Perspectives on resistance management strategies for Bt-cotton in India

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ABSTRACT

Bt-cotton has been released for commercial cultivation in India in 2002. The three transgenic hybrids MECH-12, MECH-162 and MECH-184 released by Monsanto-Mahyco, India, incorporate cry1Ac gene for bollworm control. It is anticipated that in the next few years many more seed companies would be releasing Bt-cotton hybrids for commercial cultivation in India. The technology has been found to be very useful for bollworm management in almost all the field trials conducted by research institutions and farmers. Bt-cotton expresses Cry1Ac toxins in all plant parts, albeit to a variable extent, during the entire cropping season. The toxin imposes a high selection pressure on lepidopteran pests, which are susceptible to it, thus extending the possibilities of resistance development. For the technology to be sustainable, it is imperative that appropriate resistance management strategies are developed so that resistance development can be delayed. As a pre-requisite for the designing of strategies, resistance risk assessment was made using information from the studies conducted at CICR. The information includes; frequency of resistance alleles in field populations; potential of bollworm to develop resistance; mode of inheritance of the resistance alleles; geographical variability in susceptibility of bollworms to Cry toxins and seasonal dynamics of Cry1Ac expression in the transgenic crop. Additionally, on-farm experiments were conducted to evaluate the impact of various refugia designs on the target pest survival and sustenance and to understand the influence of Bt-cotton on target and non-target pests, predators and parasitoids. Based on the data generated and the cotton cropping ecosystems prevalent in India we propose resistance management strategies for implementation in India so as to ensure a long-term sustainability of the Bt-cotton technology in India.

Introduction

Transgenic Bt-Cotton technology is probably one of the most exciting advances in cotton pest management in recent times. Transgenic Bt (*Bacillus thuringiensis*) cotton crop is expected to keep lepidopteran insect pests in check, thus leading to a reduction in the overall use of insecticides. Bt transgenic plants incorporating cry1Ac genes are known to be toxic to the American bollworm, *Helicoverpa armigera* (Hübner), Pink bollworm, *Pectinophora gossypiella* (Saunders), the spotted bollworm, *Earias vittella* (Fabricius) and the spiny bollworm *Earias insulana* (Boisd). The commercially cultivated Bt-transgenic cottons employ constitutive promoters and synthetic codon-optimized modified Crystal (cry) genes to achieve high levels of expression, continuously throughout the crop growth (Perlak, *et al.*, 1990). Thus, the Cry toxin will have a continuous effect on all the lepidopteran insects thereby leading to development of resistance in cotton bollworms and other lepidopterans to Bt toxins. For transgenic plants to be effective and for their usefulness to be extended, it is necessary to design proper proactive resistance management strategies prior to large-scale cultivation.

Bacillus thuringiensis

Bacillus thuringiensis (Bt) is a soil bacterium that produces insecticidal proteins during its sporulation. It was discovered first in Japan by Ishawata in 1901 and by Berliner in 1911 in Germany (Baum *et al*., 1999). Currently, out of the158 known Bt-genes, about 20 insecticidal crystal (Cry) proteins produced by *Bacillus thuringiensis* are recognized for their potential in plant protection. Twelve of these, the Cry1 and CryII toxins are toxic towards various pest species of lepidoptera. Bt toxins are specific to insect families but their activity often varies among species within families (MacIntosh *et al.*, 1990). Cry1Ac, Cry1Aa and Cry2Aa are the three toxins that were found to be the most toxic to cotton bollworms especially *Helicoverpa armigera*. The commercial transgenic Bt-cottons released thus far for cultivation, incorporate a single gene, Cry1Ac. Ever since the first commercialization, Bt-transgenic crops have now come to occupy about 25% of the total area under transgenic agriculture.

Bt transgenic cotton -India

Three Bt-cotton hybrids MECH-12, MECH-162 and MECH-184 were released for cultivation in 2002 in India by MAHYCO Pvt. Ltd., India. Five new Bt-cotton hybrids, (RCH-2, RCH-20, RCH-34, RCH-138 and RCH-144), from RASI Hybrid Seeds Pvt. Ltd., are in the final stages of field testing. All the above-mentioned Bt-cotton hybrids were developed by introgressing the cry1Ac gene into one of the parents of the hybrids, from an American Bt-variety, Coker 312, under license from Monsanto, USA. At least six other seed companies are in the process of developing twenty-thirty more Bt-cotton hybrids. It is expected that Bt-cotton technology would occupy about 25% of the total cultivable area under cotton in India by 2007. Cotton acreage in India is only 5% of the total cropped area, yet it consumes more than 50% of the total insecticides used in the country. Insecticides worth US $200 M, are used annually for cotton pest management alone. The pattern of usage, however, is not uniformly spread, for instance Andhra Pradesh alone, where cotton cultiva-

tion is only 0.3% of the total cropped area of the country, accounts for 17% of the pesticide use on cotton in India. Again within the average picture of the state, coastal AP uses 30% more pesticide than the state average. The approximate estimates of insecticide sales is about US $65 M in Guntur district alone, where cotton is grown in about 0.15 M hectares. Clearly areas with maximum pesticide use per hectare, necessitated mostly due to bollworms, are likely targeted market niches for the bollworm resistant transgenic cottons. Moreover, since the price of the transgenic seed in the US is nearly four times the cost of non-transgenic seed, the technology when introduced into India may appeal mostly to the high input cotton cultivators of the irrigated belt. Growing Bt cotton transgenics on marginal land is not recommended by the companies as these would not show any impact in the absence of strong insect pressure. In most areas of rainfed cottons the use of insecticides and pest pressures are low hence, it may not be economical in such belts, to encourage the use of transgenics.

Temporal variation in Cry1Ac expression

The expression of Cry1Ac in leaves and other plant parts of the eight Bt-cotton hybrids was estimated through ELISA (Enzyme Linked Immunosorbent Assay) at weekly to fortnightly intervals up to the first harvest (Figure 1). Cry1Ac expression ranged at 0.01 to 19 μg/g in various parts of the plant. The highest expression was in leaves at 75 days after sowing (DAS). A decline in expression of toxin levels was observed in all the eight hybrids. The earliest decrease was in MECH-162, with toxin levels falling off to 1-2 μg/g by 85 DAS. Expression in some hybrids such as RCH-144 and MECH-184 declined only after the 120th day after sowing. The expression levels were highly variable in different plant parts. Though younger leaves expressed highest levels of the toxin, there was a lot of variability in expression. The boll rind, buds and flowers had low expression at 0.01 to 2 μg/g. On an average the Cry1Ac expression in the eight Bt-cotton hybrids was found to be adequate for bollworm protection at least until the first 100-120 days after sowing (Figure 1). However, some plant parts such as the boll rind, square bracts, buds and flowers which express low levels of Cry1Ac, may sustain a small proportion of larvae that feed on them. In-vivo and semi *in vivo* bioassays were conducted on intact plants and isolated plant parts. The assays indicated that a small proportion of larvae survive under field conditions and majority of these grew well on flowers and boll rind. Survival of 5-10% larvae on Bt-cotton plant parts in semi in vivo bioassays is not uncommon. An overall analysis revealed that the Bt-cotton technology had a capability of reducing insect pest infestations by 60-90% under field conditions. The efficacy to a large extent was dependent on the host into which Cry1Ac was introgressed.

Bt transgenic cotton crops, which express Cry1Ac, were found to cause 100% and 75-90% mortality in susceptible *H. virescens* and *H. zea* respectively, in the U.S (Mahaffey *et al*., 1995). The same levels of expression caused far less than 90% mortality of *H. armigera* and *H. punctigera* (Wallengren) in Australia (Forrester and Pyke, 1997) indicating that *Helicoverpa* species appear to be innately tolerant to the Bt toxins when compared to the *Heliothis* species. Under field conditions in India, a small extent of survival of *H. armigera* on Bt cotton plants was regularly noticeable. Thus, tolerant individuals are still likely to survive despite high expression of the Cry1A toxins and may subsequently contribute to the resistant gene pool. Daly (1994) reported that transgenic cotton plants in Australia killed susceptible larvae early in the season but the effect declined significantly after about 95-100 days after sowing, when an increasing proportion of first instar larvae placed on transgenic leaves survived to late instars. Moreover, studies on Bt cotton in the United States and Australia have shown that Cry1Ac production decreased over the growing season and that the bio-efficacy of the residual protein was reduced by interaction with increasing levels of secondary plant metabolites (Daly and Fitt, 1998; Federici, 1998). Differential expression in plant tissues may contribute a third cause of a reduced efficacy of the Bt transgenic crops. If proper resistance management strategies are not implemented the efficacy of pest management through Bt transgenic crops will be seriously diminished due to widespread development of resistance. Such strategies have not yet been developed for the small farmer and predominantly un-irrigated cotton growing systems of countries such as India.

Geographical variability in H. armigera *susceptibility to Cry1Ac toxins in India*

The baseline toxicity of Cry1A toxins on field populations of the cotton bollworm, *Helicoverpa armigera* (Hüb.) was determined through log dose probit analysis (Table 1). The LC_{50}s for Cry1Ac ranged from 0.01 – 0.67 mg/ml of diet (67-fold tolerance) in field populations of *H. armigera* collected from various parts of the country (Kranthi *et al*., 2001). The slopes for the field strains obtained from the probit assays were generally low. The c^2 values indicated heterogeneity in response to the toxins in most of the field strains that were tested. Insect populations collected from some parts of southern India were found to be more tolerant to Cry1Ac. The overall average LC_{50} and LC_{99} deduced from the data were 0.1 and 75 mg/ml of diet for Cry1Ac. These values represent the baseline susceptibility and indicate appropriate diagnostic doses for future routine monitoring of resistance to Cry1Ac through discriminating dose assays.

Geographical variability in *E. vittella* susceptibility to Cry1Ac toxins in India

The spotted bollworm larvae, in general were found to be extremely susceptible to Cry1Ac. Results indicated that Cry1Ac was very effective against neonates with LC_{50} at 0.28–1.215 ng/cm^2. The overall average LC_{50} and LC_{99} for Cry1Ac, as deduced from the data were 0.88 ng/cm^2, and 627 respectively. These data would be useful to consider the respective LC_{50} 's as the baseline susceptibility index for resistance monitoring through the conventional log dose probit assays. The LC_{99} values represent the diagnostic doses for routine monitoring of resistance through discriminating dose assays.

Potential of H. armigera *resistance to Cry1Ac*

Resistance factors (Kranthi *et al*., 2000) in *H. armigera* to Cry1Ac were relatively low for the first four episodes of selection pressure. But, resistance increased rapidly to an LC_{50} related factor of 76 fold by end of the 10th generation and was 56 fold at the 11th generation (Table 2). Similarly, the resistance factors with respect to the EC_{50} were 34 and 13 fold at the 10th and 11th generation respectively. Resistance was more clearly indicated in the LC_{90} data. The slope, which was relatively steep at 1.8 in the first generation, declined to 0.68 by the end of the 11th generation indicating an increase in the number of resistant heterozygous individuals in the final population. A laboratory strain, that was maintained without any selection pressure for 10 generations did not exhibit any change in susceptible response to the Cry1Ac toxin.

Frequency of 'resistance alleles' to Cry1Ac in field populations of H. armigera

The frequency of resistance alleles in field populations, as deduced from F_2 screen assays using a diagnostic dose bioassay on F_2 of sib-mated progeny of iso-female lines, was found to be 2.3 x 10^{-3}. Thus one individual larva in every 440 field insects was found to harbor a resistance allele to Cry1Ac.

Genetics of resistance

Reciprocal genetic crosses were performed between susceptible and resistant strains to obtain F_1 progeny, which was tested for its susceptibility to Cry1Ac. The F_1 progeny was backcrossed to one of the parents to confirm the nature of the resistance-allele. Results indicated that *H. armigera* resistance to Cry1Ac was incompletely dominant.

Cross resistance and joint-toxic action

Resistant strains selected with Cry1Ac exhibited a broad-spectrum resistance, to a variable degree, to almost all the Cry1 toxins tested, and to a lesser extent to Cry2Aa. A near-isogenic Cry1Ac-line also exhibited some amount of cross-resistance to Cry2Aa. Joint toxic action studies indicated that none of the Cry1 toxin combinations displayed any significant synergism. However, combinations of Cry1 with Cry2 toxins showed additive toxic effect on *H. armigera*.

Ecological implications

Bt-cotton transgenic plants are expected to primarily target the bollworms. Apart from the bollworms, the cotton crop in India is affected by other lepidopteran insects, which are foliage feeders and have been reported to cause economic losses to the crop in various parts of the country. Prominent among these are the tobacco caterpillar *Spodoptera litura* (Fabricius), cotton semilooper, *Anomis flava* (Fabricius), cotton leaf folder, *Syllepte derogata* (Fabricius) and the Bihar hairy caterpillar, *Spilarctia obliqua* (Walker). Cry1Ac has a remarkable toxicity on all these insect pests except *Spodoptera* spp., In general, *Helicoverpa* species appear to be innately tolerant to the Bt toxins when compared to the *Heliothis* species. Cry1Ac is highly toxic to the other two bollworms of cotton, namely, spotted bollworm *Earias vittella* (Fab.) (Kranthi *et al*., 1999) and the pink bollworm *Pectinophora gossypiella* (Saund.) (Barlett *et al*., 1997), but only marginally toxic to the tobacco caterpillar *Spodoptera litura* (Fab.) (Kranthi *et al*., unpublished data). Moreover, the Cry1Ac toxins are not at all toxic to insect pests belonging to insect orders other than lepidoptera, importantly, the sucking pest complex, which includes jassids, aphids and whiteflies. Hence, insecticides would still have to be used early in the cropping season on Bt transgenic cotton. Insecticide application early in the season in now been recognized as being incompatible with IPM and problematic to cotton ecosystems, especially with the broad scale killing of beneficial insects through the use of broad spectrum organophosphate insecticides intended for sucking pest control.

Concerns have also been expressed on the likely effects of Bt cotton on entomophagous beneficial insects and toxicity on non-target organisms thus resulting in pest shifts due to micro-ecological changes. Cry1Ac is a broad-spectrum lepidopteran toxin that can cause significant changes in almost all populations of lepidopteran insects occurring on the cotton crop. Some of these insects, which cause negligible economic damage to the crop, harbor parasitoids that have the potential to keep the bollworm populations under check (Kranthi, unpublished data). Consequently, in the absence or low populations of natural enemies, the bollworms can emerge as stronger pests than before. In-

sects pests such as *Spodoptera litura*, which are less affected by the Cry1Ac toxin and which have been under check due to the use of pyrethroids, can resurface as major pests as pyrethroid use is likely to be reduced on transgenic Bt cottons. However, except the possibility of Cry1Ac influencing the density dependent natural enemy predator and parasite populations, all other the concerns appear to be less note-worthy. Cotton pest spectrum in the US was found to alter after the introduction of Bt cotton. Unsprayed Bt cotton sustained four times more attack of tarnished bugs, 2.4 times more with boll weevil, 2.8 times more with stink bugs and Spodoptera. Due to these changes in pest complex, farmers had to spray 3-5 times on Bollgard as compared to 6-8 times on non-Bt cottons (Bacheler and Mott, 1996).

Resistance management strategies

Development of insect resistance to a toxin is due to progressive selection and sequential propagation of individuals of a population, surviving the toxicant. Continuous selection pressure with the toxicant eventually leads to an increase in numbers of tolerant/resistant individuals in populations. Very often questions have been raised on the viability of Bt resistant transgenic cotton as the development of bollworm resistance to Bt toxins is considered to be an accepted inevitability. After the introduction and large-scale cultivation of Bt transgenic cotton it is reasonably certain that *H. armigera* will respond to the intense selection pressure through a decline in its susceptibility to Cry1Ac, the gene used frequently against it. Also, the use of a single gene may lead to a rapid resistance development in species that are more susceptible or even moderately susceptible to the toxin thus reducing the impact of the technology for effective lepidopteran insect management.

Bollworm resistance development under field conditions would be influenced by a number of factors. Notably amongst the few core issues, area under Bt-cotton, expression levels in plants, frequency of the resistance allele, genetic nature of resistance, mobility of the insect, alternate hosts and mating synchrony of Bt-cotton-surviving-resistant and susceptible insects are important.

Resistance Management strategies relevant to the Indian context are as follows:

Refugia

World over, refugia have been one of the most commonly deployed resistance management strategies. The strategy is based on the fact that if a small defined area of non-transgenic plants are cultivated in close vicinity of the toxin-expressing-transgenic plants, they serve as hosts of the target insect pests, a major proportion of which would be susceptible insects. These would then serve as reservoirs of the susceptible alleles and when mated with the survivors from transgenic plants would result in heterozygous progeny, which would express susceptibility, especially if the resistant alleles are recessive in nature. The strategy relies on several conditions:

a) That the alleles conferring resistance are recessive and/or
b) That the toxin expression levels are high enough to kill the semi-dominant heterozygous progeny.
c) That there is a random mating and mating synchrony between the resistant survivors from transgenic plants and the susceptible insects from the non-transgenic plants
d) That there is no fitness deficit associated with resistance
e) That the resistance-alleles in field populations are rare.

Though refugia is a very useful strategy under most situations, it may have only a limited influence in delaying the development of resistance in *Helicoverpa armigera* in India even if fully implemented, for the following reasons:

a) Studies (Kranthi *et al*., unpublished) have shown that the alleles conferring resistance to *Helicoverpa* are incompletely dominant and autosomal in almost all field strains tested.
b) The frequency of resistance alleles in field strains is reasonably high at an average of 2.3×10^{-3} (Kranthi *et al*., unpublished).
c) Though Cry1Ac is the best toxin available for use in transgenics, survival of *Helicoverpa armigera* on Bt-cotton and highest concentrations of Cry1Ac-diet are not rare.
d) There is a 67 fold variability in susceptibility of field strains to Cry1Ac (Kranthi *et al*., 2001).
e) The hybrids (MECH-12, MECH-162 and MECH-184) released for cultivation in India have a highly variable level of toxin expression in variable parts depending on the age and stage of the plant. In some plant parts the expression is inadequate to afford a consistent protection against *H. armigera*. On an average the toxin expression would not qualify the definition of a high-dose (25 times that of LD_{99} of a susceptible strain).
f) The development time of larvae on Bt-cotton is about 20-26 days as compared to 13-16 days on non-Bt-cotton. Hence, mating synchrony between susceptible insects from non-Bt plants and the survivors from Bt-plants may be difficult to occur. Because majority of *H. armigera* mate within three to six days of emergence and die soon thereafter, this favors assortative, not random, mating. Susceptible individuals will mate with each other before the resistant individuals even hatch. This may however depend on generation overlap if *Helicoverpa* infestations are continuous.
g) Bt-cotton and the corresponding non-Bt cotton have different phenologies. The crop maturation stage in each of the Bt and non-Bt crops is different. In

general, wherein Bt-cotton enters a reproductive phase with large numbers of fruiting parts, the corresponding non-Bt in most cases is in the peak vegetative phase. Thus, there is no synchrony in the crop maturity as a result of which there is asynchrony in pest infestation on each of the Bt and non-Bt crops. The problem gets compounded with the fact that when *H. armigera* infests cotton there is hardly any other alternative simultaneous host. Pigeon pea is usually available as a host at a time when cotton crop ceases to be attractive to *H. armigera*. This may result in scenario in which the Bt-cotton may select for resistance in the first few broods of insects, which in turn would survive on a less effective Bt-cotton crop or the subsequent non-Bt alternate hosts.

Refugia in the USA is accepted as cultivation of either a 4% area as unsprayed non-transgenic crop or a 20% area under unsprayed non-transgenic plants in close proximity of the transgenic crop. Because of a high initial frequency of resistance alleles, there have been scientific arguments that refugia needs to be increased to 30-50%. However, this was deemed uneconomical and the previous recommendations had been retained. The refugia area in each of these countries was essentially derived from simulation models based on the initial frequency of resistance alleles and the nature of resistance allele. A refugia of 20% sprayed or 4% unsprayed non-Bt-cotton was supposed to have allowed a survival of at least 500 susceptible moths per each of the resistant surviving insect from Bt-cotton fields.

The Indian perspective on the research prerequisites for development of resistance management strategies, is relatively unclear. Indian data on the genetics of resistance, the frequency of resistance-alleles in field populations and biological attributes of resistant strains, are currently inadequate to be able to arrive at a well defined refuge area. An area of 20% refugia of non-Bt with Bt-cotton (based on the US strategy) for the Indian conditions can be seen as a strategy that has been arbitrarily derived in the absence of well defined resistance management options that could be drawn from scientific data from Indian work.

The current Indian recommendation of ensuring a 5-row non-Bt crop all around an acre of Bt-cotton crop to ensure a 20% refugia, does not appear to be practical under Indian conditions. It is difficult to presume that this would be easily acceptable, given the fact that Indian farmers do not plant border rows. It is difficult not just for scouting and spray applications but for all other agricultural operations as well. Moreover, it is not difficult to understand that farmers who are already disgusted with bollworm control wouldn't want to grow non-Bt-cotton alongside, especially when Bt-cotton is available as a powerful and handy tool. Given the difficulties that they have gone through with bollworms over the past two decades, the concept of allowing damage in 20% non-Bt crop with refugia as a resistance management tool may be difficult for the Indian farmer to accept. The difficulty may aggravate when they would have to compete with farmers who may not adopt the refugia strategy.

Several studies (mostly simulation models) have indicated that refugia, as well defined rows of non-Bt crops sown along with Bt-crops, is a better option over seed mixes. Two major studies investigated the efficacy of seed mixtures in comparison with refugia. Mallet and Porter (1992) used computer modeling to show that seed mixtures would be less preferred in comparison with fields of solely toxic plants (Mallet and Porter, 1992). The arguments against seed mixes have been that they could create a halo effect wherein the susceptible larvae from non-Bt plants would migrate to Bt-cotton plants and get killed, thus depleting the susceptible reservoir. Tabashnik (1994) found that seed mixtures were preferable to pure Bt fields. Both studies agree that refugia are more successful than seed mixtures (Mallet and Porter, 1992; Tabashnik, 1994a).

But, under the Indian context a 10% seed mix of non-Bt (having phenological synchrony) with Bt-cotton can be viewed as a practical tool to overcome the farmer-dependent resistance management strategy of 20% refugia. The strategy can be useful, especially as it may augment the susceptible populations with those that would survive on Bt-cotton crop due to low expression in some of the plant parts. Moreover, the bolls of Bt-cotton hybrids have F_2 seeds, thus having 25% non-Bt seeds as inbuilt susceptible refugia. Since, boll-rind has less Cry1Ac expression and is one of the key target feeding sites of bollworms on the plant, the F_2 bolls would add to a sizeable refugia that would augment a 10% non-Bt seed-mix.

All factors considered, the proposed 10% seed-mix refugia strategy for Indian farmer conditions may be the most practical and attractive option that is available for resistance management. The strategy assumes more significance in light of the fact that susceptible population is a resource that can be depleted if proper care is not exercised. It is also important to consider that the refuge method can be more useful when used in combination with other strategies to enhance the effectiveness of both. It would certainly be useful to explore the options of cultivating other non-Bt crop plants as intercrops if their flowering synchronizes with the peak infestation period of *H. armigera* on cotton.

Low expression

Low expression of the toxin spares heterozygous individuals and helps in the conservation of the susceptible alleles in field populations. But this is likely to result in inadequate and unacceptable levels of pest control. Low-expression has been an option that has

been ruled out as a resistance management strategy in several countries. It appears providential that this may prove to be an inadvertently introduced useful resistant management strategy under Indian conditions. Hybrids are likely to express lower levels of toxin than straight varieties, due to their hemizygous nature. India has Bt-cotton hybrids, which have good Cry1Ac expression levels (up to 19 μg/g fresh weight). But this can be categorized as moderate with reference to the innate tolerance of *H. armigera* to Cry1Ac, high geographical variability, high frequency of resistance-alleles and an incompletely dominant resistant trait.

High expression

High toxin expression is a favored option from the standpoint of efficient pest control. It favors reduction of heterozygous individuals but banks on susceptible strains from refuges to maintain an overall susceptibility. The success of the high dose strategy depends on rare and recessive or partially recessive resistance alleles (Huang *et al*., 1999). But, temporal changes resulting in a reduction in toxin expression can help heterozygous individuals to overcome the toxin and thus spread resistance alleles. Additionally, heterozygotes must have no advantage over insects homozygous for the susceptibility allele, or the development of resistance can be hastened (Curtis, 1981). Another potential problem with the high dose strategy involves natural enemies. If pest populations are eliminated temporarily, natural enemies in the ecosystem, which fed upon these pests, may leave or die. This leaves room later for a secondary pest outbreak when pests return and the population is not controlled naturally by enemies (Hoy, 1998). Resistance has generally been found to be a recessive or partially recessive trait, though some evidence indicates that some resistance alleles may be dominant or co-dominant (Tabashnik *et al*., 2000). Huang *et al*. (1999) showed that the resistance allele in Ostrinia nubilalis to Bt-sprays was incompletely dominant. This evidence undermines the usefulness of the high dose strategy. However, no similar results have been found in studies of resistance to transgenic plants, which use different toxins (Tabashnik *et al*., 2000). To achieve high doses, synthetic development or enhancement of Bt toxins is possible. Another method recently studied involves expression of toxins in different parts of the cell. With tobacco plants engineered to express CryIIAa2 toxins in the chloroplasts, resistance was dramatically reduced in populations of three different species already showing significant resistance to nuclear expression of CryIIAa2 (Kota *et al*., 1999). At the given state of art of cotton-transformation technology in India, it may take a few years to achieve a high expression in straight varieties so that the technology can look really robust.

Regulating expression

Most resistant management approaches aim at reducing selection pressure in order to delay resistance development. Reduction in selection pressure can be brought about by either a temporal regulation of toxin expression as an insect inducible response or a tissue specific expression. Bollworms rarely feed on leaves. They prefer fruiting parts. Theoretically it sounds attractive to consider the option that tissue specific promoters are used to ensure only fruiting parts produce the toxin so that they are protected while the insect pest is encouraged to feed on leaves, which may at the most result in insignificant yield losses. But, conversely it is possible that young larvae survive on leaves, grow into older instars capable of surviving the toxin levels in fruiting parts, and thus cause yield losses. The toxin regulating choice however, has never reached any practical stage in any of the transgenic development programs till date. But, because, toxin expression in almost all commercial Bt-cotton crops is known to decline after a certain stage, it may be useful to consider the use of promoters (example Late Embryogenesis Abundant - LEA promoters) to ensure that the toxin is produced even at late stages in the crop life.

Gene pyramids

Gene stacks offer an attractive option of delaying resistance especially if two or more genes are available with high toxicity levels having independent modes of action and are do not have cross resistance. With these assumptions, the frequency of the occurrence of resistance alleles in any individual would be very rare (more rare than that for each of the individual toxins), hence probability of delay in resistance development. Moreover, the option is attractive because it also helps in increasing the efficacy of pest control, may qualify as high dose under Indian conditions and thereby reduce the requirement of refugia area. Hence, to combat the innately Cry1Ac tolerant *H. armigera*, it may be useful to resort to gene pyramiding through a combination of more than one gene such that together, the combination represents a high dose. Cry1Ac is highly toxic to the other two bollworms of cotton, namely, spotted bollworm *E. vittella* and the pink bollworm *P. gossypiella*, but only marginally toxic to the tobacco caterpillar *S. litura* (Kranthi *et al*., unpublished data). It is also important to prevent the re-emergence of the Cry1Ac tolerant tobacco caterpillar, *S. litura* (Fab.) as a major pest, especially in the wake of reduced pyrethroid usage on Bt cotton transgenics. One of the strategies towards this end would be to use toxins such as Cry1C or Cry1F, which is toxic to *S. litura* (Kranthi *et al.*, unpublished data) in conjunction with Cry1Ac, which was demonstrated as a synergistic combination against *H. armigera* (Chakrabarthy *et al.*, 1998). Gene stacking can be effective if the toxins are delivered simultaneously but each recognizes a different binding site in the insect midgut (Frutos *et al*., 1999). Frutos *et al*. (1999) showed that the use of Bt Cyt1Aa helped overcome 5000-fold resistance to Bt CryIIIAa toxins in *C. scripta*. However, combinations of Cry toxins to enhance toxicity have always been the most improbable task. *H. virescens* shows cross-resistance to many strains of Bt Cry toxins; *P. xylostella* and *P. interpunctella* in

the field and the laboratory readily evolve resistance to up to five or six Bt Cry toxins simultaneously (Tabashnik, 1994b). This may be partially explained by the 1997 finding that a single gene in *P. xylostella* is responsible for resistance to four individual Bt Cry toxins (Tabashnik *et al.*, 1997). However, with the inclusion of external refugia, this strategy seems more effective than mosaics or rotations of different Cry toxins. It must be noted that for even the small amount of resistance delay the gene stacking strategy may provide, refugia are necessary (Caprio, 1998). In order to effectively reduce the total insecticide use on cotton, it would be a good idea to transform cotton genotypes that are resistant to sucking pests, with Cry toxins, so that the plants would resist a wider range of the pest complex.

Spatial and temporal transgene deployment

Our studies (Kranthi *et al.*, unpublished) indicate that *H. armigera* resistance to Cry1Ac extends cross-resistance to several toxins including Cry1Aa, but not to Cry2Aa. But this may not be used as an opportunity to stack the genes together since significant additive or synergistic advantage is yet to be properly demonstrated in any field strain. However, these could be used in resistance management as two different transgenic varieties each incorporating the individual toxin gene, to be grown either as mosaics or rotated one after another every year. Though mosaics have never been shown to be practically useful in resistance management programs, rotations have certainly been used throughout for insecticide resistance management. Thus rotation of two or three different transgenic crops alternatively one after the other each year would cause a reduction in selection pressure due to each of the single toxins, and thus delay resistance development. Unfortunately, it has been shown that after removal of selection pressure for resistance to Bt (during periods where Bt is in use), the frequency of resistance in the *H. virescens* populations remains stable or only decreases very slowly (Tabashnik, 1994b).

Bt cotton transgenic crops, which are the products of intense scientific research involving high costs and efforts, indeed represent the state-of-art in pest management technology. Apart from the likelihood of reduction in insecticide use on transgenic cotton by at least 50-90%, it is also expected to ensure favorable ecological, economic and sociological returns, in contrast to the harmful effects due to the use of conventional insecticides. It is in the best interests of the farming community, that the benefits of such a technology must be conserved and extended for the longest possible time. Since development of resistance is an evolutionary eventuality, it is imperative that studies must be initiated to understand the basic nature of the phenomenon to enable combat the problem more effectively.

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Table 1. *Baseline toxicity of Cry1Ac to* H. armigera *and* E. vittella *in India.*

	H. armigera				***E. vittella***			
Strains	n	LC_{50}	95% FL	Slope + SE	n	LC_{50}	95% FL	Slope + SE
Nagpur	300	0.15	0.09 - 0.30	1.1 ± 0.1	100	1.25	0.32 –2.69	1.1 + 0.2
Wardha	310	0.012	0.01 - 0.01	1.7 ± 0.2	100	0.33	0.24-0.46	2.3 + 0.5
Parbhani	254	0.01	0.00 - 0.02	1.0 ± 0.2	100	0.28	0.17 –0.84	1.2 + 0.5
Akola	298	0.14	0.09 - 0.25	1.3 ± 0.1	100	1.21	0.67-4.77	0.9+0.3
Nanded					100	1.87	0.52-3.2	1.1 ± 0.4
Rangareddy	409	0.41	0.27 - 0.66	1.1 ± 0.1				
Prakasam	368	0.66	0.35 – 1.54	1.3 ± 0.1				
Guntur	300	0.24	0.11 - 1.31	0.9 ± 0.2				
Coimbatore	300	0.22	0.14 - 0.49	1.2 ± 0.1				
Composite	3258	0.100	0.05 - 0.25	0.8 ± 0.1				
Bhatinda	450	0.024	0.01 - 0.03	0.8 ± 0.1				
Rajasthan					100	0.907	0.52-6.6	1.1 ± 0.4
Haryana					100	0.462	0.30-1.19	2.1 ± 0.6

LC_{50} = Median Lethal concentration in µg/ ml diet

FL = Fiducial limits

n = total number of neonates released

S.E = Standard error

Table 2. *Dose mortality response of* H. armigera *to Cry1Ac.*

	Selection dose µg /ml on previous generation	LC_{50} µg/ml	(95% F.L)	LC_{90} µg/ml	Slope ± S.E	RF
F 1		0.185*	(0.065-0.356)	0.95	1.80 ± 0.21	
F 2	1.0	0.143*	(0.084-0.336)	1.143	1.42 ± 0.33	
F 3	1.0	0.186*	(0.095-0.746)	2.655	1.11 ± 0.30	
F 4	2.5	0.534*	(0.207-2.124)	7.469	1.12 ± 0.14	3
F 5	5.0	0.325*	(0.143-4.020)	22.90	0.69 ± 0.22	2
F 6	5.0	0.980	(0.413-10.25)	15.75	1.06 ± 0.28	6
F 7	2.5					
F 8	5.0	0.808	(0.340-8.515)	17.67	0.95 ± 0.25	5
F 9	10.0	2.327*	(0.656-414.6)	54.42	0.93 ± 0.30	15
F 10	10.0	11.50*	(2.07-18218)	907.4	0.67 ± 0.22	76
F 11	10.0	8.544*	(1.79-3389.6)	624.7	0.68 ± 0.21	56

*Heterogeneity $\chi 2$ significant at 5% level of significance.

LC_{50} = Median Lethal concentration in µg/ ml diet

FL = Fiducial limits

n = Total number of neonates released

S.E = Standard error

RF = Resistance factor

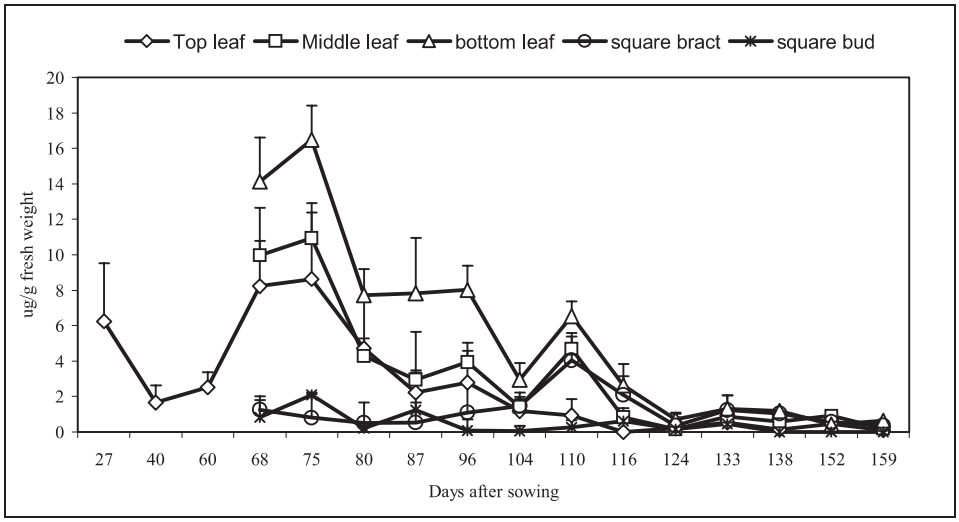

Figure 1. *Temporal variation in Cry1Ac expression in Bt-transgenic cotton (average of eight Bt-cotton hybrids).*