# Genetic engineering of crop plants for insect resistance

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TOOLS of molecular biology and genetic engineering have provided humankind with unprecedented power to manipulate and develop novel crop genotypes towards a safe and sustainable agriculture in the 21st century. Technologies and chemical inputs that have proven harmful to human health and environment need to be replaced with safer alternatives to manage insect pests in agricultural ecosystems. Many insecticidal proteins and molecules are available in nature which are effective against agriculturally important pests but are innocuous to mammals, beneficial insects and other organisms. Insecticidal proteins present in *Bacillus thuringiensis* (*Bt*), which have shown efficacy as spray formulations in agriculture over the past five decades, have been expressed in many crop species with positive results. Large scale cultivation of *Bt*-crops raises concerns about the possible development of resistant insects. Many strategies have been formulated to prevent/delay the development of resistance. These strategies have to be given serious consideration in India where the first *Bt*-crop containing resistance to insect pests, particularly *Helicoverpa armigera*, has been released for commercial cultivation in the farmers' fields. In addition to *Bt*, proteinase inhibitors present in several plant species offer a good source of resistance to insect pests. A combination of proteinase inhibitors has been suggested as a viable alternative to *Bt* to manage insects such as *H. armigera*. In recent years, several novel insecticidal proteins have been discovered in bacteria such as *Photorhabdus luminescens*. The judicious expression of multiple insecticidal proteins that differ in their mechanisms of toxicity will provide formidable barriers for insects to develop resistance. Finally, deployment of integrated pest management (IPM) strategies during the cultivation of transgenic crops will ensure durable insect resistance.

Insect pest menace is the major factor that destabilizes crop productivity in agricultural ecosystems. A variety of insect pests ranging from lepidopterans to orthopterans damage crops and stored seed. The rich biodiversity of agricultural, horticultural and forest species faces a perpetual onslaught by insect infestation because of the predominantly tropical and sub-tropical climates prevalent in India. A survey conducted among plant breeders, pathologists and entomologists shows that breeding for resistance to insect pests is at the top of the priority list of many important crops. Table 1 lists some of the important pests on major crops of India. Improvement of crop productivity by the introduction of high-yielding varieties which are more responsive to applied nitrogen and lack of proper crop rotation practices has also resulted in an enhancement of pest incidence. Insect pest management by chemicals obviously has brought about considerable protection to crop yields over the past five decades. Unfortunately, extensive and, very often, indiscriminate usage of chemical pesticides has resulted in environmental degradation, adverse effects on human health and other organisms, eradication of beneficial insects and development of pest-resistant insects. As we enter the new millennium with the objective of achieving higher and stable crop productivity to feed the burgeoning population, it is imperative to apply safe and environment-friendly strategies to attain our goals[1]. Insect pest management in an eco-friendly manner is no longer a dream. A large number of insecticidal molecules which are effective against insects and innocuous to man and other organisms have been reported. Tools of molecular biology and genetic engineering can facilitate harnessing and deployment of these molecules in crop plants in a safe and sustainable fashion[2]. In this article, we review the efficacy of various categories of insecticidal proteins for the development of insect-resistant transgenic plants and discuss the prospects of large-scale cultivation of such transgenic crops in India.

## Insecticidal proteins of *Bacillus thuringiensis*

*Bacillus thuringiensis* (*Bt*) is a Gram-positive, aerobic, sporulating bacterium which synthesizes crystalline proteins during sporulation. These crystalline proteins are highly insecticidal at very low concentrations[3]. As these proteins

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are non-toxic to mammals and other organisms, *Bt* strains and their insecticidal crystal proteins (ICPs) have acquired acceptability as eco-friendly biopesticides all over the world and have been under extensive use in agriculture, horticulture, forestry, animal health and mosquito control for the past four decades[4]. With the advent of molecular biology and genetic engineering, it has become possible to use *Bt* more effectively and rationally by introducing the ICPs of *Bt* in crop plants[5].

*Bt* strains and ICPs were first found to affect a range of lepidopteran insects, which are recognized worldwide as major agricultural pests on crops. Subsequently, discovery of new strains expanded the host range. Strains are now available which are toxic to coleopterans, dipterans, lice, mites and even nematodes[5]. Most families of Lepidoptera include species susceptible to the Cry1 and Cry2 crystal proteins produced, in particular, by *Bt* serotypes *kurstaki* and *aizawai*. Currently, the crystal toxins are classified on the basis of amino acid sequence homology. The ICPs fall under 40 different classes with some toxins exhibiting specificity to multiple insect orders[6] (www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt/). Toxicity of various ICPs towards different pests has been studied and catalogued (http://www.glfc.forestry.ca/Bacillus/Bt_Home Page/netintro99.htm). Extensive screening programmes are in progress as *Bt*–ICPs have high commercial value.

The mechanism of action of the *Bt* ICPs has been worked out in some detail[5]. The molecular structure of at least three different ICPs has been studied[3]. The crystals, upon ingestion by the insect larva, are solubilized in the highly alkaline midgut into individual protoxins which vary from 133 to 138 kDa in molecular weight, depending upon the type of protoxin. The protoxins are acted upon by midgut proteases which cleave them into two halves, the N-terminal half which is usually of 65–68 kDa is the toxin protein. The toxin protein fragment can be divided into three domains[3]. The first is involved in pore formation, the second determines receptor binding and the third is involved in protection to the toxin from proteases[5]. The toxin protein binds to specific receptors present in the midgut epithelial membranes. Upon receptor binding, the domain I inserts itself into the membrane leading to the pore formation. The disturbances in osmotic equilibrium and cell lysis lead to insect paralysis and death[5].

The delivery of *Bt* ICPs through spray formulations, engineered *Bt* and other bacteria has certain limitations. The biopesticidal sprays suffer from short half-life, physical removal (wind and rain) and inability to reach burrowing insects. Engineered bacteria very often proliferate at a rate and quantity not sufficient to kill the target insects. These disadvantages can be overcome if the ICPs are expressed in the plant cells at levels sufficient enough to kill the larvae. The first transgenic plants using *cry* genes were developed in 1987. The tobacco plants engineered with truncated genes encoding Cry1Aa and Cry1Ab toxins were found to be resistant to the larvae of tobacco hornworm[7]. However, the levels of Cry protein expression in the plant tissues were not very high. A significant breakthrough was made in 1990 by researchers at Monsanto Company (USA) who modified the *cry* genes (*cry1Ab and cry1Ac*) for better expression in plant cells[8]. The codon usage of prokaryotic genes of *Bt* was altered to resemble that of higher plants. In addition, many features like presence of putative polyA type signals and splice sites which destabilize *Bt* mRNAs in plant cells were removed without altering the amino acid sequence of the ICPs. Expression of such modified genes in crop plants, *cry1Ac* in cotton and *cry3Aa* in potato, conferred considerable protection against lepidopteran and coleopteran pests respectively. Subsequently, many crop plants which include rice, maize, peanut, soybean, canola, tomato and cabbage were transformed with various modified *cry* genes[9]. An interesting example of native gene (*cry1IA5*) expression resulting in significant resistance to *H. armigera* in transgenic tobacco was provided by Selvapandiyan *et al.*[10]. Another important landmark is the introduction of a native *cry1Ac* gene into the choloroplast genome of tobacco which expressed the Cry protein to a very high level (3–5% of leaf soluble protein)[11]. Chloroplast transformation besides providing high foreign protein expression also ensures maternal transmission of the foreign

**Table 1.** Important pests of major crops of India

| Crop | Insect pest | | Family |
|---|---|---|---|
| Rice | Yellow stem borer | *Scirpophaga incertulas* | Lepidoptera |
| | Brown plant hopper | *Nilaparvata lugens* | Hemiptera |
| Mustard | Mustard aphid | *Lipaphys erysimi* | Hemiptera |
| Chickpea | Gram pod borer | *Helicoverpa armigera* | Lepidoptera |
| Pigeonpea | Gram pod borer | *H. armigera* | Lepidoptera |
| Cotton | Cotton boll worm | *H. armigera* | Lepidoptera |
| Sugarcane | Top borer | *S. nivella* | Lepidoptera |
| Groundnut | Leaf miner | *Stomopterix nertaria* | Lepidoptera |
| Potato | Tuber moth | *Phthorimaea operculella* | Lepidoptera |
| Tomato | Fruit borer | *H. armigera* | Lepidoptera |
| Brinjal | Shoot and fruit borer | *Leucinodes orbonalis* | Lepidoptera |
| Cauliflower and cabbage | Diamondback moth | *Plutella xylostella* | Lepidoptera |

gene and therefore avoiding the spread of transgene through pollen. If extended to the important crop plants such as cotton and rice, this strategy can prove very useful in future. However, it remains to be seen if transformed chloroplast genomes will provide protection in the reproductive parts and fruiting bodies which are often the targets of insect attack.

As of now, more than 30 plant species have been transformed with *Bt cry* genes[12] (Table 2). The commercialization of *Bt*-crops started in 1996 with the introduction of bollworm-resistant cotton ('Bollgard') in USA. Subsequently, potato and maize were also commercialized[13]. In India, intensive efforts are underway to introduce *cry* genes in crop plants such as rice, potato, cotton, sorghum and vegetables. Investigations concerning evaluation of different ICPs for their relative toxicity to various target pests were made[14–18]. Transgenic crop species carrying different *cry* genes are at various stages of development. The first transgenic plants of tobacco (cv. Hema and Jayasri) developed at the Tata Energy Research Institute by using modified *cry1Ab* and *cry1C* (obtained from Dr Bert Visser, CPRO-DLO, the Netherlands) showed considerable protection against tobacco caterpillar (*Spodoptera litura*) in limited field trials conducted at the Central Tobacco Research Institute (Venkateswarlu, unpublished). Scientists at the Bose Institute (Kolkata) have introduced a modified *cry1Ac* gene in rice (IR 64) for resistance to yellow stem borer[19]. However, field evaluation of these rice transgenics has not been undertaken. A synthetic *cry1Ac* gene was introduced in rice (Pusa Basmati 1, Karnal Local and IR 64) under the control of Ubiquitin promoter and transgenic lines exhibiting total protection against neonate larvae of yellow stem borer (YSB) were identified (Khanna and Raina, unpublished). Field evaluation of these transgenics was performed in 2002 and lines resistant to YSB were identified (Raina, pers. commun.). Vegetable crops such as brinjal and tomato were transformed by synthetic/modified *cry1Ab* and *cry1Ac* genes, respectively, to confer resistance to fruit borers[20,21]. Limited field trials of *Bt*-brinjal and *Bt*-tomato were conducted for three and two growing seasons respectively. The degree of insect protection was 75% and 94% in brinjal and tomato respectively (Kumar, unpublished). Four genotypes of potato were transformed by modified *cry1Ab* to achieve considerable protection against tuber moth and *H. armigera*[22]. In addition to the work described above, many public and private sector institutions are involved in the development of insect-resistant rice, cotton, sorghum, groundnut, sunflower, castor and tobacco. In the private sector, MAHYCO in collaboration with Monsanto introduced the modified *cry1Ac* gene originally used to transform Coker 312 variety of cotton into parental lines of hybrids that have been bred specially for Indian agronomic conditions. These transfers required four back-crosses and two selfed generations[23]. The hybrids were field evaluated at different locations. Various experiments related to gene flow, effects of pollen and plants on non-target organisms, etc. were conducted. The results showed that *Bt*-cotton required no or minimal pesticide sprays while the non-transgenic plants required nine to twelve sprays to manage bollworms. Commercial release of *Bt*-cotton has been approved by the Government of India in March 2002. Another seed company Nunhems-ProAgro Seeds has been conducting field trials of *Bt*-vegetables such as tomato and cauliflower, which carry modified *cry* genes and the results are awaited.

**Table 2.** Some important *Bt*-transgenic crops

| Crop | Gene | Target pests | Ref. |
|---|---|---|---|
| Cotton | *cry1Ab/cry1Ac* | Bollworms | 72 |
| Corn | *cry1Ab* | European corn borer | 73 |
| Potato | *cry3a* | Colorado potato beetle | 74 |
| Rice | *cry1Ab/cry1Ac* | Stem borers and leaf folders | 19 |
| Tomato | *cry1Ac* | Fruit borers | 21 |
| Potato | *cry1Ab* | Tuber moth | 75 |
| Eggplant | *cry1Ab/cry1B* | Shoot and fruit borer | 20 |
| Canola | *cry1Ac* | Diamondback moth | 76 |
| Soybean | *cry1Ac* | Soybean looper | 77 |
| Corn | *cry1H/cry9C* | European corn borer | 78 |

Other crop species carrying various *cry* genes include peanut, alfalfa, cranberry, rutabaga, apple, white clover, white spruce, broccoli, grapevine, walnut, pear and sugarcane.

There is a need for systematic evaluation of the insecticidal efficacy of *Bt* ICPs to pests such as *S. litura*, *Earias insulana*, *Chilo partellus*, *Spilosoma obliqua*, *Maruca testulalis*, etc. as has been done in the case of *H. armigera*[14,15] and *L. orbonalis*[18]. Biochemical analysis of receptor binding *vis-à-vis* δ-endotoxins could provide valuable information that can help design suitable toxin combinations to be expressed in transgenic plants.

## Vegetative insecticidal proteins of *Bt*

Research efforts in the past five years have led to the discovery of novel insecticidal proteins which are produced by certain isolates of *B. thuringiensis*. These proteins unlike well-characterized crystal proteins are produced during vegetative growth of cells and are secreted into the growth medium. These proteins have been termed as vegetative insecticidal proteins (Vip). Sequences encoding for a Vip have been cloned, sequenced and the protein has been expressed in *E. coli*[24].

The 88 kDa vegetative insecticidal protein has a putative bacillar secretory signal at the N-terminal which is not processed during its secretion. It does not show any homology with the known crystalline insecticidal proteins. This structural dissimilarity is indicative of a possible divergent insecticidal mechanism than the other known *Bt*-toxins. In experiments wherein the expressed receptor to *Bt*-toxin of polyphagous pest *S. litura* was titrated against Vip toxin no interaction between these

ligands was observed (Agrawal *et al.*, unpublished). These preliminary results together with the observed structural divergence of Vip with other toxins make them an ideal candidate for deployment in insect management programmes together with the other category of *Bt*-toxins described earlier. Individually *vip* has been successfully expressed in monocots and dicot plants (Selvapandiyan *et al.*, unpublished) and efforts to pyramid *vip* in the *Bt*-transgenic crops are under way in several laboratories.

## Other insecticidal proteins from bacteria, plants and animals

### *Proteinase inhibitors*

Plants have a wide array of defense proteins including the proteinaceous proteinase inhibitors and lectins induced in response to insect attack[25]. Proteinase inhibitors (PIs) represent the most well studied class of plant defense proteins and are abundantly present in the storage organs (seeds and tubers)[26]. Their role against herbivory was hypothesized due to their abundance and their lack of activity against endogenous proteases. They were first shown as plant defense proteins in 1972 when the induction of PIs in potato and tomato was observed due to wounding and insect herbivory[25]. Subsequently, Gatehouse and co-workers demonstrated the resistance of a cowpea variety to the bruchid beetle due to the elevated trypsin inhibitor (TI) levels in the seeds[27]. Extensive studies have shown that PIs are induced as components of many defense cascades under various stress-prone conditions such as insect attack[28], mechanical wounding, pathogen attack and UV exposure. PIs have been found to cause inhibition of growth (among other deleterious effects) when fed to several insect pests in their diet[29]. PIs inhibit the gut proteinases of the insect which adversely affects the protein digestion in the gut and force the insect to synthesize alternative proteases to compensate for the inhibited activity. This leads to deficiency of essential amino acids and exerts physiological stress on the insect, leading to growth retardation[29]. This mechanism of action minimizes the possibility of developing resistance in the insects and reduces crop damage.

**Table 3.** Some examples of transgenic plants expressing genes encoding proteinase inhibitors, α-amylase inhibitors and lectins

| Crop | Gene | Target pest | Ref. |
|---|---|---|---|
| Tobacco | *Cowpea serine PI* | Tobacco bud worm | 79 |
| Tobacco | *Potato serine PI* | Tobacco hornworm | 80 |
| Rice | *Cowpea serine PI* | Stemborer | 81 |
| Potato | *Cowpea serine PI* | *Lacanobia* | 40 |
| Potato | *Oryzacystatin* | Potato beetle | 35 |
| Tobacco | *Hornworm PI* | Whitefly | 82 |
| Pea | *Bean α-AI* | Bruchids | 43 |
| Potato | *Snowdrop lectin* | Potato aphid | 40 |
| Rice | *Snowdrop lectin* | Brown plant hopper | 41 |

A direct proof of the protective role of PIs against insect herbivory was provided by Hilder *et al.*[30] who showed that the transgenic tobacco plants expressing cowpea trypsin inhibitor (TI) were resistant to the tobacco bud worm (*Heliothis virescens*). Following the cowpea TI, several serine PIs have been expressed in transgenic plants for resistance against insect pests of the order Lepidoptera while cysteine PIs have been expressed against the coleopteran pests[7,12]. However, in many cases, the transgenically expressed PIs have not demonstrated any resistance against insects (Table 3). This is because the insects have an ability to adapt to the ingested PIs by producing proteinases which are insensitive to the PI[31] or which degrade the PI[32,33]. Polyphagous insect pests like *H. armigera* adapt to various host plants by regulation of a complex complement of gut proteinases of different specificities[34]. Girard *et al.*[33] have shown that a complex proteolytic system consisting of serine, cysteine, aspartyl proteinases and leucine aminopeptidase in the insect gut confers a high level of resistance to oryzacystatin I and Bowman birk inhibitor in beetle larvae. Recently Cloutier *et al.*[35] have demonstrated that hypertrophic behaviour and production of inhibitor-insensitive proteinases are responsible for the adaptation of the Colorado potato beetle to transgenically expressed oryzacystatin I.

In a coevolving system of plant–insect interactions, insects have adapted to the PIs of their host plants and hence the non-host plants represent one of the best sources of identifying effective PIs. At the National Chemical Laboratory, Pune, scientists have studied the potential of three non-host plant PIs (winged bean, groundnut and potato) against the polyphagous pest *H. armigera* and have found them to be very effective inhibitors of the *H. armigera* gut proteinases and larval growth[36]. Additionally, the use of PIs from sources other than plants has also been considered and exploited[12]. Besides the selection of proper PIs, the efficacy of the selected PI can be improved using protein engineering to improve its inhibitory activity[37] and the affinity of the PI can be studied using the phage display technique[38].

Considering the adaptation of the insects to a single PI, it would be advantageous to express a combination of PIs for effective resistance. An appropriate combination of PIs targeted to inhibit the complete spectrum of insect gut proteinases improves the stability of each and thus efficiently impairs digestion of dietary proteins in the insect gut. Additionally, a targeted statement of such a combination, specifically the differential temporal statement, will ensure exposure of the insect to different PIs in succession, forcing the insect to alter its mid-gut composition more than once leading to an additional physiological stress. On the basis of results on non-host PIs, Harsulkar *et al.*[36] have proposed a strategy to use a combination of PIs involving tissue-specific statement of potato PI-II and winged bean PIs in a transgenic crop for control of *H. armigera* infestation. Since *H. armigera* is a foliar

feeder at lower instars and later shifts to the developing seeds, expression of potato PI-II in foliage and winged bean PIs in developing seeds could effectively counteract *H. armigera* infestation. Such transgenics have still to be produced and tested on a large scale.

### *Plant lectins*

Lectins are proteins having affinity for specific carbohydrate moieties. They bind to glycoproteins in the peritrophic matrix lining the insect midgut to disrupt digestive processes and nutrient assimilation. A lectin from snowdrop (*Galanthus nivalis*) when expressed in transgenic tobacco and potato has been found to be toxic to aphids[39] and the tomato moth *Lacanobia oleracea*[40]. Foissac *et al.*[41] have expressed the snowdrop lectin in transgenic rice. Engineered plants showed resistance against brown plant hopper (*Nilaparvata lugens*) and green leaf hopper (*Nephotettix virescens*). Wheat germ agglutinin, pea lectin, jacalin and rice lectin have been expressed in plants like tobacco, maize, and potato mainly against aphids[12]. However, many lectins are toxic/allergenic to mammals, and thus might put major restrictions on their usage in developing transgenics safe for human consumption[42]. Given the importance of developing transgenic crops resistant to insect pests, it is of paramount interest that lectins from different edible plants (particularly that are consumed raw without any influence on human health) are tested for their effectivity against major pests.

### α-*amylase inhibitors*

The common bean (*Phaseolus vulgaris*) contains a family of related seed proteins called phytohemagglutinin, arcelin and α-amylase inhibitor (AI). AI forms a complex with certain insect amylases and is supposed to play a role in plant defense against insects. The introduction and expression of the bean α-*AI* gene in pea confers resistance to the bruchid beetles[43]. Transgenic Azuki bean carrying α-*AI* gene was resistant to three species of bruchids[44]. Higgins and his group at CSIRO, Australia introduced α-*AI* gene in an Indian genotype of chickpea (C-235) and derived significant protection against bruchids (Sarmah *et al.*, unpublished). However, bruchids such as *Zabrotes* can feed on plants producing α-*AI* because they possess a serine proteinase able to cleave some kinds of α-*AI*. It is therefore difficult to evaluate the long-term benefits of the expression of these genes in plants.

### *Insect chitinases*

Chitin is an insoluble structural polysaccharide that occurs in the exoskeletal and gut lining of insects. It is believed to protect the insect against water loss and abrasive agents. Because of critical function of chitin it has been considered as a potential target for insecticidal proteins. Dissolution of chitin by chitinase is known to perforate peritrophic matrix and exoskeleton and make insects vulnerable to attack by different pathogens. Expression of proteins which will interfere with chitin metabolism is likely to have a serious effect on the growth and moulting of insects. In this aspect chitinase produced by insects themselves has been used as an insecticidal protein. Expression of cDNA for chitinase obtained from the tobacco hornworm, *Manduca sexta*, in tobacco plants offered partial protection against *Heliothis virescens*[45]. The larvae feeding on the transgenic plants exhibited several growth aberrations and died prematurely. In addition, it has been demonstrated that including chitinase protein together with insecticidal proteins of *B. thuringiensis* potentiates the effect of these toxins[12]. Consequently, chitinases of fungi and insects have been receiving increased attention and are being evaluated as potential insecticides in combination with *Bt*-toxins. It is believed that exposure of insect larvae to high levels of chitinases in conjunction with *Bt* toxins would enhance their vulnerability to *Bt*-toxins and would lead to more effective control of insect pests.

### *Plant metabolic enzymes*

Tryptophan decarboxylase from periwinkle was expressed in tobacco wherein it induced synthesis of tryptamine and tryptamine-based alkaloids[12]. Pupal emergence of whitefly decreased as a result of feeding on such plants. The mechanism by which tryptamine interferes with insects is not known. Other enzymes such as polyphenol oxidase and lipoxygenase have been shown to be toxic to insects[12] but to date, no report has described over-expression of these genes in transgenic plants.

### *Insecticidal viruses*

There are many viruses pathogenic to insect pests. These viruses are used in insect pest management programmes. Genomes of small viruses can be introduced into crop plants, which will synthesize the viral particles and acquire entomocidal property. For instance, *H. armigera* Stunt Virus (HaSV) is a tetravirus specific to lepidopteran insects and is very remotely related to viruses of plants and animals. HaSV is harmless to beneficial insects and the environment and its deployment in transgenic plants would not pose any risks[46]. A bio-prospecting approach is needed in India to identify such entomopathogenic viruses whose genomes can be manipulated in plants.

### *Genes from bacteria other than Bt*

Another bacterium which aroused interest in recent years is *Photorhabdus luminescens* that dwells inside the gut of

entomophagous nematodes[47], which belong to the family Heterorhabtidae. The nematodes invade the insect hemocoel and release the bacteria from their gut. The bacteria proliferate and kill the host within 24 to 48 h. The nematodes feed on the bacteria and the host cadaver. It was found that the bacteria synthesize high molecular weight protein complexes toxic to insects ranging from Lepidoptera, Coleoptera to Dictyoptera. The insect toxicity of the proteins was observed at nanogram concentrations similar to *Bt* toxins and the mode of action was different from that of *Bt*. Four genes encode the toxin complex and this may pose some complications for their expression in plant cells. However, this complex will provide an effective alternative to *Bt* and can also serve as a good candidate to be expressed along with *Bt* in transgenic plants.

### *Novel genes of plant origin*

Cloning of genes from higher plants resistant to insect pests is feasible by a molecular breeding approach. Recent example of cloning of the *Mi-1* gene from wild tomato (*Lycopersicon peruvianum*) has given an opportunity to control root-knot nematode and potato peach aphid simultaneously[48]. The vast biodiversity of Indian flora can yield rich dividends in this respect.

## Resistance management

One of the primary concerns of deployment of genetically engineered insect-resistant crops in a developing country like India is the durability of resistance. Engineering with *Bt* genes for insect resistance in crops has been a commercially successful technology. While the *Bt* toxins are mainly targeted against lepidopteran, coleopteran and dipteran crop pests, many species in these three orders have evolved resistance to *Bt* toxins[42,49]. As in the case of *Bt* toxins, insect species show adaptation or resistance to protease inhibitors mainly by altering their complement of secreted proteases[50–52]. Hence it is reasonable to assume that other insecticidal candidate genes and their products may not be beyond the adaptive range of insect pests. On the other hand, the transgenic insect-resistant plants, by their characteristic pattern of toxin expression may even hasten the selection process and facilitate development of resistance in the target pest population. Deployment of a particular insecticidal gene in multiple crops targeting the same insect pest would also lead to faster emergence of resistant insects. Development of resistance in an insect population against any class of toxins would mean loss of a non-renewable resource. Hence, experts have been debating alternative resistance management strategies to slow down the evolution of pest resistance in the past. Now it is generally agreed that one strategy – the 'high dose/refuge' strategy is the most promising and practical[42].

In order to judge the merit of high dose/refuge strategy we need to understand the process of evolution of resistance in the pest population[53]. Resistance may be conferred by either genetic or non-genetic component of variation in the population, though additive genetic component of this variation coupled with fitness advantage drives the selection process. Alleles of genes conferring adaptive advantages may always be present in the population or may appear at low frequencies by mutations. In an ideal population, in absence of selection pressure, the allele and genotype frequencies are maintained in Hardy–Weinberg equilibrium. Considering *Bt* toxins as an illustrative case, in many cases resistance in insects is conferred by a recessive or partially recessive allele[42] and initially this allele is found at a very low frequency. Hence resistant insects are rare and not easily detectable but heterozygous individuals will be much more abundant than the homozygous-resistant insects. When selection pressure results in a slightly increased fitness value for the heterozygotes in relation to homozygous-susceptible individuals, frequency of alleles for resistance could build up rapidly. Many simulation models based on population genetics principles have been developed to understand factors affecting this build up of frequencies[54–58]. Gould[59] has critically illustrated the merits and limitations of different strategies of resistance management as applicable to deployment of transgenic crops in developing countries. As more recent reviews[60–62] have exhaustively covered different aspects of resistance management, we propose to confine ourselves to the high dose/refuge approach, which has been widely held to be most efficient, promising and practical.

In the high dose/refuge strategy, high dose is aimed to kill almost all the heterozygous insects. High dose will also change a partially recessive resistance trait into practically recessive nature as illustrated in Figure 1 showing

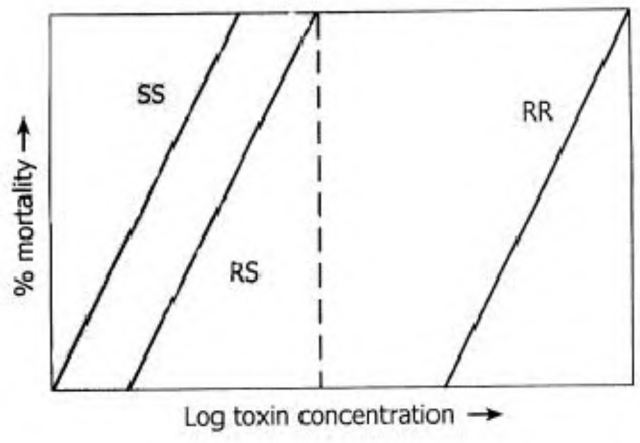


**Figure 1.** Dose response lines indicating the mortality of three insect genotypes at increasing concentrations of an insecticidal toxin. The dotted line indicates the concentration required for a high dose. S, allele conferring susceptibility; R, allele conferring resistance. (Reproduced from ref. 64.)

dose response lines for different genotypes. It may appear that high dose plants, by killing all the susceptible and heterozygous-resistant insects, lead to resistance build up in one step. The second component, refuge consisting of non-*Bt* plants, provides for survival of susceptible insects with which the surviving heterozygous and any homozygous-resistant insects would mate to produce heterozygous or completely susceptible insects. Second generation heterozygous insects will be again killed by the toxin in the plant. Thus high dose strategy coupled with refugia would lead to more durable resistance.

To adopt the strategy, one needs to define a high dose plant and means to identify these plants. A high dose plant, theoretically, would express toxin at a level enough to kill almost all the heterozygous insects. However, in practice, we need a colony of such insects to determine the high dose. Alternately, a high dose has also been defined as one that is 25 times higher than that required to kill 99% of homozygous-susceptible insects[63]. If precise dose mortality response for the purified toxin can be determined for the target insect and dose for 99% mortality can be worked out, then defining high dose would be more practical. Even this task may turn out to be difficult for some of the crop pests. More empirical values like toxin levels in order of 0.1 to 0.2% of soluble protein have been suggested for rice transformation with *Bt* genes[64]. Nevertheless, the task of identifying the transformed line with consistently high level of toxin expression could be complicated by the observations that different derived lines from the same transformation event vary in expression level and even the same transgenic plant might show changes in level of expression during different growth stages[65–68].

The second task is to define a refuge and determine the most suitable pattern and composition of refuge. As stated earlier, refuges are non-*Bt* crop plants that serve to maintain *Bt* susceptible insects in the population. Refuge can be a field of non-*Bt* plants interspersed with *Bt* fields or non-*Bt* plants within fields of *Bt* plants. These non-*Bt* plants support susceptible insects and provide them in enough numbers for ready mating with the insects developing from the *Bt* plants (Figure 2). Conditions to ensure random mating between adults emerging from *Bt* and non-*Bt* plants are essential for refuge to be effective. Hence, when field-to-field refuge is to be opted, the distance between them needs to be maintained within the flight range of the target insects and insects need to move out before mating. Mixtures of *Bt* and non-*Bt* plants within fields can be established by sowing seed mixtures or by planting rows of refuge plants within fields of *Bt* plants. But within field refuge may not be the best option for insect pests, which move from plant to plant during their active feeding stage. Such movements will 'dilute' the dose of toxin as insect may ingest sub-lethal dose of toxin from *Bt* plant and move to a non-*Bt* plant to complete its development. Thus spatial arrangement of refuge depends upon the biology of the target pest and needs to be carefully selected.

Spatial or temporal refuge within a plant can also be conceived in the form of tissue/part of the plant or growth stage of plant where/when the toxin is not expressed. While promoters with selective expressions are being used to drive the insecticidal genes in transgenic plants, an important consideration is the 'tapering' effect. If the toxin titer gradually drops down as the gene shuts off, the effect would be similar to the dilution effect of insect movement.

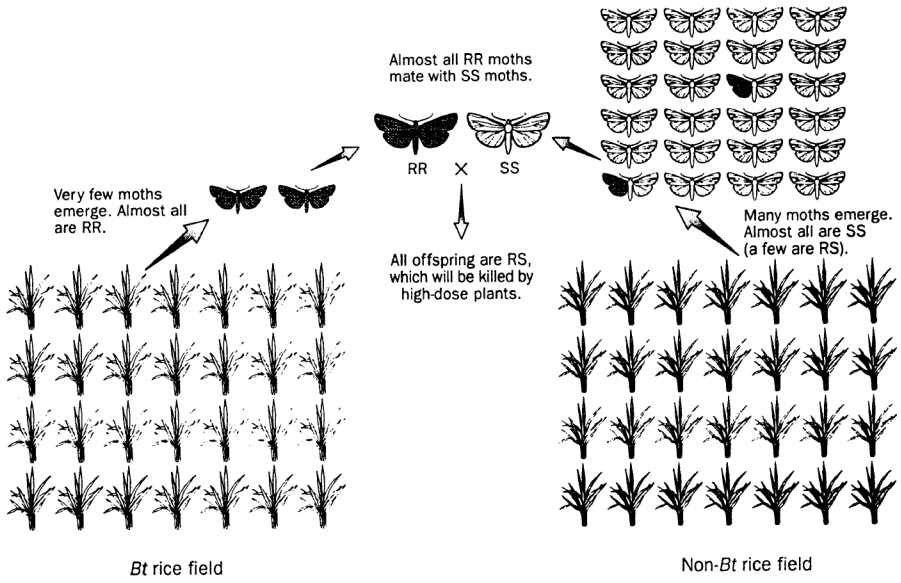


**Figure 2.** Mechanism of high-dose/refuge strategy to delay the increase in highly resistant (RR) insects in a pest population. (Reproduced from ref. 64.)

Implementing the resistance management strategy in a country like India may prove as formidable as selection of the most suitable option. In a developed country like USA, farmers growing *Bt* crops must plant 4–20% of their land to non-*Bt* cultivars, and these refuge fields must be within approximately 0.8 km of their *Bt* fields[69]. However, unstructured refuge may be maintained in small land holdings of Indian farmers by their diverse choice of cultivar, cost of seed and market demand. The government can also positively intervene by restricting the release of *Bt* varieties with specific agronomic background such that the entire area is not saturated with these. In highly productive regions farmer may not be willing to encounter insect damage in refuge fields and may even be apprehensive of higher damage in such fields. However, there are more evidences now to suggest that due to 'halo' effect of *Bt* crops, non-*Bt* crop may actually suffer less damage[70,71].

Another significant approach towards durable deployment of transgenic crops is to promote only such transformations which involve expression of at least two unrelated insecticidal genes with high levels of expression. It is obvious that if insects that are able to survive on a plant with one high dose toxin are rare, then insects that are able to survive on plants with two high dose toxins will be very rare indeed. This may call for smaller proportion of refuge fields. However, it is still important to have some refuge fields to fully harvest the benefit of this useful and environment-friendly technology on a sustainable basis.

## Perspectives

The management of insect pests in agriculture is feasible in a safe and effective manner. Molecular tools give us an opportunity to develop genotypes that carry resistance traits. The resistance needs to be protected by taking lessons from our past experiences with chemical pesticides. *Bt* has rightly emerged as a powerful tool of plant protection in agriculture in a sustainable manner. Although not universal in its application and total in its protection, *Bt* will play a central role in protecting the crop from its major insect pests. In combination with other powerful biopesticidal proteins such as proteinase inhibitors, *Bt* will drastically reduce the consumption of chemical pesticides and thus protecting the environment. Proteinase inhibitors and lectins have a major role to play in the management of secondary pests which are not susceptible to *Bt* and also as part of gene pyramiding strategies. It would also be appropriate if a particular *Bt* gene highly specific to a target insect is not deployed in multiple crops.

Considerable progress has been made in developing pest-resistant transgenic crops in India. However, the task is enormous because of the vast crop biodiversity and the number of pests prevalent in Indian agriculture. Commercial cultivation of *Bt*-cotton in 2002 and its perceived benefits would certainly spur more interest and activity in public and private research institutions. Very few attempts have been made towards isolation and characterization of *Bt*s in India. Discovery of novel *Bt*s and Cry proteins/genes will enhance our repertoire of insect protection measures in future. Cloning of *Mi*-1 gene from wild tomato (*L. peruvianum*) and its use in transgenic plants for insect protection exemplify the need for molecular breeding research aimed towards the discovery of resistance genes in wild species. There is an urgent need to test various *Bt* δ-endotoxins, Vips, proteinase inhibitors, lectins, etc. for their toxicity to pests endemic to India. Special attention must be given to *H. armigera* which is the major pest on important crops and which can survive on more than 130 plant species. The propensity of *H. armigera* to develop resistance to every known pesticide is an aspect to be considered while designing transgenic strategies. Basic plant molecular biology research is necessary to identify effective promoters which can sustain foreign protein expression during the late reproductive phase of crop plants such as cotton boll development. The durability of insect resistance in transgenic crops can only be ensured if integrated pest management (IPM) practices are followed. *Bt* as a biopesticidal formulation will continue to play an important role as a component of IPM in crop species which are not amenable to the attempts of genetic transformation.


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