Switching on *Bacillus thuringiensis* to reduce selection for resistance

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The scope of plant genetic engineering in allowing genes from Bacillus thuringiensis encoding natural endotoxin is well illustrated by the production of insect-resistance plants. There is great concern over increasing evidences of field resistance of insect pests to this endotoxin. Efforts are on for prevention of the spread of this resistance and identification of strategies which give the best protection for the lowest cost in terms of increased resistance in the pests as well as environment-friendliness.

DAMAGE to crops by insects is a major agriculture–economic factor in tropical and temperate regions of the world. Despite large-scale investment in the chemical control of pests, insects cause great economic loss by damaging or destroying the crops. This is achieved by i) direct consumption and ii) facilitating secondary infection by plant pathogens. The losses so created range from 5 to 15% of the world's annual agricultural production[1]. Alternatives to chemical pesticides are available, and have been successfully employed in the control of major pests. A suitable alternative apparently without any drawbacks has been available for more than 30 years. This is a biological insecticide from the soil bacterium, *Bacillus thuringiensis* (Bt). Bt. subsp. *Kurstaki* was discovered as early as 1902, although its commercial potential was largely ignored until 1951 (ref. 2). This biological insecticide seems to be a choice product for environmentally-sensitive applications (e.g. insect control prior to harvest, applications in forests and home gardens). Despite its remarkable safety and efficacy, Bt has not garnered a great proportion of the market for insecticides. This is largely due to the low cost and longer persistence of synthetic organic insecticides. With a tremendous development in techniques to engineer crops genetically, we now have the ability to make broader use of 'natural' insecticides. Isolating Bt insecticidal genes from the original bacterium and introducing them into the plant genome allows crop protection from insect attack without the aid of externally-applied synthetic pesticides. In fact, within two years of the first demonstration of plant transformation in 1984, control of pests in the fields was demonstrated with plants containing a Bt insecticidal protein in different parts of the plant. The use of Bt as an insecticide, offers advantages over chemical control agents, in that the species-specific action of its insecticidal crystal proteins makes it harmless to non-target insects, to vertebrates, and to the user.

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Insect-control agents

Two types of insect-control agents have been developed and proven effective following introduction into plants. These are (1) genes encoding delta endotoxins from Bt which have been studied widely in transformed plants and (2) a class of proteins known as proteinase inhibitors; which when present in relatively high levels in the diet, are effective against certain insects[1]. Besides using the two separately or individually, a combination of Bt endotoxins and proteinase inhibitors is being studied in detail, for controlling the damage caused by different insects and without causing any harm to the environment[3].

The choice of a Bt endotoxin, as the first insecticidal protein for introduction into plants, was based on the extensive knowledge gathered/accumulated about this class of crystal proteins since 1902. The entomocidal bacterium Bt upon sporulation, normally produces a number of insect toxins, including crystalline delta toxin[4]. Upon ingestion by a susceptible insect, high pH and proteinases of the insect's midgut, believed to be responsible for the solubilization of the crystal result in a rapid cleavage of protoxin to yield an active toxin. The effects of the toxin occur within minutes of ingestion, beginning with midgut paralysis and ending with the disruption of midgut cells, with the result the insect slowly starves to death[5].

Because of increasing pressure from the environmentalists to reduce the use of chemical insecticides the industrial effort to develop improved Bt products is increasing. At present, use of Bt sprays is still very limited because of low stability of the protein in the field,

relatively high production costs and the limited insecticidal spectrum. Microbial insecticides account for only 1.6% share of the world insecticide market, but 95% of these sales involve products based on isolates of Bt which amounts to only 1% of the global insecticide market. Applicability of Bt products should expand keeping in view the recent development of insect-resistant crops. This is possible by producing crops transformed with insecticidal crystal protein genes. The use of these insecticidal transgenic plants will eliminate stability problems and should significantly reduce the cost of insect control. More than 50 crop species, including major field crops such as corn, cotton, soybean, potato and rice have now been successfully transformed[6]. Insect-resistant plants containing Insecticidal Crystal Proteins (ICPs) coding genes are expected to be among the first transgenic varieties to be commercialized during this decade.

Strains of Bt have been registered as commercial insecticides for over 30 years beginning with 'Thuricide' (Sandoz label) in 1957. The crystal within the bacterium contains one or more types of toxin protein, each of which can have its own specificity[7]. On the basis of their structure, antigenic properties and activity spectrum, crystal protein and their genes have been classified into four major groups: Cry I (Lepidoptera-specific), Cry II (Lepidoptera- and Diptera-specific), Cry III (Coleoptera-specific), and Cry IV (Diptera-specific)[7–9].

Introduction of Bt endotoxin genes into plants

A Bt endotoxin with insecticidal activity against lepidopteran larvae was introduced successfully into tobacco plants in July 1987 by Vacek and co-workers (Plant Genetic System, a Belgian biotechnology company)[10]. Truncation of the full-length gene to a size encoding the toxin protein did not reduce insecticidal activity of Bt but improved its expression. The success of Vacek *et al.* can be attributed to their choice of promoter (wound-inducible upon insect feeding) and to the fusion of Bt endotoxin with the gene for kanamycin resistance. This allowed them to use kanamycin resistance for selecting plants expressing high levels of the fusion gene product. The levels of Bt endotoxin so produced were sufficient to kill first-instar *Manduca sexta* larvae, and this insecticidal property was heritable. A second report of successful transformation with Bt followed shortly. In August 1987, Fischhoff and co-workers (Monsanto Company[11]) tried the expression of Bt in tomato. For this purpose, a 35 S CaMV promoter was used to direct expression of insecticidal Bt toxin gene. As a result, sufficient protein was produced to kill *Manduca sexta* larvae. The best insecticidal activity was found in plants expressing a truncated Bt endotoxin. Later in 1987, Agracetus Co. reported expression of Bt endotoxin in tobacco,[12] with 35 S CaMV promoter linked to an AMV (alfalfa mosaic virus) leader sequence, giving Bt mRNA enough to be easily detectable on Northern blot analysis. Using immunoblot techniques, they identified a peptide in a resistant plant corresponding in size to that expected for a truncated Bt endotoxin. In these three initial reports, the Bt endotoxin genes chosen generated insecticidal activity against a lepidopteran insect.

Modifications to the bacterial gene sequence of Bt endotoxin to make it more readily expressible in plants have been reported and have been shown to increase concentration up to 500-fold. Consequently, host range includes less sensitive pests such as the tomato fruit worm (*Heliothis zea*) and tomato pin worm (*Keiferia lycopersicella*)[13]. Majority of Bt strain and toxin proteins are active against the caterpillar larvae (lepidopteran insects) while others have been found to be active against dipteran or coleopteran larvae.

The long history of safe use of Bt endotoxin as an insecticide, the relatively narrow range of target insects effected by a single protein toxin, and the strong insecticidal activity of this toxin against the larva of susceptible insect are clear advantages for transferring this insecticidal protein gene into plants using different vectors. In addition, the presence of Bt endotoxin inside a plant should provide a cost-effective strategy for the grower by precluding the need for repeated insecticide spraying in the field and should kill the insect before pests cause any significant crop damage. Another potential advantage would be the expression of insecticidal proteins in internal and underground regions of the plant that are inaccessible by traditional regimes. The advantages of trangenic Bt plants, however, are also accompanied by certain disadvantages: (1) the bacterial gene under consideration might be difficult to express at effective concentration in the entire plant, and (2) insect resistance might develop with time.

Structure of toxin

The insecticidal activity of Bt is associated with a parasporal glycoprotein crystal that is synthesized within the organism during its sporulation cycle. Bt subsp. Kurstaki, berliner, alesti and tolworthi, all produce crystals that are similar to each other structurally, biochemically, immunologically and functionally. The parasporal crystal comprises approximately 20–30% of dry weight of the sporulated culture and consists mainly of protein (~ 95%) and a small amount of carbohydrates (~ 5%) (ref. 13). These four subspecies synthesize bipyramidal crystals (~ 1 μ in size) and usually each bacterium contains only one such crystal. Subspecies israelensis forms crystals of various shapes and each bacterial cell contains two to four crystals 0.1 to 0.5 μm,

varying in shape from cuboidal to bipyramidal, ovoid or amorphous[14–17]. Solubilized crystal proteins from the five subspecies reveal a common electrophoretic profile[18]. Several polypeptides are extractable from these inclusions, one of which, a major protein 26 to 28 kDa is the actual toxin. Chemical formula worked out by Gustafson *et al.*[19] for Bt.t. insecticidal protein from *E. coli* after correction for the presence of water and sodium is $C_{3053}H_{4615}N_{791}O_{928}S_{11}$. 300 H_2O · 50 $NaHCO_3$ and was calculated to 48.27%C; 68.7% H and 14.35% N.

Parasporal crystal contains the full-length 644 residue protoxin as the minor component, and a product of bacterial processing with 57 residues removed from the N-terminus as the major component[19,20]. Delta endotoxin comprises three domains which are, from N- to C-terminus D I, II and III. Domain I, from the N terminus of the 67 K toxin to residue 290, is a seven-helix bundle in which a central helix is completely surrounded by six outer helices tilted at about +20° to it. Domain I is hexaplanal in cross section. Domain II, from residues 291 to 500, contains three antiparallel β sheets packed around a hydrophobic core with a triangular cross section. Domain III, from residues 501 to 644 at the C terminus is a sandwich of two antiparallel β sheets. The entire molecule is wedge shaped with domains I and III at the bulky end of the molecule. Through their contact, one of the two β sheets in domain III is almost entirely buried[21,22] (Figure 1). The core of the endotoxin molecule encompassing the domain interfaces is built from the five sequence blocks that are highly conserved throughout the delta endotoxin family[8]. The high degree of conservation of internal residues implies that homologous proteins would adopt a similar fold.

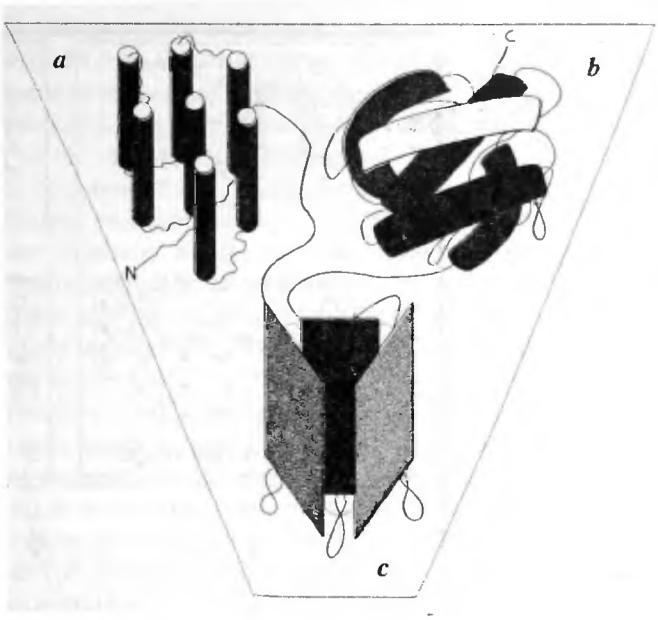

Figure 1. Schematic representation of the beetle toxin depicting domain organization. The three domains are: ***a***, a seven-helix bundle; ***b***, a three-sheet assembly and ***c***, a β sandwich. (Based on structure worked out by Li *et al.*, *Nature*, 1991, 353, 815–821).

Mode of action

As already mentioned, Bt comprises a number of different subspecies, each of which produces a different insect-specific toxin. For example, Bt subsp. *Kurstaki* is toxic to lepidopteran larvae including moths, butterflies, skippers, cabbage worm and spruce budworm. Bt subsp. *israelensis* kills diptera such as mosquitoes and black flies. Bt subsp. *tenebrionis* is effective against Coleoptera such as the potato beetle and the boll weevil[23–28].

When the parasporal crystal is ingested by a target insect, the protoxin is activated within its gut by the combination of alkaline pH (7.5 to 8.0) and the specific digestive proteases, which converts the protoxin into an active toxin with a molecular mass of approximately 65,000–1,60,000 which is proteolytically processed by midgut proteases to yield smaller toxic fragments (5 to 80 kDa)[29,30]. The most widely-accepted model for the mechanism of the toxin insecticidal action involves: binding of the activated toxin to specific receptors on the villi of midgut epthelial cells; insertion and piercing of the toxin into the membrane which results in the formation of an ion channel (Figure 2). This channel disrupts the normal midgut ion flow causing paralysis and death of the insect, which is the most accepted model of the mechanism of action[28,31–35] of the insecticidal toxin.

Domains II and III are helpful in binding to specific receptors on the midgut epithelial cells[36,37]. Domains I and III insert into membrane[38] of the midgut epithelial cell apical. β17 of domain III is involved in the ion channel activity for which it is presumed that domain III may insert into the membrane. Domain I is also responsible in the ion channel function[39]. In general, interdomain interactions influence the capacity of specific receptor binding, mainly determined by the C-terminal toxin domain (II).

Development of Bt resistance in insects

Despite the wide use of Bt over the last 25 years, no case of resistance development in the field has been reported. For a long time, proponents of Bt suggested that microbial insecticides would be free of insect resistance. Concern has been voiced over a large-scale introduction of crops containing monogenic resistance trait which could rapidly lead to the development of resistance within insect populations. Also, transformation of plants with individual toxins would appear to bypass advantages offered by a mixture of toxins normally produced by the bacteria. For instance, under laboratory conditions, significant resistance to Bt was developed by

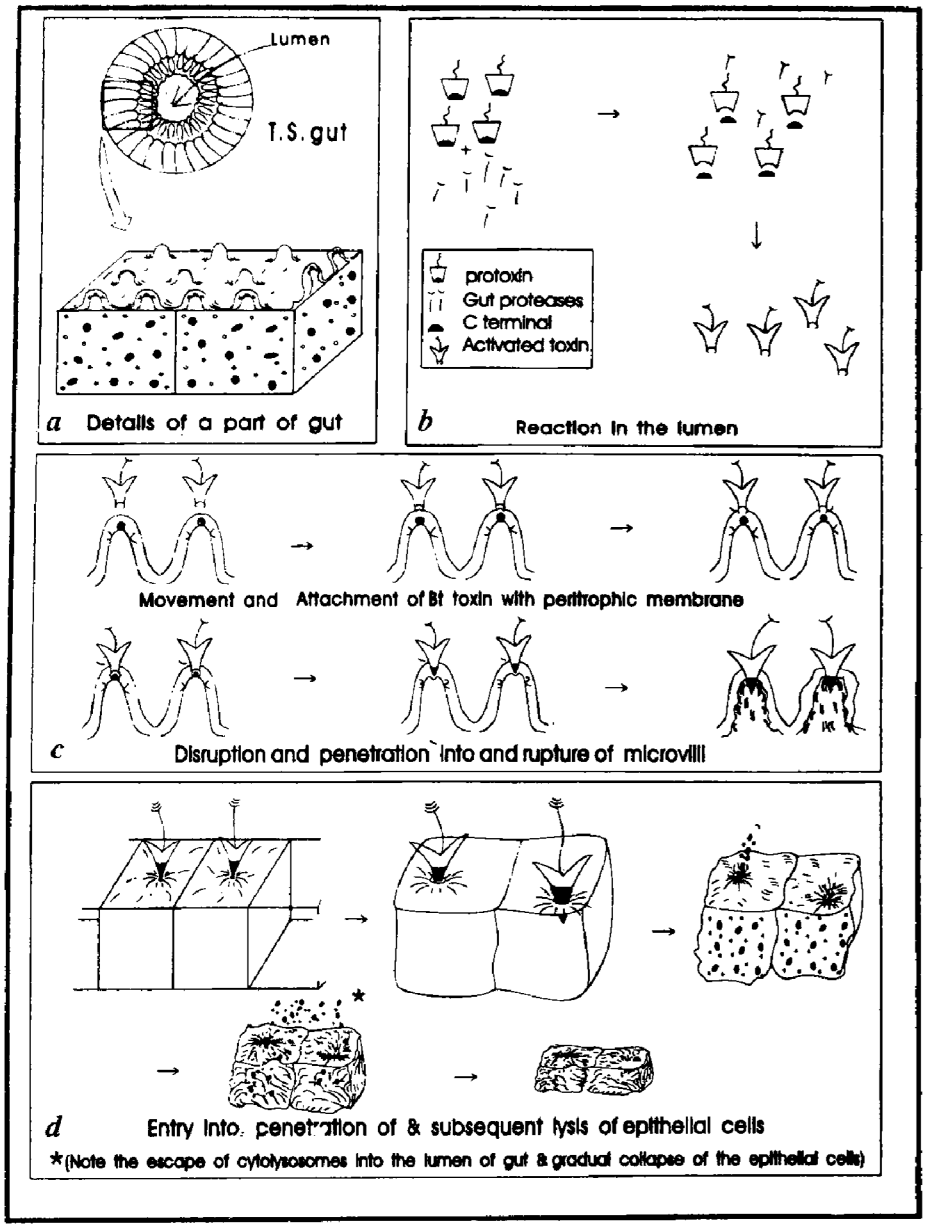

Figure 2. Schematic presentation of the mode of Bt toxin action on the microvilli of insect leading to its death.

Plodia interpunctella strains[40] and a *Heliothis virescens* strain[41]. Moreover, the development of field resistance by *Plutella xylostella* (diamondback moth) to Bt particularly in areas where the moths have been sprayed with high doses of the toxin has brought forth the limitations[42,43]. In *Plodia interpunctella* and *Plutella xylostela*, resistance is partially recessive and apparently due to one or few major loci[40,44,45]. Resistance in *Heliothis virescens* may be partially recessive[46,47], but may involve multiple factors and be inherited as an additive trait. Resistance in *Leptinotarsa decemlineata* is due to one incompletely dominant gene and several genes that interact with it[48]. The mechanism of resistance can be related to disruption of the steps involved in the mode of Bt toxin action such as solubilization, activation of protoxin to toxin, binding with the receptors, and pore formation. The resistance is also often related to a change in receptor binding properties on the brush border membrane vesicles of the insect midgut[42,49–51]. Moreover, the greater presence of the ICPs in transgenic plants as compared to sprays could increase selection pressure and risk of resistance. Experience with chemical insecticides demonstrates that given adequate selection pressure, most insect species will develop some

level of tolerance to a given insecticide[40,48]. The US Environmental Protection Agency (EPA) is now facing a coalition of consumers, environmentalists and organic farmers, who intend to sue EPA for allowing planting of genetically-engineered crops to produce Bt toxin. They claim that commercial planting of the crops threatens to create widespread resistance to Bt toxin, on which the farmers rely heavily[52]. High level expression of toxin genes throughout the life of all the plants of a crop would exert a tremendous pressure on the insect population to evolve a new biotype, resistant to Bt toxin[53].

Avoiding insect adaptation

For the continued success of Bt, we should anticipate problems of insect resistance and develop strategies to check the possibility of resistance arising among crop pests. Some recent reports can provide a basis for devising strategies. First, a correlation between toxicity and binding to specific receptors on the brushborder membrane of the insect's midgut (the target site of ICPs) has been shown for different sets of ICP–insect combinations[7,8,54,55]. For example, Cry IA(b) and Cry IB are both toxic to *Pieris brassicae*, while only Cry IA(b) is active against *Maduca sexta*. Both toxins bind to high affinity receptors of *P. brassicae*, but only Cry IA(b) toxin binds to *M. sexta* membrane receptors. This indicates that receptor binding to a great extent accounts for the spectrum of susceptible insects. This receptor binding is characteristic of each individual type of ICP. Moreover, binding studies have shown that one insect may be susceptible to several different ICPs, depending on the presence of different receptors on the midgut wall. For example, it was demonstrated that Cry IA(b) and Cry IB recognize different receptors in *P. brassicae*. Tobacco and tomato plants were generated which exhibit insect resistance due to introduction of modified *Cry* IA(b) and *Cry* IC genes of Bt. Limited modification at selected regions of the coding sequences of genes is sufficient to obtain resistance against *Spodoptora exigua, H. virescens* and *M. sexta*[1]. These receptor molecules seem to be glycoproteins. Uptil now, seven different Lepidoptera-specific ICP types have been defined, and for each type, at least one amino acid sequence is known. A few attempts have been made to locate the regions responsible for species specificity within the sequence of ICPs. These studies indicate that (at least in the case of the Cry IIA (ref. 56) and the Cry IA(a) (ref. 57) ICP type) the specificity-determining region is located within the C-terminal half of the toxic fragment of ICP. It has been possible to detect apparent correlation between sequence or structural motifs and the binding or toxicity spectrum of these ICPs. Indeed, by performing binding studies with hybrid ICPs, it has been demonstrated that, at least for the Cry ICP type, the polypeptide segment responsible for binding is located within the C-terminus.

Secondly, it has been demonstrated that resistance of *P. interpunctella* strain mentioned above, is correlated with 50-fold reduction in the affinity of an ICP for its membrane receptor[58]. Interestingly, the resistance strain remained sensitive to another ICP type, which was not present in the selecting agent (i.e. Dipel, a commercial formulation of Bt). Surprisingly, the resistant strain exhibited an increased sensitivity to ICP, as compared to the sensitive strain, apparently due to an increased binding capacity. In addition, in this case, the two ICP types recognized different binding sites. It is very unlikely that the only function of the ICP membrane receptor would be to bind ICPs. Therefore, one can assume that they have some (as yet unknown) physiological role in the insect midgut. Possible physiological mechanisms of resistance to Bt endotoxin including a change in gut pH or in enzymes that would effect dissolution and activation, revealed no obvious change while studying resistant *P. interpunctella*[59] and another level of resistance can build up at Bt toxin activation step[60]. It has been observed that *P. interpunctella* have lower *N*-α benzoyl L-arginine *p*-nitroanilide hydrolysing and prototoxin activating compounds than a susceptible or resistant strain. It is interesting therefore, to note the modifications that have occurred in the resistant strain: (i) a reduction in affinity of one ICP receptor, (ii) an increased concentration of another type of ICP receptor and absence of the trypsin-like enzyme in insect midgut. (The second modification had not occurred in a *P. interpunctella* strain that had developed less resistance.) This would have resulted in a change not only in the ICP-binding function but the normal physiological function of the first receptor as well. This loss in function would then be compensated for by an increase in concentration of the other receptor type. Recently, it has been demonstrated that the mechanism of resistance in the field, as in the laboratory selected *P. interpunctella* strain, is due to a change in ICP membrane receptor[41]. The strain had remained sensitive to other ICP types, not present in the Bt variety used in field. These ICPs were shown to bind to other receptors and to have virtually identical binding characteristics in both the sensitive and resistant strains. Thus, the biochemical mechanism of resistance is the same in field resistant *P. xylostella* strain and the laboratory-selected *P. interpunctella* strain. This suggests that such alteration in membrane ICP receptors represents a general mechanism by which insects can develop resistance to Bt. Initial frequency of alleles for resistance to Bt toxin in field population of *H. virescens* was worked out to quantify the risk of insect pests adapting to this ecologically-valuable class of toxins. The field frequency of these alleles was worked out to be 1.5×10^{-3}. This underscores the need for caution in deploying transgenic insect resistance plants in the field to control insect pests. In *Scirpophaga orcertulas* and *Chilo suppresalis* (yellow and striped rice stem borers),

heterologous competition-binding arrays were performed to investigate Bt toxin binding cross reactivity. The results showed that Cry 1Aa and Cry 1Ac recognize the same membrane-binding site, which is different from Cry 2A or Cry 1C-binding site in yellow stem borer or striped stem borer[51,61,62]. This report suggests that development of multitoxin systems in transgenic plants with different toxin combinations ought to be developed. This will be used to the recognizing ability of different binding sites with the receptors on villi of midgut of different insect pests. The multitoxin systems will prove useful in implementing deployment strategies, thereby decreasing the rate of insect resistance against Bt toxin. Understanding the biochemical basis for resistance development in insect population exposed to different Bt formulations will provide far more effective toxin selection.

In conclusion, these results suggest that the use of transgenic plants that express different ICPs and which bind to different receptors having wide range of receptor binding specificity may be a valuable strategy to delay resistance. It is generally accepted that the acquisition of resistance will be slowed down by using mixtures of different insecticides which have different target sites[63,64]. According to theoretical models, the use of insecticide mixtures would be more effective than other tactics such as sequences rotations or mosaics of insecticides[65]. These models are based on the following assumptions:

- Resistance to each pesticide is controlled independently (i.e. there is no cross-resistance), is monogenic and functionally recessive.
- Doubly-resistant individuals are rare.
- Part of the plant population is untreated.
- The pesticides have equal persistence.

Resistance towards the selecting agent (Dipel) or Bt toxin was suggested to be monogenic and is at least partially recessive[3,40]. ICPs are expected to have equal persistence, certainly when expressed in plants. A mixed plant population of susceptible and resistant plants could be provided by sowing a mixture of seeds of the two types.

Whether using trangenic plants with single or a variety of ICP genes, one should try to keep selection pressure as low as possible. Restriction of ICP expression to those plant tissues which are most susceptible to pest damage could decrease selection pressure while still providing adequate protection. In cotton for example, ICP expression might be limited to bolls by using tissue-specific regulatory sequences to express the gene. There is evidence for the anti-feedant properties of ICPs[66]. Hence, insect larvae might migrate to unprotected parts of the plant. In cotton, this would probably not result in significant yield loss, as cotton plants can withstand considerable leaf damage[66]. Another option might be to provide refuge in a temporal fashion by activating ICP gene expression, only at a particular period of the growth season. Evolution of resistance to genetically-engineered crop varieties expressing insecticidal crystal (Cry) proteins of Bt, can be slowed down by minimizing Cry-induced pest mortality and maximizing pest mortality attributable to other causes[67]. Patchworks of Bt-treated and untreated fields can delay the evolution of pesticide resistance, but the untreated refuge fields are likely to sustain heavy damage due to large scale attack by insects[68].

Transgenic plants with fine-tuned ICP expression could become an integral/indispensable part of integrated pest management (IPM) programmes, in which intense pest-control measures are taken only after consideration of economic damage thresholds. These techniques usually reduce insecticidal input and hence selection pressure. For example, cotton cultivars with boll specific ICP expression might need the aid of chemical insecticides when pest densities are very high. As field-scouting programmes to estimate pest densities currently exist in cotton, these cultivars would fit in IPM programmes[69]. A different approach consists of the development of plants that express ICPs only in response to specific damage thresholds. The identification of wound-inducible promoters is an important step in this direction. The alternative is to deny potential risks for resistance development and to hope that by the time ICP genes are 'lost' to insects, new resistance factors will be available. It is not known how long it will take to expand the assortment, however. Over the next ten years we will go for field tests of genetically-engineered crops for insect control. Pest management strategies will be developed to incorporate a new insect control agent, the plant itself[70].

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