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Segregation of Bt Genes in Cotton and Development of Pink Bollworm Resistance

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ABSTRACT

About 90% of India's total cotton cultivated area is occupied by hybrid Bt-cotton to utilize hybrid vigor. Hybrids with both *Cry1Ac* and *Cry2Ab* genes were found to be susceptible to pink bollworm (PBW) attack. Research was conducted to analyze the segregating pattern of these genes in selfed bolls of different generations with PBW damage. The average number of seeds per boll containing *Cry1Ac* in F1 was 14.8 ± 1.303 (2022) and 13.8 ± 2.16 (2023), and *Cry2Ab* gene in F1 was 12.4 ± 0.894 (2022) and 17.8 ± 1.64 (2023). In F2 single-boll selfed seeds, the number of seeds was reduced compared to F1 for *Cry1Ac* gene, whereas for *Cry2Ab*, the seed number was similar to F1. In advanced breeding lines, the number of *Cry1Ac*-positive seeds was high compared to F1 and F2. As for the segregating pattern for *Cry1Ac* and *Cry2Ab* genes in the F2 generation, an expected ratio of 3:1 was observed for two years. The highest field incidence of PBW infection was noted for the Bt variety Rajat Bt in 2022 and 2023. Results can vary based on the zygosity of the parents used in hybrid development. Research revealed that gene segregation lowers Bt toxicity and increases PBW infestation thereby decreasing the viability of Bt plants. Another way to overcome resistance is by stacking these *Cry* genes in a variety rather than a hybrid. As all the genes will be in homozygous condition, the toxicity produced will be higher and uniform throughout the entire population.

Genetically modified Bt cotton has been widely adopted by cotton farmers around the world due to its effectiveness in controlling bollworm pests and its potential environmental and economic benefits. In 2002, India approved the first genetically modified cotton for commercial cultivation (James, 2003). The first transgenic cotton was developed using cotton cultivar Coker 312 through *Agrobacterium tumefaciens* (Smith and Townsend) Conn transformation with *Cry1Ac* and named event Mon531, which has been bred with many different cotton varieties and successfully introduced for commercial cultivation around the world. Later, event Mon531 was retransformed using particle bombardment technology with a gel-purified linear DNA fragment that contained *Cry2Ab* and β-glucuronidase (*uidA*) coding regions (Huber et al., 2002; John, 1997). The insertion of the cassette containing *Cry2Ab* and *uidA* coding sequences into cotton event Mon531 gave rise to event Mon15985 (BG-II), which comprises both the *Cry1Ac* coding sequence as well as the cassette encoding the *Cry2Ab* coding sequence (Huber et al., 2011).

In India, pink bollworms [*Pectinophora gossypiella* (Saunders)] were successfully controlled by Bt cotton hybrids until 2008. The first reports of resistance to pink bollworm infections were made in 2009 in Gujarat state. *Cry2Ab* resistance also surfaced (Naik et al., 2018). Gene pyramiding, selection pressure, inadequate refuge compliance, cross-resistance, genetic diversity, and other characteristics were some of the causes that led to the development of resistance. Throughout India's cotton-growing regions, resistance quickly expanded. States that produce a lot of cotton, such Maharashtra, Telangana, and Andhra Pradesh, saw widespread resistance by 2017–2018. In India, 90% of the total cotton cultivated area is planted in Bt hybrids. The F1 hybrid plants segregate seeds in their bolls, which accelerated the development of resistance. According to Kranthi (2012), F1 plants that bear F1 bolls have seeds that separate into a 9:3:3:1 ratio: *Cry1Ac* + *Cry2Ab* in 9, *Cry2Ab* in 3, *Cry1Ac* in 3, and no Bt gene in 1. A single boll thus contains a range of seeds: seed containing no Bt, seeds containing *Cry1Ac* alone, seeds contain-

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ing *Cry2Ab* alone, and seeds containing *Cry1Ac* + *Cry2Ab* together. Because of selection for resistance to specific poisons, this environment is perfect for the development of resistance (Kranthi, 2015). Cotton hybrid seeds in India are produced through controlled cross-pollination of two distinct parental lines. The selection of parental lines is often based on their heterotic value. These lines are carefully selected based on their genetic characteristics, especially for Bt traits as well as traits for yield potential, disease resistance, fiber quality, and adaptability to local growing conditions.

In BG II cotton hybrids, the *Cry1Ac* and *Cry2Ab* genes typically behave as dominant traits under hemizygous conditions: if the hybrid plant has only one copy of the Bt gene (hemizygous), it can still express the Bt toxin and exhibit resistance to targeted pests. Importantly, the segregation of *Cry1Ac* and *Cry2Ab* genes is independent of each other. This implies that the segregation pattern of one Bt gene is unaffected by the presence or absence of the other Bt gene. As a result, offspring segregate each gene individually in a 3:1 ratio, guaranteeing the genetic features linked to *Cry1Ac* and *Cry2Ab* are inherited independently.

In Bt cotton, the *Cry1Ac* and *Cry2Ab* genes normally are found on separate chromosomes, thus show independent assortment during genetic inheritance. When two genes are located on separate chromosomes, the distribution of features among the progeny is described by the segregation of alleles in the ratio 9:3:3:1, but genetically, it is 1:2:1:2:4:2:1:2:1, giving rise to nine distinct types of segregants representing the mix of recessive and dominant alleles at both loci. After hybrid plants selfed in the second generation, three out of 16 plants expressed *Cry2Ab* alone, lacking the *Cry1Ac* gene (Edpuganti, 2018).

Heuberger et al. (2008) reported segregation of *Cry1Ac* and *Cry2Ab* in a mixture of Bt and non-Bt seeds in every boll of the Bt plants. Their model had a small proportion of bolls on non-Bt refugia plants that were contaminated by Bt pollen. In India, 12.9 million hectares are planted with Bt cotton hybrids potentially segregating to both genes, and there is gross non-compliance of the refugia strategy. In the present investigation, segregation pattern of two genes, *Cry1Ac* and *Cry2Ab*, in Bt cotton varieties/hybrids were studied. These genes are located on the same chromosome but are relatively far apart; thus they can assort independently due to genetic recombination or crossing over that occurs during meiosis.

MATERIALS AND METHODS

Plant Materials. Four different cotton types: a variety, hybrid (F1), F2, and advanced breeding line (F6-F8), were selected from the Central Institute for Cotton Research (CICR) field based on combinations of *Cry* proteins. The genotypes were Rajat-Bt (*Cry1Ac*), F1 hybrid developed at CICR (*Cry1Ac* and *Cry2Ab*), F2 (*Cry1Ac* and *Cry2Ab*) segregating population, and advanced breeding line (*Cry1Ac* and *Cry2Ab*). The four different types were grown in the main field of the Indian Council of Agricultural Research (ICAR) CICR, Nagpur, India during two years: 2021-2022 and 2022-2023. Leaf tissue samples were taken from each plant 30d after the seeds germinated, and each plant was separately subjected to enzyme-linked immunosorbent assays (ELISA) to measure the expression of the two Bt toxins. Only toxin-positive plants were considered further for the study. Observations were made for five selfed bolls from each plant and five plants from each generation were used to minimize the error. One day before flower opening, 10 flowers on each plant were bagged to prevent cross-pollination. A week later, the bags were removed and the developing squares labelled. When completely opened, these selfed bolls were chosen for additional research. All seeds from four locules of each boll were analyzed for *Cry1Ac* and *Cry2Ab* protein expression through ELISA test. The suggested fertilizer dose and plant protection measures were followed for proper crop management. Open bolls damaged by pink bollworms were collected from all plants in the field after 150 d. The damaged and undamaged bolls were recorded separately, from which the percentage of open boll damage was calculated to assess pink bollworm damage.

Enzyme-Linked Immunosorbent Assays (*ELISA*). The expression of *Cry1Ac* and *Cry2Ab* genes in the segregating population was studied using ELISA, which provides valuable insights into the presence and levels of Bt proteins in seeds. Tissue samples (seeds) from segregating populations of Bt cotton plants were collected. The samples were prepared by extracting proteins from the tissue as per protocol (Agrisure ELISA Kit, Maharashtra, India) to preserve the integrity of the *Cry* proteins. Antibodies specific to *Cry1Ac* and *Cry2Ab* proteins were immobilized on a microplate. The extracted protein sample (from cotton seeds) was added to the microplate wells. The samples containing *Cry1Ac* or *Cry2Ab* proteins were

bound to the immobilized antibodies. After washing away unbound proteins, a secondary antibody linked to an enzyme (e.g., horseradish peroxidase) was added. This enzyme catalyzes a color change reaction. The intensity of the color change was proportional to the amount of *Cry* protein present in the sample. The development of color was read in the ELISA Reader (BioTek, USA) at 450nm, and the presence or absence of the color indicated the expression of *Cry1Ac* and *Cry2Ab* proteins.

Statistical Analysis. According to the Mendelian Law of Independent Assortment, the two *Cry* genes segregate independently. Chi-square test was used to compare Mendelian monohybrid ratios with the segregation pattern of seeds from each hybrid, that is, positive for both *Cry1Ac* and *Cry2Ab* genes, positive only for *Cry1Ac*, positive only for *Cry2Ab*, and negative for both genes. The hybrid (F1) percentage of seeds that represented various gene combinations was calculated, and the coefficient of variance (CV %) for observed differences between sampled bolls for each combination of the *Cry* genes was computed. The percentage of seeds that represented various gene combinations within the bolls for the hybrids and the segregating lines were studied during both years for the two genes separately.

RESULTS

The analysis of variance determined a significance difference among the lines for *Cry* gene expression (Table 1). The average number of seeds per boll containing *Cry1Ac* gene in F1 was 14.8 ± 1.303 in 2022 and 13.8 ± 2.16 in 2023. The number of seeds containing *Cry2Ab* was 12.4 ± 0.894 in 2022 and 17.8 ± 1.64 in 2023. In F2 single-boll selfed seeds, the *Cry1Ac* seed numbers were reduced compared to F1, whereas for *Cry2Ab*, the average numbers were similar to F1. In advanced breeding lines, the number of *Cry1Ac*-positive seeds was high compared to F1 and F2. The CV was high in the F2 generation for both genes compared to F1 in 2022. In 2023, the CV was 10.98% for *Cry1Ac* and 9.23% for *Cry2Ab* (Table 2). For both *Cry1Ac* and *Cry2Ab* genes, an expected ratio of 3:1 was observed for the two years (Fig. 1).

According to data shown in Table 2, the highest incidence of pink bollworm infection was noted in

Table 1. Analysis of variance for the *Cry* gene expression

S.No.	Year	Genes	Mean sum of square		
			Replication	Treatment[z]	Error
1	2022	*Cry1Ac*	3.575	20.316*	3.441
2		*Cry2Ab*	2.825	205.733**	2.358
3	2023	*Cry1Ac*	0.925	32.266**	3.725
4		*Cry2Ab*	5.325	371.383**	1.591

[z]*significant at 5%; **significant at 1%

Table 2. Expression of *Cry* genes in seeds produced by variety, F1, F2 and advanced breeding lines

	2022					2023				
	Average number of seeds with *Cry1Ac* per boll		Average number of seeds with *Cry2Ab* per boll		Open Boll Damage/ Plant	Average number of seeds with *Cry1Ac* per boll		Average number of seeds with *Cry2Ab* per boll		Open Boll Damage/ Plant
Genotypes	Mean ± sd	CV	Mean ± sd	CV	Mean	Mean ± sd	CV	Mean ± sd	CV	Mean
Rajat-Bt	17.4±1.51	8.715	*[z]	*	17±1.14	18.2±1.788	9.82	*	*	22±1.31
F1 Hybrid Selfed Seeds	14.8±1.303	8.809	12.4±0.894	7.213	9±1.58	13.8±2.16	15.709	17.8±1.64	9.231	14±1.01
F2 Single Boll Selfed Seeds	13.2±2.58	19.60	13.4±1.516	11.317	13±2.01	12.2±1.303	10.98	17.8±1.64	9.231	17±1.47
Advanced Breeding Lines	17.2±1.787	10.40	12.98±2.47	19.49	11±1.87	15 ± 1.58	10.54	15.8±2.167	13.72	15±0.98

[z*]Rajat-Bt does not have *Cry2Ab* gene

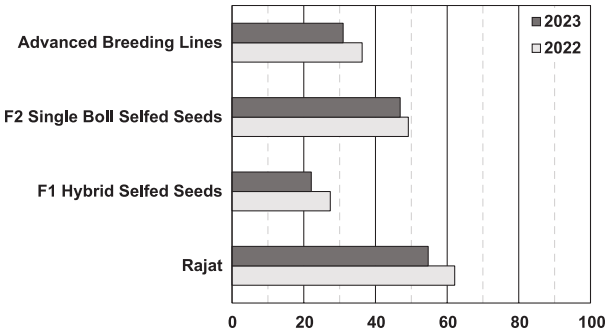

Figure 1. Percentage of boll damage in population caused by pink bollworm (PBW) during 2022 and 2023.

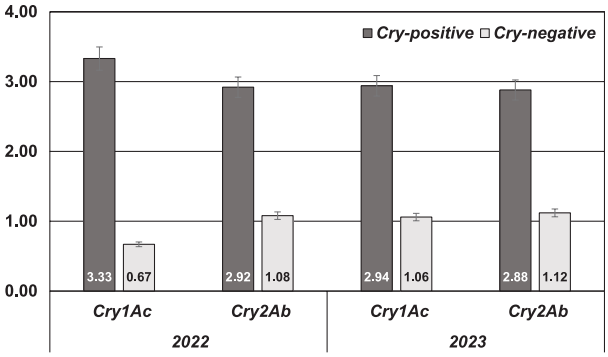

Figure 2. Segregating ratio of *Cry1Ac* and *Cry2Ab* genes in F2 seeds during 2022 and 2023.

the field for Bt variety Rajat-Bt in 2022 and 2023, followed by F2 hybrid selfing. The results also revealed that selfed F1 hybrids showed the least amount of damage. But in every instance, maximum damage was seen in a single locule of each boll, followed by damages to two and three locules. Damage to all four locules found the least. Overall boll and locule damage due to pink bollworm infestation was less in 2022 than 2023 (Fig. 1).

The segregation patterns of seeds for F1 selfed seeds/F2 lines followed the Mendelian monohybrid ratio for *Cry1Ac* and *Cry2Ab* (Table 3, Fig. 2). In 2022, out of 89 seeds, 74 were positive and 15 were negative for *Cry1Ac*, and among 85 seeds, 62 were positive and 23 were negative for the *Cry2Ab* gene. During 2023, 69 seeds had *Cry1Ac* gene out of 94 seeds, whereas for *Cry2Ab*, 64 seeds were positive among 89 seeds studied. The chi-square test showed no significant differences between the observed 3:1 ratios for the two genes during two years. The chi-square value for *Cry1Ac* was 3.149 and 0.127 for 2022 and 2023, respectively and 0.192 and 0.453, respectively for *Cry2Ab* (Table 3).

DISCUSSION

A variety of Bt genes, each with a unique mode of action, have been inserted into different crops, including cotton, to extend and boost the effectiveness of a single Bt toxin. Combining the *Cry2Ab* and *Cry1Ac* genes has been shown to have synergistic effects against cotton bollworms, *Helicoverpa armigera* Hübner, and to delay the development of resistance in target insects (Wei et al., 2015). Nevertheless, the commercially produced transgenic cotton events offered by multinational corporations combine two different events into a single cotton cultivar. Even with the stacking of two genes, pink bollworm has developed resistance to Bt cultivars. The toxicity expression level *Cry* protein concentrations were found to be highest at 100 d after sowing in research by Likhitha et al. (2023), then at 75, 125, and 40 d after sowing. According to a study conducted by Mahon et al. (2004), the expression of *Cry2Ab* in Bollgard-II was highest in squares, providing strong support for the varying expression of *Cry* genes for both one and two genes in a pyramid arrangement. Boll-wise segregation of both the *Cry* genes combined in the seeds of Bt cotton hybrids and the segregation patterns of seeds in hybrids followed the Mendelian dihybrid that was reported by Mahesh and Muralimohan (2023).

In the present study, no seasonal variation was observed across both years i.e. the average number of seeds showing positive to both the *Cry* genes. This is because both genes are qualitative and do not interact with the environment to create variation. The Rajat-Bt variety, which was developed

Table 3. Segregation pattern of *Cry* genes in seeds produced by F1

Year	Genes	No of Seeds Tested	Positive	Negative	Ratio	Chi-square	*p*-value
2022	*Cry 1Ac*	89	74	15	3:1	3.149	3.84
	Cry 2Ab	85	62	23	3:1	0.192	
2023	*Cry 1Ac*	94	69	25	3:1	0.127	
	Cry 2Ab	89	64	25	3:1	0.453	

and released by CICR in 2020, carries a single-gene *Cry1Ac* and fortunately it, still, can control the cotton bollworm. Rajat-Bt is homozygous for *Cry1Ac* and has no *Cry2Ab* gene. Thus, all seeds from each locule of individual boll were positive for *Cry1Ac* toxin during both seasons. For the hybrid F1, the 3:1 ratio was found to be stable across the years with a slight change in the number of seeds expressing the *Cry* proteins. Considering parental status, hybrids can be homozygous or hemizygous for one or both *Cry* genes (Fig. 3).

The hybrids exhibit zygosity in three different conditions: (1) both parents have the *Cry* genes in homozygous condition, (2) only one parent has the *Cry* genes, and (3) both parents are heterozygous for the *Cry* genes. In the first condition, the hybrids will be homozygous for the *Cry* genes and the seeds will be positive for the toxic protein. Whereas for the second and third conditions the F1 hybrid will be hemizygous/heterozygous for the gene, and the seeds are expected to segregate in the Mendelian mono- and dihybrid ratio. In the third condition where both parents are heterozygous, the F1 hybrids will segregate for the *Cry* genes i.e. the hybrid will have the *Cry* gene expression in 9:3:3:1 where 9 parts of the plant population will be positive for both the genes, 3 each positive of either of the *Cry* genes and 1 part will be negative for both the *Cry* genes. In the third condition, there is an advantage considering refugia, the hybrids segregate such that one-fourth of the population will be negative for the *Cry* gene when considering a single gene, and one-sixteen of the population will be negative for *Cry* toxins when two genes are taken into account. A similar pattern is observed for the two genes *Cry1Ac* and *Cry2Ab* (Fig. 4). When two genes are considered the segregation pattern also varies. Most commercially available hybrids have genes either in homozygous-homogeneous or hemizygous-homogenous condition. Whereas in the public sector all hybrids mentioned in Figs. 1 and 2 are usually found.

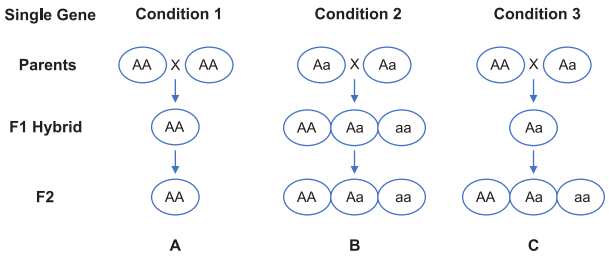

Figure 3. The zygosity of the single *Cry* genes is shown diagrammatically in the parental lines (Parents), Bt cotton hybrid F1 plant, and seeds found in the hybrid plants' bolls (F1 boll). A. Represents both parents in homozygous condition for a single gene, therefore F1 and F1-selfed seeds are homozygous naturally. B. When both parents are hemizygous for single gene, F1 will segregate for positive and negative plants in a 3:1 ratio in F1, and all possible combinations occur in F1 selfed without selection. C. When one parent alone has the gene in homozygous condition F1 will be hemizygous for the gene still expressing the toxicity and F2 will segregate in a 3:1 ratio.

The overall efficacy of Bt cotton hybrids against bollworms was shown by exhibiting far less locule damage than their counterparts (Vennila et al., 2004). Compared to non-Bt hybrids, Bt hybrids exhibited substantially less locule damage, which varied from 17.83 to 25.4% (Sharma et al., 2001). Pink bollworm infestation was lowest in timely-sown (June 25) arboreum variety plants, whereas the greatest infestation was noted at harvest in early-planted (June 5); non-Bt hirsutum hybrid plants (Ingole et al., 2019). The present investigation found that a single locule had the highest level of pink bollworm infestation, followed by two and three locules. Four locules had the least damage in 2022 and the most damage in 2023.

In the F2 population, the selfed seeds were found to have all the *Cry* genes in combination. Most of the seeds showed *Cry* gene expression for either one of the genes. In this population as there is no selection pressure operating, Hardy Weinberg equilibrium is achieved, and all possible combinations of genes are expressed. In this F2 population, bollworms were found on plants negative for both *Cry* genes. Similarly, in the advanced breeding lines, the seeds with toxic protein expression were in a higher number when compared to the seeds of negative plants. This is due to selection pressure, where plants were selected for both genes throughout the generation advancement. Following selection of the heterozygote plants, the remaining plants will express the protein uniformly, whereas the segregating plants compete for the genes (Figs. 1 and 2). The findings imply that segregation can increase variance and decrease mean boll toxicity. Reduced mean toxicity is predicted to boost individual pink bollworm survival, but higher variance permits the pink bollworm population to consume bolls with a variety of Bt toxicities (Mahesh and Muralimohan, 2023). The ability of several Bt hybrids planted across a large geographic area to survive longer with a variety of toxicities could enable the selection of several resistance-conferring alleles in pink bollworm field populations. The development of resistance in a variety of pest species

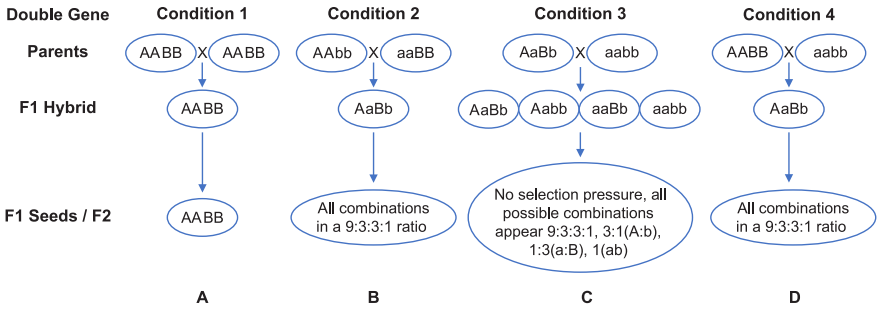

Figure 4. The zygosity of the single *Cry* genes is shown diagrammatically in the parental lines (Parents), Bt cotton hybrid F1 plant, and seeds found in the hybrid plants' bolls (F1 boll). A. Both parents have two dominant genes in homozygous condition, B. Both parents have one dominant gene in homozygous condition. C. One parent expresses *Cry* genes in hemizygous condition and absent in other parent. D. One parent has both the genes in dominant homozygous and other parent is homozygous recessive.

can be accelerated by feeding on both Bt and non-Bt plant tissues (Brévault et al., 2015).

CONCLUSIONS

According to this study, segregation has the potential to decrease Bt toxicity and enhance the survival rate of pink bollworm on Bt plants. Other bollworm species might not have this opportunity, particularly *H. armigera*, which feeds on tissues from the F1 generation, such as the rind and lint of bolls. Here inclusion of refugia becomes crucial for controlling bollworms. As more individuals survive on Bt plants, refugia might not be able to control the development of resistance in pink bollworms. We suggest that the parental lines involved in developing hybrids should be homozygous for insect-resistant transgenes. For resistance management measures like the refugia to be successful, segregation might be impossible to avoid in any other way. Another way to overcome the problem is to stack these *Cry* genes in a variety rather than a hybrid. As all the genes will be homozygous, the toxicity produced will be higher and uniform throughout the entire population. Along with Bt variety, the addition of refugia becomes essential to overcome resistance against *Cry* genes developing in other bollworms species.

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