Occurrence of molecularly diverse *Bt* Cry toxin-resistant mutations in insect pests of Bt^+ corn and cotton crops and remedial approaches

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Cultivation of Bt^+ genotypes has dispensed with insecticidal sprays and thereby corn and cotton farmers have hugely benefited worldwide. Recent recordings of genetically diverse Cry-resistance in insect pests of Bt^+ corn and cotton fields have raised grave concern. Curiously, bulk of Cry-resistant pink bollworms found in certain Bt^+ cotton fields in India proved homozygous for multiple linked mutations. Besides, dominantly inheritable Cry-resistance and cross resistance between different Cry-proteins have also been noted. To stem evolution of resistance against anti-insect protein-toxins, new nematology research on IPM procedures, complementary to refuge and Cry stacking technologies is imminently needed.

Keywords: *Bt* corn and cotton, complementary pest-management practices, cry-resistant variants, insect pests.

Cry-protein variation and its insect killing mechanism(s)

CRY toxins, produced in the spores of soil bacteria *Bacillus thuringiensis* (*Bt*), are a large family of Cry1 to Cry 70 well-characterized, crystal-forming, water-soluble, delta-endotoxin, three-domained globular insecticidal proteins (Figure 1)[1,2]. Independent evolution of their three domains and swapping of domain III between different Cry toxins have been responsible for the variety in their specificities[3]. Besides, a series of CryMod mutant toxins have been developed that overcome resistance in insects to conventional Cry toxins[4,5]. Cry proteins have been observed to kill the larval stages of a narrow range of insect species, such as of the order Lepidoptera (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Ab), Coleoptera (Cry3Aa, Cry3Bb, Cry8Ea) and Diptera (Cry4Aa, Cry4Ba), by disruption of their midgut, the details of which are beginning to be understood (Figure 2)[6,7]. Cry toxin binds to its receptors on the epithelial cells of midgut which includes cadherin(s), aminopeptidase(s), alkaline phosphatase(s) and glycolipids, and thereby two modes of killing action get initiated. Mode 1 culminates in the osmotic lysis of cells via formation of transmembrane pores[8,9]. In mode 2, cells die apoptotically, following stimulation of G protein, increase in synthesis of cAMP and functionalization of the protein kinase A effector-mediated apoptotic metabolism[10]. Cry proteins, on account of specificity in their insecticidal activity, serve as an important genetic tool for the management of important agricultural pests.

Cry protein(s) in the control of corn and cotton insect pests

Several of the *Cry* genes have been transferred into certain industrial and food crops, individually and in combination, to construct Bt^+ varieties that produce Cry toxin(s) constitutively and are therefore resistant to the major chewing and hole-making insect pests of each crop, in the respective areas of their cultivation. Commercially utilizable Bt^+ transgene varieties have been developed in broccoli, corn (maize), cotton, egg plant (brinjal), potato, soybean and tomato. Among these, Bt^+ corn and Bt^+ cotton have been adopted for cultivation in many countries. Bt^+ crops are grown in more than 76 m ha worldwide[11]. India grows Bt^+ cotton in 10.4 m ha, which was 98% of all the cotton grown in the country in 2013 (ref. 12). Bt^+ crops have benefited farmers from diverse geographical areas in reducing their dependence on the use of chemical insecticides. Bt^+ cotton began to be grown in USA

Figure 1. (Left) Pink bollworm (*Pectinophora gossypiella*) larva and (right) a cotton boll spoiled by infestation of pink bollworm.

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and India in 1996 and 2002 respectively. In the Bt^+ cotton varieties cultivated in the two countries, *Cry1Ac* gene had been used to impart resistance against the major pests, *Pectinophora gossypiella* and *Helicoverpa armigera*. While *Cry1Ac* Bt^+ cotton continues to be cultivated in USA[13], in India development of large-scale resistance against *Cry1Ac* in the pests within six years necessitated replacement of *Cry1Ac* Bt^+ cotton genotypes by those possessing two *Cry* genes, namely *Cry1Ac* + *Cry2Ab* (ref. 14).

Use of refuge for enduring recessive Cry-resistance mutations

Introduced for cultivation in USA in 1996, Bt^+ varieties of corn and cotton kept the populations of their respective pests (pests for corn = *Ostrinia nubilasis* (European corn) borer and *Diabrotica virgifera* (western corn rootworm); pests for cotton = *Helicoverpa armigera* (cotton bollworm) and *Pectinophora gossypiella* (pink bollworm)) susceptible to Cry toxin for more than a decade and a half. This was enabled by compliance to the refuge/high dosage-based pest resistance management procedure. Before the release of Bt^+ crops, experimentation on the laboratory strains of pests had confirmed the expectation that pests will evolve resistant mutations against the Cry toxins. In one such study, four Cry1Ac-resistant mutations recovered in *P. gossypiella* were found to be recessive and all of them mapped in the *PgCad* (*cadherin*) gene[15,16]. Based on the then known model of killing action of Cry on insect larvae[17], it was presumed that Cry resistance will be due to mutations in gene(s) for Cry receptor proteins in the midgut of insects and will be recessive. To check increase in the population of homozygous recessive Cry-resistant insects in the Bt^+ fields, co-cultivation of Bt^- pest-susceptible variety in ≥ 20% of area as refuge, alongside the Bt^+ pest-resistant main crop, was proposed[18]. It was thought that the refuge will make available a population of susceptible insects, which upon mating with the homozygous recessive resistant insects will produce heterozygous progeny that will be susceptible to the Cry toxin. Further, use of high Cry dose Bt^+ varieties, which accumulated Cry toxin with 25 times higher concentration over the dose that killed 99% of the Cry susceptible insects, ensured that the resistance development in pests was rare[19]. Evolution of Cry resistance in pests due to recessive mutations, in the Bt^+ crop fields, has been mostly due to non-use of sufficient refuge area and/or high-dose Bt^+ varieties. Indian farmers abstained from using refuge because of their individually small land holdings. Recently observed instances of Cry-resistance suggest that refuge technology is an insufficient measure to counter Bt^+ resistance development in the field populations of insect pests.

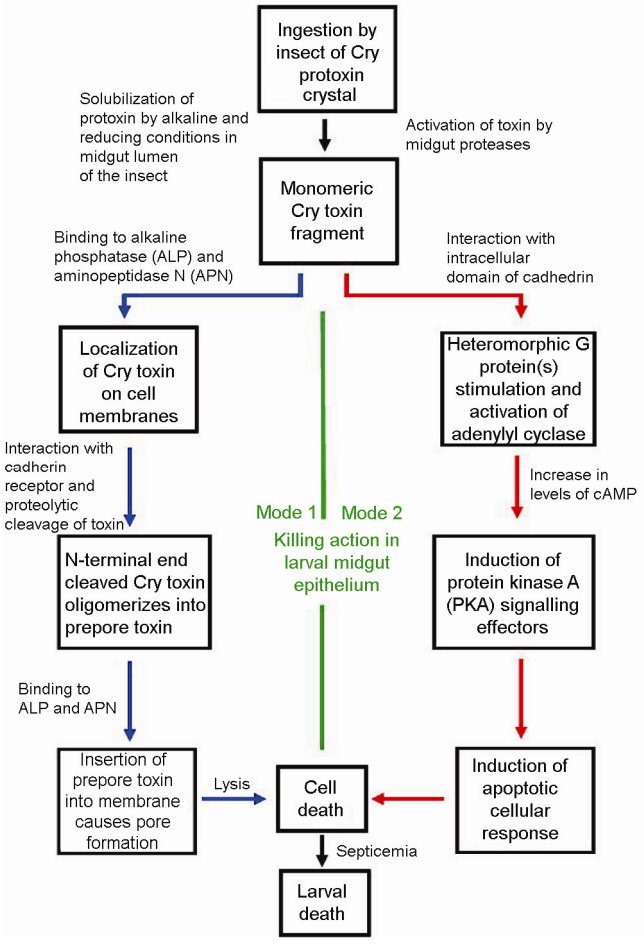

Figure 2. The scheme of the killing action of Cry toxin, via the complementary modes 1 and 2, on the larvae of lepidopteran insects.

Cry-resistance mutations in insect pests

Table 1 lists genetic alterations in the mutants selected against a variety of individual Cry toxins, in laboratory populations of several insect species. It may be noted that midguts of larvae of Cry-resistant mutants are able to tolerate Cry toxins in high concentrations because mutations in them have: (a) decreased the quantities of proteases or increased the quantities of glycolipids and esterases such that the availability of free and active toxin for its killing action is non-optimal; (b) down-regulated the expression of Cry toxin receptors, including aminopeptidase(s), alkaline phosphatase(s) and cadherin-like proteins, via interference with the gene network that controls the expression of Cry receptors; (c) abolished the synthesis of active Cry receptors such as those mentioned above and ATP-binding cassette transporter C2, via mutations in the body of the genes specifying them; or (d) led to non-availability of products of certain as yet uncharacterized genes essential for killing action of Cry on insects. Only some of these genetic deficiencies have been observed to be correlated with Cry resistance that has been noted in the pest

Table 1. Properties of the laboratory selected mutations in model insects that impart resistance to *Bt* Cry toxin(s)

Bt Cry toxin	Pest/insect	Model plant host(s) for the pest	Nature of mutation(s) that impart toxin resistance in the pest	Reference
Cry1Ac	*Plodia interpunctella*	Stored grains	Reduction in trypsin-like gut protease activity[a]	57
Cry1Ac	*Heliothis virescens*	Tobacco, cotton	Recessive retrotransposon insertional mutation in a gene for a cadherin-like protein (*HevCaLP*) that prevents synthesis of full length cadherin	27
Cry1Ac	*Helicoverpa armigera*	Cotton	Autosomal mutation that causes up-regulation of esterase expression leading to esterase sequestration of toxin[b]	58
Cry1Ac	*Helicoverpa armigera*	Cotton	Dominant mutation in an unknown autosomal gene	59
Cry1Ca	*Spodoptera exigua*	Cotton	Non-expression of gene(s) for aminopeptidase N1 (*APN1*), on account of mutation in unknown gene(s)	60
Cry2Ab	*Pectinophora gossypiella*	Cotton	Recessive mutation in an unknown gene that also gave cross resistance against Cry1Ac	25
Cry1Ac	*Helicoverpa armigera*	Cotton	Recessive deletion mutation in the *APN1*	61
Cry1Ac and *Cry1Ab*	*Heliothis virescens*	Cotton	Recessive deletion mutation in the gene for ATP-binding cassette transporter (ABC transporter) sub-family C, member 2 (*ABCC 2*)	62
As above	*Plutella xylostella*	Crucifers	As above, but independent	63
As above	*Trichoplusia ni*	Cabbage	As above, but independent	63
As above	*Pectinophora gossypiella*	Cotton	Recessive insertion of the retrotransposon *CRI* in the *PgCad* gene	20
Cry1Ac	*Heliothis virescens*	Cotton	Unknown mutation downregulates the gene for alkaline phosphatase (*HvmALP*)[c]	64
Cry1Ab	*Ostrinia nubilalis*	Corn	Two different and independent base substitution mutations in gene for amino peptidase-P like gene (*OnAPP*)	65
Cry1Ac	*Trichoplusia ni*	Cabbage	Unknown mutation downregulates APN1	66, 67
Cry1Ab	*Diatraea saccharalis*	Sugarcane and rice	RNAi mediated low expression of Cad gene(s)	68
Cry1Ab	*Bombyx mori*	Mulberry	Recessive base substitution mutation in the *ABCC2* gene	69
Cry1Ac	*Pectinophora gossypiella*	Cotton	Four independent mutations in the *PgCad* gene	16
Cry1Fa	*Plutella xylostella*	Crucifers	Recessive mutation in the gene *ABCC2*	70
Cry1Ab	*Ostrinia nubilalis*	Corn	Mutation(s) in unknown gene(s) that reduce expression of the genes for aminopeptidase 1 and 3	71
Cry	*Bombyx mori*	Mulberry	Mutation in the gene *ABCC2*	72

[a]Cry resistant strains of *Ostrinia nubilalis* were observed to possess less active serine proteinases by Li *et al.*[73]; [b]Ma *et al.*[34] have reported similar sequestration of Cry1Ac and Cry2Ab toxins by lipophorin glycolipids leading to toxin resistance in *Helicoverpa armigera*; [c]*Helicoverpa armigera* and *Spodoptera frugiperda* Cry resistant insects also carry this mutational defect.

populations of Bt^+ corn and Bt^+ cotton crop fields. Among the 19 reports about the evolution of field genetic resistance to Cry in insect pests listed in Table 2, in 14 of them *Bt* resistance had developed due to mutation(s) in the Cry-receptor *Cadherin* gene(s), while in the remaining the mutated gene(s) need identification. Together, Tables 1 and 2 summarize the properties of 56 Cry-resistant mutant alleles in insects. Several lessons and questions delineated by the available information on these 56 Cry-resistance events are discussed below.

Characteristics of Cry-resistance in Bt^+ crop fields

The technology of crop protection against pests by the use of Bt^+ crop varieties needs modification in terms of the following newly discovered properties about mutations that impart resistance to Cry toxins in insects. (a) There are two complementary pathways of killing action of Cry on the larvae of susceptible insects, the pore forming and apoptosis signalling pathways, each specified by a number of genes. This suggests that Cry resistance will often emerge as a polygenic trait. Some instances of involvement of mutations in more than one gene in Cry resistance development have been reported[10,15,20–23]. In studies such as that of Fabrick *et al.*[14], where *PgCad* mutations have been revealed by sequencing of *Cad* gene, detection of any co-mutation(s) on other genes will require comparative sequence analysis of the entire genomes of the representative resistant and susceptible members of the pest population. The assumption that mutation(s) in the *Cadherin* gene are mostly responsible for *Cry* resistance evolution in insects such as the cotton bollworm and pink bollworm is no longer valid. (b) Presence of combinations of point, deletion and insertion mutations in the *Cad* gene of Cry-resistant insects of

Table 2. Properties of the mutations responsible for the acquirement of field resistance against Cry toxins in pest populations infesting Bt^+ corn and Bt^+ cotton crops in North America, South Africa, China and India

Crop	*Bt* transgene	Pest	Nature of *Bt* resistance imparting mutation in the pest population(s)	Reference
Corn (maize)	Cry1Ab	*Busseola fusea* (Stem borer)	Dominantly inherited mutation in an unknown gene	32, 75
As above	Cry3Bb1	*Diabrotica virgifera* (Western corn root worm)	Dominant mutation in an unknown gene[a]	33, 76
As above	Cry1Ab	*Ostrinia nubilalis* (European corn borer)	Recessive point and deletion mutations in the extracellular domain of the *OnCad* (*Cadherin*) gene together impart *Bt* resistance	24
As above	Cry1F	*Spodoptera frugiperda* (Fall armyworm)	Autosomal recessive mutation in unknown gene(s)	77
Cotton	Cry1Ac	*Heliothis virescens* (Tobacco budworm)	Recessive LTR-type retrotransposon insertion mutation in *HvCad* gene	28
As above	As above	*Helicoverpa armigera* (Cotton bollworm)	Premature stop codon mutation in the extracellular domain of *HaCad* gene	78
As above	Cry2Ab	As above	Autosomal recessive mutation in unknown gene(s)	79
As above	Cry1Ac	As above	Recessive deletion mutation of exons 8 to 25 in the *HaCad* gene	80
As above	As above	As above	Insertion of LTR of the retrotransposon *HaRT1* and independent insertion of the whole *HaRT1* inherited recessively, both in the exon 8 of *HaCad* gene	29
As above	As above	As above	Dominant mutation in an *HaCad* gene	34
As above	As above	As above	A mis-sense recessive mutation in the extracellular domain of the *HaCad* gene; an independent dominant mutation in an unknown gene	21
As above	As above	As above	A dominant mutation in an unknown gene; and an independent dominant mutation in the *HaCad* gene	35
As above	As above	As above	A dominant deletion mutation in the intracellular domain of the *HaCad* gene	10
As above	As above	As above	Two independent dominant mutations which demonstrate cross resistance to Cry2Ab	26
As above	As above	As above	A complex recessive insertion-cum-deletion-cum base substitution mutation in the *HaCad* gene	30, 81
As above	As above	*Pectinophora gossypiella* (Pink bollworm)	Three independent recessive mutations in the extracellular domain of *PgCad* gene (r^1 = 24 bp deletion; r^2 = 202 bp deletion creating a stop codon; r^3 = 126 bp deletion and an insertion of a retrotransposon); combination of two mutations imparts resistance	15, 20
As above	As above	As above	Two independent recessive mutations in the extracellular domains of *PgCad* gene	82
As above	As above	As above	Recessive mutations in more than one autosomal gene governs toxin resistance	22, 23
As above	As above	As above	Eight different recessive alleles in *PgCad* gene, each comprising one or more base substitution, deletion and/or transposon-like insertional mutations, which altogether produce 19 different kinds of *Cad* transcripts	14

[a]Mutations originally called as non-recessive, incompletely recessive or semi-dominant have been treated as dominant here.

different species[14,24], indicates that the closely linked mutations co-occurred. (c) On the one hand, a recessive mutation in an unknown gene that got selected for providing resistance to Cry2Ab in a laboratory population of *Pectinophora gossypiella*, demonstrated cross-resistance against *Cry1Ac*[25]. On the other hand, different dominant mutations, one in each of two field populations of *Helicoverpa armigera* that got selected in Bt^+ cotton fields for resistance against Cry1Ac, also imparted cross-resistance against Cry2Ab[26]. These observations indicate that there are some step(s) in the pathways of killing action of Cry proteins against insects that are not Cry toxin-specific. (d) Both in the laboratory-selected Cry-resistant insects and *Bt*-resistant insects that got selected in Bt^+ cotton and Bt^+ maize field environments, there are instances of Cry resistance caused by insertion element/transposon/retrotransposon insertions[14,20,27–30]. Movements of transposons are known to be indicative of loss of their silence and presence of stress that induces their transpositions[31]. (e) Dominant mutations have been observed to be responsible for the evolution of Cry resistance in field populations of several insect species[10,26,32–35]. Some of the mutations reported to be dominant and not yet molecularly characterized may turn out to be a result of transposon insertion. The dominant mutations jeopardize the use of refuge to stop build-up of the resistance development in Bt^+ crop fields.

Why are mutations occurring at high frequencies in *Bt*$^+$ cotton fields?

The following three observations have raised questions about the molecular mechanism(s) by which mutations imparting resistance to Cry in insects may be arising, under the selective pressure of high doses of Cry protein(s). (a) Many of the Cry-resistance mutations, having their origin in the laboratory or in the field, are insertional, especially resulting from retrotranspositions[14,20,27–29]. There are instances of co-occurrence of mutations of different kinds (base substitutions, extended deletions and insertions in different combinations) at different sites in the same gene rendering the mutated insect Cry-resistant[14,30]. (c) Among the *Bt*-resistant insects occurring in *Bt*$^+$ crop fields, there was predominance of insects in which specified mutant alleles imparting Cry-resistance were in homozygous condition[14]. These properties of Cry resistance are indicative of high levels of (spontaneous) mutagenesis occurring in insects. Was it related to the presence of Cry toxin in the food of insects is an important question that has arisen?

It is known in plants, animals and man that a variety of stress conditions lead to demethylation of cytosines in the DNA and result in the induction of transposons residing in the genome. The demethylated cytosines serve as hot spots for base substitution, deletion and insertion mutations to occur and thereby enhance genetic variability[31,36–39]. There is considerable correspondence in the mechanism(s) of epigenetic DNA methylation in plants, vertebrates and insects[31,40,41]. The genome of lepidopteran insect *Mamestra brassicae* has vertebrate-like content of methylated cytosines in its genome[42]. The genomic DNA of coleopteran insect *Tribolium castaneum* is relatively more densely methylated in genes and repetitive DNA than those of *Apis mellifera*, *Bombyx mori*, *Mamestra brassicae* and *Medauroidea extradentata*[43–46]. The adults of *Tribolium castaneum* undergo extensive genomic demethylation during development. Like in plants, the adaptive response of this insect exposure to high temperature involves loss of methyl groups from the DNA[46]. The response to different kinds of stress is similar in plants[31]. Therefore, co-occurrence of more than one mutation observed in *Cad* gene of different Cry-resistant insects[14,30] is in conformity with the known fact in a variety of organisms that there is increase in the frequency of mutations in and around the locations of demethylated cytosines in gene/DNA[36,37]. Fabrick *et al.*[14] have reasoned that homozygosity for the disrupted allele at the *Cad* locus in six out of eight *Bt*-resistant insects that they studied was a result of assortative mating. Such matings would occur if there was dearth of insects possessing different genotypes and preponderance of insects possessing same/similar genotypes. The mutation frequency in the mother populations of the insects studied by Fabrick *et al.*[36,37] may have been so high that the bulk of males and females possessing distinct genotypes was sterile/inviable (see review on dioecy[47]). Such situations in *Bt*$^+$ cotton fields at Anand, Gujarat and Khandwa, Madhya Pradesh, have been thought to be suitable for the evolution new species in living organisms in general[48]. A relevant question that has arisen is: what is the cause of extremely high mutation rate in the populations of *Pectinophora gossypiella* sampled by Fabrick *et al.*[14]? It is possible that in the presence of Cry, the PKA signalling or some other process is non-apoptotically functional in adult insects, especially in their reproductive systems, such that the genomic DNA undergoes extensive demethylation. Consequently, the gametes acquire all kinds of mutations in and about the sites of cytosine demethylation. It is hypothesized that under such conditions the insect populations of Anand and Khandwa became loaded with mutations, including those that imparted Cry resistance.

New combinations of anti-insect proteins

In recent years, the insect pests of *Bt*$^+$ cotton crops in India and *Bt*$^+$ corn crops in South Africa, Puerto Rico and USA have developed resistance against Cry toxin, by genetically/mutationally subverting the biochemical process that mediates the killing action of Cry toxin on insect larvae. The Cry resistance that the insects developed in their populations was inheritable as recessive alleles, dominant alleles or a combination of both. With the emergence of dominant Cry resistance in insects, the refuge-based technology to delay Cry-resistance development in insects was no longer tenable. As an immediate countermeasure, *Bt*$^+$ crops transferred with more than one Cry gene, each with different modes of killing action against the same insect, such as Cry1Ac + Cry2Ab, Cry1F + Cry2Ab or Cry1Ab.105, Cry1Ab + Cry3Bb or Cry34Ab/Cry35Ab + Cry3Bb, have been constructed and a few of them released for cultivation. Such cotton genotypes are under extensive cultivation in India and Australia. Since development of resistance against multiple Cry toxins is possible (Tables 1 and 2)[49], there is interest in using *Cry* genes in a combination of genes for insecticidal toxins synthesized in heterologous bacteria such as *Serratia*, *Xenorhabdus* and *Photorhabdus* and insecticidal proteins/peptides synthesized in the venoms of centipedes, spiders and tarantulas[1,2,50]. The peptide OAIP-1 specified by Australian tarantula has been found to be effective against cotton bollworm[51]. Irrespective of the source and diversity of genes for pest resistance in crops, complementary approaches are required to minimize the chances of genetic resistance development in pests.

Need for development of suitable IPM practices

The use of suitable integrated pest management (IPM) practices[52–54] in the crop fields of pest-resistant genotypes

requires intensification. In Arizona, sporadic releases of reproductively sterile populations of *Pectinophora gossypiella* in *Bt* cotton fields have contributed significantly to Cry-resistance-free cotton cultivation since the introduction of *Bt* cotton cultivation in 1996 (ref. 55). Liu *et al.*[56] have reported that the release of populations of the ladybird beetle *Coleomegilla maculata*, a natural enemy of *Plutella xylostella*, in the Bt^+ broccoli crops delayed the evolution of Cry1Ac resistance in *Plutella xylostella*. Each pest of Bt^+ corn or Bt^+ cotton has its own viral, bacterial and fungal pathogens and insect and nematode predators. For example, *Campoletis chlorideae* the parasitoid of *Helicoverpa armigera* and *Apanteles angaleti* and *Bracon greeni* the parasitoid of *Pectinophora gossypiella* occurring in Indian cotton fields. There is a need to develop pest management technologies based on local pathogens and predators of crops constructed to resist pests with the use of gene(s) for peptide/protein toxins of diverse origins. This field of entomology needs to be intensified further for continued beneficial use of Bt^+ type of pest-resistant crops. Complementary use of crop varieties bearing pest resistance imparting heterologous genes, agronomic refuge technology and new integrated pest management practices will hopefully prevent crop losses due to pest infestation.

Summary

The genetically engineered Bt^+ plant accumulates Cry toxin(s) such that the larvae of susceptible pests ingesting their organs get killed and the plant safely concludes its life cycle. Cultivation of Bt^+ corn and cotton crops, started in 1996 and now occupying 76 m ha worldwide, has hugely benefited farmers by dispensation of insecticidal sprays. Recently, a cause of concern has been the evolution of resistance to Cry in pest populations in many geographical areas of Bt^+ crop cultivation. Dominantly or recessively inherited mutations, including transposon insertions in different genes were found responsible for *Bt*/Cry resistance in field populations of the pests of both Bt^+ corn and Bt^+ cotton. Curiously, bulk of Cry-resistant *Pectinophora gossypiella* sampled from Bt^+ cotton fields in India was homozygous for multiple mutations in their *Cadherin* gene, indicating that the frequency of mutations in pest(s) against the selection pressure of Cry has been high. To stem *Bt* resistance development, crop genotypes carrying one *Cry* gene are being replaced with those possessing two or more *Cry* genes whose killing mechanisms for the same insect are different. Observations of dominant mutations and of cross-resistance between Cry1Ac and Cry2Ab suggest that new strategies are required to delay evolution of Cry resistance against stacked crop genotypes. Release of sterile homologous pests and selected predators and/or pathogens of pests and deployment of agronomic measures to control pests are possible means to delay evolution in pests of resistance against protein toxins. Nematology research on IPM procedures complementary to refuge and Cry-stacking technologies also needs strengthening.

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