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Frequency of Cry1Ac and Cry2Ab resistance alleles in pink bollworm, *Pectinophora gossypiella* Saunders from Andhra Pradesh, India

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Abstract Since 2002, transgenic crops that produce Cry1Ac and Cry2Ab toxins have been used in India to control bollworms. From 2002 to 2009, Bt-cotton effectively controlled the pink bollworm (PBW). However, since 2009 numerous studies have reported high survival of PBW on Bt-cotton expressing Cry1Ac and Cry2Ab toxins, indicating reduced susceptibility to Cry toxins expressed in Bt-cotton. In the current study, we attempted to estimate the frequency of resistance alleles to Cry1Ac and Cry2Ab toxins in a field collected population of pink bollworms from Andhra Pradesh. Resistance allele frequency was estimated using an F_2 screen methodology for the first time after 17 years of Bt-cotton cultivation. Our study finds that the allele frequency for Cry1Ac is 0.082 and for Cry2Ab is 0.054, with a detection probability of greater than 97%. In our survey conducted in 2018–19 and 2019–20, we also noticed high survival and damage by PBW on Bt-cotton expressing Cry1Ac and Cry2Ab. Our survey report reveals, > 30% flower damage, > 90% green boll damage and > 80% locule damage by PBW on Bt-cotton expressing Cry1Ac and Cry2Ab toxins.

Keywords Field-evolved resistance · Pink bollworm · F_2 screen · Cry toxins · Bt resistance

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Introduction

Pink bollworm (PBW), *Pectinophora gossypiella* Saunders (Lepidopetra: Gelechiidae), one of the world's most important cotton pests, has been controlled by transgenic cotton producing Bt toxins in several countries for over a decade. Bt-cotton expressing *cry1Ac* gene was introduced in 2002, and in 2006 Bt-cotton pyramided with *cry2Ab* gene was introduced in India for commercial cultivation. Bt-cotton was mainly introduced to manage bollworms, American bollworm, *Helicoverpa armigera* (Hubner), pink bollworm, *P. gossypeilla* and spiny and spotted bollworms, *Erias* spp. Since the introduction of Bt-cotton in India, it has successfully managed the bollworm complex, providing higher yields of high-quality lint, reduced insecticidal sprays, and higher cost benefits (Choudhary & Gaur, 2015; Ramasundaram et al., 2014). On the other hand, the evolution of resistance to Cry toxins expressed in Bt-cotton may impede its performance and deprive farmers of its benefits (Matten & Reynolds, 2003). The

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Genetic Engineering Appraisal Committee (GEAC) mandated a high dose with 5% structured refuge as insect resistance management (IRM) plan in India to delay the evolution of resistance in target insect pests. The effectiveness of the high dose/refuge strategy depends on several key assumptions (Bourguet et al., 2003). One of these assumptions requires Bt resistance frequencies in target insect populations to be rare (e.g., < 0.001) (Andow & Alstad, 1998). Several studies have been conducted across the globe that measured the frequency of resistance alleles to Cry toxins in target pests (Andow & Alstad, 1998; Huang et al., 2007; Mahon et al., 2007). Estimating the frequency of resistance alleles to Cry toxins expressed in GM crops in target pests has helped to detect, measure, and monitor the evolution of resistance in field populations. Several techniques have been proposed to detect and measure resistance in field populations (Huang, 2006). Among the several methods, the F_2 screen has been adopted in the present study due to its sensitivity and accuracy in detecting resistance alleles (Andow & Alstad, 1998). We estimated resistance allele frequency in PBW collected in South India.

Materials and methods

Field survey

A comprehensive survey was conducted in the Guntur, Prakasam and Kurnool districts of Andhra Pradesh during 2018–19 and 2019–20 on the incidence of PBW. In three districts, 12 major Bt-cotton growing mandals were selected for the survey. A survey was initiated during flower initiation stage (45–50 Days after sowing (DAS) and continued till the end of the crop (170 DAS) capturing important crop growth stages. Samples were collected at every 15 days interval from each field to assess the PBW damage. In each mandal, five villages were selected, from each village five fields were surveyed for assessing PBW damage. Green bolls were collected from the farmers' fields and brought to the laboratory for estimating boll and locule damage.

Rosette flowers were counted on five randomly selected plants per field and expressed as rosette flowers (%). Similarly, five bolls per plant were randomly picked to estimate locule and boll damage, and 300 green bolls were sampled from each Mandal and brought to the laboratory. The percent damage was expressed as damaged bolls or locules to total bolls or locules observed.

Field collection and rearing

For F_2 screen studies, PBW larvae were sampled from various Bt-cotton fields in the Guntur, Prakasam, and Kurnool districts of Andhra Pradesh in 2019–20 (Fig. 1). Sampling was carried out in Bt-cotton fields as non-Bt-cotton fields were scarce. Bolls were randomly gathered at a rate of one boll per plant and up to 125–175 per hectare. Collected bolls were brought to the laboratory for extracting PBW larvae. After dissecting the bolls, live larvae were placed onto 24 well trays (1 larva/well) containing an artificial feed. All PBW larvae were grown on an artificial diet as stated in (Kranthi, 2005) and kept in the insect growth chamber at 27 °C ± 1 °C and 75% RH until pupation. Only one larva per boll was transferred to the diet to avoid screening several larvae of a single female.

Single pair family lines

All the field-collected larvae were maintained by location for establishing single pair family lines. Fifth instar larvae were sexed based on the visual marking on the dorsal side of the seventh or eighth abdominal segment. Larvae having a pair of dark-colored testes were separated as males, while larvae without such markings were separated as females (Ramya et al., 2020). Sexed larvae were reared separately to pupation on an artificial diet. Healthy, well-developed pupae were isolated and kept for adult emergence in plastic containers (1 L). Newly emerged male and female moths (< 12 h) belonging to the same location were paired in a plastic jar (1 L) for mating and oviposition. A tiny twig with cotton squares and a few leaves immersed in a 2% agar solution was supplied as the substratum for egg laying for adults. The absorbent cotton dipped in honey solution (50 ml honey + 50 g sucrose + 2 g methyl para-hydroxy benzoate in 1000 ml) was provided as an adult feed. Mating pairs were kept in incubation chambers and checked at regular intervals (2 d) for egg laying and hatching. Hatched neonates were transferred onto an artificial diet using a fine brush.

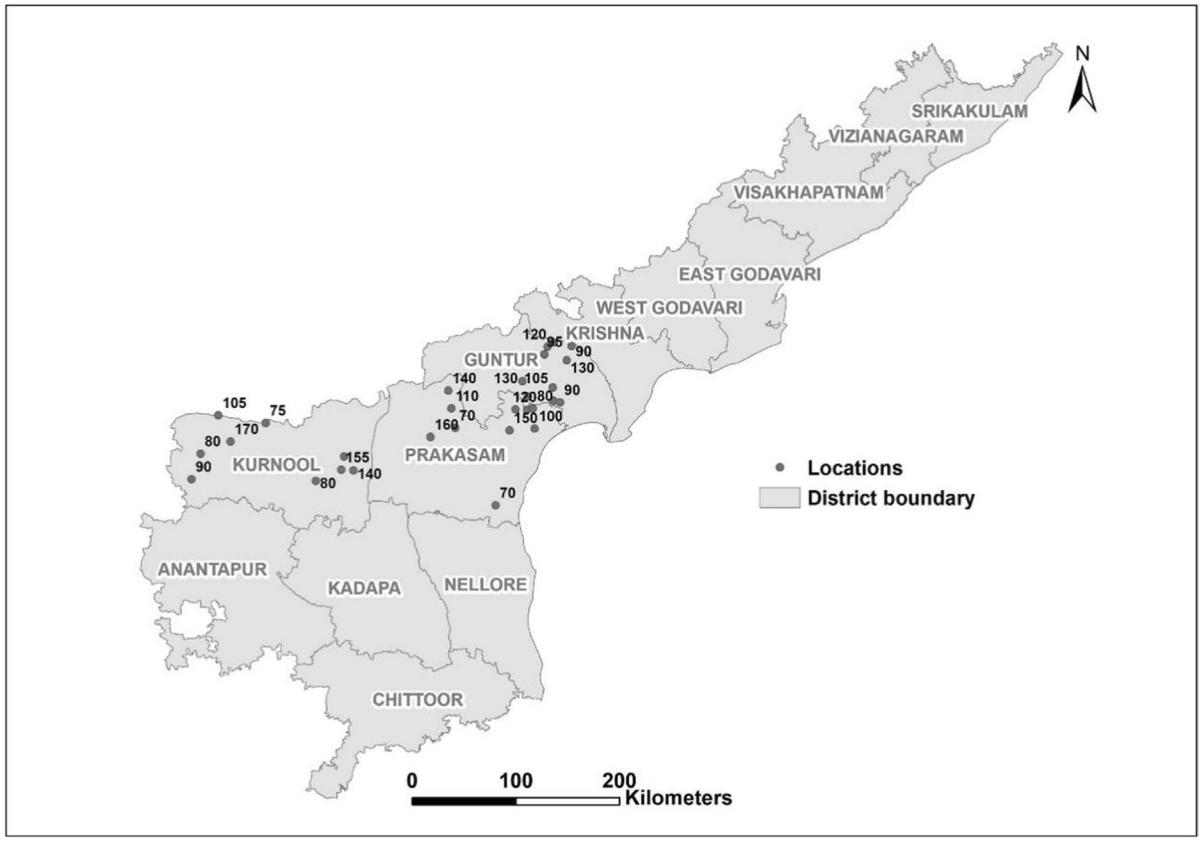

Fig. 1 Pink bollworm larvae collected from different Bt-cotton growing regions of Andhra Pradesh

F_1 generation

Neonates (F_1) that hatched were maintained by the family line and reared up to pupation. The visual marks indicated in previous sections were used to sex late instar larvae. Pupae were isolated, washed with 2% sodium hypochlorite, and kept in plastic jars for adult emergence (1L). The sib-mating of F_1 moths was accomplished by releasing at least five male and female moths into 5-L plastic jars. 15 ± 0.34 (Mean $\pm$ SEM) sib-mating pairs were produced on average per family line. 2–3 sib-mating jars per family line was maintained for production of F_2 neonates.

F_2 generation and screening

Neonates that hatched from sib-mating pairs were considered as the F_2 generation. F_2 neonates hatched on a single day from different sib-mating pairs within the family line were combined. The F_2 neonates were reared for two days on an artificial diet, and on third-day, larvae were exposed to a diet containing a discriminating dose of Cry toxins. Cry1Ac and Cry2Ab toxins were provided by Central Institute for Cotton Research (CICR), Nagpur India. Cry1Ac was obtained as MVP II (*Pseudomonas* encapsulated Cry1Ac 19.7%) formulation containing 1 ml of 2% MVP II + 1 ml of 1X PBS and Cry2Ab as lyophilized corn leaf powder containing 6 mg Cry2Ab toxin per gram. Two different diets were prepared with Cry1Ac toxin and Cry2Ab toxin for screening. Discriminating dose of Cry1Ac and Cry2Ab (Fabrick et al., 2014, 2015; Mohan et al., 2016; Reynolds, 2006; Tabashnik et al., 2005, 2006, 2012, 2013) were used to screen the F_2 population for resistance alleles (Andow et al., 1998; Blanco et al., 2008; Kukanur et al., 2018; Mahon et al., 2007). An artificial diet was prepared and allowed to cool. When the temperature was around 40–45 °C, MVP II containing Cry1Ac toxin was

added to get the concentration of 10 µg/ml of diet. Similarly, the Cry2Ab toxin diet was also prepared by mixing the required quantity of corn leaf powder to get the concentration of 10 µg/ml of diet. The toxin diet was poured into 24-well multi-cavity trays that contained approximately 1 ml of toxin diet and allowed to cool and solidify. F_2 neonates were transferred at one larva per cell, sealed with a parafilm sheet to avoid larvae escape. All the diet assays trays were transferred to an incubator maintained at 27 ± 1 °C and 70 ± 5% RH. Larvae were scored based on the survival and developmental ($\geq L_3$) stages on the 21st day after treatment as described by (Tabashnik et al., 2005). If the treated F_2 larvae survived and reached L_3 or later stages by the 21st day, such lines were considered positive for carrying resistance alleles. All the surviving F_2 larvae after the 22nd day were transferred to a normal diet and reared to pupation for further reconfirmation for carrying resistance allele in the F_3 generation.

Resistance confirmation

Neonates of single-pair family lines that survived and completed larval development after treatment with Cry1Ac and Cry2Ab toxins were suspected of carrying resistance alleles. Therefore, in the F_3 generation, surviving family lines were retested with discriminating doses of Cry1Ac and Cry2Ab to see if they carried resistance alleles at discriminating dose of 10 µg/ml of diet. This procedure was followed to eliminate the false positives from the diet assay. If the larvae survived and reached the third instar by the 21st day, they were considered as true positive for carrying resistant alleles. Those lines were considered false positive if treated F_3 larvae recorded 100 percent mortality or took more than 22 days to reach the third instar.

Statistical analysis

Resistance allele frequencies (E[pR]) for Cry1Ac and Cry2Ab and the likelihood of missing a resistance allele existing in a line (P_{No}) were estimated using the equations provided by (Andow & Alstad, 1998) and (Stodola & Andow, 2004). All the calculations were performed in R-program (R Core Team, 2021).

Results

Survey to assess the damage caused by PBW on Bt-cotton

Survey conducted in three major Bt-cotton growing districts of Andhra Pradesh to assess the PBW incidence showed a higher incidence of PBW on flowers, green bolls and locules. The PBW damage ranged from 4.25% to 92.50% in two years study (2018–19—2019–20). PBW incidence was noticed from 45–50 days after sowing of the crop and continued till the end of the crop (till 170 days). A lower incidence of PBW was noticed at the flowering stage and incidence increased as crop stage advanced. During 2018–19, per cent damage on flowers ranged from 4.25—31.50%, lowest at 60 DAS and highest at 120 DAS. Among the regions, highest incidence was noticed in Guntur followed by Prakasam and Kurnool. Similar trend was noticed during 2019–20 per cent damage on flowers ranged from 4.75% to 37.75%. Lowest damage was noticed at 60 DAS and highest damage was recorded during 120 DAS. Highest damage was noticed in Guntur region followed by Kurnool and Prakasam.

PBW damage on green bolls was noticed from 75 DAS in all the regions and was noticed upto 135 DAS. Per cent boll damage ranged from 13.75% to 92.50%, lowest damage was noticed at 75 DAS and gradually increased at 135 DAS. During 2018–19, Prakasam recorded highest green boll damage (89.50%) followed by Kurnool and Guntur. Similarly, during 2019–20 lowest boll damage was noticed at 75 DAS in Gutnur region and highest damage (92.50%) was noticed in Prakasam district (Table 1).

Per cent locules damage was assessed from the field collected samples. Per cent locule damage by PBW ranged from 4.25% to 80.00% during 2018–19 and 4.50% to 82.75% during 2019–20. Lowest damage was noticed at 75 DAS and highest damage was noticed at 135 DAS during both the years. Among the regions, Kurnool recorded highest locule damage during 2018–19 and 2019–20 followed by Guntur and Prakasam (Table 1).

Development of the F_2 population

The Table 2 provides information on the larvae collected, the single pair family lines established, the

Table 1 Pink bollworm incidence and damage on flower, bolls and locule during 2018–19 and 2019–20

District	Stage	60 DAS		75 DAS		90 DAS		105 DAS		120 DAS		135 DAS	
		2018–19	2019–20	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20
Guntur	Flower	4.25	5.25	7.00	9.00	8.75	10.75	16.00	16.50	31.50	37.75	15.50	18.50
Prakasam	Flower	5.00	5.25	7.75	8.25	10.75	12.00	13.00	15.25	31.00	32.00	22.00	23.75
Kurnool	Flower	4.75	4.75	6.00	6.00	9.00	10.75	15.75	18.50	27.00	33.00	23.75	27.50
Guntur	Green bolls	0.00	0.00	13.75	14.25	22.25	22.75	53.25	49.50	78.50	79.25	86.25	89.00
Prakasam	Green bolls	0.00	0.00	17.75	16.75	25.00	25.00	57.00	58.00	75.00	75.25	89.50	92.50
Kurnool	Green bolls	0.00	0.00	18.00	17.75	27.50	36.00	56.25	57.75	76.75	79.75	86.75	89.75
Guntur	Locules	0.00	0.00	4.25	5.25	8.25	7.88	17.50	19.63	54.00	59.25	76.50	80.13
Prakasam	Locules	0.00	0.00	4.94	4.50	9.13	8.75	15.81	25.50	50.88	57.75	72.25	75.50
Kurnool	Locules	0.00	0.00	4.88	5.63	9.38	12.25	14.19	16.75	51.13	57.50	80.00	82.75

Table 2 A detailed breakdown of PBW larvae collected, families established, and lines reached F_2 screen

Location	No. larvae collected	Family (IF) lines established	IF reached F_1 generation	No. F_1 pupae/family line (Mean ± SEM)		IF reached F_2 generation	Cry1Ac screen		Cry2Ab screen	
				Male	Female		Lines subjected	Lines survived[a]	Lines subjected	Lines survived[a]
Guntur	1090	227	49	27.38 ± 0.57	33.29 ± 0.56	21	21	13	21	17
Prakasam	1220	290	63	19.23 ± 0.57	23.73 ± 0.61	26	26	25	26	18
Kurnool	1065	166	41	16.37 ± 0.66	20.95 ± 0.66	19	19	8	19	10
Total	**3375**	**683**	**153**	**21.00 ± 0.37**	**25.97 ± 0.39**	**66**	**66**	**46**	**66**	**45**

[a]family lines survived at 21 days after treatment

development of the family lines up to the F_3 generation, the family lines exposed to screening, and the F_2 screen survival rates. Three locations in Andhra Pradesh yielded 3375 field-collected larvae, from which 683 family lines were formed. 153 family lines (22.4%) out of the 683 family lines produced viable eggs and finished the F_1 generation. Only 66 (43.13%) of the 153 lines produced F_2 populations, allowing us to examine the resistance alleles for Cry1Ac and Cry2Ab. For each family line that met the requirements of the F_2 screen of > 10 females and males contributing to the F_2 population, a total of 21.00 0.37 F_1 males and 25.97 0.39 F_1 females were obtained in the F_1 generation. The F_2 population from sib-mating F_1 pairs was sufficient for screening. A total of 11,455 F_2 neonates were tested on a diet containing a discriminating dose of Cry1Ac toxin, and 11,355 F_2 neonates were tested on a diet containing a discriminating dose of Cry2Ab toxin. Sixty F_2 larvae of each single pair family line were transferred to a normal diet for calculating control morality (μ). An average of 173.56 ± 12.66 and 172.05 ± 12.77 F_2 larvae per family line were subjected to F_2 screen for Cry1Ac and Cry2Ab toxin diet assays respectively. Many of the lines were lost during P_1 and F_1 generation due to failure in mating, mating that resulted in nonviable eggs or eggs that failed to hatch, larvae died due to unknown reasons, larvae that failed to pupate and adult that failed to emerge from pupae.

Screening for Cry1Ac resistance

F_2 neonates from each family line were transferred onto a toxin diet containing Cry1Ac toxin for screening resistance alleles. Out of 66 family lines subjected to the screen, F_2 neonates of 20 family lines showed no survival on 21st d after treatment. Among the 20 family lines, four lines (G-130, 152, 186, 196) from Guntur, one line (P-100) from Prakasam, and six lines (K-49, 60, 77, 84, 101, 107) from Kurnool had survivals (< 4 larvae) on 21st day, but larvae did not reach L_3 stage and died after 25th d. F_2 larvae of the remaining 46 (69.70%) family lines survived, reached the L_3 stage by 21st d, and were suspected of carrying resistance alleles for Cry1Ac toxin. F_2 larvae on a normal diet (toxin free) showed 12.4% mortality and were used for calculating P_{No}. To rule out the false positives in the F_2 screen, surviving larvae were transferred to a normal diet on 22nd d and progressed to F_3 generation for reconfirmation. F_3 neonates obtained from sib-mated F_2 pairs were subjected to a toxin diet containing discriminating doses of Cry1Ac. Out of the 46 surviving family lines, larvae of 26 lines died in later instars or in the pupal stage, or adults did not lay viable eggs, hence could not re-test for carrying resistance alleles. In comparison, F_3 progenies of 20 family lines yielded sufficient larvae and were subjected to reconfirmation. F_3 larvae of all the 20 family lines survived the reconfirmation test and were considered true positives (Table 3).

Screening for Cry2Ab resistance

F_2 neonates of 66 family lines were also evaluated for Cry2Ab resistance alleles. Out of 66 family lines, neonates of 21 family lines showed no survival on a diet containing a discriminating dose of Cry2Ab toxin on 21st d after treatment. Out of 21 family lines, F_2 larvae of 3 family lines (G-130, 186, 190) from Guntur, five family lines (P-67, 100, 145, 176, 186) from Prakasam, and six family lines (K-49, 52, 65, 77, 102, 103) from Kurnool showed survivals (< 4 larvae) on 21st d but died after 25th d. F_2 larvae of 45 (68.18%) family lines survived the screen and reached the L_3 stage on 22nd d. All the neonates of surviving family lines were subjected to reconfirmation for Cry2Ab resistance in the F_3 generation. F_2 larvae of each family line maintained on a normal diet (toxin free) showed 11.2% mortality (μ). Many of the surviving family lines did not reach the F_3 generation. Out of 45 surviving family lines, progenies of 32 family lines died due to reasons mentioned in an earlier section, and only neonates of 13 family lines produced sufficient F_3 population for the reconfirmation test. Progenies of all the 13 family lines survived the reconfirmation test and were scored as true positives (Table 3).

Resistance allele frequency

As family lines were established from field-collected larvae, we considered the single pair as two-parent family lines where both male and female originated from the field-collected population. A total of 264 alleles were screened for Cry1Ac and Cry2Ab resistance in the field-collected pink bollworm population. A total of 20 and 13 family lines were reconfirmed as true positives in F_3 generation for carrying Cry1Ac and Cry2Ab resistance alleles, respectively. F_2 larvae

Table 3 The F_2 and F_3 survival rate and P_{No} of family lines

District	Family	Cry1Ac		Cry2Ab		P_{No}
		F_2 survival rate (%)	F_3 survival rate (%)	F_2 survival rate (%)	F_3 survival rate (%)	
Guntur	G-3	0.60	0.67	0.30	0.47	0.000194
	G-7	0.83	0.83	0.57	0.33	0.006246
	G-9	0.80	0.67	0.37	0.00	6.63×10^{-05}
	G-12	0.40	0.00	0.33	0.00	0.00158
	G-25	0.47	0.50	0.70	0.17	0.000861
	G-49	0.87	0.57	0.47	0.17	2.58×10^{-05}
	G-50	0.77	0.47	0.63	0.17	0.000314
	G-60	0.63	0.33	0.40	0.13	0.000236
	G-65	0.53	0.43	0.20	0.33	0.000114
	G-79	0.10	0.00	0.17	0.00	0.000298
	G-84	0.13	0.00	0.13	0.00	0.00076
	G-101	0.17	0.00	0.33	0.00	0.000833
	G-108	0.00	0.00	0.00	0.00	0.000573
	G-130	0.00	0.00	0.00	0.00	0.005906
	G-152	0.00	0.00	0.17	0.00	0.000247
	G-153	0.00	0.00	0.17	0.00	0.125255
	G-186	0.00	0.00	0.00	0.00	0.00149
	G-190	0.00	0.00	0.00	0.00	0.000143
	G-196	0.00	0.00	0.17	0.00	0.001008
	G-203	0.10	0.00	0.13	0.00	0.000687
	G-212	0.00	0.00	0.17	0.00	0.001128
Prakasam	P-1	0.33	0.37	0.23	0.00	4.91×10^{-05}
	P-10	0.20	0.40	0.13	0.00	8.97×10^{-05}
	P-12	0.33	0.33	0.33	0.17	0.000321
	P-17	0.40	0.00	0.33	0.00	0.00913
	P-18	0.40	0.00	0.17	0.00	0.014871
	P-21	0.53	0.00	0.23	0.00	0.015359
	P-29	0.13	0.13	0.30	0.47	0.000146
	P-31	0.37	0.03	0.10	0.00	0.000143
	P-50	0.57	0.10	0.53	0.67	3.28×10^{-05}
	P-56	0.13	0.00	0.13	0.00	0.007262
	P-60	0.20	0.00	0.07	0.00	0.003244
	P-67	0.17	0.00	0.00	0.00	0.007031
	P-91	0.23	0.00	0.17	0.00	0.015359
	P-100	0.00	0.00	0.00	0.00	0.00849
	P-107	0.30	0.00	0.17	0.00	0.012697
	P-111	0.13	0.00	0.13	0.00	0.007748
	P-145	0.17	0.00	0.00	0.00	0.031505
	P-146	0.33	0.00	0.00	0.00	0.022259
	P-176	0.27	0.00	0.00	0.00	0.009522
	P-186	0.13	0.00	0.00	0.00	0.031505
	P-200	0.37	0.00	0.00	0.00	0.027003
	P-212	0.20	0.00	0.00	0.00	0.130479
	P-230	0.23	0.00	0.13	0.00	0.019511
	P-280	0.13	0.00	0.13	0.00	0.010006
	P-286	0.33	0.00	0.33	0.00	0.013764
	P-288	0.13	0.00	0.13	0.00	0.006605

Table 3 (continued)

District	Family	Cry1Ac		Cry2Ab		P_{No}
		F_2 survival rate (%)	F_3 survival rate (%)	F_2 survival rate (%)	F_3 survival rate (%)	
Kurnool	K-5	0.10	0.47	0.33	0.00	0.000452
	K-15	0.17	0.27	0.30	0.00	0.000578
	K-21	0.37	0.07	0.53	0.40	0.000334
	K-39	0.00	0.00	0.17	0.00	0.028242
	K-40	0.53	0.33	0.63	0.50	0.000338
	K-42	0.47	0.00	0.37	0.00	0.025949
	K-44	0.47	0.63	0.33	0.80	0.000924
	K-49	0.00	0.00	0.00	0.00	0.060831
	K-52	0.17	0.00	0.00	0.00	0.02054
	K-60	0.00	0.00	0.00	0.00	0.500015
	K-65	0.57	0.70	0.00	0.00	0.001112
	K-77	0.00	0.00	0.00	0.00	0.031172
	K-84	0.00	0.00	0.33	0.00	0.051573
	K-101	0.00	0.00	0.00	0.00	0.067329
	K-102	0.00	0.00	0.00	0.00	0.019006
	K-103	0.00	0.00	0.00	0.00	0.026815
	K-107	0.00	0.00	0.10	0.00	0.009522
	K-146	0.00	0.00	0.20	0.00	0.055145
	K-152	0.00	0.00	0.00	0.00	0.012853

of 36 family lines against Cry1Ac and 21 family lines against Cry2Ab toxin had a very high survival rate (> 0.25), possibly indicating more than one R allele in the surviving family lines (Table 3).

The frequency of Cry1Ac resistance alleles in PBW collected from Andhra Pradesh was estimated to be 0.082 with 95% CI of 0.051–0.105, and the frequency of Cry2Ab resistance alleles was estimated to be 0.054 with 95% CI of 0.029–0.077 (Table 4).

We document a high level of prevalence of Cry1Ac and Cry2Ab resistance alleles in the field population collected from Andhra Pradesh. There was a < 3% probability of missing a resistance allele in 66 lines screened for Cry1Ac and Cry2Ab. The experiment-wise detection probability was > 97%.

Discussion

In 2002, Bt-cotton producing Cry1Ac toxin (BG) was introduced to manage American bollworm (ABW), pink bollworm (PBW), and spotted bollworm (SBW). In 2010, Monsanto India limited ascertained the breakdown of BG resistance in the PBW population collected from four districts of Gujarat. In 2015, the Central Institute for Cotton Research (CICR) survey reported higher infestation and survival of PBW on BG-II cotton in populations collected from certain parts of Gujarat. The studies reported about 40–80% boll damage, and the results of resistance monitoring showed that PBW had developed resistance to Cry1Ac, Cry2Ab, and Cry1Ac + Cry2Ab toxins. In 2015–2016, similar reports of higher infestation and survival of PBW on BG-II cotton were reported from Andhra Pradesh, Telangana, Karnataka, and Maharashtra (Mohan, 2017). A field survey conducted in 2018 also reported very high flower and boll damage by PBW in Andhra Pradesh. The infestation levels of 29.62% on flowers and 83% boll damage were recorded on BG-II cotton (Naik et al., 2021; Raju et al., 2021). In our survey conducted during 2018–19 and 2019–2020, shows high survival

of PBW larvae on Bt-cotton squares and bolls. We found out that 98% PBW larvae survived on BG-II cotton collected from farmer's field. Thus, the survival of PBW on BG-II cotton in many parts of the country indicates the evolution of resistance to Cry1Ac and Cry2Ab expressed in BG-II cotton (Kranthi, 2016; Mohan, 2017). Resistance monitoring of Bt-cotton is considered to be an essential prerequisite for resistance management of target pests of Bt-cotton. Since the introduction of Bt-cotton, resistance to Cry toxins has been monitored in the target pests by CICR, Nagpur. However, none of the prior investigations attempted precisely estimate resistance allele frequency. In the present study, for the first time we attempted to estimate the frequency of Cry1Ac and Cry2Ab resistance alleles in PBW collected from various Bt-cotton growing regions of Andhra Pradesh. The present study results reveal a high frequency of Cry1Ac and Cry2Ab resistance alleles in the PBW population collected from various districts of Andhra Pradesh. The results show decreased pink bollworm susceptibility to Cry1Ac and Cry2Ab toxin expressed in BG-II cotton. We estimated the allele frequency to be 0.082 with a 95% CI of 0.051- 0.105 for Cry1Ac and the frequency of Cry2Ab resistance alleles to be 0.054 with a 95% CI of 0.029–0.077 based on the discriminating dose assay. On the Cry1Ac toxin diet assay, 46 lines survived the F_2 screen. Of those, 20 lines were reconfirmed as true positives in the F_3 generation. The remaining 26 lines that survived the F_2 screen were lost in the F_3 generation. Similarly, on the Cry2Ab toxin diet assay, 45 lines survived at F_2 generation, and only 13 lines were reconfirmed as true positives. The remaining 32 lines were lost in the F_3 generation. All the lines that survived the F_2 screen but could not reach the F_3 generation for reconfirmation may also carry resistance alleles for Cry1Ac and Cry2Ab toxins. Hence, we consider our estimate to be robust and conservative as we have considered only lines that were reconfirmed in the F_3 test in calculating allele frequency. However, considering the entire surviving lines in the F_2 screen as true positives, the estimated allele frequency will be 0.184 with a 95% CI of 0.1443–0.086 for Cry1Ac and 0.180 with a 95% CI of 0.1404–0.01954. Thus, warranting the evolution of resistance in the field population and indicating a decrease of pest susceptibility to Bt-cotton expressing Cry1Ac and Cry2Ab toxins. The results of the present study corroborate with field conditions, wherein a heavy incidence of PBW on Bt-cotton was noticed during the study period, a survey was conducted before conducting F_2 screen analysis. We recorded > 90% boll damage in the fields surveyed during 2018–19 and 2019–20. Our estimates also corroborate with reports of Kranthi, 2016; Mohan, 2017; Naik et al., 2021, wherein, field evolved resistance to Cry1Ac and Cry2Ab was reported. Naik et al. (2021) reported the highest magnitude of 371.8-fold resistance to Cry1Ac and 4214.3-fold resistance to Cry2Ab in the field population. The difference in allele frequency to Cry toxins might be due to their difference in duration of exposure, Bt-cotton expressing *cry1Ac* was introduced in 2002 while BG-II cotton expressing *cry1Ac* and *cry2Ab* was introduced later in 2006. Earlier introduction of Bt-cotton with a single toxin and large-scale adoption of Bt-cotton might have selected the population against Cry1Ac toxin. Further long-term exposure to Cry1Ac toxin might have resulted in higher allele frequency compared Cry2Ab. Moreover, the allele frequency to Cry toxins is independent of each other, as Cry1Ac and Cry2Ab resistant genes are independently segregated (Gould, 1998).

Table 4 Frequency of Cry1Ac and Cry2Ab resistance alleles after F_3 reconfirmation test

Toxin	Allele frequency	Lower CI	Upper CI	P_{No}
Cry1Ac	0.082	0.051	0.105	97.78%
Cry2Ab	0.054	0.029	0.077	97.78%

The evolution of resistance in target pests to insecticides and Cry toxins is inevitable. However, with the proper implementation of management strategies, the evolution of resistance in target pests can be delayed. Monitoring studies have established that the refuge strategy has helped to delay the evolution of resistance in target pests to Bt-cotton (Cerda & Wright, 2004; Gould, 1998). In many instances, field evolved resistance has been reported in many target pests of Bt-crops that have been grown without following proper insect resistance management strategies. The field evolved resistance to Bt crops has been reported in bollworm, *Helicoverpa zea* (Boddie) to Cry1Ac in cotton in the USA in 2003 (Luttrell et al., 2004), fall armyworm, *Spodoptera frugiperda* (Smith) to Cry1F in corn in Puerto Rico in 2006 (Matten et al., 2008),

stem borer *Busseloa fusca* (Fuller) to Cry1Ab in corn in South Africa in 2006 (van Rensburg, 2007). The two key conditions that govern the long-term effectiveness of Bt-crops are the availability of sufficient refuge and the expression of high dose of toxins in Bt-crops (Gryspeirt & Grégoire, 2012). Many reasons may be attributed to the failure of the Bt technology in India (Kranthi, 2016). The major factors that contributed to the failure of Bt-cotton are a) Non-compliance with refuge by farmers; almost all the farmers do not sow the non-Bt-cotton seeds provided with Bt-cotton seeds; b) large and unconventional areas were brought under Bt-cotton cultivation with scant regard to scientific practices, which might have hastened the process of selection. Since the introduction of Bt-cotton in India, the area under Bt-cotton has rapidly increased. Around 99% of the cotton area is under Bt-cotton, and around one percent is under other kinds of cotton (*G. arborium* and *G. barbadense*). In 2019, Bt-cotton was cultivated on 11 m ha. This large-scale cultivation of Bt-cotton might have exerted high selection pressure on target pests. c) The quality of seeds supplied with Bt-cotton seeds was not suitable as a refuge (Kranthi et al., 2017). d) The genetic analysis of resistance in PBW to Cry1Ac revealed that resistance is a recessive trait and a monogenic mode of inheritance. The progenies between the survivals of homozygous recessive individuals will yield maximum survival. e) Compared to the American bollworm, *H. armigera* is polyphagous in nature and has many other hosts that act as natural refuges. These natural refuges act as a pool reservoir for susceptible alleles and aid in diluting resistance alleles to the toxins of Bt-cotton. In the case of PBW, which is monophagous or feeds on limited hosts, entirely depends on the primary host (Cotton) for its growth and development. Thus, lack of refuge and large-scale cultivation of Bt-cotton expressing Cry toxins has triggered an arms race between the PBW and Bt-cotton, resulting in faster resistance evolution than other bollworms. f) In India, Bt-cotton is available as a hybrid with a variable expression of Cry toxins. The Cry toxins expression in Bt-cotton varies with age, plant parts, and season (Kranthi et al., 2005; Olsen et al., 2005). Thus, exposing the larvae to sublethal doses aids in the faster build-up of resistance. g) Other agronomic practices have also been selected for resistance to PBW, like the cultivation of Bt-cotton beyond its period, i.e., 180 days with extra irrigation, which favors the multiplication of PBW for the next season. Thus, other management options should be integrated instead of entirely relying on the refuge to manage pests effectively.

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Author contribution GMV & VCB – study conception and design of the research, ARA – implementation and data collection, KVS – data analysis and manuscript preparation. Ch. C, PAK, and VSR- reviewed the manuscript, GMV – reviewed and edited the manuscript. All the authors read and approved the manuscript.

Data Availability The data that support the findings of this study are available from the corresponding author, (GMVP), upon request.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval Not applicable.

Conflict of interests The authors declare that they have no conflict of interest.

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