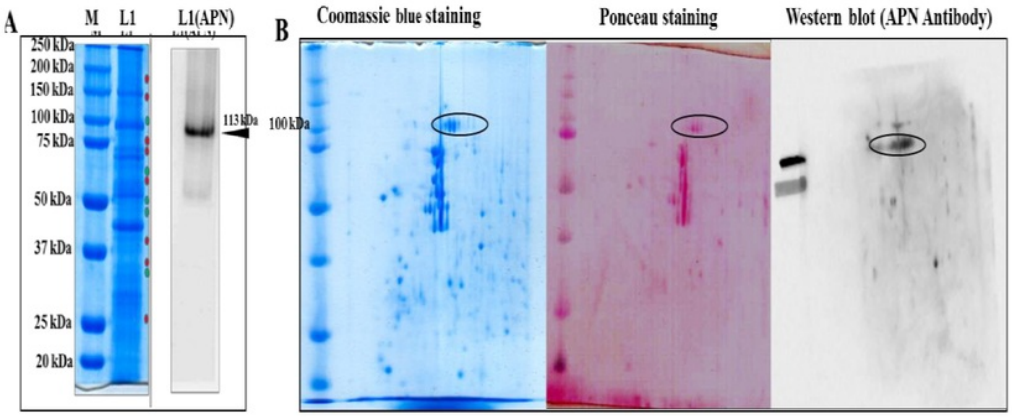


Fig. 42 Identification of membrane bound APN. (A) Detection of APN in membrane proteins with molecular weight 113 kDa (please note that APN membrane proteins in marked (⬭) zone. (B) Two-dimension SDS-PAGE and immunoblotting with APN antibody showed the presence of multiple cross reacting protein isoforms of APNs.

From DIGE analysis mid-gut of both the 6th generation control and toxin exposed insects (24 h), a total of 1552 spots were analysed, and it was found that out of them 225 protein spots were differentially expressed. For statistical analysis we took a cut off of ±1.2 (average ratio). Then student t-test (>0.05) and one way ANOVA (>0.05) was performed. Finally 55 protein spots were identified, which showed significantly differential expression. For the present study we focused on the spots which interacted with APN antibody (Fig. 43). The differential expression analysis showed up-regulation of most of the spots (protein), expect spot no. 4 (Table 4). This down-regulated spot was further analysed through MALDI-TOF/TOF, and identified as aminopeptidase 2 (APN2) by comparing the theoretical values generated through the same parameters used for protein identification through MS/MS analysis using through Mascot (www.matrixscience.com) software (Figs. 44 and 45).

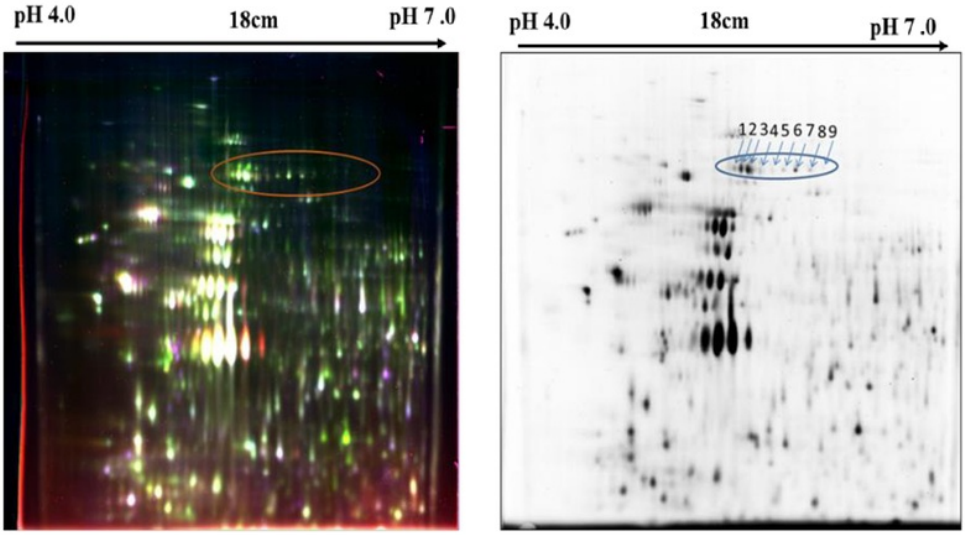
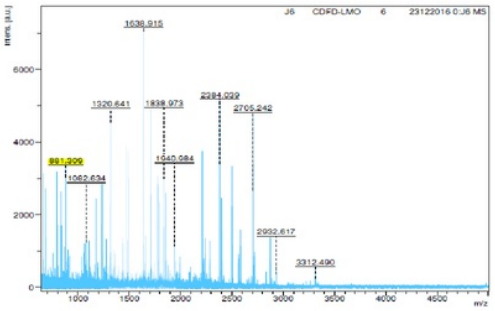


Fig. 43 DIGE expression analysis of membrane bound APN isoforms. BBMVs prepared from the 6th generation control and 24 h toxin exposed (sub-lethal) larval mid-gut was used. Please note the presence of multiple APNs spots (⬭) in the expected zone range.

Spot No	Appearance	T- test	AV.Ratio	One-way ANOVA
1	18/18	0.61	1.21	0.61
2	15/18	0.060	1.66	0.060
3	18/18	0.33	1.41	0.33
4	18/18	0.56	-1.07	0.56
5	18/18	0.58	1.77	0.58
6	18/18	0.41	1.26	0.41
7	18/18	0.017	1.61	0.017
8	18/18	0.031	1.83	0.031
9	18/18	0.39	1.28	0.39

Table 4 DIGE Expression analysis of protein spots which were detected by APN antibody cross reaction. All the protein spots were up- regulated statistically ($p>0.05$) expect spot no.4 which was down regulated.



Search Parameters

```
Type of search         : Peptide Mass Fingerprint
Enzyme                 : Trypsin
Fixed modifications    : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values            : Monoisotopic
Protein Mass           : Unrestricted
Peptide Mass Tolerance : ± 210 ppm
Peptide Charge State   : 1+
Max Missed Cleavages   : 1
Number of queries      : 74
```

m/z	S/N	Quality Fac.	Res.	Intens.	Area
662.276	19.1	2340	4120	803.64	212
678.334	16.4	4191	4133	686.57	184
683.206	17.9	1635	3707	751.76	225
685.215	6.6	372	3527	279.14	89
704.309	28.3	10517	4541	1180.32	311
706.373	6.4	468	3409	267.71	90
721.335	31.2	26678	4434	1300.83	359
729.250	6.4	197	3313	264.28	96
795.182	8.5	221	2743	350.76	174
797.370	6.7	219	2000	275.34	185
810.433	18.8	8082	4231	782.17	260
828.441	76.9	95225	4311	3197.18	1102
832.338	22.2	7137	3989	927.55	347
842.524	10.9	5966	4249	453.74	163
870.564	8.1	697	3480	332.53	147
881.298	6.6	393	2982	270.45	143
895.520	43.6	51106	4524	1791.87	663
943.489	8.8	1961	3908	361.16	162
954.550	322.3	74127	4568	13175.18	5382
960.524	6.2	3697	5089	254.05	92
966.570	6.1	1095	5241	252.72	88
968.568	12.0	2930	4225	488.99	211
982.590	11.4	3406	4258	464.00	206
1064.648	6.2	247	2694	252.02	197
1109.603	13.1	5692	5373	537.80	237
1133.679	15.9	4607	5406	652.54	296
1139.674	226.8	31245	5022	9300.03	4619
1179.691	9.9	2236	6014	410.87	179
1204.700	30.1	17246	5512	1252.09	616
1232.728	6.9	591	4260	288.71	185
1234.759	18.3	4274	5543	760.52	388
1238.713	28.6	4619	5656	1191.17	605
1307.745	7.7	597	5906	319.00	169
1317.813	15.3	893	5472	626.86	363
1319.734	12.3	353	5108	513.15	312
1371.713	120.5	52680	5950	5083.40	2934
1385.737	8.8	3392	6042	372.10	212
1399.753	10.5	3623	5411	443.39	278
1463.828	11.9	2661	6390	504.14	302

m/z	S/N	Quality Fac.	Res.	Intens.	Area
1475.866	6.1	1715	5871	259.30	167
1483.821	6.9	520	5927	292.33	190
1493.836	14.3	14514	6115	605.65	392
1547.820	11.6	8424	5821	487.65	351
1560.889	6.7	1369	4662	280.14	250
1574.841	147.1	42137	6020	6148.95	4499
1588.879	28.3	20481	6479	1179.06	809
1690.965	32.6	37337	6191	1322.54	1080
1707.890	7.3	1620	5099	295.47	288
1717.012	18.4	10076	6109	741.91	621
1739.963	234.2	60038	6311	9358.47	7986
1754.943	11.0	502	4554	434.88	503
1771.945	7.6	922	4407	300.80	362
1777.011	17.3	2479	4786	684.17	780
1781.961	6.1	522	4918	239.19	264
1785.383	7.7	2146	3123	303.95	554
1824.975	44.6	27819	6773	1712.00	1502
1840.986	22.0	1774	5187	842.34	950
1845.880	37.3	1015	6512	1435.09	1335
1848.108	179.8	14756	5402	6869.59	7690
1918.037	12.4	830	4514	447.55	611
1926.427	21.2	1757	3276	738.99	1432
1947.040	9.0	1025	4944	316.28	401
1967.054	143.6	5690	5953	4969.61	5296
1969.112	43.2	1039	4592	1494.24	2044
1982.018	65.6	11473	6618	2232.47	2266
1996.090	13.5	1379	4571	455.88	641
2090.035	19.3	7809	6358	580.92	636
2147.189	8.3	911	6421	236.28	265
2211.229	8.3	357	5347	220.97	300
2234.203	41.3	8204	7044	1031.43	1109
2384.147	7.4	2229	5793	157.87	243
2501.399	6.9	880	7008	135.53	174
2874.540	13.6	1884	7255	205.35	325
3072.744	20.6	756	9093	276.49	420

Fig. 44 MS/MS analysis of down regulated protein spot using Mascot software. The peptide peaks of M/Z values (highlighted) matched with the theoretical peaks generated with APN2 sequence.

APN2_ACJN Mass: 107367 Score: 8 Expect: 1.2 Matches: 5

Probable Protein APN2 OS=Achaea janata GN=APN2 PE=1 SV=1

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
683.3030	682.2958	682.3326	-54.00	288 -	292	0	K.YEFPK.M
881.4221	880.4148	880.4290	-16.11	868 -	874	0	K.VDELYSR.H
943.6217	942.6144	942.5134	107	562 -	570	0	R.SVDIPGLSR.A
1782.2090	1781.2017	1780.8970	171	172 -	186	1	R.AFPCFDEPALKAIFR.T
1785.6317	1784.6244	1784.8403	-120.96	791 -	805	0	R.DFYMNQVIADDSLVR.E

Aj.APN2 sequence

MYLLTLLALI GTAFCGPLST QVRNVDNRAE LYVLPTTIKP TFYDVRLFLD PGNPELFYGQ
VSIRILPQVE TNEIVLHAME MLITENAIQV YTDREPTENI VTGFTQAGDD THFLRIQLSR
QLTVQQPATI HIDYVGHFAE NMFGVYRSTY TENGNTIALI TSQLQPTFAR RAFPCFDEPA
LKAIFRTTIY APPQYNVVRS NMPLRTDTLK EDVAGYTKHE FQDTLIMSTY LIAYLVSNFE
YIDNNVDPIF NIPFRVYSRP GTQDTAEFAL DFGQKNMVAL EDYIDFKYEF PKMDKVAVPD
FAAGAMENWG LVIYREVALL VTEGVTTTSV RQNIGRIICH ENLHQWFGNE VGPASWTYTW
LNEGFANFFE NYATNLVVPE WRMMDQFVIA LQNVFQSDAV LSVNPMTHPV YTPSEILGTF
NAVAYQKSGS VIRMMQHFLT PEVFRQGLIY YVKAMSRQAA APTHLYSSLQ QALTESSHSI
PYQLSTVMTR WTEQGGFPVV TITRSADTAE SVVVAQERFL TDSTLSSDDR WHVPLNWALS
TNPDFSDTRP MEWIPPNFPA RSVDIPGLSR AEWFVFNKQQ TGYYRVNYDD GNWEALAKVL
NENHELIHLL NRGQILDDAF NLARNGRTNY RHPFEISRYL VNEQDYIPWA AVNPAFTYLD
VVLSESPVYD EFRYYILNLT APLYNSLGFE VNSGEEFVTP YHRNIILDLN CRLGNSHCIS
TAQDLLDSFR NNPAQRLNPD IQTLVYCSGL RGGDADNFNF LWNQYANTDD SSEQSILLNA
LGCTSNVELR DFYMNQVIAD DSLVREQDRH TILVSVTNAS PDNMNAALDF IIENFHLIQP
RVQGLTGTTN ILNALARRLT SQEHSAKVDE LYSRHQAIFT AGELASIAAI RENIAASITW
SNENAATVED WLRQNYGDGD GDGAAAMTAS LVLLISALVA IFNY

Fig. 45 Identification of down regulated protein spot. The five peptides obtained from MS/MS analysis marched with the amino acid sequence (highlighted) present in the aminopeptidase isoform 2 (APN2).

4 Discussions

Mid-gut of Lepidopteran insect larvae is a multifaceted tissue which performs variety of function ranging from digestion, absorption, immune function, transmission of pathogens including baculovirus and interaction with various molecules (Kliot *et al.*, 2014; Wu *et al.*, 2016; Kim *et al.*, 2016). The proteins present in the apical brush border of mid-gut epithelial tissues are digestive proteases, which play important role in digestion and variety of transporters which facilitate transport and absorption. Among the large number of proteins which have been identified in BBMV of mid-gut such as cadherin-like proteins (Nagamatsu *et al.*, 1999; Xu *et al.*, 2005), GPI-anchored aminopeptidase-N (APNs) (Knight *et al.*,1994; Rajagopal *et al.*, 2003; Budatha *et al.*, 2007) and alkaline phosphatases (ALPs) (McNall and Adang,2003; Jurat-Fuentes and Adang, 2004). Glycolipids and glycoconjugates (Valaitis *et al.*, 2001) also bind and/or interact with Cry toxin produced by various strains of *Bt*. Recently ATP-binding cassette transportor subfamily C2 (ABCC2) has also been characterized as Cry toxin functional receptor (Tanaka *et al.*, 2013; Zhou *et al.*, 2016).

Ligand blot analysis (toxin overlay assay) with DOR *Bt*-1 showed the prescence of multiple intearcting proteins in the membrne fractions isolated from mid-gut, fat body as well as in tissue homogenates of salivery gland and Malpighian tubules of *A. janata* larvae. Among them two proteins, one with molecular weight around of 100-110 kDa and another one with 60-70 kDa were shown to interact in all four tissues. On the basis of existing literature, the protein with higher molecular weight ranged closure to reported aminopeptidase N (110 kDa) and the one with lower molecular weight matched with alkaline phophatase (68 kDa). Earlier studies from our group have already demostrated them as Cry toxin binding protein/receptor. In *A. janta* the full length APN was cloned and confirmed by lignad blot analysis using Cry 1Aa and DOR 5 toxins, from mid-gut, fat body, Malphighian tubules and salivary glands (Budatha *et al.*, 2007; Ningshen *et al.*, 2013).

In presnt study toxicity of DOR *Bt* -1 strain was analyzed primarily keeping in the view that the Cry toxins produced from this strain have high insecticidal activity towards *A. janta* as well as other Leipidopteran pest (Vimaladevi *et al.*,2006; Reddy *et al.*, 2013). Finding of this study revealed binding of 5-6 membarane protiens with activated toxin obtained from DOR *Bt*-1 and recombinant Cry1Ac (BGSC) in the larval mid-gut of *A. janata.* The prominent interaction was seen primarily with membrane protein of 110 kDa, which was confimed by immunodetection using anti-APN antibody generated against a small frgament of recpmbinant APN from *A. janta.* Furthermore, the sequece obtained through MALDI-TOF/TOF -MS/MS analysis matched with the alanyl aminopeptidase of *Heliothis virescens* with molecular weight 113 kDa (Gill *et al.*, 1995). Colocalization studies were perfromed further to confirm whether or not the Cry toxins really interact with APN presnt in inner apical brush border (BBMVs). Once again it was found that both DOR *Bt*-1 and Cry 1Ac toxins interact with membrane vesicles, where APN was localized using anti APN antibody.The results obtained in present study corroborated well with earlier reports where brush border membrane region was shown to interact and accumulate toixns in intoxicated larvae (Bravo *et al.*, 1992; Yi *et al.*,1996; Valaitis, 2011). The findings presented in chapter 1 clearly showed that Cry intoxication caused a significant changes/ damge in the microvilli mid-gut BBMVs of mid-gut epithelial cells of *A. janata* larvae. Hence, this study cleraly indicateed that mid-gut membane bound protein aminopeptidase N is a major Cry toxin receptor in the larval forms of *A. janata.*

The molecular weight of APNs in various insects ranged between 100-150 kDa. (Crava *et al.*, 2013). In insects they were shown to be produced in multiple forms (isoforms), and same has already been demonstrated in Lepidipteran insects like *Bombyx mori* (Nakanishi *et al.*, 2002); *Trichoplusia ni* (Wang *et al.*, 2005); *Ostrinia nubilalis* (Crava *et al.*, 2013) and *Heliothis virescens* (Perera *et al.*, 2015). As the genome sequences of *A. janta* is not available for finding out the presence of APN isoforms, next generation RNA sequencing (*de novo* trnscriptome) of

larval mid-gut of *A. janata* was carried out (unpublished data). The screening of data revealed the presence of five different isoforms which were further cloned and confirmed by Sanger sequencing. *In silico* analysis of identified sequences revealed the presence of characteristic aminopeptidase activity motif "GAMENEG" and zinc binding motif "HEXXHX18H". The isoforms were named based on the homology found with reported sequences in NCBI data base as APN1 (3054 bp, 1017aa, ~114 kDa, pI 4.73); APN2 (2835 bp, 944 aa, ~107 kDa, pI 4.81); APN4 (2853 bp, 950 aa, ~108 kDa; pI 4.81); APN6 (2868 bp, 955aa, ~109 kDa, pI 4.94) and APN9 (2280 bp; 759 aa, ~88 kDa, pI 5.43). The molecular weight of all the isoforms of APN identified in *A. janata* ranged between 100-120 kDa, except APN9 which was much smaller than others and it was around 88 kDa. A similar low molecular weight aminopeptidase of 90 kDa was also reported in *Bombyx mori* (Nakanishi *et al.*, 2002), further this showed 37% sequence identity with others. Detailed analysis also suggested that these isoforms were paralogs to each other and were expressed by different genes of single clusters. Phylogenetic review also suggested that most of the aminopeptidases arose from gene duplication, from a recent common ancestor of Lepidopteran and Dipteran gene (Hughes, 2014). In addition, conserved domain analysis of the identified sequences revealed that they belonged to peptidase M1 aminopeptidase N family with endoplasmic reticulum aminopeptidase 1 (ERAP1) [M1_APN_2 and ERAP1_C domain-containing protein], which are type 2 integral membrane proteases (Kola *et al.*, 2016). *In silico* analysis showed the presence of transmembrane peptide signal at C-terminal end of APN 1, APN2 and APN4, while APN2 did not have any signal peptides at N-terminal. Further, APN6 showed signal peptides at N-terminal but not the transmembrane peptide at C-terminal. APN9 lacked both the signal sequences.

In *A. janta,* all the above identified isoforms were expressed exclusively in mid-gut tissue and none of the other tissues analysed exhibited their expression, suggesting the tissue

specific expression of these isoforms, and most likely they were important proteases, which play role in physiological function of the mid-gut during larval development.

For the management of this pest (*A. janta*), once pest appears in the field (late 2nd or early 3rd instar) two sprays of *Bt* formulations are applied with a gap of three days. Same strategy was followed in the laboratory to find out the role of aminopeptidase N as Cry toxin receptor. In the present study upon sub-lethal dose of Cry toxin exposure, the process of development of Cry toxin tolerance was monitored in a population of insect using a generation wise analysis. During the continuous exposure to toxin for 72 h the expression of all the APN isoforms gradually declined in the larval mid-gut in time dependent manner. Further, each isoform was differentially regulated. The expression analysis of APN isoforms from generation wise analysis (for 6 continuous generations) revealed no significant difference in APN1, APN6 and APN9 expression, but the expression of APN2 declined while APN4 showed inclined expression. These findings suggest that upon Cry toxin exposure larval mid-gut has ability to modulate the expression of various proteases including APNs (Budatha *et al.*, 2007).

The detailed DIGE expression analysis carried out with BBMVs proteins isolated from 6th generation control as well as 24 h toxin exposed (sub-lethal dose) revealed up regulation of all the proteins spots, which were detected using anti-APN antibody expect the one. A detailed structural analysis of the down regulated protein spot further confirmed it as APN2 using MALD-MS/MS analysis. As it is mentioned in previous section that APN2 lacked signal peptide but showed the presence of transmembrane domain, the reduced expression of APN2 might lower the interaction of Cry toxin with apical membrane of mid-gut epithelial cells and block the cellular damage thus providing toxin tolerance in the larvae. Further, inclined expression seen for APN4 might, compensate the physiological function of down regulated APN2 and facilitate not only survival of the insects but also its growth. In cabbage looper, *Trichoplusia ni* Cry resistant insects were shown to down regulate APN1 (Tiewsiri and Wang, 2011).

Findings of the present study clearly suggests that APN2 most likely acted as a functional Cry toxin receptor in *A. janta* and mediated Cry toxin based damage in susceptible insects. Further it's down regulation upon sub-lethal Cry toxin exposure during generation wise analysis facilitated the tolerance of toxin by the larvae. Finally, this study also suggests that in *A. janata* larvae exploit variety of strategies to avoid the deleterious effects of Cry intoxication, which in long run would make them resistant to *Bt* formulations and/or Cry toxin based transgenics.

Summary

The lack of homogeneity in field application of *Bt* formulation may often result in ingestion of sub-lethal doses of the biopesticide by a fraction of the pest population and thereby they develop toxin tolerance and resistance in long term. Present laboratory study evaluated various larval mid-gut responses to sub-lethal dose of Cry toxin in Lepidopteran pest *A. janata*.

Prolonged exposure (0-72 h) of sub-lethal dose of DOR *Bt*-1 formulation to seven-day old or 3rd instar larvae showed deleterious effects on larval growth and development. Histomorphological analysis of the toxin exposed mid-gut epithelium showed degeneration of epithelial cells with condensed nucleus and fairly low cytoplasmic content. In addition, large number of vacuoles were also observed within the epithelial cells. Maximum cell death was observed between 24 to 36 h. Further the pattern of cell death was evaluated using AnnexinV and Propidium Iodide staining with confocal microscopy, flow-cytometery and DNA fragmentation analyses and the results obtained indicated that necrosis was the most likely reason for cell death upon Cry intoxication.

Larvae avoided feeding on toxin coated leaves, and made an attempt to overcome toxin mediated damage in gut epithelium by extensive cell proliferation. This was monitored using a toluidine staining and kit-based BrdU labelling. Few pockets of BrdU-positive cells were identified from the mid-gut collected after 24 h toxin exposure, and after 48 h, the number of small cells labelled with BrdU increased considerably. These cells were mostly observed in the basal region of the mid-gut epithelium, and they could be ascribed to be mid-gut stem cells/regenerative cells. The events of cell necrosis and regeneration occurred simultaneously during the toxin exposure. Hence, cell proliferation might predictably play a role in compensating the cell death during Cry intoxication.

Arylphorin, a hexamerin protein, is known to be predominantly synthesized by the fat body tissue and released into the haemolymph during active feeding phage of larvae. Detectable but low expression of arylphorin in the mid-gut as compared to the fat body was observed which was further confirmed through *in situ* hybridization and localization of arylphorin transcript in the gut epithelium primarily in the basal cells. Expression analysis using Cry intoxicated larval mid-gut further showed time dependent increase in endogenous arylphorin gene expression during active cell proliferation, which was not only seen upon Cry intoxication but also during molting in control larvae where regeneration is well documented. Presence of arylphorin transcript and its altered expression in the mid-gut during Cry toxin exposure, indicated a plausible role of arylphorin in rapid gut regeneration, which might facilitate and promote Cry toxin tolerance in the Lepidopteran larvae.

Aminopeptidase N(s) are well known digestive proteases and they cleave the neutral amino acids from N terminal of protein/ peptide. By default they also interact/ bind with Cry toxins and act as interacting/ receptor proteins in Lepidopteran insects including *A. janata.* In the present study five different isoforms (APN isoforms) were identified and named as APN1, APN2, APN4, APN6 and APN9. The expression profile analysis of all the APN isoforms upon continuous sub-lethal dosage of toxin exposure revealed that it gradual declined in time dependent manner and various isoforms were differentially regulated. The field based application strategy for development of toxin tolerant insect populations (6 generations) showed a non- significant difference in APN1, APN6 and APN9 expression but a decline in the expression of APN2 and incline in expression of APN4. Further, DIGE expression analysis of APN isoforms (detected through anti APN antibody) also confirmed the lower expression of APN2 isoform. The present study clearly suggested that APN2 might act as a functional receptor of Cry toxin in *A.janata,* and its binding to the toxin initiated extensive cell damage in mid-gut. Further the analysis revealed that upon long-term generation wise exposure the larval forms

altered the expression profile of various APN isoforms and reduced the expression of APN2 to avoid insecticidal action and develop tolerance against Cry toxin. The upregulation APN4 most likely facilitated and compensated the digestive physiological function of down regulated APN2 for the survival of the larvae.

Conclusion

Present studies revealed that prolonged exposure to sub-lethal doses of Cry toxin formulation have deleterious effect on larval growth and development. Cry toxin intoxication promotes necrotic form of cell death. Further larvae reduced toxin feeding to minimize the toxin based mid-gut damage and tried to replenish the injured cells through enhanced proliferation (regeneration) of mid-gut stem cells. The observation that the enhanced mid-gut cell proliferation coincided with the elevated endogenous arylphorin expression suggested a plausible role of arylphorin in rapid gut regeneration, which might facilitate and promote Cry toxin tolerance in the Lepidopteran larvae. Among all the isoforms of aminopeptidase N identified *in A. janata*, APN2 might act as a functional receptor of Cry toxin, hence insect host system tried to reduce its expression in order to survive from toxin based damage and lethality. This strategy might help the insects not only to overcome toxin toxicity but also in development of tolerance against *Bt* biopesticides during long term generation wise field applications.

Larval mid-gut responses against sub-lethal dosages of Cry toxins in Lepidopteran pest, Achaea janata

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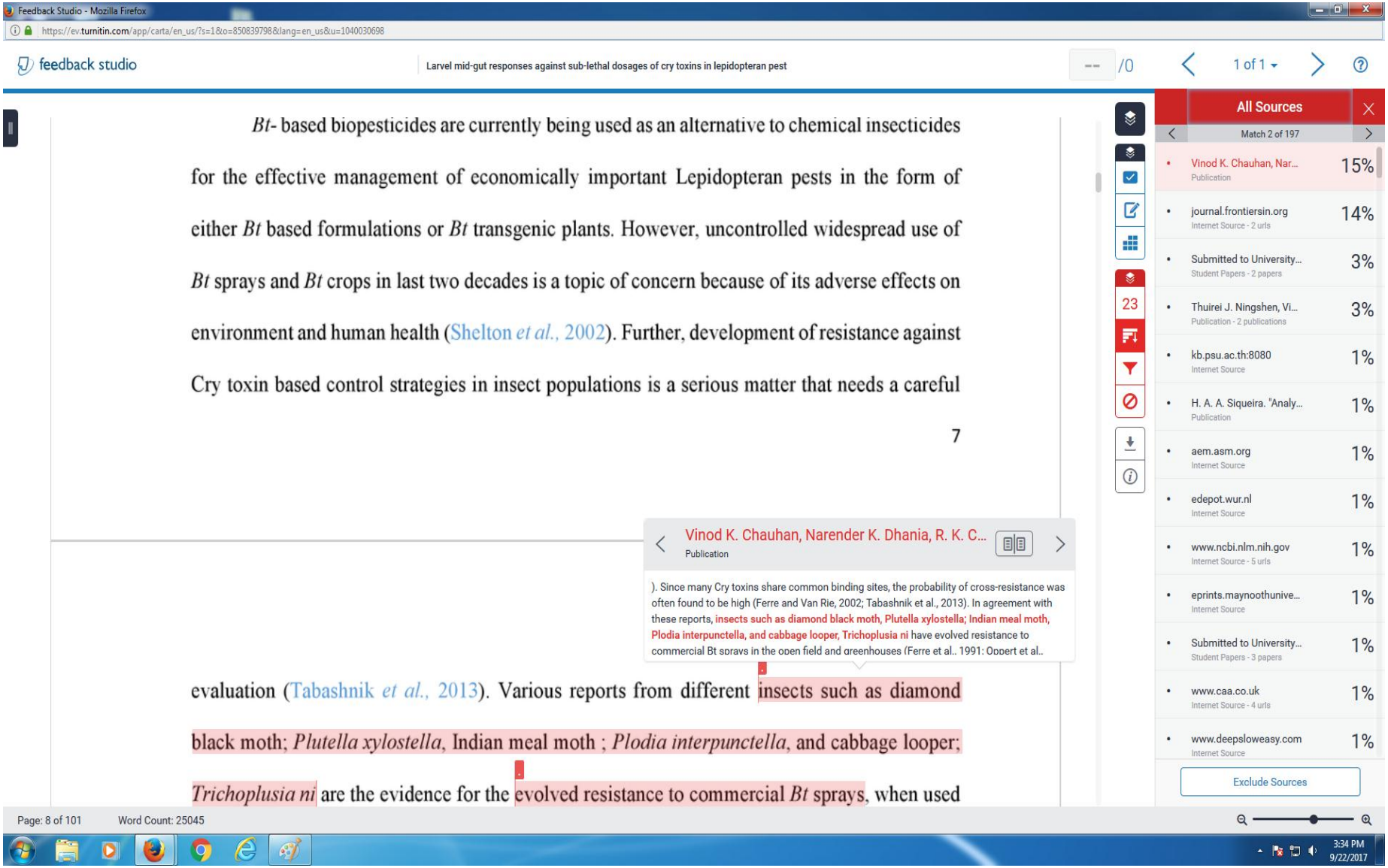
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Bt- based biopesticides are currently being used as an alternative to chemical insecticides for the effective management of economically important Lepidopteran pests in the form of either *Bt* based formulations or *Bt* transgenic plants. However, uncontrolled widespread use of *Bt* sprays and *Bt* crops in last two decades is a topic of concern because of its adverse effects on environment and human health (Shelton *et al.*, 2002). Further, development of resistance against Cry toxin based control strategies in insect populations is a serious matter that needs a careful

evaluation (Tabashnik *et al.*, 2013). Various reports from different insects such as diamond black moth; *Plutella xylostella*, Indian meal moth ; *Plodia interpunctella*, and cabbage looper; *Trichoplusia ni* are the evidence for the evolved resistance to commercial *Bt* sprays, when used