Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(4): 198-204

Received: 10-05-2020
Accepted: 14-06-2020

Kennedy Ningthoujam
College of Post Graduate Studies in Agricultural Sciences, Umiam, Meghalaya, India

Mareena Sorokhaibam
College of Agriculture, Imphal, Manipur, India

Rojeet Thangjam
College of Agriculture, Thanzawl, Mizoram, India

GT Gujar
Indian Agricultural Research Institute, New Delhi, India

Corresponding Author:
Kennedy Ningthoujam
College of Post Graduate Studies in Agricultural Sciences, Umiam, Meghalaya, India

Association of alkaline phosphatase activity with inheritance of Cry1Ac resistance in *Helicoverpa armigera* (Hübner)

Kennedy Ningthoujam, Mareena Sorokhaibam, Rojeet Thangjam and GT Gujar

Abstract
A number of putative Cry toxin receptors have been identified in various insect midgut epithelia, of which alkaline phosphatase activity has been proposed to be closely associated with the resistance of *Helicoverpa armigera* to Bt toxin. In the present study, midgut brush border membrane vesicles (BBMV) preparations from resistant and susceptible population of *H. armigera* were assayed for marker enzyme specifically alkaline phosphatase so as to understand the association of the levels of alkaline phosphatase activity and the expression of Cry 1Ac resistance. Also, the association of the levels of alkaline phosphatase activity in relation to the mode of inheritance of Cry1Ac through their F_1, backcrosses and F_2 progenies were examined. Identification and characterization of Cry toxin receptors in the midgut of *H. armigera* has a very important role in clarifying the Bt toxicology and its resistance mechanism so as to sustain long term use of Bt cotton. In our study, the specific acitivity of alkaline phosphatase was 108.10 and 160.93 µM/min/µg of protein, respectively for resistant and susceptible population. Alkaline phosphatase activity was observed to be more in Cry1Ac susceptible population as compared to the resistant population. The mean larval alkaline phosphatase of the resistant and susceptible parents (p=0.025) differed significantly, thus suggesting the association of decreased APN activity in resistant population. Maternal effects on inheritance of Cry1Ac resistance development was observed in F_1 progenies while nuclear effect was observed in backcrosses and F_2 progenies. Hence, it became clear from the study that the Cry1Ac resistance in the *H. armigera* population is conferred by alteration of the larval midgut binding to the Cry toxins.

Keywords: Alkaline phosphatase, *H. armigera*, Cry1Ac, Bt cotton, Resistance

1. Introduction
The cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), is a major polyphagous pest of cotton and other crops, and the introduction of Bt cotton expressing Cry1Ac has effectively suppressed regional outbreaks of *H. armigera*, not only on cotton but also on other host crops [1]. Transgenic cotton expressing *Bacillus thuringiensis* (*Bt*) insecticidal proteins is the primary strategy for controlling lepidopteran insects on cotton in India. It remains the only transgenic crop released successfully in India for commercial cultivation in the year 2002. *Bt* cotton provides benefits in suppressing bollworm, reducing use of insecticides, increasing yield and thus, giving higher profits to farmers [2].

However, similar to the history of synthetic pesticides, long-term application of *Bt* crops exerts high selection pressure on their target insects, resulting in some insects evolving resistance to Bt insecticidal proteins. As a result, the durability and effectiveness of *Bt* crops have diminished [3]. The evolution of resistance has become a primary threat to the continued efficacy of *Bt* toxin [4].

Therefore, understanding the mode of inheritance of resistance to Cry1Ac and studying the levels of activity and binding affinities of bio-marker enzymes *viz.*, alkaline phosphatase and aminopeptidase etc. in association with other physiological traits to *Bt* toxin in *H. armigera* becomes a prime importance to overcome the resistance development.

Insect pests resistance to Cry toxins results from alternations in the intoxication process including osmotic cell death and disruption of the midgut [5]. Of these, altered receptor like cadherin, alkaline phosphatase and aminopeptidase-N present on the brush border membrane vesicles (BBMV) of the midgut are considered important factors to reduced toxin binding and resistance development [6].

Cry toxin monomer after proteolytic action of its protoxin binds with highly abundant glycosylphosphatidylinositol (GPI)-anchored aminopeptidase and alkaline phosphatase on the midgut epithelium surface. Later, Cry toxin monomer binds with cadherin and further gets proteolyzed in domain-I after which oligomer formation takes place. The toxin oligomer after binding with either aminopeptidase or alkaline phosphatase receptor inserts itself into epithelial memberane to disrupt osmotic balance thereby leading to its toxicity [5].

Chen *et al.* [7] first reported the evidence of synergism of a Bt toxin in *H. armigera* by a toxin-binding fragment of alkaline phosphatase against resistant insects. The reduced activity and reduced transcription of an alkaline phosphatase protein that binds Cry1Ac was associated with resistance to Cry1Ac in resistant strains.

Hence, the present study was undertaken to understand the expression of Cry 1Ac resistance with the level of alkaline phosphatase activity in *H. armigera*. For this, the association of the levels of alkaline phosphatase activity in relation to the mode of inheritance of Cry1Ac through their F_1, backcrosses and F_2 progenies were examined. Midgut brush border membrane vesicles (BBMV) preparations from resistant (DNDT-R) and susceptible (M4-S) population were assayed for marker enzyme specifically alkaline phosphatase. Based on our results, we observed that ALP function as a receptor for the Cry1Ac toxin in *H. armigera* and play important functional role in the mechanism of action of the Cry1Ac toxin.

2. Materials and Methods

2.1. Insect rearing. The susceptible (M4) and resistant (DNDT-R) of *H. armigera* strain was initiated from populations (eggs, larvae, adult) collected from Bangalore, Dharwad, Raichur and New Delhi (IARI) in 2013. The larvae were reared in the laboratory on a chickpea-based semi-synthetic meridic diet of Nagarkatti and Prakaash [8] adapted by Gujar *et al.* [9] until pupation. The adults emerging from pupae were fed with 10% honey solution fortified with multivitamins throughout their egg-laying period. The pairing of adults was carried out by keeping six male and six female moths from the same place in mating jars. The eggs collected from these jars were kept at conducive conditions until the neonates emerged, which were used for screening of tolerance. Bioassays (at different doses ranging 0.001 to 3.0 μg of Cry1Ac/g of diet and at a diagnostic dose of 1 μg of Cry1Ac/g of diet) were carried out of these neonates using pure toxin of Cry1Ac. During bioassay the neonate larvae were fed on the toxin treated diet and the per cent survival at 7^{th} day was recorded. The larvae which survived were considered as tolerant. These tolerant larvae were considered as the parental generation. A constant temperature of 28±1ºC and 80±1% RH was maintained throughout the rearing period.

2.2. Toxin and bioassays

For bioassays MVPII liquid formulation (Monsanto India Ltd., Bangalore) containing 19.7% Cry1Ac, encapsulated by *Pseudomonas fluorescens* was used [10]. Cry1Ac in MVPII is 99% identical to the active toxin sequence of Cry1Ac gene in the Monsanto's 531event *Bt* cotton [11].

Susceptibility of F_1 neonates of *H. armigera* to Cry1Ac was evaluated using diet incorporation method [12]. Different concentrations of Cry1Ac ranging from 0.001 to 3.0 μg /g of diet were prepared, mixed thoroughly into an aliquot of diet and transferred to small plastic containers (5.5 cm in diameter; 1.5 cm in height). Each concentration had three replicates and each container of 3g diet served as one replicate. Ten neonate insects were released in each of the replicate. The control consisted of untreated diet. Observations for mortality were taken at 7^{th} day and LC_{50} was calculated by using MLP 3.01 [13].

2.3. Screening by giving selection pressure at 1µg of Cry 1Ac/g of diet.

One hundred and forty six isofemale lines (Batch I) were made in which pairings were done among tolerant strains, among normal strains and also between tolerant strain and normal population.

F_1 generation: Jars housing the single pairs were regularly monitored, eggs were collected and the neonates were reared in the laboratory under conducive condition. On pupation, pupae were collected, washed and sexed and equivalent numbers of males and females were kept in a single jar and allowed to sib-mate in bulk.

2.4. Collection of F_2 generation from the isofemale lines (Batch I)

F_2 generation: Eggs were collected daily and stored at 10ºC. When at least 200 eggs had accumulated over an interval of 4 days the eggs were placed in incubator at 25ºC to promote hatching. The neonates obtained from these eggs were the F_2.

F_2 Screen Assay: Assays were conducted in circular plastic containers (5.5 cm in diameter and 1.5 cm in height) which contain 3 g of rearing diet mixed with Cry 1Ac toxin of concentrations of 1 and 10 µg of Cry 1Ac/g of diet.

From this screening, four promising lines which showed higher percentage of survivals were selected in order to obtain F_3 generation. The F_3 generations obtained from these selected lines were subjected to 10 µg of Cry1Ac/g of diet and the line with the highest percentage of survival was selected. This selected line was considered as resistant line (DNDT-R) for this study.

2.5. Collection of F_2 generation from the isofemale lines (Batch II)

F_2 generation from the isofemale lines (batch II) is obtained by following the same procedure as was done in isofemale line (batch I).

For the selection of most susceptible line a total of 65 isofemale lines were made of which 45 isolines were from mixed normal population and 20 isolines were from the remaining survival lines of F_2 screening other than the four selected promising lines.

F_2 screening: F_2 neonates were screened using Cry1Ac toxin of concentrations of 1 and 10 µg of Cry1Ac/g of diet. From this screening, the line which showed least survival percentage in 1 µg of Cry1Ac/g of diet and 0% survival in 10µg of Cry1Ac/g of diet were selected. This selected line is the susceptible line (M4-S).

2.6. Crosses and backcrosses

Reciprocal F_1 crosses were performed by mating susceptible ♀ × resistant ♂ (SR) and resistant ♀ susceptible ♂ (RS) by using F_6 adults of resistant DNDT-R and F_4 adults of susceptible M4-S strains. Sibmating was also done for maintaining both the resistant and susceptible pure lines. All the crosses were conducted in masses using six pairs of adults in mating jars with four replications. The F_1 neonates of

hybrid crosses were assayed with Cry1Ac treated diet. The hybrids of each cross were reared to adults, which were further used for performing backcross with resistant parent. The progenies of all the backcrosses were subjected to bioassay and their mortality responses were observed. The backcrosses were conducted by mating adults of the hybrid cross with the F_6 resistant DNDT parent *viz*., ♀ of SR × ♂ of DNDT-R; ♀ of DNDT-R × ♂ of SR; ♀ of RS × ♂ of DNDT-R and ♀ of DNDT-R × ♂ of RS. Adults of the F_1 reciprocal crosses were further sibmated to obtain the reciprocal F_2 generation (fig. 1).

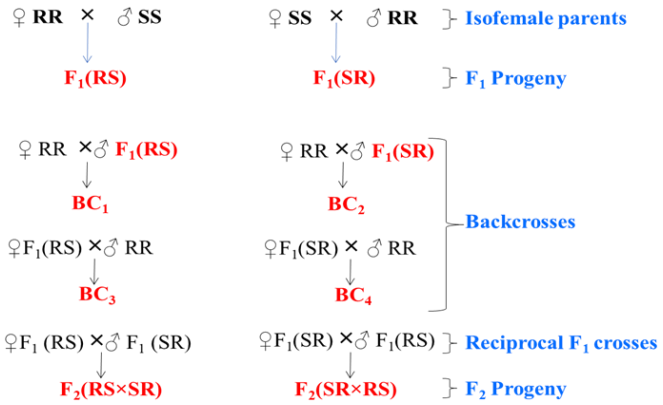

Fig 1: Diagrammatic representation of different types of mating of *H. armigera* in the present studies

2.7. Preparation of Brush Border Membrane Vesicle (BBMV) from larval midgut

The brush border midgut membrane vesicles from midguts were prepared as per method described by Wolfersber *et al.* [14]. Final instar larvae of *H. armigera* were immobilsed by keeping at –20 ºC for 10 minutes and dissected in ice cold MET buffer (300mM mannitol, 5mM EGTA (Ethylene Glyco-0-0'- bis-(2-Aminoethyl) NHNA-Tetraacetic acid). 17mM Tris-HCl, pH 7.5). Midguts were pulled out after making longitudinal cut on dorsal surface of larvae using good quality blade. Food particles were removed from midgut by longitudinally cutting midguts and rinsing in MET buffer. The weight of midguts thus prepared was taken, to which MET buffer was added @ 9 times the weight of midguts. Midguts were homogenized using glass homogenizer and to the homogenate same volume of 24 mM magnesium chloride was added and kept on ice for 15 minutes. The mixture was centrifuged at 1856 g for 15 min at 4º C. The supernatant was decanted and centrifuged at 23470 g for 30 minutes at 4ºC. The pellet was taken and resuspended in half the original volume of MET buffer and magnesium chloride and kept on ice for 15 minutes and centrifuged as the original homogenate. The pellet obtained at 23470 g was resuspended in half the original strength of MET buffer and stored at –80ºC as aliquots until use. At time of analysis the storage buffer was removed and the pellet was dissolved in 20mM Tris-HCL (pH 8.0) containing 1% CHAPS for 24 h at 24^0 C. After centrifugation the supernatant containing membrane receptor was used for analysis.

2.8. Estimation of marker enzymes of BBMV preparation:

Assays of activity of alkaline phosphatase from BBMV preparation were conducted as marker enzymes for the BBMV. The activity of alkaline phosphatase was assayed according to the procedure of Lowry *et al.* [15].

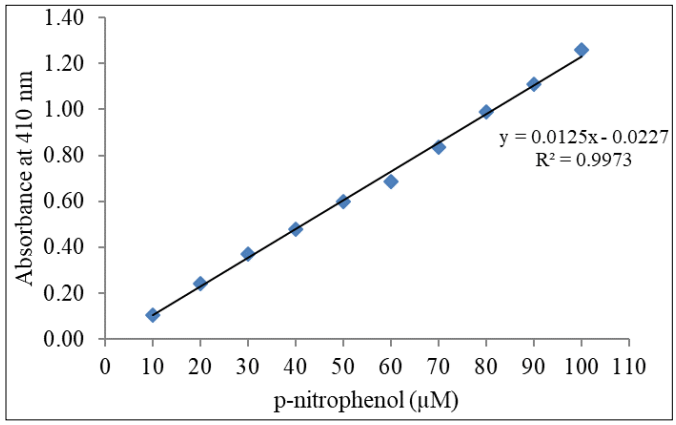

Fig 2: p-nitrophenol standard curve

2.9. Characterization of Alkaline Phosphatase

Alkaline phosphatase converts substrate p-nitrophenyl phosphate to yellow coloured product p-Nitrophenol. The product formed is measured spectrophotometrically at 410 nM (fig. 2).

Total protein content of larval gut homogenate was determined by Bradford method [16]. From the stored samples, 10 µl protein per sample was taken for the alkaline phosphatase activity. Standard curve was prepared using *p*-nitrophenol. 600 µl substrate buffer (3 mM *p*-nitrophenylphosphate, 5mM $MgCl_2$, 0.1% Triton X-100 and 50 mM Glycine-NaOH buffer, pH 10.4) was added to 1.5 mL eppendorf. Pre-calculated volume of each containing 10 µg of protein was added to the ependorf. Total reaction volume was made up to 700 µl by adding 1% Triton X-100. The reaction was incubated in at 37ºC for 1 hr. The reaction was stopped by adding 300 µL of 6 mM KOH. Standard curve was prepared by adding 10-100 µl of 1mM *p*-nitrophenol to 600 µl substrate buffer (0.1% Triton X-100 and 5 mM $MgCl_2$, pH 10.4) and the volume was made up to 700 µL with 1% Triton X-100. To this reaction, 300 µL of 6M KOH was added. Each sample and standard was prepared in triplicates and 200 µL of each was transferred to the microplate reader, SPECTRA max PLUS 384®, (Molecular Devices, Sunnyvale, California, United States) and observed at 410 nM.

The alkaline phosphatase activity was calculated from regression equation obtained from standard curve using SoftMax Pro®. The isoenzymes detected by gradient gel electrophoresis and alkaline phosphatase specific staining were grouped as per their molecular weight in different ranges. Frequency distribution of isozymes among *H. armigera* individuals was done with Microsoft Excel®. Per cent individuals having isozymes specific molecular weight range were plotted against molecular weight range. Statistical analysis was done with SAS® software.

2.10. Electrophoresis for separating ALP isozymes:

Alkaline phosphatase isozymes were separated on non-denaturing gradient (5-10%) SDS-PAGE as per Suurs *et al.* [17]. Ten µg of alkaline phosphatase protein in appropriate volume for each sample was measured for the experiment. Samples were loaded using sample buffer (60 mMTris buffer, 2% SDS, 20% glycerol and 0.02% bromophenol blue). Ten samples of each group were separated on 5-10% acrylamide concentration gradient gel at 30 mA/gel current along a lane of BLUeye preatained broad range proteins (Genedirex®). The gel was stained using alkaline phosphatase specific BCIP-NBT colour development substrate of Promega Corporation®,

USA in alkaline phosphatase buffer (100 mMTris-HCl pH 9.0, 150 mMNaCl and 1 mM $MgCl_2$) as per manufacturer's instructions.

3. Results and Discussion

Our study focuses on the expression of Cry 1Ac resistance with the level of alkaline phosphatase activity in *H. armigera*. For this, the association of the levels of alkaline phosphatase activity in relation to the mode of inheritance of Cry1Ac through their F_1, backcrosses and F_2 progenies were examined. Midgut brush border membrane vesicles (BBMV) preparations from resistant (DNDT-R) and susceptible (M4-S) population were assayed for marker enzyme specifically alkaline phosphatase.

The current understanding of Bt-toxin resistance in insects is associated generally with either conversion of Bt protoxins to activated toxins by insect midgut proteases [18] or by altered binding capacity of toxins to midgut proteins [19].

In our earlier study of F_2 screening done to detect major alleles conferring resistance to Cry 1Ac in *H. armigera*, fold resistance between resistant (DNDT-R) and susceptible (M4-S) population was observed to be 231.33 (table 1).

Table 1: Fold resistance of resistant strain, (DN×DT) against susceptible strain, (M Line)

Population	7th day				
	LC_{50}	Fiducial Limit (95%)	Slope ± SE	Chi square	Resistant Ratio
DN×DT (Resistant line)	6.94	1.15 – 9.46	4 ±1.47	2.44	231.33
M4 line (Susceptible)	0.03	0.005 – 0.09	0.46 ± 0.12	7.02	1

Four main functional receptors of the Cry1Ac toxin have been identified and verified from the brush border epithelium, including alkaline phosphatase [20], Cadherin [7], aminopeptidase [21].

Various studies with glycosyl phophatidylinositol (GPI)-anchored APN1 from lepidopteran insects consistently demonstrated that APN1 is one of the midgut receptors for Cry1Ac and related to the resistance to *Bt* toxins [22], which is in agreement with our findings. In our study, the specific acitivity of alkaline phosphatase was 108.10 and 160.93µM/min/µg of protein, respectively for resistant and susceptible population (table 2). Alkaline phosphatase activity was observed to be more in Cry1Ac susceptible M4 population as compared to the resistant DNDT population. The mean larval alkaline phosphatase of the resistant and susceptible parents ($p = 0.025$) differed significantly, thus suggesting the association of decreased APN activity in resistant population.

Takesue *et al.* [23] reported the role of the alkaline phosphatase as the binding anchor via phosphatidylinositol-specific phospholipase in *Bombyxmori* larvae. A high resistance to Cry1Ac is correlated with reduced level of alkaline phosphatase activity in *H. virescens* [24]. Later, Krishnamoorthy *et al.* [25] identified the role novel Cry1Ac binding proteins in the midgut of *H. virescens* to elucidate resistance. Ning *et al.* [26] reported the involvement of alkaline phosphatase with the Cry1Ac binding in cotton bollworm, *H. armigera*.

Jurat-Fuentes *et al.* [27] reported that the reduced level of membrane bound alkaline phosphatase expression might be considered as biomarker for Cry toxin resistance in lepidopteran pests, as monitoring of resistance based on reduction in alkaline phosphatase activity would allow detection of resistance to *Bt* toxins. Similarly our current data also showed the reduction in alkaline phosphatase activity in resistant parents as compared to susceptible parents. Wei *et al*., 2018 opined that ALP2 and APN1 may be used as markers to monitor and manage pest resistance in transgenic crops as the down regulation of ALP2 and APN1 affected the Cry1Ac resistance in both lower and higher levels of resistance in the LF-resistant H. armigera strains, when the ALP gene is downregulated, C. suppressalis was also reported to become resistant to Cry1A-, Cry2A- and Cry1C-transgenic rice lines [28].

Table 2: Inheritance of Cry 1Ac resistance with the level of alkaline phosphatase activity in *H. armigera*.

Mating			ALP activity		Δ Specific Activity (Percentage)	Theoretical Parental contribution (Genome)	Effects
Female ♀	Male ♂	Progenies (genotypes)	Range (µM /min/µg of protein)	Specific Activity (µM/min/µg of protein) ± SE			
RR		-	93.78 – 123.93	108.10 ± 8.74	-52.82 ± 7.81 (32.82%)*		R (nuclear)
SS		-	129.61 – 185.89	160.93 ± 16.55			
RR	SS	F_1 (RS)	289.99 – 348.10	320.49 ± 16.48	-327.16 ± 16.44 (0.50%)**		Maternal effect (cytoplasmic)
SS	RR	F_1 (SR)	582.04 – 685.36	647.65 ± 32.92			
RR	F_1 (RS)	Back cross 1	51.19 – 76.45	61.00 ± 7.82		75% R: 25% S	Effect is controlled by Nuclear gene
RR	F_1 (SR)	Back cross 2	46.26 – 54.95	50.53 ± 2.51		84.5% R:12.5% S	
F_1 (RS)	RR	Back cross 3	502.81 – 623.26	582.48 ± 39.84		92.2% R: 6.25% S	
F_1 (SR)	RR	Back cross 4	609.01 – 694.17	665.73 ± 28.36		96.2% R: 3.12% S	
F_1 (RS)	F_1 (SR)	Reciprocal F_1 crosses	609.73 – 701.22	670.36 ±30.32	27.13 ± 0.18 (0.04%)*		Effect is controlled by Nuclear gene
F_1 (SR)	F_1 (RS)		582.91 – 681.33	643.23 ± 30.5			

*, ** Significance of Student's t test at the level of *P* value <0.05 and 0.001 respectively.

The larval alkaline phosphatase activity of the resistant parent was found to be significantly different from both the susceptible parent and F_1 and F_2 progenies. The larval alkaline phosphatase activity of the resistant and susceptible parents ranged from 93.78 to 123.93 µM/min/µg of protein and 129.61 to 185.89 µM/min/µg of protein respectively. The mean larval alkaline phosphatase of the resistant and susceptible parents ($p = 0.025$), resistant and F_1 ($p = 0.002$), resistant and F_2 ($p = 0.002$), susceptible and F_1 ($p = 0.001$), susceptible and F_2 ($p = 0.001$) were found to be significantly different (table 3).

Table 3: ALP activity of *H. armigera* resistant and susceptible parents and their crosses at *P* value of significant difference at 0.005

	Resistant (R)	**Susceptible (S)**	**F_1 (R♀×S♂)**	**F_1 (S♀×R♂)**	**F_2 (RS♀×SR♂)**	**F_2 (SR♀×RS♂)**
Resistant (R)	--	0.025	0.002	0.002	0.002	0.002
Susceptible (S)			0.001	0.000	0.001	0.001
F_1 (R♀×S♂)				0.003	--	--
F_1 (S♀×R♂)						--
F_2 (RS♀×SR♂)						0.031
F_2 (SR♀×RS♂)						--

There was a stiff increase in alkaline phosphatase activity in F_1 (S♀×R♂) than F_1 (R♀×S♂) suggesting the influence of maternal effects (cytoplasmic) in the inheritance of Cry1Ac resistance in *H. armigera*; while studying the ALP activity in the backcrosses and F_2 progenies, nuclear genes were observed to contribute to the inheritance of Cry1Ac resistance (table 1). Maternal effects on Cry1Ac resistance development has been noted in *H. armigera* [29] and in *P. xylostella* [30]. However, maternal effects and sex linkage were not evident in Cry1Ac resistance in pink bollworm [31] and in Cry2Ad resistance in *P. xylostella* [32]. Our earlier studies on the inheritance of Cry1Ac resistance in *H. armigera* [33] observed autosomal inheritance and similar inheritance models on different cry toxins have been recorded for the cotton bollworm *H. armigera* [34], the pink bollworm *P. gossypiella*[35], the Asian corn borer *Ostrinia furnacalis* [36] and the southern house mosquito *Culex quinquefasciatus* [37].

However, little fluctuation was observed in the alkaline phosphatase activity in F_2 progenies. Since Cry1Ac has multiple binding sites in *H. virescens* BBMV [23] and their mode of inheritance of Cry1Ac in association with alkaline phosphatase has not been examined to date, it is possible that changes in Cry1Ac level due to reduced alkaline phosphatase level are masked by binding to the alternate receptors in the BBMV.

The limitation of using reduced alkaline phosphatase levels in monitoring for resistance to Cry toxins is, considering that in most cases Bt resistance is recessive, heterozygotes larvae present similar levels of membrane bound alkaline phosphatase activity as susceptible parents. Secondly, it may need to combine with additional biomarkers to assure accurate detection of resistance [26].

However, Caccia *et al.* [38] reported that the increased alkaline phosphatase activity in the midgut lumen was associated with Cry1Ac resistance in *H. zea*. In another findings, Anilkumar *et al.* [39] reported no binding differences between the susceptible and the Cry1Ac-resistant AR strain of *H. zea*, subsequent backcrossing of this resistant colony to a laboratory-susceptible colony that had received an influx of wild individuals warranted a repeat of the binding experiments. This indicated that resistance was not linked to a reduction in binding.

Wang *et al.* [40] reported that the Cry1Ac resistance in *T. ni* is conferred by loss of affinity for midgut binding site in the *T. ni* midgut. The level of resistance to Cry toxins in the resistant *T. ni* larvae is associated with the lack of the Cry1Ab/ Cry1Ac shared binding site in the larvae. Consistently, the direct binding analysis demonstrated that the midgut BBMV preparation from the resistant *T. ni* larvae lacked affinity for binding to Cry1Ab/ Cry1Ac.

Moreover, the F_1 larvae from the cross of the susceptible and resistant parents, which were susceptible to Cry1Ac, exhibited affinity for the midgut binding to the toxins, further confirming that loss of the Cry 1Ab/Cry1Ac binding affinity in the larval midgut is the mechanism conferring the resistance.

This result is in agreement with the detected reduction in irreversible binding, as ALP is considered to be responsible for localizing Cry1A toxin oligomers to lipid rafts [41] and the subsequent toxin insertion into the membrane (irreversible binding).

Further experiment conducted on the separation of the *H. armigera* protein on non-denaturing gradient SDS-PAGE and staining with BCIP-NBT revealed the distribution pattern of alkaline phosphatase isozyme in resistant, susceptible, F_1 hybrids, backcrosses and F_2 hybrids. The distribution pattern of alkaline phosphatase isozyme in resistant and susceptible population is almost similar and most of the isozymes seem to be predominant in molecular weight range 65-85 kDa (Fig. 3 & 4).

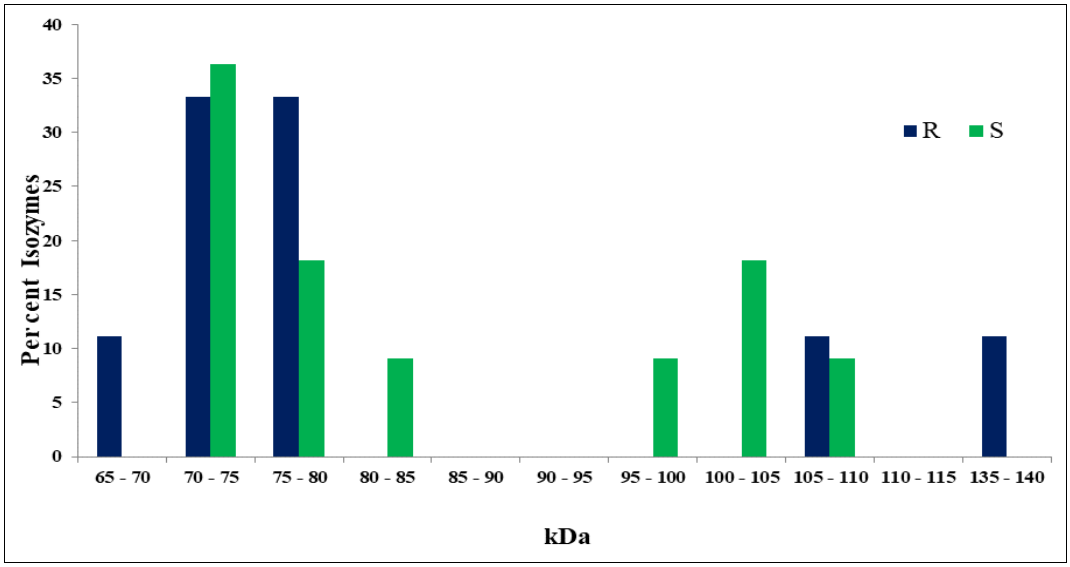

Fig 3: Distribution of isozymes in resistant and susceptible with respect to Cry1Ac

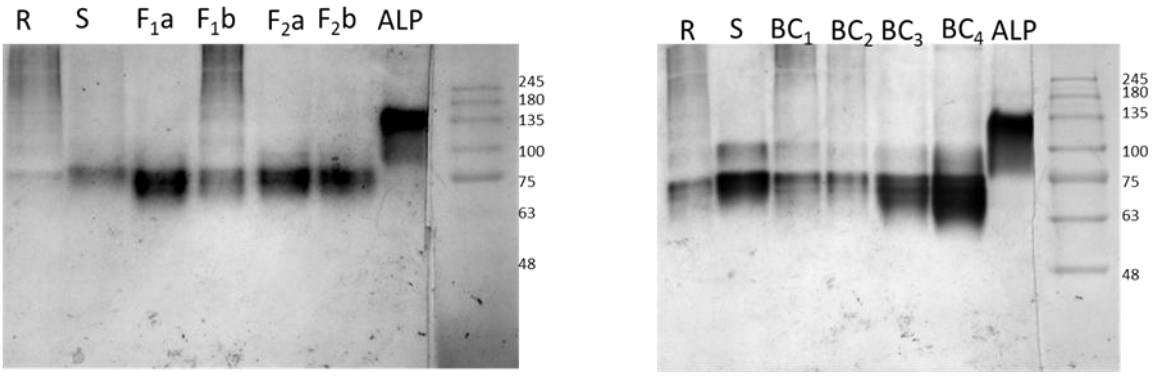

Fig 4: ALP specific staining of PAGE for detecting ALP isozymes

In pinkbollworm, the frequency of alkaline phosphatase isozyme in the molecular weight range 70-75 kDa was more in resistant larvae whereas equal frequency of isozymes in the molecular weight range 65-70, 70-75 and 100-105 kDa was found in control. It may be due to differential role of alkaline phosphatase isozymes (soluble or membrane bound) in the binding toxin molecules in lumen and thereby preventing oligomer formation essential for insertion in epithelial membrane, thereby imparting resistance to Cry2Ab in pink bollworm [42].

4. Conclusion

In this study, it became clear that the Cry1Ac resistance in the *H. armigera* population is conferred by alteration of the larval midgut binding to the Cry toxins. Maternal effects on inheritance of Cry1Ac resistance development was observed in F_1 progenies while nuclear effect was observed in backcrosses and F_2 progenies. What serves as the receptor for the Cry1Ac in *H. armigera* larvae and what alteration has occurred to the receptor in the resistant population require further investigation in order to understand the molecular genetic basis for the field evolved resistance in *H. armigera* populations.

5. Acknowledgements

This research was supported by Division of Entomology, Indian Agricultural Research Institute (IARI), New Delhi as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Entomology of Kennedy Ningthoujam (Registration No. 9672) under the guidance of Dr. G.T. Gujar, Ex- Head, Division of Entomology, IARI, New Delhi, India.

6. References

1. Lu Y, Wu K, Jiang Y, Guo Y, Desneux N. Widespread adoption of Bt cotton and insecticide decrease promotes biocontrol services. Nature. 2012; 487:362–365.
2. Kathage JK, Qaim M. Economic impacts and impact dynamics of Bt (*Bacillus thuringiensis*) cotton in India. Proceedings of the National Academy of Sciences, USA. 2012; 109:11652–11656.
3. Jakka SR, Liang G, James H, Rahul B, Joel JS, Kenneth N *et al*. Field-evolved mode 1 resistance of the fall armyworm to transgenic Cry1Fa-expressing corn associated with reduced Cry1Fa toxin binding and midgut alkaline phosphatase expression. Appl Environ Microbiol. 2016; 82:1023–1034.
4. Tabashnik BE, Jeffrey AF, Gopalan CU, Alex JY, Luke M, Jie Z et al. Efficacy of genetically modified Bt toxins alone and in combinations against pink bollworm resistant to Cry1Ac and Cry2Ab. PloS one. 2013; 8:e80496.
5. Deist BR, Michael A, Fernandez-Luna RMT, Adang MJ, Bonning BC. *Bt* Toxin Modification for Enhanced Efficacy. Toxins. 2014; 6(10):3005–3027.
6. Likitvivatanavong S, Chen J, Bravo A, Soberón M, Gill SS. Role of cadherin, alkaline phosphatase and aminopeptidase N as receptors of Cry11Ba toxin from *Bacillus thuringiensis* subsp. *jegathesan* in *Aedes aegypti*. Appl. Environ. Microbiol. 2011; 77(1):24–31.
7. Chen W, Liu C, Xiao Y, Zhang D, Zhang Y, Li X *et al*. A Toxin-Binding Alkaline Phosphatase Fragment Synergizes *Bt* Toxin Cry1Ac against Susceptible and Resistant *Helicoverpa armigera.* PLoS One. 2015; 17:10(4):e0126288.
8. Nagarkatti S, Prakash A. Rearing of *Heliothis armigera* (Hübner) on an artificial diet, Technical Bulletin of the Commonwealth Institute of Biological Control, Bangalore 1974, 17.
9. Gujar GT, Mittal A, Kumari A, Kalia V. Host crops influence on the susceptibility of the American bollworm, *Helicoverpa armigera* (Hübner) (Noctuidae: Lepidoptera) to *Bacillus thuringiensis* Berliner var. *kurstaki* HD-73. Entomol. Exp. Appl. 2004; 113:165–173.
10. Gilroy TE, Wilcox R. Hybrid *Bacillus thuringiensis* gene, plasmid and transformed *Psuedomonas fluorescens*. US Patent. 1992; 5:128–130.
11. Tabashnik BE, Liu YB, Dennehy TJ, Sims MA, Sisterson M, Biggs MR *et al*. Inheritance of resistance to Bt toxin Cry1Ac in a field derived strain of pink bollworm (Lepidoptera: Gelechiidae). J Econ. Entomol. 2002; 95:1018–1026.
12. Gujar GT, Kumari A, Kalia V, Chandrashekar K. Spatial and temporal variation in susceptibility of American bollworm, *Helicoverpa armigera* (Hübner) to *Bacillus thuringiensis* var. *kurstaki.* Curr. Sci. 2000; 78:995–1001.
13. Ross GES. Maximum likelihood programme. The numerical algorithms Gr. Rothamsted Experiment Station, Harpenden, UK, 1977.
14. Wolfersberger MG, Luthy P, Maurer A, Parenti P, Sacchi VF, Giordana B *et al*. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comparative Biochemistry and Physiology. 1987; 86:301-306.
15. Lowry OH, Roberts NR, Wu M, Hixon WS, Crawford EJ. The quantitative histochemistry of brain. II. Enzyme measurements, J Biol Chem., 1954; 19:207-215.
16. Bradford MM. A rapid and sensitive method for the

quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976; 72:248.
17. Suurs LCJM. Jongedijk E, Tan MMC. Polyacrylamide gradient-gel electrophoresis: a routine method for high resolution isozyme electrophoresis of *Solanum* and *Lycopersicon* species. Euphytica. 1989; 40:81-186.
18. Wei J, Liang G, Wang B, Zhong F, Chen L, Khaing MM *et al*. Activation of Bt protoxin Cry1Ac in resistant and susceptible cotton bollworm. PLOS ONE, 2016. DOI: 10.1371/journal.pone.0156560
19. Xiao Y, Zhang T, Liu C, Heckel DG, Li X, Tabashnik BE *et al*. Mis-splicing of the ABCC2 gene linked with Bt toxin resistance in *Helicoverpa armigera*. Scientific Reports. 2014; 4:6184.
20. Flores-Escobar B, Rodríguez-Magadan H, Bravo A, Soberón M, Gómez I. *Manduca sexta* aminopeptidase-N and alkaline phosphatase have a differential role in the mode of action of Cry1Aa, Cry1Ab and Cry1Ac toxins from *Bacillus thuringiensis*. Appl Environ Microbiol. 2013; 79:4543-50.
21. Wei J, Zhang M, Liang G, Wu K, Guo Y, Ni X *et al*. APN1 is a functional receptor of Cry1Ac but not Cry2Ab in *Helicoverpa zea*. Scientific Reports. 2016; 6:19179. DOI: 10.1038/srep19179.
22. Valaitis AP. Localization of *Bacillus thuringiensis* Cry1A toxin-binding molecules in gypsy moth larval gut sections using fluorescence microscopy. J Invertebr Pathol. 2011; 108:69-75.
23. Takesue Y, Yokota K, Miyajima S, Taguchi R, Ikezawa H. Membrane anchors of alkaline phosphatase and trehalose associated with the plasma membrane of the larval midgut epithelial of the sil*kworm, Bombyx mori.* J Biochem. 1989; 105:998-1001.
24. Jurat-Fuentes JL, Adang MJ. Importance of Cry1delta-endotoxin domain II loops for binding specificity in *Heliothis virescens* (L.). Appl. Environ. Microbiol. 2001; 67:323-329.
25. Krishnamoorthy M, Jurat-Fuentes JL, McNall RJ, Andacht T, Adang MJ. Identification of novel Cry 1 Ac binding proteins in midgut membranes from *Heliothis virescens* using proteomic analyses. Insect Biochemistry and Molecular Biology. 2007; 37:189-201.
26. Ning C, Wu K, Liu C, Gao Y, Jurat-Fuentes JL, Gao X. Characterization of a Cry1Ac toxin-binding alkaline phosphatase in the midgut from *Helicoverpa armigera* (Hubner) larvae. J Insect Physiol. 2010; 56:666–672.
27. Jurat-Fuentes JL, Karumbaiah L, Jakka SR, Ning C, Liu C, Wu K *et al*. Reduced levels of membrane-bound alkaline phosphatase are common to lepidopteran strains resistant to Cry toxins from *Bacillus thuringiensis*. PLoS One. 2011; 6:e17606.
28. Qiu L, Wang P, Wu T, Li B, Wang X, Lei C *et al*. Downregulation of *Chilo suppressalis* alkaline phosphatase genes associated with resistance to three transgenic *Bacillus thuringiensis* rice lines. Insect Mol Biol. 2018; 27:83-89.
29. Kranthi KR, Dhawad CS, Naidu SR, Mate K, Behere GT, Wadaskar RM, Kranthi S. Inheritance of resistance in Indian *Helicoverpa armigera* (Hübner) to Cry1Ac toxin of *Bacillus thuringiensis*. Crop Prot. 2006; 25:119-124.
30. Tabashnik BE, Cushing NL, Finson N, Johnson MW. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J Econ. 1990; 83:1671-1676.
31. Liu YB, Tabashnik BE, Meyer SK, Crickmore N. Cross-resistance and stability of resistance to *Bacillus thuringiensis* toxin Cry1C in diamondback moth. Appl. Environ. Microbiol. 2001; 67:3216-19.
32. Liao J, Xue Y, Xiao G, Xie M, Huang S, You S *et al*. Inheritance and ftness costs of resistance to *Bacillus thuringiensis* toxin Cry2Ad in laboratory strains of the diamondback moth, *Plutella xylostella* (L.). Scitific Reports. 2019; 9:6113.
33. Ningthoujam K, Sharma K, Gujar GT. Inheritance of resistance to *Bacillus thuringiensis* toxin, Cry1Ac in *Helicoverpa armigera* (Hübner). Annals of Plant Protection Science. 2015; 23:205-209.
34. Nair R, Kalia V, Aggarwal KK & Gujar GT. Inheritance of Cry1Ac resistance and associated biological traits in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). J Invertebr Pathol. 2010; 104:31-38.
35. Malthankar PA, Gujar GT. Association of Cry2Ab resistance with alkaline phosphatase in pink bollworm (Saunder) (Lepidoptera: Gelichiidae). Biopest. Internl. 2014; 10:143-151.
36. Zhang S, Cheng H, Gao Y, Wang G, Liang G, Wu K. Mutation of an aminopeptidase N gene is associated with *Helicoverpa armigera* resistance to *Bacillus thuringiensis* Cry1Ac toxin. Insect Biochem. Mol. Biol. 2009; 39:421-429.
37. Wirth MC, Walton WE, Federici BA. Inheritance, stability, and dominance of cry resistance in *Culex quinquefasciatus* (Diptera: Culicidae) selected with the three cry toxins of *Bacillus thuringiensis* subsp. *israelensis*. J Med Entomol. 2012; 49:886-94.
38. Caccia S, Moar WJ, Chandrashekhar J, Oppert C, Konasale J, Anilkumar KJ *et al*. Association of Cry1Ac Toxin Resistance in *Helicoverpa zea* (Boddie) with Increased Alkaline Phosphatase Levels in the Midgut Lumen. Appl Environ Microbiol. 2012; 78:5690-5698.
39. Anilkumar KJ, Pusztai-carey M, Moar WJ. Fitness costs associated with Cry1Ac-resistant *Helicoverpa zea* (Lepidoptera: Noctuidae): a factor countering selection for resistance to *Bt* cotton? J Econ. Entomol. 2008; 101:1421-1431.
40. Wang P, Zhao JZ, Rodrigo-Simi A, Kain W, Janmaat AF, Shelton AM *et al*. Mechanism of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a greenhouse population of the cabbage looper, *Trichoplusiani*. Appl Environ Microbiol. 2007; 73:1199-1207.
41. Zhuang M, Oltean DI, Gómez I, Pullikuth AK, Soberón M, Bravo A *et al*. *Heliothis virescens* and *Manduca sexta* lipid rafts are involved in Cry1A toxin binding to the midgut epithelium and subsequent pore formation. J Biol Chem. 2002; 227:13863-72.
42. Malthankar PA, Gujar GT. Toxicity of *Bacillus thuringiensis* Cry2Ab and the inheritance of Cry2Ab resistance in the Pink bollworm, *Pectinophora gossypiella* (Saunders). Indian Journal of Experimental Biology. 2016; 54:586-596.