The Risk of Resistance Evolution in Insects to Transgenic Insecticidal Crops

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Abstract

The evolution of resistance in target pests to transgenic insecticidal crops is a significant environmental risk. Resistance is the phenotype of an individual that gives the individual the ability to survive on a transgenic insecticidal plant from egg to adult and produce viable offspring. The goal of insect resistance management (IRM) is to delay or prevent the occurrence of control failures from resistance by delaying or preventing the evolution of resistance. A practicable IRM strategy is necessary to attain this goal, which means that the costs associated with implementing IRM should also be considered. In addition, because of the uncertainty in IRM strategies, it is essential to allow the IRM strategy to be changed as new information becomes available.

It is widely agreed that resistance evolution can be successfully managed. The simplest approach is to reduce selection pressure by maintaining refuge habitats. The high-dose/refuge strategy is by far the most widely considered and used. This strategy requires that the transgenic insecticidal crop produces a sufficiently high toxin concentration that the resistance allele is rendered recessive, and that a host plant other than the transgenic insecticidal crop is growing nearby as a refuge for the target pest or pests. The strategy works by reducing the selection pressure favouring the resistance alleles. This is done by having a larger refuge and a higher dose. The larger the refuge, the smaller the proportion of the population exposed to selection. The higher the dose, the smaller the fitness advantage of the resistant/susceptible heterozygote over the susceptible homozygote in the transgenic field. A third and quantitatively smaller effect is caused by the mingling and mating between individuals from transgenic and refuge fields, which reduces the rate of formation of resistant homozygote offspring.

IRM strategies begin with resistance risk assessment to identify the pest species most at risk. Resistance monitoring is essential to track the progress of resistance evolution and to determine the success of the IRM strategy. Phenotypic monitoring methods are best suited for low-dose events and genic methods are best suited for high-dose events. Resistance risks are real, but they can be managed.

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Riassunto

Un significativo rischio ambientale è rappresentato dallo sviluppo della resistenza alle colture transgeniche ad azione pesticida negli insetti bersaglio. Tale resistenza è data dal fenotipo di un individuo che gli conferisce la capacità di sopravvivere, dalla fase uovo a quella adulta, su una pianta transgenica ad azione pesticida e di produrre progenie fertile.

Lo scopo delle strategie di gestione della resistenza (insect resistance management, IRM) è di ritardare o prevenire la possibilità che il controllo venga meno cercando di ritardarne o prevenirne l'evoluzione. Per raggiungere questo obiettivo è necessaria una stategia IRM praticabile, il che significa che devono essere considerati anche i costi associati alla sua adozione. In aggiunta, a causa dell'incertezza insita in questo tipo di tecniche, è essenziale consentirne la modifica non appena nuove informazioni si rendano disponibili.

È largamente accettato il concetto che lo sviluppo della resistenza possa essere gestito con successo. L'approccio più semplice è di ridurre la pressione di selezione mantenendo degli habitat rifugio. La strategia che prevede l'impiego di alti dosaggi e di aree rifugio è quella più ampiamente considerata e usata. Questa tecnica richiede, da parte della pianta transgenica insetticida, la produzione di una quantità di tossina sufficientemente alta da fare in modo che l'allele della resistenza sia reso recessivo, e che un pianta ospite diversa dalla pianta transgenica insetticida venga fatta crescere nelle sue vicinanze come rifugio per l'insetto bersaglio o gli altri insetti. La tecnica funziona riducendo la pressione di selezione che favorisce l'allele della resistenza. Questo è ottenuto adottando un'ampia area rifugio e un alto dosaggio. Più ampio è il rifugio, più piccola è la porzione della popolazione esposta alla selezione. Più alta è la dose, minore è il vantaggio nell'adattamento dell'eterozigote resistente/sensibile rispetto all'omozigote sensibile nel campo transgenico. Un terzo e quantitativamente più piccolo effetto è causato dal mescolamento e dall'accoppiamento tra individui proveneinti dal campo transgenico e dall'area rifugio, fattore che riduce il rapporto nella formazione di progenie omozigote resistente.

Le tecniche IRM hanno inizio con la valutazione del rischio di resistenza, per identificare le specie più a rischio. Il monitoraggio della resistenza è essenziale per tracciare la progressione dell'evoluzione della resistenza e per determinare il successo della strategia IRM. Metodi di monitoraggio fenotipico sono più adatti per eventi a basso dosaggio, mentre metodi genici meglio si adattano per eventi ad alto dosaggio. I rischi di resistenza sono reali, ma possono essere gestiti.

1. INTRODUCTION

Many kinds of transgenic crops have been or are being considered for commercial use. The crops that have been commercialised include herbicide tolerant crops, insect resistant crops, virus resistant crops, crops producing chemicals for use in industrial applications, and crops producing pharmacologically-active compounds. In the future, a greater diversity of chemicals will likely be produced by crops, and crops producing vitamins, drought-tolerant crops, other stress-tolerant crops, and many others may become commercialised.

Of these crops, it is likely that all insect resistance crops will require resistance management to maintain their usefulness into the future. In addition, herbicide tolerant crops and virus resistant crops will also need some degree of resistance management. In the United States of America (USA), resistance management is not conducted on either herbicide-tolerant or virus resistant crops. Herbicide tolerance is considered a passive trait in the USA that does not exert direct selection for resistant weeds by itself. The herbicides applied to the herbicide tolerant crop are considered the selection agent, so according to this reasoning, the resistance management should be associated with the registration of the herbicide, which is not conducted in the USA. In the European Union (EU), by contrast, resistance management for herbicide tolerant crops is considered. The herbicide tolerant crop is considered an indirect agent of selection, and can be regulated to provide effective weed resistance management. This review will not address weed resistance management associated with herbicide tolerant crops. Resistance to the herbicide glyphosate (RoundUp®) has begun to be reported in herbicide tolerant soya bean in the USA and Argentina, so this is becoming a significant problem. Transgenic virus resistant crops have been exempted from most environmental regulatory oversight in the USA. There has been virtually no scientific consideration of resistance management for these virus resistant crops anywhere in the world.

This review will focus only on transgenic insect resistant crops. Since the mid-1990s when these crops were first commercialised in the USA and Canada, resistance risks were considered, so that by the late 1990s, mandatory risk management for resistance risk was a required part of the registration of these crops for commercial use.

This review provides the present state of resistance risk assessment and management, with a look at some of the challenges facing developing countries. It begins by examining the adverse effects of resistance and the social justification for a focus on resistance risk analysis and showing that most of the transgenic insecticidal crops now available are so-called '*Bt*' crops, in which the transgene is derived from the bacterium *Bacillus thuringiensis*. Resistance is defined and methods for identifying resistant individuals are reviewed, and the goals and experiences of insect resistance management (IRM) are described. Four different approaches to IRM are discussed and the high-dose/refuge strategy is defined, its assumptions clarified, and the keys to its success are specified. The definition of dose has been commonly misunderstood, so it is described in detail to dispel some of this confusion. IRM planning begins with resistance risk assessment, and a simple method for resistance risk assessment is presented and illustrated with an example from Viet Nam. In the past few years and increasingly in the future, new transgenic insecticidal crops will have multiple toxins against particular target pests. These are called pyramided transgenic traits, and they present some challenges that will become increasingly complex in the future. This review closes with a discussion on resistance monitoring. Because most transgenic insecticidal genes are expected to be high-dose events, new monitoring methods have been required. The reasons for this and the kinds of methods now available are described. Resistance risks are significant, but IRM can be used to manage these risks.

2. ADVERSE EFFECTS OF RESISTANCE

Resistance in insects to pest control is a serious problem worldwide. Adverse effects from resistance include: resistance is common and costly to society, farmers and companies that sell insecticides and transgenic crops; resistance can lead to increased insecticide use, and may compromise other pest-control products; and resistance destabilises pest control and pesticide regulation.

Although resistance problems have been known for nearly 100 years, resistance became a significant agricultural problem after World War II (Figure 1), when modern, intensive agricultural technologies proliferated. Resistance has occurred quickly when there has been strong, uniform selection on a pest population for sufficiently long periods of time over

spatially extensive areas. Modern intensive agriculture, with its reliance on pesticides, monoculture and uniform production practices has provided these conditions, and resistance has proliferated. It took most of the 20th century before an entomological consensus was reached about the seriousness of the problem (NRC, 1986). Whalon *et al*. (2008) now report 7470 cases of resistance in insects to particular pesticidal products. Using these data, 16 species of arthropods account for 3237 (43 %) of these cases (Table 1). These include three mites, a cockroach, two aphids, a whitefly, two beetles, three Lepidoptera, three mosquitoes and the housefly. Resistance to *Bt* toxins has been documented in 17 insect species (Tabashnik, 1994; Huang *et al*., 1999), so it is now widely assumed that resistance to transgenic insecticidal crops, such as *Bt* maize and *Bt* cotton can occur.

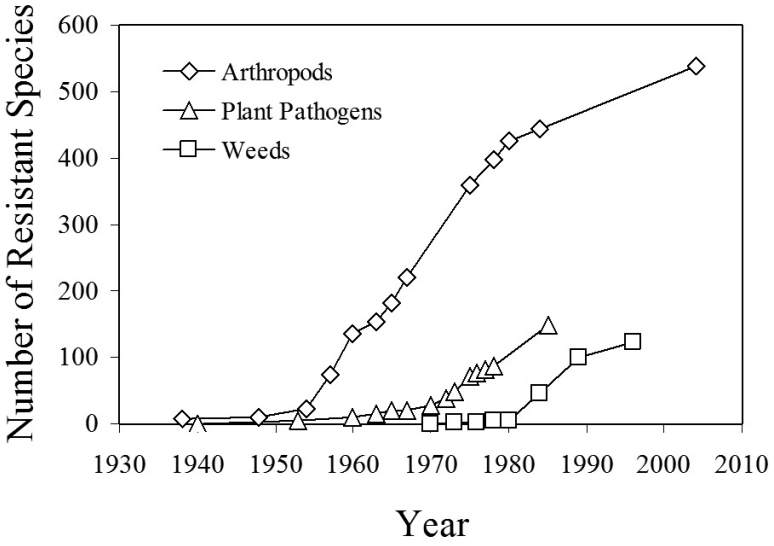

Figure 1. Number of species resistant to agricultural pest control chemicals. Source of data: Georghiou, 1986; Holt and Labaron, 1990; Heap, 1997; Whalon 2008.

Table 1. Species with the highest reported number of cases of resistance (Whalon *et al.*, 2008; http://www.pesticideresistance.org/DB/index.html; cited February 2008)

Species	Family-Order	Common name	Cases
Helicoverpa armigera	Noctuidae-Lepidoptera	Cotton bollworm	435
Tetranychus urticae	Tetranychidae-Acari	Two-spotted spider mite	327
Myzus persicae	Aphididae-Homoptera	Green peach aphid	293
Plutella xylostella	Plutellidae-Lepidoptera	Diamondback moth	278
Culex quinquefasciatus	Culicidae-Diptera	Southern house mosquito	229
Blattella germanica	Blattellidae-Orthoptera	German cockroach	213
Aedes aegypti	Culicidae-Diptera	Yellow fever mosquito	196
Musca domestica	Muscidae-Diptera	House fly	183
Panonychus ulmi	Tetranychidae-Acari	European red mite	178
Leptinotarsa decemlineata	Chrysomelidae-Coleoptera	Colorado potato beetle	175
Bemisia tabaci	Aleyrodidae-Homoptera	Sweet potato whitefly	167
Boophilus microplus	Ixodidae-Acari	Southern cattle tick	127
Culex pipiens pipiens	Culicidae-Diptera	House mosquito	119
Tribolium castaneum	Tenebrionidae-Coleoptera	Red flour beetle	108
Heliothis virescens	Noctuidae-Lepidoptera	Tobacco budworm	106
Aphis gossypii	Aphididae-Homoptera	Melon and cotton aphid	103

In the United States of America alone, the social cost of resistance insects has been about US$133 million annually in extra insecticide applications, measured in 1980 dollars (Pimentel *et al.*, 1980). Unexpected yield losses from resistance have not been estimated, but are likely to be a similar order of magnitude. For some pests, such as Colorado potato beetle (*Leptinotarsa decimlineata*) and diamondback moth (*Plutella xylostella*), resistance is so extensive that few effective pest control alternatives remain. In northeastern Mexico and the Lower Rio Grande of Texas, resistance to insecticides evolved in tobacco budworm (*Heliothis virescens*), a pest of cotton, in early 1970. This caused about 700,000 acres of cotton to be lost (Adkisson, 1971; 1972), devastating

many local communities, some of which have never recovered.

Effective resistance management will allow farmers to use a transgenic insecticidal crop for a long period of time. For example, *Bt* maize can provide yield benefits to farmers up to between 7 and 18 bushels/acre in the northern maize belt of the USA (Rice and Pilcher, 1998). Its cost is typically an additional US$10/acre, so *Bt* maize can net a farmer US$4-26/acre even at very low maize prices. Now with increased demand for maize to produce fuel alcohol in the USA, net gains can be as high as US$11-62/acre. Loss of this income because of resistance evolution could have significant detrimental effects on farm families. *Bt* maize, however, is not without risk to farmers. If there is little insect pest damage, there may be no yield increase, and the farmer can lose the US$10/acre paid for the *Bt* maize seed. Most USA maize receives no insecticide applications, but on the small amount that does, the potential benefits from using *Bt* maize are less certain. In much of this "high-insecticide" use area, spider mites, a leaf-feeding pest, are a problem and miticides are commonly applied. *Bt* maize does not control these mites, but the miticides do control the main target pests controlled by *Bt* maize. Consequently, it is not yet clear if farmers in these regions will receive substantial benefits from the use of *Bt* maize and if insecticide use in the region will decline.

Needless to say, resistance management is also beneficial to the companies that sell transgenic insecticidal crops. For example, the maize seed market is a highly competitive, ~US$4 billion a year market in the USA, and now that most major seed companies are selling *Bt* maize hybrids, prolonging the life of this product will enable the companies to make additional profits. Indeed, seed companies have been able to use *Bt* maize to increase their share of the maize seed market. A shift of only 1 % is equal to US$40 million/year and is a substantial gain to the company. If profits of seed companies were the only reason for resistance management, there would be little need for society to intervene to ensure that effective resistance management occurs. The major beneficiaries of the use of transgenic insecticidal crops would be the major beneficiaries of resistance management, and they would suffer the costs of poor stewardship and resistance failures. However, seed companies are only one of the many stakeholders, and their concerns do not fully match the concerns of the other important stakeholders, including consumers and farmers, hence the need to regulate resistance management.

Several other reasons have compelled society to take an active role in ensuring

that effective resistance management is implemented. First, there are other farmers who depend on *Bt*-based insecticides and do not or will not use transgenic insecticidal *Bt* crops. For example, under present guidelines, *Bt* sprays, but not *Bt* crops, can be used as part of organic agricultural production. If pests evolved resistance to a *Bt* crop, the other *Bt*-based insecticides would likely become ineffective against those pests, and organic farmers would suffer higher pest control costs. In some countries, such as Brazil, *Bt* insecticides are widely used in conventional agricultural production. In these countries, the evolution of resistance could harm other conventional farmers. In other words, farmers who experience no benefit from a transgenic insecticidal crop might have to pay the costs of poor stewardship by others. Resistance management helps protect the interests of those farmers who do not use a transgenic insecticidal *Bt* crop but who rely on *Bt* insecticides.

In addition, resistance management of transgenic insecticidal *Bt* crops is important because it preserves a pest control method that results in less harm to the environment and human health than many other insecticides. *Bt*-based pest control has several significant advantages over traditional synthetic insecticides. *Bt* toxins have a narrow range of non-target species effects, very low mammalian toxicity, and no record of carcinogenicity. Loss of *Bt*-based controls because of the evolution of resistance would probably increase the use of insecticides that are more harmful to the environment or human health.

Finally, effective resistance management can help stabilise pest control in the future. For example, the USA Environmental Protection Agency (EPA) registers pesticides only after in-depth risk assessment and review, but unregistered pesticides can be used under emergency exemptions with very little review. Use of unregistered pesticides under emergency exemptions may cause unanticipated environmental or human health risks. During 1991-1994, about 30 % of all emergency exemptions requests in the USA were made, at least in part, because of resistance (Matten *et al.*, 1996). With effective resistance management, the need for emergency exemptions could be significantly reduced.

3. KINDS OF TRANSGENIC INSECTICIDAL CROPS

Many insecticidal transgenic crops have been experimentally developed, but most of the commercially available transgenic insect resistant crops are *Bt* crops, either in maize or cotton. *Bt* crops contain a gene that has

insecticidal properties from the soil bacterium, *Bacillus thuringiensis*, from which they derive their *Bt* moniker. These genes produce proteins that fall into one of several classes: Cry proteins, which are the most widely used (**cry**stal proteins); Vip proteins, which are being developed for commercial use (**v**egetative **i**nsecticidal **p**roteins); and several others, which are not yet close to commercial use. There are a wide variety of Cry proteins, with over 40 major classes, and hundreds of subclasses, and each has its own unique spectrum of activity against insects. A relatively small number of these have been used in transgenic crops.

Cry toxins kill insects by a complex process. After ingestion, the crystals must dissolve in the insect midgut. This occurs readily when the pH of the midgut is alkaline, but occurs hardly at all under acidic conditions. In the presence of certain enzymes, the crystal releases a 130-135 kDa biologically inactive protoxin of Cry1Ab or Cry1Ac. In a series of poorly understood reactions, this protoxin is processed by proteolytic enzymes to yield a 65 kDa activated toxin that can bind to receptors on the midgut epithelium. All of the commercialised *Bt* crops using Cry toxins produce a soluble, activated Cry toxin, circumventing all but the final step of this process, because these activated toxins bind directly to the receptors in the insect midgut. The receptor-toxin complex somehow induces pore formation in the midgut wall, lysis of the midgut, septicemia, and rapid death of the insect. In short, the insect dies of stomach ulcers. Vip toxins act by binding to other receptors on the insect midgut epithelium, but less is known about the mode of action for Vip toxins than Cry toxins.

In addition to these *Bt* proteins, a few proteinase inhibitors have been commercialised or are nearing commercialisation. These include cowpea trypsin inhibitor (CpTI), which has been used in several Chinese transgenic cottons. The proteinase inhibitors act by inhibiting protein digestion, which results in starvation and death to the insect.

Some of these transgenic events are combined together in a single variety. When the two transgenes are targeted against the same pest, and each is toxic by itself, the variety is "pyramided". When the two transgenes are unrelated and not both targeted against the same pest, the variety is "stacked".

3.1. Maize

Two kinds of *Bt* maize varieties have been commercialised. The first has Cry toxins targeting larvae of certain pest moths. These include two kinds

of Cry1Ab, Cry1F, Cry1Ac, and Cry9C. Of these, only the Cry1Ab and Cry1F events are still commercially available. Since 2003, varieties targeting root-feeding Chrysomelid beetles have been commercialised, using Cry3Bb or the binary toxin Cry34Ab and Cry35Ab. The binary toxin requires both proteins to be toxic, and neither is toxic by itself. It is therefore quite different from "pyramided" varieties. In many cases, *Bt* maize events are stacked with herbicide tolerant traits. Also very common are varieties stacked with both *Bt* resistance to a moth pest and *Bt* resistance to the Chrysomelid beetle pest. More recently, some true pyramided varieties are being considered for commercial use.

Typically, the different events result in phenotypic differences in expression of the Cry toxins (Table 2). Bt11 (OECD unique identifier SYN-BTØ11-1) and MON810 (Yieldgard™ corn; OECD unique identifier MON-ØØ81Ø-6) are virtually the same transformation event, but have different levels of *cry1Ab* expression. Bt11 has higher expression in the grain, and MON810 has higher expression in the leaves. Event 176 (OECD unique identifier SYN-EV176-9) also uses *cry1Ab*, but because it relies on different promoters, expression of Cry1Ab toxin is lower in grain and the whole plant and much higher in pollen than Bt11 and MON810. DBT418 (OECD unique identifier DKB-89614-9) expresses Cry1Ac toxin, which is not as toxic as Cry1Ab toxin to the major pests of maize in the USA, and also expresses this toxin at a low concentration. Both Event 176 and DBT418 had substantial declines in expression during the maturation period of maize, which made them both susceptible to pest damage during an important period of maize growth, and increases resistance risk, as discussed later. CBH-351 (Starlink™ corn; OECD unique identifier ACS-ZMØØ4-3) expresses Cry9C, which is more toxic than Cry1Ab against the main pests in the USA. These variations have important implications for resistance management. In a later section, the concept of dose as a key component in resistance management is introduced. It is critical to note that dose ≠ concentration. A transgene expressing a higher concentration of Cry toxin is not necessarily also expressing a higher dose!

Bt maize is presently grown in Argentina, Canada, the Philippines, Spain, South Africa and the USA. The target pests differ in different regions in the world (Table 3). The Lepidopteran pests targeted by Cry1Ab and Cry1F are all members of the families Crambidae and Noctuidae. These two Cry toxins also kill many other species of Lepidoptera, including some valued butterfly species, such as monarch butterfly, *Daneus plexippus*. The Coleopteran pests

targeted by Cry3Bb and Cry34/35 are all in the tribe Diabroticini. These Cry toxins appear to be more narrowly targeted, as they have no detectable effect on several beneficial Coleoptera in the ladybird beetle and ground beetle families (Coccinellidae and Carabidae).

Table 2. Expression of Cry toxin in *Bt* maize plants. Data summarised in Andow (2001) from numerous sources and USA EPA (2005b; 2007). All values are expressed per fresh tissue weight unless otherwise noted. [1] Not available (NA). [2] Dry weight basis (DW). [3] Not detectable, below the detection limit of available methodology (ND).

Event	Bt protein	Grain (µg/g)	Leaf (µg/g)	Pollen (µg/g)	Pith (µg/g)	Root (µg/g)	Whole plant (µg/g)
Event 176	Cry1Ab	<5	4.4	7.1	NA[1]	NA	0.6
BT11	Cry1Ab	1.4	3.3	<0.09 DW[2]	NA	2.2-37.0	6.3
MON810	Cry1Ab	0.19-0.39	10.34	<0.09 DW	NA	NA	4.65
CBH 351	Cry9C	18.6	44	0.24	2.8	25.87	250
DBT 418	Cry1Ac	43	1.2	ND[3]	NA	NA	0.15-1.0
TC1507	Cry1F						
MON863	Cry3Bb	49-86	30-93	30-93	NA	3.2-66	13-54
DAS-59122-7	Cry34Ab	50 DW	50-220 DW	74 DW	33 DW	37-50 DW	32-77 DW
DAS-59122-7	Cry35Ab	1 DW	41-85 DW	0.02 DW	10 DW	3-8 DW	7-14 DW

3.2. Cotton

All of the commercialised *Bt* cottons have been targeted to control pest moths, especially those that feed on the developing boll, and utilise several *Bt* toxins, usually in tandem, including two kinds of Cry1Ac toxins, synthetic Cry1A and Cry1F toxins, and the Cry2Ab and Vip3A toxins. All of these *Bt* cottons are still commercially available. *Bt* cotton varieties have become complicated in some parts of the world, as the transgenes appear to have been deliberately introgressed into native germplasm resulting in "unofficial" *Bt* cotton varieties. This seems to be common in both China and India. In addition, stacking of traits has proceeded much further in cotton than any other crop. Presently, *cry1Ac* is stacked with *cry2Ab*, and *cry1F* is stacked with *cry1Ac*. In Australia, the single gene varieties are no longer registered and only stacked varieties are allowed.

Table 3. Main target pests or potential target pests of *Bt* maize. NA = not available in the country.

Bt protein	USA & Canada	Spain	The Philippines	South Africa	Argentina
Cry1Ab	*Ostrinia nubilalis, Diatraea grandiosella, Helicoverpa zea, Diatraea saccharalis*	*O. nubilalis, Sesamia nonagriodes*	*O. furnacalis*	*Chilo partellus, Sesamia calamistis, Buseola fusca, Eldana saccharina*	*D. saccharalis*
Cry1F	*O. nubilalis, D. grandiosella, H. zea, D. saccharalis, Spodoptera frugiperda*	*O. nubilalis, S. nonagriodes*	NA	NA	*D. saccharalis, S. frugiperda*
Cry3Bb	*Diabrotica virgifera, D. barberi, D. mexicana*	NA	NA	NA	NA
Cry34Ab/ Cry35Ab	*D. virgifera, D. barberi, D. mexicana*	NA	NA	NA	NA

As discussed below, this decision was made largely to reduce the risk of resistance evolution in *Helicoverpa armigera*, the key pest of cotton in Australia.

As was true in *Bt* maize, the different *Bt* cotton events result in phenotypic differences in expression of the Cry toxins (Table 4). Concentrations of toxin in pollen, leaf, root and seed tissues may vary by two orders of magnitude in the different events. Concentrations in flowers and bolls are less variable, and toxin has generally not been found in cotton nectar. The cry1Ac events show a significant decline in expression during boll maturation, while the other events do not. This decline increases resistance risk and will be discussed later, to reinforce the point that dose ≠ concentration. Although there are three kinds of *vip3A* cotton (COT102 [OECD unique identifier SYN-IR1Ø2-7], COT202 and COT203), with the latter two appearing to be more suitable commercial events, very little information is available on expression levels in COT202 or COT203. The COT202 and COT203 events contain only the insect resistance gene, *vip3A*, but under the control of a different promoter than that in COT102, which also contains an antibiotic resistance gene.

Table 4. Expression of Cry toxin in *Bt* cotton plants. Data summarised in more detail in Tran *et al*., (2008) from multiple sources. All values are expressed per fresh tissue weight unless otherwise noted. Abbreviations are the same as in Table 2. Data were not available for CpTI.

Event	Bt protein	Flower (µg/g)	Leaf (µg/g)	Pollen (µg/g)	Nectar (µg/g)	Boll (µg/g)	Root (µg/g)	Seed (µg/g)
MON531	Cry1Ac	2.2-3.1	0.3-5	0.012	ND	17 DW	0.2-43 DW	0.49-4.3
15985	Cry2Ab	8.4-26.2	5.5-40.1	ND	ND	6.4-22.9	NA	43.2
COT 102	Vip3A	NA	3-22	1.1 DW	ND	0.3-1.9	0.2-2	2-4 DW
Cry1A (Chinese)	Cry1A	0.2-0.8	0.06-2.3	NA	NA	0.1-0.4	1.12-1.33	NA
281-24-236	Cry1A	1.6-6.5 DW	5.3-18.8 DW	0.06-0.7	<0.05	1.4-7.6 DW	0.36-1.6 DW	4.13-7.5
3006-210-23	Cry1Ac	0.9-2.2 DW	1.31-1.92 DW	1.45	ND	0.33-0.75 DW	0.05-0.2 DW	0.55-0.57

Bt cotton is grown in many parts of the world, including Argentina, Australia, Brazil China, India, South Africa and the USA. Although the target pests differ in different parts of the world, there are some strong similarities among the species (Table 5). In all parts of the world with extensive cotton production, there is a *Helicoverpa* or *Heliothis* species that is a key pest. These are sister genera in the tribe Heliothinini in the family Noctuidae [Lepidoptera]. In addition, *Pectinophora gossypiella* {Gelichiidae: Lepidoptera] is another common pest that occurs worldwide. Of course there is some regional differentiation of the cotton pest fauna and some transformation events control a wider range of species than others (e.g., *Spodoptera* and *Agrotis* {Noctuidae: Lepidoptera]). Field trials of Vip3A cotton have shown that it provides effective control of *Helicoverpa armigera* in Australia (Llewellyn *et al*., 2007) and *Heliothis virescens* in the USA (Cloud *et al*., 2004). Field trials with Cry1F + Cry1Ac cotton found that it can provide effective control of tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*) and pink bollworm (*Pectinophora gossypiella*) in the USA (Haile *et al*., 2004).

Table 5. Main target pests or potential target pests of *Bt* cotton. NA = not available in the country.

Bt protein	USA	China	India	Australia	Argentina	Brazil	South Africa
Cry1Ac	*Heliothis virescens, Helicoverpa zea, Pectinophora gossypiella*	*Helicoverpa armigera, P. gossypiella*	*H. armigera, P. gossypiella, Earias vittella*	NA	*Helicoverpa gelotopoeon, H. zea, H. virescens, Alabama argillacea, P. gossypiella*	*H. armigera, A. argillacea, P. gossypiella*	*H. armigera, P. gossypiella*
Cry1Ac + Cry2Ab	*H. virescens, H. zea, P. gossypiella, Spodoptera spp., Pseudoplusia includens*	NA	NA	*H. armigera, E. cupreoviridis, E. vitella, Anomis flava*	NA	NA	NA
Vip3A	NA	*H. armigera, P. gossypiella, Ostrinia furnacalis, Earias vitella , E. cupreoviridis, E. insulana, Anomis flava, Sylepta derogata*	NA	NA	NA	NA	NA
Cry1F + Cry1Ac	*H. virescens, H. zea, P. gossypiella, P. includens, Trichoplusia ni, Spodoptera spp., Agrotis ipsilon*	NA	NA	*H. armigera*	NA	NA	NA

3.3. Other Crops

Bt rice, *Bt* soya bean, and a few other *Bt* crops have been developed, but not yet commercialised. Both *Bt* rice and *Bt* soya bean are targeted against Lepidopteran pests, stem borers of rice and pod borers of soya bean. In addition, *Bt* poplar has been developed against several Chrysomelid beetle pests. *Bt* potato was commercially available for several years in the USA until it was withdrawn from the market. This was based on *cry3Ab* and was targeted against Colorado potato beetle (*Leptinotarsa decimlineata*). It was withdrawn because processors would not purchase it. Many other *Bt* crops have been made, but most are not near commercialisation and many have never been intended for commercial use. These include *Bt* oilseed rape, *Bt* broccoli and many others. *Bt* eggplant (also known as aubergine or brinjal) may be commercialised soon in India.

4. DEFINITION OF RESISTANCE

Resistance is caused by genes in the target insect that reduce susceptibility to a toxin, and is a trait of an **individual**. Resistance is defined as a phenotype of an individual that can survive on the transgenic insecticidal plant from egg to adult and produce viable offspring. For *Bt* crops, this means that an individual must grow and mature feeding only on the *Bt* crop, and then mate and produce viable offspring. There is much confusion in the scientific literature over the definition of resistance. However, from a genetic or an evolutionary perspective, it is essential to define resistance as a trait of an individual. A consequence of this definition is that if only one individual in a population is resistant, the population contains resistance.

Often researchers will use the term "tolerance" instead of resistance. There are several reasonable definitions of tolerance, but some of them overlap strongly with the definition of resistance and lead to confusion. In this paper, a "tolerant" individual is one that is not resistant, but has the ability to grow on toxin concentrations that are higher that that possible for a typical individual. This definition can be made more precise and quantitative, but the definition is intended to enable identification of "partial resistance" – individuals that survive better than susceptible individuals, but are not fully resistant.

Much of the confusion with the term "resistance" stems from the fact that it is used to describe a characteristic of a population. Specifically, it is used to describe a field population with enough resistant individuals to cause economic damage to the target crop. However, it is confusing and illogical to use the same term to describe both individuals and populations. Hence, it is necessary to have a term to describe such a field population, and that term is **control failure from resistance** (aka field resistance). An operational definition of control failure from resistance is necessary so that we know what we want to avoid during resistance management and we know when to admit failure and move on. A control failure from resistance occurs when the pest causes significant economic damage to the crop. There are several reasonable operational definitions. For example, a control failure could be defined as occurring when the pest causes detectable economic damage to the crop, when the pest causes economic damage that is similar to that caused by susceptible insects on a non-resistant crop variety, or when the economic damage is considered unacceptable to the grower.

It will often happen that resistance is not yet known in a target species at the pre-release stage of development of the transgenic crop. Thus, it may be important to define resistance operationally, so that resistance can be looked for in advance. This is discussed from a methodological perspective later in Section 6. For a variety of logistical reasons, it may be difficult to evaluate every individual from egg to adult on plants growing in the field. Instead, it may be necessary to use *Bt* plant tissues from the field or a glasshouse in laboratory assays (e.g., Huang *et al.*, 2007). In some cases, it may be necessary to use chemically purified Cry protein toxin, such as might occur if it is difficult to use tissues from whole plants. For example, root tissue may be difficult to collect, and excised root tissue may deteriorate quickly. In addition, the seed company marketing the *Bt* crop may not allow use of the *Bt* plant tissue for identifying resistance. This can happen if local patent law gives the company the right to disallow such research work. In either event, it may be necessary to conduct considerable research to identify a method for identifying resistant individuals.

5. RESISTANCE EVOLUTION CAN BE MANAGED

5.1. Goal of Insect Resistance Management (IRM)

The goal of insect resistance management (IRM) is to delay or prevent the occurrence of control failures from resistance by delaying or preventing the evolution of resistance. A practicable IRM strategy is necessary to attain this goal. This means that the IRM strategy should not place undue burdens on farmers and other parties who will implement the strategy, or such burdens should be at least partially offset by implementation incentives. In other words, the costs associated with implementing IRM must be considered in setting the IRM strategy.

Although preventing resistance and control failures would seem the more sustainable goal, prevention requires active management or evolutionary selection pressures against resistance alleles in a population (Gould and Tabashnik, 1998; Andow and Hutchison, 1998). Although some such management measures have been implemented in IRM for *Bt* cotton in Australia and *Bt* sweet maize in the USA, the efficacy of these measures in preventing resistance has not been evaluated. For both crops, *Bt* crop residues are required to be destroyed after harvest. Because these residues are more likely to harbour resistant insects, residue destruction selects against resistance. Without a substantial cost of resistance, either

via management or genetics and physiology, it is not possible to prevent resistance and control failures. Until all relevant resistance alleles are discovered and the cost of each is quantified, it is foolhardy to presume that resistance and control failures can be prevented. Therefore, IRM starts by aiming to delay resistance far enough into the future.

IRM strategies can be broadly characterised as either responsive or pre-emptive. Responsive strategies react to the occurrence of control failures from resistance, while pre-emptive strategies attempt to avoid or delay resistance before a field failure occurs (Brown, 1981; Dennehy, 1987; Sawicki and Denholm, 1987). Historically, most IRM strategies for insecticides have been responsive, although some have become pre-emptive in recent years. All IRM strategies for transgenic *Bt* crops have been pre-emptive strategies.

5.2. Adaptive IRM

Under adaptive IRM it is possible to change IRM strategies and tactics as new information and experience becomes available (Andow and Ives, 2002). Although it has been emphasised that IRM strategies must be dynamic and adaptive (Forrester, 1990), static IRM is more common, where there is no planned process to change it. Based on the Australian experiences in cotton, increasingly pointed pleas for adaptive resistance management have been made (Forrester, 1990; Denholm and Rowland, 1992; Forrester *et al.*, 1993; Forrester and Bird, 1996). An adaptive strategy relies on an effective and sensitive resistance monitoring system, which remains a significant constraint on implementing adaptive IRM strategies (NRC, 1986; Denholm, 1990; Sawicki, 1996).

In many cases, IRM plans for transgenic insecticidal crops are developed before resistance has been discovered and before much of the important evolutionary and implementation data have been collected. This means that there is often considerable uncertainty associated with these initial plans; they may be insufficient to guard against control failures from resistance, or they may be too restrictive. For example, IRM for *Bt* cotton in southeast USA has been adaptive IRM. The plan relied initially on a refuge of non-*Bt* cotton (USA EPA, 2001). Recent evidence suggested that non-cotton host plants provided an extensive refuge, so the refuge requirement for non-*Bt* cotton was reduced in parts of the USA (Gould *et al.*, 2002; Abney *et al.*, 2004; Jackson *et al.*, 2006). IRM for *Bt* maize in the USA corn belt relies on a 20 % non-*Bt* maize refuge,

but there is still no effective monitoring system that would provide information to adapt IRM (Andow and Ives, 2002). In general, the granting of temporary registrations by the USA EPA for all *Bt* crops has enabled the development of adaptive IRM. When the temporary registration is about to expire, the USA EPA can review all of the available information to determine how to change the IRM requirements. In the presence of uncertainty, adaptive resistance management provides some safety margin to increase the durability of resistance.

Adaptive IRM requires time to implement. When new information becomes available, there will be a time lag before IRM can be changed. Some of this time lag is due to the need to confirm the veracity of the new information, and some of it is due to the fact that bureaucratic decisions take time. In either event, it is important that the new information or experience comes quickly enough that changes to IRM can be effective. For example, when monitoring the frequency of a recessive resistance allele in an insect population, the response time to adapt IRM must be less than 2 to 12 years, depending on the resistance frequency detected (Andow and Ives, 2002). An F_2 screen is a cost-effective method for monitoring recessive resistance alleles that would provide adequate detection sensitivity (Andow and Alstad, 1998).

IRM can be adapted in two main ways when information about increased resistance allele frequency is obtained. One approach is to reduce the selection differential between resistant and susceptible genotypes, and a less explored approach is to manipulate the movement of male and female moths among fields (Andow and Ives, 2002). Simulations suggest that contrary to expectation, reducing the selective differential does not result in very large gains in time to delay control failure due to resistance. Instead, manipulating movement among *Bt* and refuge fields may result in the greatest gains. While both approaches may have some utility in adaptive resistance management, management practices based on changing movement patterns of moths could be particularly effective at prolonging the efficacy of *Bt* crops through adaptive resistance management.

5.3. Experience with IRM (Andow *et al.*, 2008)

Since 1990, there has been an increased development of pre-emptive IRM strategies (Denholm and Rowland, 1992) for both insecticides and transgenic insecticidal crops. This occurred first in southern USA (Plapp *et al.*, 1990) and Australia with pyrethroid IRM in cotton during the 1980s, which in Australia led to the creation of the Transgenic and Insect Management Strategy

Committee (TIMS) committee, which in turn has guided IRM for *Bt* cotton in Australia. In the USA, pre-emptive IRM for insecticides and transgenic crops has developed in parallel, continuing from the mid-1990s with the creation of the EPA-Insecticide Resistance Action Committee and several EPA-Science Advisory Panels.

All IRM strategies depend first and foremost on methods to reduce the selection pressure of the insecticide or insecticidal crop on the target pests. However, the methods used differ substantially for insecticides versus insecticidal crops. For insecticides, rotation of product mode of action and reducing the need for insecticide application through effective alternative control practices in an Integrated Pest Management system have essential roles, while refuges, when present, are typically unplanned and unstructured. For insecticidal crops, planned, structured refuges are sometimes the sole method for IRM, although practices that minimise the need to use the insecticidal crop and methods that select against resistance are also components of some of the IRM strategies, most notably in Australia.

Ecological and economic factors may explain some of these differences. Specifically, IRM must be practicable for farmers, which means that it is ecologically effective and the least cost alternative for farmers. Although refuge strategies have been proposed for some time (e.g., Comins, 1977; Georghiou and Taylor, 1977), they have been considered too complicated and costly for insecticide IRM. In particular, when insecticides are used with scouting and economic thresholds, it is difficult to convince a grower to leave some of the crop as an unsprayed refuge. Instead, it is more cost-effective to rotate modes of action and to minimise the need for insecticide sprays, as both of these IRM tactics are consistent with grower goals to increase profit, minimise risk, and/or reduce management time. In contrast, when pest control is pre-emptive (insecticidal crops, insecticides applied at planting), refuges may be implemented as a part of planting, and planned for areas less likely to suffer economic losses. In addition, while average expected pest losses to the refuge can be calculated, in any given year these losses may or may not be incurred. In this context, refuges do not necessarily reduce profits, especially when longer time frames are considered (Hurley *et al*., 2001).

IRM for *Bt* cotton has developed very differently in Australia and the USA. Australia requires structured cotton or non-cotton crop refuges, requires larger refuge populations, specifies a planting window, requires use of economic

thresholds to manage pests on refuges, and requires control of volunteer plants and destruction of crop residues. The USA requires smaller structured cotton refuges or allows unstructured non-cotton wild plant and crop refuges, and does not require any other IRM measure. The Australian requirements are more risk averse than the USA requirements. These differences are in part due to the history of resistance failures in Australia that have sensitised growers to the resistance problem, convincing them to aggressively manage resistance. Probably more significantly, however, is the TIMS process used in Australia, which involves the growers in the development of the IRM strategy. By doing this, growers are informed of the need for IRM, can influence the development of IRM so that it is consistent with their production goals, and are prepared to implement and comply with the requirements. In contrast, the USA uses a regulatory process that focuses on the seed company registrant and limits grower inputs to the decision because the growers are not the product registrants. This means that growers are less invested in the IRM strategy and must be convinced of the need and benefits after it has been decided.

It is not yet possible to know for certain how effective IRM has been at delaying the onset of resistance for any *Bt* crop (e.g., Tabashnik *et al*., 2003). However, the *Bt* cotton IRM strategy in Australia has surely delayed the rate of resistance evolution. Based on the rate of resistance evolution in cotton pests with no IRM strategy, and on present knowledge about the commonness and inheritance of resistance to single-gene *Bt* cotton in *H. armigera* (Akhurst *et al*., 2003; Downes *et al*., 2007), indications are that some resistance failures would have been likely had its widespread cultivation occurred in Australia. In addition, this would have jeopardised the IRM strategy for the two-gene *Bt* cotton.

5.4. Possible Complications

Several factors have been suggested to complicate the ability to develop practicable IRM plans. These include the evolutionary cost of resistance, quantitative resistance, multigenic resistance, farmer opposition to IRM, and problems implementing IRM in small-scale production systems. While there are many possible complications, IRM can be planned to address them all.
The first three are similar in one respect. Typically IRM plans assume that there is no evolutionary cost to resistance, and that resistance is determined by a single gene locus with a single resistance allele. These assumptions result in a more robust IRM plan. The empirical evidence suggests that resistance in insects is usually determined genetically by a single allele at a single locus (McKenzie, 1996). However, the evidence also suggests that most resistance

alleles have an associated fitness cost (McKenzie, 1996). A fitness cost means that the resistance allele is less fit than a wild-type allele in the absence of selection by the toxin, such as might occur in a refuge field. Hence, it would seem logical to assume a fitness cost than to assume no fitness cost. A major problem arises in IRM planning at this point. What fitness cost should be assumed? If it is too large, then we risk rapid control failures, so how do we choose a value that is not too large? Unfortunately, the empirical literature is not very helpful, because fitness costs are frequently poorly quantified and depend on the resistance mechanism. Consequently, IRM plans assume no fitness cost, but can be adapted to take into account a fitness cost when resistance is discovered and the cost is quantified.

Because IRM for transgenic insecticidal crops has relied on a non-*Bt* refuge, it has been argued that farmers will not implement the IRM plan because they will not tolerate economic losses associated with the refuge. Such a perspective takes too narrow a perspective on farmer interests (Hurley and Mitchell, 2007). In addition to short term economic goals, farmers have a long-term interest to preserve a transgenic insecticidal crop, and even more significantly, they have a motivation to cooperate with their neighbours for the benefit of the community. These long-term and broader social goals partially mitigate the cost of implementing IRM. In addition, IRM can sometimes be implemented with non-crop refuges (e.g., wild plants for *Bt* cotton in southeastern USA) or other crop refuges (e.g., tobacco for *Bt* cotton in southeastern USA and pigeon pea for *Bt* cotton in Australia). In addition, pests can be controlled on the non-*Bt* crop refuges, something which is explicitly planned for *Bt* cotton in Australia, and is not expected to compromise IRM (Ives and Andow, 2002).

It is widely suggested that IRM in small-scale cropping systems will be difficult. Small-scale farmers have little land, capital or economic flexibility to bear the costs of IRM individually. As a consequence, community level action has been suggested for small-scale rice systems (Cohen *et al.*, 1996), and naturally occurring crop and non-crop refuges have been suggested for small-scale cotton systems in Viet Nam (Fitt *et al.*, 2008) and Brazil (Fitt *et al.*, 2006).

6. IDENTIFICATION OF RESISTANT INDIVIDUALS

To manage resistance effectively, it is essential to be able to identify resistant individuals. This may seem obvious, but there are many complications that make identification difficult. The most definitive test is to rear the individual

from egg to adult on the *Bt* crop. However, when resistance is being first identified, normally only one or a limited number of individuals is identified as potentially resistant. Because there are many ways for a developing insect to die that are unrelated to toxin consumption, it is very risky to subject these few individuals to the definitive test immediately. Imagine the consternation if the one putative resistant individual was inadvertently crushed in a leaf axil or eaten by a predator while it was developing on the *Bt* plant. Alternatively, imagine the concern if some of the resistant individuals escaped into the natural population to accelerate the evolution of resistance! Despite these concerns, using the *Bt* plant is the definitive test for resistance.

Alternatively, bioassays are used to determine discriminating concentrations, which can be used to identify resistant individuals. A discriminating concentration is defined as the concentration of toxin in a laboratory assay that discriminates between resistant and susceptible individuals. The concentration kills nearly all susceptible individuals and allows nearly all resistant individuals to survive. Ideally, resistant individuals are needed to determine the discriminating concentration, but in the absence of resistant individuals, some multiple of the LC_{50} or LC_{99} is commonly used. The LC_{50} is the lethal concentration at which 50% of the susceptible individuals die, and the LC_{99} is the concentration causing 99% mortality of susceptible individuals. A discriminating concentration set to several multiples of the LC_{50} concentration or a few multiples of the LC_{99} concentration would provide a concentration at which <<1% of susceptible individuals would survive. If it were also true that resistant individuals would survive this high concentration, then it could be used as a discriminating concentration. Such bioassays might require the use of purified toxin equivalent to that produced in the transgenic plant. Purified toxin is often expensive, so bioassay methods should be considered to minimise the use of toxin and cost of the assay.

Although use of *Bt* plant tissue typically will not allow the estimation of an LC_{50} or LC_{99}, freshly excised *Bt* plant tissue can, in many circumstances, be used as a discriminating concentration to separate resistant and susceptible phenotypes, for example the use of excised maize leaf tissue (Huang *et al.*, 2007). Directly feeding on intact plants in a glasshouse may be less suitable as a discriminating concentration (Zhao *et al.*, 2002).

The USA EPA (2001) uses a method for identifying resistance that corresponds to an incorrect definition of resistance -

"Progeny from the sampled target pest population will exhibit both of the following characteristics in bioassays initiated with neonates: (1) An LC_{50} in a standard Cry toxin diet bioassay that exceeds the upper limit of the 95 % confidence interval of the mean historical LC_{50} for susceptible populations; and (2) > 30 % survival and > 25% leaf area damaged in a 5-day bioassay using Bt leaf tissue under controlled laboratory conditions."

The main problem with this method is that it defines resistance as a characteristic of a population. As emphasised above, resistance is a trait of an individual that is under genetic control in the individual. It appears that the USA EPA definition has confounded the definitions of "resistance" and "field control failures" caused by resistance. There are further technical problems with the USA EPA definition. Because resistance evolves gradually, the historical LC_{50} will rise as resistance becomes more common. Thus, it might be expected that the LC_{50} of a population will be difficult to distinguish from its historical LC_{50}. In addition, coupling survival and leaf damage means that the insects must be large enough to cause the required damage. Because all larvae start the assay as neonate larvae, they may still be very small at the end of the 5-day assay. Thus, it will be necessary to assay many of them together to meet the leaf damage criterion. Under these crowded conditions, survival rate is likely to be low, making it difficult to meet criterion 2. The simpler definition of survival from egg to adult on the *Bt* plant followed by reproduction is more rigorous and less costly to conduct experimentally than the USA EPA experimental protocol.

7. MEANS TO MANAGE RESISTANCE EVOLUTION

Four general approaches can be used to delay resistance evolution (Fitt *et al.*, 2008). The approach most widely used is to reduce the selection pressure (exposure) on the pests to *Bt* cotton by maintaining refuge plants. By reducing selection pressure, resistance evolution can be delayed substantially. Specific issues to be considered include: size, placement, time of planting and management of refuges. Certainly, the simplest approach by far is to reduce selection pressure by maintaining refuges.

A second approach is to reduce the fitness differential between resistant and susceptible insects. The fitness differential is the fitness advantage of resistant phenotypes over susceptible phenotypes when both are exposed to the

transgenic plant. This can be accomplished by suppressing pests emerging from the transgenic crop with other control tactics such as insecticides, cultural controls, or more effective biological control. High control efficacy results in a high fitness differential. Low efficacy results in a low fitness differential.

A third approach is to reduce *RS* heterozygote fitness. When resistance is rare, the rate of evolution of resistance is mainly determined by the fitness of heterozygotes. Heterozygotes may have a susceptible or a resistant phenotype. If they are phenotypically susceptible, then they have low fitness on the *Bt* plant (resistance is recessive), and the rate of resistance evolution is slow. It is possible that natural enemies can alter heterozygote fitness, however, little is known about potential selective feeding by natural enemies in *Bt* crops. As will be discussed in Section 8, a high-dose event has low *RS* heterozygote fitness, and a low-dose event has higher *RS* heterozygote fitness.

The fourth approach can be used only with high-dose IRM strategies (see below). For some target species it may be possible to manage the sex-specific movement and mating frequencies to delay resistance evolution (Andow and Ives, 2002). By using chemical and environmental attractants, it may be possible to enhance the movement of males and simultaneously reduce the movement of females from refuges to transgenic fields limiting the impact of source-sink dynamics (Caprio, 2001).

A "seed mixture" is often considered as a possible resistance management tactic, particularly for smallholder systems. It would involve mixing the seeds of a *Bt* and a non-*Bt* crop variety in the seed bags or planters so that a fine-scale mixture of *Bt* and non-Bt plants occurs in each field and the farmer no longer controls the deployment of the refuge. While it is true that seed mixtures are better than no IRM at all (Tabashnik, 1994), they can seriously compromise IRM by the movement of larvae between plants (Mallet and Porter, 1992). The worst case would occur when resistant heterozygotes, which are phenotypically susceptible when feeding on *Bt* plants, can survive on the *Bt* plant long enough to move to a neighbouring non-*Bt* plant, where they can complete development. By doing so, these heterozygotes become functionally resistant, and resistance evolution is greatly accelerated. In an analogous way, susceptible larvae and resistant heterozygotes feeding initially on non-*Bt* plants where they develop to older growth stages, and then move to *Bt* plants where the susceptibles are killed but the heterozygotes survive (because the older growth stages are more tolerant of the *Bt* crop than younger ones).

This also makes heterozygotes functionally resistant, accelerating resistance evolution.

Research has suggested that larval movement of most key target pests of all available *Bt* crops (Tables 3 and 5) is sufficient to suggest that seed mixtures should not be used. Larvae of *Helicoverpa armigera* move from plant to plant as they mature (King, 1994), suggesting that seed mixtures of *Bt* cotton would speed up resistance evolution. Larvae of *Heliothis virescens* (Parker and Luttrell, 1999), *Ostrinia nubilalis* (Davis and Onstad, 2000), and *Helicoverpa armigera* (Zhang *et al.*, 2004) all move too much to allow seed mixtures. Larvae of *P. gossypiella*, on the other hand, are very sedentary and rarely move between bolls on a plant. If this species were the only pest of cotton, seed mixtures might be a feasible tactic. However, except for southwestern USA, there is no region in the world where this species is the only Lepidopteran pest of cotton. Consequently in nearly every case, seed mixtures should not be used as an IRM tactic.

8. HIGH-DOSE/REFUGE STRATEGY

8.1. Key Components

Of all of the various strategies and tactics considered for IRM (Georghiou and Taylor, 1977; NRC, 1986; Roush, 1994; Gould, 1998), the high-dose/refuge strategy is by far the most widely considered and used (Andow and Hutchison, 1998; Gould and Tabashnik 1998; USA EPA, 2001; Box 1). This strategy is relatively simple to develop and implement, and provides the ready means to monitor compliance in the field.

The high-dose/refuge strategy requires that the *Bt* crop produces a sufficiently high toxin concentration and that a host plant other than the *Bt* crop is growing nearby as a refuge for the target pest or pests. A high-dose renders resistance recessive, which can greatly delay resistance evolution. A refuge provides unselected pests, which will mate with resistant individuals emerging from *Bt* fields, thereby making all offspring heterozygous and phenotypically susceptible.

BOX 1. KEY ASSUMPTIONS OF HIGH-DOSE/REFUGE STRATEGY

The high-dose/refuge strategy requires that *Bt* maize produce a high-dose of toxin and that non-*Bt* host plants are growing nearby as a refuge for pests. This strategy relies on three essential assumptions (Andow and Hutchison, 1998; Andow, 2001). Computer simulation models show that if these assumptions hold, the evolution of resistance will be substantially delayed (Comins, 1977; Tabashnik and Croft, 1982; Gould, 1986; Roush, 1994, 1997; Alstad and Andow, 1995).

I. High-dose

Plant tissue must be sufficiently toxic that any resistance allele in the target population is functionally recessive (Tabashnik and Croft, 1982). High-dose is a property of both the *Bt* plant and the target pest, and is not merely based on the concentration of toxin in the plant. Thus, it can only be determined when resistance alleles have been found in the natural population. Prior to finding resistance alleles, dose can be hypothetically determined based on data from other pests and/or other *Bt* crops.

II. Resistance is rare

The resistance alleles must be sufficiently rare (the frequency should be $<10^{-3}$ [Roush, 1994]- even lower is better) so that nearly all resistance alleles will be in heterozygote genotypes. If nearly all resistance alleles are in heterozygotes, they can be eliminated by the *Bt* crop if it expresses a high-dose. For example, suppose the frequency of resistance alleles is 0.001. Under random mating, about 2×10^{-3} individuals will be *RS* heterozygotes and 1×10^{-6} will be *RR* homozygotes. If the frequency of resistance alleles is 0.0001, about 2×10^{-4} individuals will be *RS* heterozygotes and 1×10^{-8} will be *RR* homozygotes. A 50 ha non-*Bt* maize field may have between 300,000 - 15,000,000 maize borers, so the number of *RR* homozygotes in the field might be 0.3-15 when the *R* allele is 1×10^{-3}, and 0.003-0.15 when the *R* allele is 1×10^{-4}.

III. Sufficient mating between refuge and transgenic crop

The non-*Bt* refuges must be interspersed sufficiently among the *Bt* crop fields, so that there is sufficient mingling and mating between individuals emerging from refuges and *Bt* fields. Mating should be random within fields, but mating does not have to be random between fields. Mating may be more likely to occur within fields than between fields (some inbreeding within fields), without compromising IRM (Ives and Andow, 2002). There must be sufficient mingling and mating so that any *RR* female emerging in a *Bt* field is more likely to mate with a male from the refuge than a male from the *Bt* field. Assuming that the refuge is large enough, the populations in the refuge will be much larger than populations in the *Bt* fields, and relatively small amounts of movement from the refuge to the *Bt* field is needed to ensure that refuge males are more likely to mate with females in the *Bt* fields. If this occurs, then nearly all resistant homozygotes will mate with susceptible homozygotes, producing heterozygous progeny that cannot survive on the *Bt* crop.

The dose of the insecticidal toxin in a *Bt* crop is a major factor determining the level of resistance risk. Dose depends on both the concentration of the Cry toxin in the *Bt* plant and the genetic characteristics of the target pest. A "high-dose" is defined as one that kills a high proportion (>95 %) of heterozygous resistance genotypes, so that the heterozygotes have a similar mortality as the homozygous susceptible genotypes (Georghiou and Taylor 1977; Roush, 1997; Gould, 1998). For a high-dose, resistance is recessive or nearly so. A "low-dose" is anything that is not a high-dose.

Dose is a measure of the relative fitness of the three possible genotypes associated with resistance evolution. These genotypes are the *RR* homozygotes (with two resistance, *R*, alleles), the *SS* homozygotes (with two susceptibility, *S*, alleles), and the *RS* heterozygotes (with one of each kind of allele). Dose is a measure of the relative fitness of the *RS* heterozygote relative to the difference between the *RR* and *SS* homozygotes. If the fitness of the *RS* heterozygote is similar to the *RR* homozygote, resistance is said to be dominant, and resistance evolution can be extremely fast. If the fitness of the *RS* heterozygote is similar to the *SS* homozygote, resistance is said to be recessive, and resistance evolution can be delayed for a long time with the appropriate management.

A refuge is a habitat in which the target pest can maintain a viable population in the presence of *Bt* cotton fields, where there is no additional selection for resistance to *Bt* toxins and insects occur at the same time as in the *Bt* fields (Ives and Andow, 2002). Refuges can be structured [deliberately planted in association with the *Bt* crop] or unstructured [naturally present as part of the cropping system]. The refuge can comprise the non-*Bt* crop, another crop that is a host for the target pest or pests, or wild host plants. The refuge can be managed to control pest damage, as long as the control methods do not reduce the population to such low levels that susceptible populations are driven to extirpation (Ives and Andow, 2002). The effectiveness of any refuge will depend on its size and spatial arrangement relative to the *Bt* crop, the behavioural characteristics [movement, mating] of the target pests and the additional management requirements of the refuge.

Resistance management will differ for high-dose versus low-dose plants. Simulation models clearly show that a high-dose can delay the evolution of resistance more effectively than a low-dose (Roush, 1994; Alstad and Andow, 1995; Gould, 1998; Caprio, 1998; Tabashnik *et al.*, 2003). A high-dose may also allow greater options for resistance management with less restrictions on how

non-transgenic refuges are managed (Carrière and Tabashnik, 2001; Ives and Andow, 2002; Onstad *et al.*, 2002; Storer *et al.*, 2003), and so may be more readily implemented than for low-dose events. Low-dose events will require larger non-transgenic refuges and/or restrictions on the management of these refuges. Indeed, in Australia, growers agreed to cap the area of single-gene *Bt* cotton cultivation [low-dose for *H. armigera*] to 30 % of the total crop in addition to management requirements for refuges (50 % sprayed cotton refuge or 10 % unsprayed cotton refuge) (Fitt, 2004). In the USA, it has been argued that a 50 % refuge may be needed for low-dose plants (Gould and Tabashnik, 1998), and elsewhere, larger refuges have been suggested (Fitt *et al.*, 2006, 2008). Simulations have indicated that a 50 % refuge was needed for low-dose plants (Fitt *et al.*, 2006).

8.2. How the High-Dose/Refuge Strategy Works

The high-dose/refuge strategy delays the evolution of resistance primarily by reducing the selection pressure favouring the resistance alleles (Ives and Andow 2002). Resistance alleles are favoured in the *Bt* fields, so the overall selection pressure is related to the proportion of *S* alleles that are exposed to the *Bt* fields. This proportion is determined mainly by the size of the refuge. When the refuge is larger (or equivalently, preferred by females for egg laying), fewer *S* alleles end up in the *Bt* fields, and more of the susceptible individuals remain in the refuge to reproduce. This reduction in selection pressure also occurs for low-dose events with a refuge.

The second most important effect of the high-dose/refuge strategy is that it reduces the fitness advantage of the *RS* heterozygote over the *SS* homozygote. As indicated previously, when *R* alleles are rare, they occur mainly as *RS* heterozygotes in the field, and the fitness advantage of the *R* allele over the *S* allele is primarily determined by the fitness advantage of the *RS* heterozygote over the *SS* homozygote genotype. A high-dose event is one in which the *R* allele is nearly recessive, which means that the fitness of the *RS* heterozygote is nearly the same as the *SS* heterozygote. Specifically, >95 % of the *RS* heterozygotes should be killed by the *Bt* crop, which means that the *RS* heterozygote has a <5 % fitness advantage over the SS homozygote in the *Bt* field. The lower the fitness advantage, the slower the rate of evolution. A low-dose event does not enjoy this advantage.

Finally, a third and quantitatively smaller effect is related to the mingling and mating promoted between individuals from a *Bt* field and a refuge

field. This reduces the rate of formation of *RR* offspring in *Bt* fields. Because *Bt* fields will select against *S* alleles, the resulting adult population in a *Bt* field will have a high frequency of *R* alleles. Without the mingling and mating with individuals from the refuge, the *Bt* population would mate among themselves. With a high *R* allele frequency, a high proportion of the offspring of such matings would be *RR* homozygotes, which would have a very large fitness advantage over the *SS* homozygotes, negating the second advantage of the high-dose/refuge strategy described in the previous paragraph. If refuge adults mingle and mate, nearly all of the offspring of adults emerging in a *Bt* field will be *RS* heterozygotes or *SS* homozygotes, minimising the fitness advantage of the *R* allele.

When resistance is rare, the population size of adults that emerge in *Bt* fields is expected to be very small. Specifically, if p is the resistance allele frequency, and the *RS* heterozygotes have a 5 % fitness advantage over the *SS* homozygotes in the *Bt* field, then the expected emergence rate in a *Bt* field is ~2*0.05*p times the emergence rate in a refuge. If p = 0.001, this implies that the emergence rate in a *Bt* field is expected to be 1×10^{-4} that in a refuge field. Hence, it takes a relatively small proportion of the adults emerging from the refuge (<5 %) to provide enough individuals to the *Bt* field to reduce the rate of formation of *RR* offspring in the *Bt* fields.

Other papers have suggested that the high-dose refuge strategy works by "diluting" *R* alleles in the *Bt* field (e.g., Kranthi and Kranthi, 2004; Bates *et al.*, 2005, Cameron *et al.*, 2005). This is one possible metaphor to explain the third effect described above, although it ignores the first two more significant effects. This metaphor, however, does not really describe how the third effect works, which is to enhance mingling and mating between *Bt* and refuge fields to reduce the rate of formation of *RR* offspring.

8.3. Determining Dose and Efficacy

To determine the "dose" of a *Bt* crop, it is essential to have insects resistant to that *Bt* crop. If resistance is determined by allelic variation at a single locus, dose is determined by the fitness of *RS* heterozygotes compared to *SS* homozygotes, so it is essential to be able to compare experimentally the fitness of each. This, of course, requires having resistant insects that can be crossed to create *RS* heterozygous individuals to be challenged with the *Bt* crop and compared to the *SS* homozygotes.

Plant tissue must be sufficiently toxic that any resistance allele in the target population is functionally recessive (Tabashnik and Croft, 1982). One way to determine this is to conduct a concentration-response bioassay using *SS*, *RS* and *RR* genotypes. A bioassay would vary the concentration of the insecticidal toxin exposed to the three genotypes and estimate survival rate (or some other measure of fitness) of each genotype at each toxin concentration. Each genotype would have a characteristic concentration-response curve (downward sloping lines; Figure 2). If the transgenic insecticidal plant expresses the toxin at a concentration *h* or higher (Figure 2), then it can be considered a high-dose event, because the survival rate of the *RS* heterozygotes is about the same as that of the *SS* homozygotes. If the plant expressed the toxin at concentrations <*h*, then it would be considered a low-dose event.

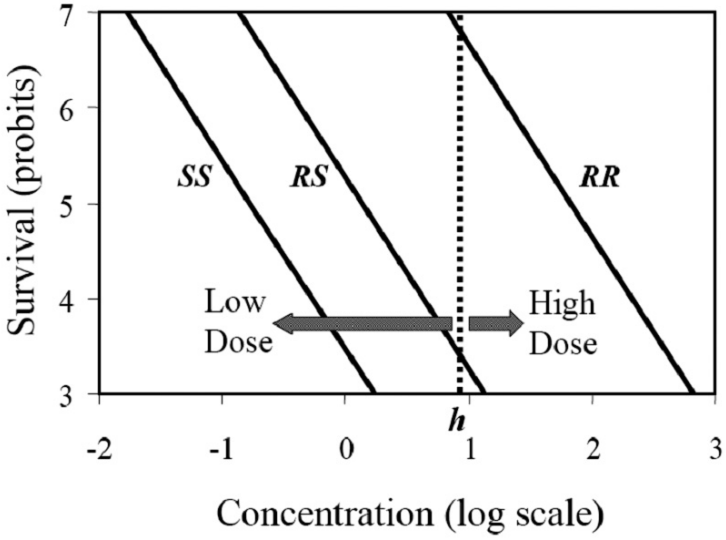

Figure 2. Illustration of high-dose from a bioassay. Downward sloping lines are hypothetical concentration-response curves for a resistant homozygote (RR), susceptible homozygote (SS) and the heterozygote (RS). If a transgenic plant expresses toxin at concentrations greater than h (dashed line), it would be high-dose. In this case, the RS heterozygote has <5 % advantage over the SS homozygote. If the plant expresses toxin at lower concentrations, it would be low-dose.

There are many ways to conduct bioassays. Several possible carriers of the toxin can be used. The carrier can be a natural food source (plant tissue) or artificial diet. Generally, plant tissue is treated with a surface application of the toxin in a series of toxin dilutions. With an artificial diet, toxin can either be incorporated into the diet (Gould *et al.*, 1997; Hilbeck *et al.*, 1998) or applied to the surface of the diet (Marçon *et al.*, 1999). A surface application conserves toxin, and is acceptable when only small amounts of toxin are available. It can be difficult to conduct if the diet surface is not level and smooth - even tiny bubbles will interfere - and the method tends to underexpose larvae that bore into the diet (Bolin *et al.*, 1999).

Transgenic plants can also be used directly as a bioassay, although this will not allow the estimation of a concentration-response curve or the LC_{50}. As previously stated, undiluted tissue can in many circumstances be used as a discriminating concentration to separate resistant and susceptible phenotypes. One advantage of this method is that it avoids the necessity of estimating *h* (Figure 2), as the concentration in the plant tissue is defined as *h*. By using the transgenic plant tissue, it is necessary only to estimate survival (or other fitness measure) of the genotypes at the one plant tissue concentration. As a consequence, it is not necessary to estimate genotype survival at many toxin concentrations, nor is it necessary to purify toxin or to purchase purified toxin, and neither is it necessary to develop and validate a separate laboratory bioassay system. The main drawbacks with this method are that concentrations in plant tissue may depend on environmental conditions, and secondary plant compounds may interfere with the results, confounding the source of mortality (Olsen and Daly, 2000). The first concern can be addressed by measuring toxin concentration in the plant tissue and increasing experimental replication to test the range of variation in expression of the toxin in the plant. It might be argued that the second concern represents the actual selection pressures on the three genotypes in the field, as they will have to survive all mortality factors associated with the plants, and therefore, is not really a concern.

In most cases prior to the field release of the *Bt* crop, resistant insects will not have been discovered. When resistance in a target species has not yet been found, it is not possible to evaluate heterozygous genotypes, so it is impossible to determine if a transgenic plant is high-dose or not.

Instead, a temporary, provisional definition of "high-dose" must be used. One such definition is: a plant is provisionally high-dose if it expresses toxin at a

concentration that is 25 times the LC_{99} of the target pest (Gould and Tabashnik, 1998). This operational definition has been accepted for use by the USA EPA, even though the supporting scientific evidence is weak. One alternative definition is a high-dose produces at least 99.99 % mortality of homozygote susceptibles relative to a non-*Bt* control (ILSI, 1999). Unfortunately, both of these definitions link dose with efficacy, which is the kill rate of susceptible *SS* homozygotes. Although they may be adequate provisional definitions, the definitive methods that have just been described are necessary to evaluate the effectiveness of IRM.

Efficacy is the kill rate of *SS* homozygotes, compared to *RR* homozygotes, and does not directly provide information about dose. However, the scientific literature on resistance in insects to *Bt* toxins, suggests that there is a correlation between efficacy and dose (Caprio *et al.*, 2000). Caprio *et al.* (2000) found that dominance and *SS* survival were correlated, such that higher *SS* survival was associated with higher dominance (low-dose). They showed that a concentration that is 50 times the LC_{99} is high enough that all known resistance alleles would be functionally recessive and high-dose. Even though this is a scientifically defensible provisional definition of high-dose, it has not been widely used. In general, estimation of the LC_{99} is technically difficult, as it is necessary to screen large numbers of SS individuals to estimate accurately the concentration at which 1 % survive. One way to do this is to extrapolate from the LC_{50}, which is easier to estimate. Another way to estimate an LC_{99} is to dilute the toxin in the plant until a concentration allowing ~5 % survival can be determined, and then repeat the experiment with high replication at several concentrations higher than this approximate LC_{95}. A third way to determine if the *Bt* plant expresses at a high enough concentration is to dilute the tissue 25X (definition of Gould and Tabashnik, 1998) or 50X (definition of Caprio *et al.*, 2000) and determine if *SS* survival is >1 % or <1 % at this diluted concentration. If *SS* survival is <1 %, the plant provisionally is a high-dose event. Otherwise, it is a low-dose event.

9. DEVELOPING AN IRM STRATEGY – RESISTANCE RISK (Fitt *et al.*, 2008)

For any given crop there are usually multiple pest species that require control, and any given pest control tactic usually affects multiple pest species. This has also been true for all transgenic insecticidal crops that have been commercialised to date (Tables 3 and 5). So, as a first step in developing an IRM strategy, it is important to assess which species are at risk of resistance and of these, which is most at risk.

To assess the relative resistance risk of a *Bt* crop, it is necessary to have a list of species that occur on the crop and are susceptible to the *Bt* proteins in use. Resistance risk can then be assessed by considering:

- the likely dose of the transgenic toxin to which each species is likely to be exposed [influenced by characteristics of the transgene, interactions with plant chemistry and variety, climatic and agronomic factors]. Because dose is a property of both the transgenic crop and the pest species, a plant may be high-dose for some pests, but low-dose for others. For example, MON810 and Bt11 appear to be high-dose against *Ostrinia nubilalis*, but low-dose against *Helicoverpa zea* in the USA (Andow, 2001). Cry1Ab cottons are likely to be high-dose against *Pectinophora gossypiella* and *Alabama argillacea*, but possible low-dose against *Helicoverpa armigera* and *Spodoptera frugiperda* in Brazil (Fitt *et al.*, 2006).
- potential exposure of each species to the dose that may lead to selection in favour of resistance [influenced by association of the species with the crop relative to other host plants, generations per crop cycle, other hosts in the farming system, pest mobility and behaviour]

which together allow a determination of pest species at risk of evolving resistance to the transgene. Dose strongly influences the rate of resistance evolution, and coupled with information on potential exposure, the relative resistance risk of the various pest species can be assessed and the species identified that is most likely to evolve resistance before the others – which might be the main target for pre-emptive IRM.

Resistance management first focuses around the biological attributes of this main target, or weak link, species. Afterwards, it is important to confirm that the resistance management strategy constructed around the weak link species would also delay resistance evolution in the other species at risk.

While doing this, it is essential that the resistance management plan be practicable, that is, growers can actually implement it. The resistance management plan builds on the information from the previous risk assessments, using the following three steps:

- Determination of the likely requirements for resistance management,

including refuges
- Development of the likely requirements of a potentially workable resistance management plan
- Specification of monitoring needs and development of potential contingency responses

In the following section, the approaches toward determining resistance risk are described and illustrated using *Bt* cotton in Viet Nam. For a more detailed description of this case, including the development of a practicable IRM plan, the reader is referred to Fitt *et al*. (2008).

9.1. Identification of Pest Species Potentially at Risk

Identification of key pest species that could evolve resistance to the transgenic crop first involves identifying the key target pests in each of the major geographic regions where the transgenic crop is likely to be grown, and then evaluating the resistance history of each species. In some cases, identification of the key target species can be difficult because the transgenic crop has not been tested against all relevant species. For many *Bt* crops, there is considerable information about the key pests that are known to be susceptible to *Bt* toxins, but there is almost no quantitative information from specific developing countries, such as Viet Nam, on the efficacy of potential *Bt* crops to control the pests in Vietnamese cropping environments (Fitt *et al*., 2008). The potential risk of resistance evolution to *Bt* cotton in Viet Nam will be used in this section as an extended example to illustrate the principles of resistance risk assessment.

For example, seven Lepidoptera can be identified as potential targets of *Bt* cotton in Viet Nam (Fitt *et al*., 2008). Based on evidence of relative susceptibility to a range of *Bt* proteins (Cry1Ac, Cry2Ab, Cry1F, Vip3A) from studies elsewhere, the key target species are probably *Helicoverpa armigera*, *Spodoptera exigua* and *Pectinophora gossypiella*. The latter species is highly susceptible to Cry1A and Cry2A toxins in particular, while the other two species vary in susceptibility to different proteins. All seven Lepidopteran species occur across all cotton production regions in Viet Nam, although only *H. armigera* causes serious damage in all regions.

A prior history of resistance evolution to conventional pesticides can also provide considerable insight into the risks associated with transgenic insecticidal crops. Species with a history of repeated resistance evolution

should be prioritised in any risk assessment, because their population ecology, host relationships, genetic structure and behaviour may predispose them to respond rapidly to selection pressure from a *Bt* crop. In most developing countries, only very limited information is available to assess the past history of resistance in pests, because there have been only sporadic monitoring programs in place. Available knowledge is based largely on perceived field failures and anecdotal information plus results from some monitoring activity. Even this limited information is sufficient to identify risks. Continuing with the example from Viet Nam (Fitt *et al.*, 2008), *H. armigera* and *S. exigua* have developed resistance to all classes of pesticides available in Viet Nam, whereas the remaining five Lepidopteran species have no known examples of pesticide resistance. Thus, of the seven potential target Lepidopteran pests, *H. armigera* and *S. exigua* are likely to be at greater risk of resistance evolution than the others.

9.1.1. Likely Dose

As discussed above, many methods can be used to determine the dose of a transgenic insecticidal crop. However, in many developing countries, this information will not be available for the specific pests and transgenic events in the country. For example, comprehensive laboratory or field information is not available for any combination of the Lepidopteran pests and specific *Bt* cotton varieties being considered in Viet Nam. However, based on research on some of these pests elsewhere in the world and some preliminary confirmatory research in Viet Nam on some species, it is likely that Cry1Ac + Cry2Ab *Bt* cotton expresses a moderate- to high-dose for *H. armigera* and *S. exigua*, and a high-dose for *P. gossypiella*. Sufficient information is not available for Vip3A or most of the Cry1Ac/Ab *Bt* cottons to fully evaluate their dose. It is possible that Vip3A could be expressed at a high-dose for both *H. armigera* and *S. exigua*, and the single gene Cry1Ac/Ab *Bt* cottons are low- to moderate-dose events for both species.

9.2. Potential Exposure of Target Pests to the Bt Crop

9.2.1. Association with Bt crop

The greater the association of the target pest with the *Bt* crop, the greater the potential exposure and the greater the selection pressure. To rapidly assess resistance risk with minimal required information, the association of the target species with the *Bt* crop is the **maximum period of overlap** of the species on the target crop, in terms of area, spatial distribution, and seasonal availability of the crop. Overlap can be evaluated on the basis

of presence/absence and general knowledge about the species. Rapid assessment procedures are particularly cost-effective. Because it is widely agreed that resistance will evolve given enough time, it is critical to quickly focus on the mostly likely species to evolve resistance, so that critical knowledge gaps can be identified and practicable resistance management strategies can be developed. More precisely, quantitative evaluations may become necessary to develop realistic resistance management plans, but it is important not to wait for such studies at these initial stages.

For *Bt* cotton in Viet Nam, the three main pest species differ markedly in host range and association with cotton (Fitt *et al.*, 2008). *Pectinophora gossypiella* is a specialist on *Gossypium* species. While some other malvaceous hosts may be used, most of the *P. gossypiella* population is probably associated with cotton in Viet Nam and crop hygiene between seasons is an important pest management tactic. *P. gossypiella* may complete several generations per year in cotton and probably has the tightest association with cotton in Viet Nam of the three species. *Helicoverpa armigera* has a wide range of recorded hosts, including crops and wild hosts, but in the coastal region of Viet Nam, where both rainfed and irrigated cotton is grown, it is possible that multiple generations will occur on cotton throughout the year. During the dry season, *H. armigera* may complete 3-4 generations on cotton and another 2-3 during the rainy season crop. However, cotton currently makes up only 5 % of the crop area in this region. Many of the other crops, such as maize, groundnut, soya bean, mung bean and tobacco are also suitable host plants and in some cases are more highly preferred than cotton (eg. maize), so the association of *H. armigera* populations with cotton may be loose. Likewise, *Spodoptera exigua* has a wide host range incorporating not only the hosts listed above, but also a broad range of vegetable crops, where it is likely to be exposed to even greater pesticide pressure than in field crops. Based on its association with cotton during the rainy season, *P. gossypiella* is likely to be exposed to more intense selection in *Bt* cotton than *H. armigera* or *S. exigua*. However, the lack of evidence for past resistance of *P. gossypiella* to pesticides may suggest the overall risk is lower. This species is also more likely to have high-dose expression than the other two species, which decreases the resistance risk for it and increases the resistance risk for the other species.

9.2.2. Association of other plants with Bt toxin

Whether the new *Bt* crop under consideration is the first or the tenth being

considered for commercial use in a country, it is important to consider how it will affect, and be affected by, resistance evolution associated with previous and future pest control tactics. In some instances, *Bt* crops may already have been commercially introduced and/or commercial *Bt* sprays be in common use. The new *Bt* crop may affect resistance evolution of the previously used pest control tactics, and these tactics may influence resistance evolution on the new *Bt* crop. For example, extensive use of a Cry1Ac-based *Bt* spray could select for resistance so that resistance to a Cry1Ac-*Bt* crop is more common than expected. This would result in faster resistance evolution. Specifically in Viet Nam, *Spodoptera exigua* is sometimes controlled on vegetable crops and maize with *Bt* sprays. Hence *S. exigua* may experience greater exposure through the vegetable crops production sector (Fitt *et al.*, 2008).

In addition, future *Bt* crops may interfere with the resistance management strategy for the new *Bt* crop under consideration. Although it is not fully possible to anticipate future developments, it is possible to anticipate the most likely developments and plan for their eventual execution. For example, Viet Nam plans to commercialise *Bt* cotton, *Bt* maize and *Bt* soya bean. Maize and soya bean are important alternative crops for *Helicoverpa armigera*, and maize is a significant crop in the central coastal region of Viet Nam, representing 3-5 times the area of cotton, with both extensive rainy season and dry season crops. As a highly preferred host plant for *H. armigera* and also a host for *S. exigua*, *Bt* maize could severely compromise the stability of a *Bt* cotton system. Soya bean is not cropped as extensively as maize in Viet Nam, so it represents a smaller risk. The risk from either *Bt* maize or *Bt* soya bean depends largely on the exact proteins deployed. Cotton may be transformed to express Vip3A, Cry1Ac, Cry2Ab or various combinations. The Cry1 proteins are also likely candidates in maize and soya bean. It is anticipated that 50-70 % of hybrid maize may eventually be planted to *Bt* maize, expressing either Cry1Ab or Cry1F. Because Cry1Ab shares similar binding sites in the insect midgut with Cry1Ac, Cry1Ab maize would significantly heighten the risk of resistance through increasing exposure and selection in two host plants of both *H. armigera* and *S. exigua*, which overlap with both crops extensively in time and space. By contrast, *Bt* rice, which may be considered for Viet Nam sometime in the future, would not provide an added risk to *Bt* cotton because none of the target pests are common across those two crops. Overall, when these other *Bt* crops are considered, the resistance risk of *H. armigera* is high.

9.2.3. Scale of adult movement

Adult movement, mating and oviposition will affect exposure among plants in a field, and between fields. Estimates of adult female movement should be separated into pre-mating and post-mating movement, while estimates of adult male movement should concentrate on pre-mating movement. Post-mating movement by males is irrelevant for resistance evolution because males that have completed mating will not contribute to future generations.

The scale of adult movement determines how much mixing and mating can occur between individuals emerging from different fields. For the purposes of relative resistance risk assessment of the target species, it is not necessary to have precise quantitative data on the species. In general, the less dispersive a species, the greater the risk for resistance evolution (Caprio, 2001; Carrière *et al.*, 2004a). This occurs because sedentary species will be more likely to mate with individuals from the field in which they emerged, and to oviposit in the same fields, which is likely to lead to greater selection pressure on that local part of the population. Hence, in assessing the resistance risk, it can suffice to rank the relative dispersiveness of the target species by relying on informal sources of information, including expert judgement.

Vietnamese cotton production systems are typified by small field sizes (in 2006 the 21000 cotton farmers grew on average less than 0.7 ha of rainy season cotton each, and 8204 farmers grew dry season cotton on an average of 0.35 ha each), considerable levels of intercropping and high crop diversity set in a matrix of diverse natural vegetation (Le *et al.*, 2008). There is little information specific to Viet Nam on adult movement of the three main target species of *Bt* cotton, although *H. armigera*, *P. gossypiella* and *S. exigua* have been studied extensively elsewhere. *Helicoverpa armigera* is highly mobile and capable of extensive inter-regional movements, while at times populations appear quite sedentary (Fitt, 1989; King *et al.*, 1990; Feng *et al.*, 2005). *Spodoptera exigua* is capable of extensive local and inter-regional movement, although it is markedly less mobile than *S. litura* (Saito, 2000). *Pectinophora gossypiella* is probably the most sedentary of them all (Tabashnik *et al.*, 1999; Carrière *et al.*, 2001, 2004a,b). For *P. gossypiella* in Arizona, it was determined that refuges should not be further than 0.75 km away from *Bt* cotton fields (Carrière *et al.*, 2004a,b). Given this and knowledge from elsewhere, it seems reasonable to rank

the dispersiveness as: *P. gossypiella* < *S. exigua* < *H. armigera*. However, because Viet Nam field sizes are so small, it can be concluded that all species are likely to move sufficiently to leave the field where they emerge at high rates. Vietnamese researchers report that *H. armigera* moths will move from a variety of neighbouring crops and from relay and intercrops onto cotton (Le *et al.*, 2008). In the most popular cotton cropping system, cotton is relayed after maize and intercropped with pulses and other crops, providing a continuous series of suitable host plants for *H. armigera*. These alternative intercrops can provide an important refuge for *Bt* susceptible genotypes of *H. armigera*.

9.3. Species with Greatest Risk

The result of a risk assessment for resistance risks will identify the pest species at greatest risk for resistance evolution, based on available and incomplete knowledge. Considering dose, efficacy, association with cotton and association with other crops, Fitt *et al.* (2008) concluded that the species most at risk for evolving resistance to *Bt* cotton in Viet Nam is *Helicoverpa armigera*.

10. PYRAMIDED TWO-TOXIN RESISTANCE

Pyramided, two-toxin resistance has two different toxin genes in the transgenic plant that affect the same target pest. Different target pests on the same transgenic crop may be affected by one or both toxins. If the target is affected by one of the toxins, then even if the additional toxin is present, the plant is not a pyramided variety for that pest. For example, Cry1F in cotton is toxic to *Spodoptera frugiperda* but Cry1Ac is not especially toxic to this species (USA EPA 2005a). Both Cry1F and Cry1Ac are toxic to *Heliothis virescens* (USA EPA 2005a). Thus, a combination of Cry1Ac and Cry1F would be a pyramided variety for *H. virescens* but not for *S. frugiperda*.

Pyramided, two-toxin resistance may provide a stronger basis to plan and implement IRM. It is strongly recommended as a condition for commercial use in developing countries (Fitt *et al.*, 2004; 2006; 2008), in part because regulatory oversight on compliance to IRM requirements may be weak, and compliance may be poor.

Evolutionary models (Fisher, 1958; Wright, 1968; Crow and Kimura, 1970; Mani, 1985) show that if insect resistance to the two toxins in the plant is determined

by independent genetic loci in the insect, then the time to resistance failure may be as long as the product of the times to failure to each gene. For example, if the time to resistance to toxin A is 20 generations, and the time to resistance to toxin B is 30 generations, it may take as long as 600 generations for resistance to evolve to both toxins. As a consequence, it has been suggested that IRM requirements, such as refuge size (Roush, 1998), could be reduced.

The key assumption to allow reduction in IRM requirements such as refuge size is that resistance to toxin A has no influence on the level of resistance to toxin B and *vice versa*. This means that there is no cross-resistance between the two toxins. Unfortunately, it is not possible to know if there is or is not cross-resistance prior to the evolution of resistance in the field. Based on known independent modes-of-action of the toxins, it has been commonly concluded that no cross resistance should occur (McKenzie, 1996). In the cases where history has allowed such conclusions to be tested, they have often been erroneous, and cross-resistance has evolved (McKenzie, 1996). The likely reason is that selection will select first and strongest for any allele or mutation in the natural population that provides some level of cross-resistance. Thus, suggestions that a pyramided plant will provide more durable resistance (Roush, 1998) should be treated with considerable skepticism, and any thought of reducing refuge requirements should be rejected unless other non-biological factors intervene.

11. MONITORING RESISTANCE

The goal of resistance monitoring is to obtain timely information that can be used to avoid or lessen the adverse consequences of pest resistance (Andow and Ives, 2002). Specifically, this translates into using monitoring information to change the IRM strategy for a *Bt* crop, prior to widespread control failures due to resistance, or to justify continuation of the current IRM strategy. Necessary steps in achieving this goal will include (1) monitoring the frequency of resistance to determine if it is changing and when it might lead to control failures, (2) investigation of putative field control failures, and (3) documenting the use of *Bt* cotton and compliance with the resistance management plan. This discussion focuses on the first of these. As discussed below, for low-dose events, phenotypic methods may be appropriate for monitoring resistance frequency, and for high-dose events, genic methods are necessary.

Monitoring methods (reviewed in Andow and Alstad, 1998) that could be used are:

phenotypic methods, including

(a) screening field-collected egg masses,

(b) screening field-collected larvae, and

(c) an in-field *Bt* maize screen (Andow and Hutchison, 1998; Venette *et al.*, 2000), and

genotypic methods, including

(d) an F_2 screen (Andow and Alstad, 1998; 1999),

(e) an F_1 screen (Gould *et al.*, 1997; Yue *et al.*, 2008), and

(f) DNA-based methods (none of which have yet been validated).

Phenotypic methods screen genotypes, while genic methods screen individual alleles. Phenotypic methods are most efficient for monitoring resistance alleles that are additive or dominant whilst genic methods are most efficient for monitoring recessive resistance alleles.

The relative advantages of the two approaches depend on the phenotypic expression of the *R* allele (Andow and Ives, 2002). As discussed above, in a natural population, three genotypes may occur, *RR* homozygotes, *RS* heterozygotes and *SS* homozygotes. Both phenotypic and genic methods will detect the *RR* homozygotes. The difference is in the detection of *RS* heterozygotes. Genic methods will detect all *RS* heterozygotes regardless of the expression of the *R* allele. Detection by phenotypic methods depends on the expression of the *R* allele. If the *R* allele is dominant, then heterozygotes will be phenotypically resistant and phenotypic methods will detect them. If an *R* allele is recessive, then heterozygotes will be phenotypically susceptible, and phenotypic methods will not detect any of them. If the *R* allele has in-between expression (additive), then phenotypic methods will be able to detect half of the heterozygotes. When resistance is rare, nearly all of the *R* alleles will be in heterozygote genotypes. Clearly genic methods will be far superior to phenotypic methods when resistance is recessive.

An example may illustrate this difference more concretely. Suppose the *R* allele frequency is 0.001. What is the expected number of individuals that must be sampled to detect one *R* allele? If resistance is dominant, 500 individuals must be sampled with either phenotypic or genic methods. In some cases, a genic method requires only 250 individuals. If resistance is recessive, phenotypic methods require 1,000,000 individuals, while genic methods require 250 or 500 individuals. If resistance is minimally high-dose (5 % fitness advantage), phenotypic methods require at least 20,000 individuals screened, while genic

methods still require only 250 or 500 individuals screened.

All of these methods require a bioassay to distinguish resistant from susceptible individuals. Some bioassay approaches are to use toxin in artificial diet at a discriminating concentration on either neonate or older larvae (Roush and Miller, 1986), using artificial diets with toxin incorporated into the diet (Gould *et al.*, 1997) or toxins applied to the surface of the diet (Marçon *et al.*, 1999). Another bioassay approach is to use the *Bt* plant itself, because by definition, the plant expresses a discriminating concentration. These include using whole plants in the field (Andow *et al.*, 1998), whole plants in the glasshouse (Zhao *et al.*, 2002), and excised leaf tissue (Huang *et al.*, 2007). For its ease of use, excised *Bt* plant tissue is probably the most convenient bioassay, because it avoids potentially expensive purified toxin, and takes up less space than whole plant methods.

Whatever method is used, it is critical to maintain the identity of the field-collected individuals. Many older methods combine the field-collected individuals into a single colony prior to testing. When this is done, it is no longer possible to know how many individuals are being tested. All of the genic methods require individuals to be maintained separately. Many of the phenotypic methods have not been as carefully carried out.

11.1. Phenotypic Methods

Two approaches are to collect from field populations either eggs or larvae and bioassay them or their offspring. Eggs can be convenient because the hatching neonates can be tested directly, while larvae must be reared to produce an F_1 generation before they are tested. If the eggs occur in an egg mass or are clustered by the ovipositing female, then the number of tested individuals is closer to the number of egg masses rather than the number of larvae bioassayed (Andow and Ives, 2002). This is because larvae from the same egg mass are siblings and are not independent samples of the natural population. Because larvae collected from the field often suffer high mortality from handling, many individuals are lost prior to screening, reducing the efficiency of the method.

An in-field maize screen is a novel approach to phenotypic screening and involves planting the *Bt* crop at a time and place where many of the target insects will accumulate (Venette *et al.*, 2000). The main advantage of this method over the previous ones is that it is considerably less expensive,

because whole *Bt* plants are used to screen insects in the field.

11.2. Genic Methods

An F_2 screen is done by collecting mated adult females from the field, transporting them to the lab, collecting the eggs of those females, rearing the F_1 larvae, sib-mating the F_1 families, collecting egg masses, and screening the F_2 neonates against a discriminating concentration (Andow and Alstad, 1998). F_2 screens have been used in Australia (Downes *et al*., 2007), France (Bourguet *et al*., 2003; Génissel *et al*., 2003), Spain and Greece (Andreadis *et al*., 2007), and the USA (Stodola *et al*., 2006; Huang *et al*., 2007). An F_1 screen requires a resistance colony. Unmated field-collected insects are individually mated to the resistant laboratory colony, and the F_1 offspring of these matings are screened with a discriminating concentration assay (Gould *et al*., 1997). This method is less expensive than an F_2 screen, because it is not necessary to rear the F_2 generation for a bioassay. However, because resistance to *Bt* crops have not been recovered, except for a few pest species, this method has not been possible to use more widely. Finally, DNA-based methods can be developed into a screening method once resistance has been found. However, these methods have not yet been verified for their accuracy and precision, so they have not yet been used for monitoring.

11.3. Cost of Monitoring

The cost of monitoring R allele frequency will probably depend on the target insect and the *Bt* crop. However, many of the major costs may remain relatively similar across several crops and pests (Andow and Ives, 2002; Huang and Leonard, 2008). Using cost estimates for *Ostrinia nubilalis* in the USA, it is possible to estimate the cost of each monitoring method in relation to its ability to detect rare alleles (Andow and Ives, 2002). For dominant *R* alleles, there is an intriguing cost-detection limit crossover among the monitoring methods. For high detection limits (*R* allele frequency >0.01), which would be useful to document control failures, the least expensive methods are the phenotypic screens based on laboratory testing of larvae or eggs. At lower detection limits (*R* allele frequency <0.01) the in-field phenotypic method is the least expensive. This occurs because the costs of planting and maintaining the field plots of the *Bt* crop is expensive per tested individual until sufficient numbers of individuals are sampled to achieve the desired detection limit.

For recessive alleles, the least expensive method for all desired detection limits is the F_2 screen when no resistance colony is available (Figure 3). This

occurs because the phenotype methods require much larger sample sizes than the F_2 screen to attain a similar detection limit. For example, a detection limit of 0.05 requires only 5 iso-female lines for an F_2 screen and 400 larvae for a phenotypic screen. The most cost-effective range of detection using an F_2 screen is for allele frequencies of 0.001 and up. If a resistance colony is available, monitoring costs can be greatly reduced for a given detection level.

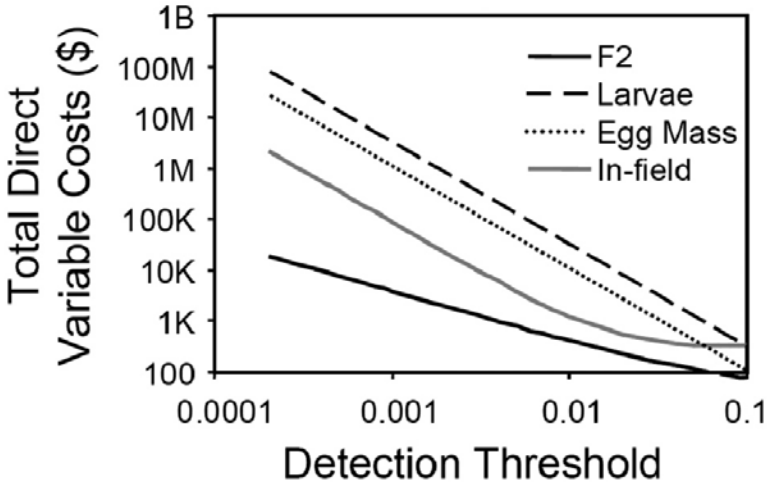

Figure 3. Cost of monitoring. Total direct variable costs (US$) for a given detection threshold for a recessive resistance (R) allele. The detection threshold is the R allele frequency that can be detected. Sampling methods are the genic F2 screen, and the phenotypic larval, egg, and in-field screens. From Andow and Ives (2002).

An additional issue that must be addressed is how to distribute monitoring efforts geographically to provide the greatest return for investment in monitoring. Monitoring is probably impractical if conducted uniformly and sparsely over large geographic areas because by the time the monitoring system detected resistance, it would be geographically widespread (Andow and Ives, 2002). Consequently, monitoring must be stratified by risk (or benefit), with most monitoring effort invested at the areas of high risk (or high potential benefit). For resistance monitoring of a *Bt* crop, it may be useful to stratify risk according to the proportion of farm area planted with the *Bt* crop.

12. CONCLUSIONS

The evolution of resistance in target pests to transgenic insecticidal crops is a significant environmental risk that could affect multiple stakeholders, including those outside of agriculture. Many kinds of transgenic insecticidal crops have been developed, but the commercially available ones, mainly cotton and maize, rely on a limited number of transgenic events, mostly crystal proteins from the bacterium *B. thuringiensis*, including Cry1A, Cry1Ab, Cry1Ac, Cry1F, Cry2Ab, Cry3A, Cry3Bb, and Cry34/35. Additional events based on vegetative insecticidal proteins of *B. thuringiensis* (Vip3A) and some proteinase inhibitors (Cowpea trypsin inhibitor, CpTI) are also available.

Resistance is defined as the phenotype of an individual that gives the individual the ability to survive on a transgenic insecticidal plant from egg to adult and produce viable offspring. Resistance should not be confused with "control failures caused by resistance". Control failures are a characteristic of a pest population (not an individual), and can occur when a sufficiently high proportion of individuals in a pest population is resistant, but control failures should not be called resistance.

The goal of insect resistance management (IRM) is to delay or prevent the occurrence of control failures from resistance by delaying or preventing the evolution of resistance. A practicable IRM strategy is necessary to attain this goal, which means that the costs associated with implementing IRM must be considered in setting the IRM strategy. In many cases, IRM plans for transgenic insecticidal crops are developed prior to the discovery of resistance in the target insect pest and before much of the important data have been collected. This means that there is often considerable uncertainty associated with these initial plans. In the presence of this uncertainty, adaptive resistance management provides a way to change IRM strategies and tactics as new information and experience becomes available.

It is widely agreed that resistance evolution can be successfully managed. Four general approaches can be used to delay resistance evolution. The approach most widely used is to reduce the exposure to selection by maintaining refuge habitats where the insect can survive and reproduce. A second approach is to reduce the difference in fitness between resistant and susceptible insects, such as by suppressing insect pests emerging from the transgenic crop, which are mostly resistant. A third approach is to

reduce *RS* heterozygote fitness, however, little is known about managing this factor. The fourth approach can be used only with high-dose IRM strategies, by managing sex-specific movement and mating frequencies to delay resistance evolution. The simplest approach by far is to reduce selection pressure by maintaining refuges. To manage resistance effectively, it is essential to be able to identify resistant individuals. The most definitive test is to rear the individual from egg to adult on the *Bt* crop, but there are some technical complications that can make identification challenging.
It is widely suggested that IRM in small-scale cropping systems characteristic of many developing countries will be difficult. Small-scale farmers have little land, capital or economic flexibility to bear the costs of IRM individually. As a consequence, community level action and naturally occurring crop and non-crop refuges may be important to ensure effective IRM.

Of all of the various strategies and tactics considered for IRM, the high-dose/refuge strategy is by far the most widely considered and used. This strategy is relatively simple to develop and implement. The high-dose/refuge strategy requires that the *Bt* crop produces a sufficiently high toxin concentration that the *R* allele is rendered recessive, and that a host plant other than the *Bt* crop is growing nearby as a refuge for the target pest or pests. A refuge provides unselected pests, which will mate with resistant individuals emerging from *Bt* fields, thereby making all offspring heterozygous and phenotypically susceptible. Although it cannot be conclusively stated that the high-dose/refuge strategies implemented in Australia, Canada and the USA have delayed resistance, the available evidence supports the conclusion that they have succeeded in delaying resistance evolution. The high-dose/refuge strategy works primarily by reducing the selection pressure favouring the resistance alleles. This is done by having a larger refuge and a higher dose. The larger the refuge, the smaller the proportion of the population exposed to selection in the *Bt* field. The higher the dose, the smaller the fitness advantage of the RS heterozygote over the *SS* homozygote in the *Bt* field. Both factors result in slower evolution. A third and quantitatively smaller effect is caused by the mingling and mating between individuals from *Bt* and refuge fields, which reduces the rate of formation of *RR* offspring in *Bt* fields. When determining the dose of a transgenic insecticidal crop, it is critical to distinguish dose from efficacy. Dose is the advantage of the *RS* heterozygote over the *SS* homozygote when both are feeding on the *Bt* plant. Efficacy is the mortality rate (or survival rate) of the *SS* homozygote when it is feeding on the *Bt*

plant. Although there tends to be a correlation between high efficacy and high dose, the relationship is weak and only partially predictive.

IRM strategies can be developed by beginning with resistance risk assessment. For any given crop there are usually multiple pest species that require control, and any given pest control tactic usually affects multiple pest species. A first step in developing an IRM strategy is to assess which species are at risk of resistance and of these, which pest species is most at risk. This can be done by:

- Identifying the pest species on the crop that are susceptible to the *Bt* toxins in use.
- Characterising the likely dose of the transgenic toxin for each species.
- Characterising the potential exposure of each species to the *Bt* crop.

With this information, the relative resistance risk of the various pest species can be determined and the species that is most likely to evolve resistance before the others can be identified.

Resistance monitoring is essential to track the progress of resistance evolution and to determine the success of the IRM strategy. One of the main goals of resistance monitoring is to obtain timely information that can be used to change the IRM strategy to avoid or lessen the adverse consequences of pest resistance. Several monitoring methods have been proposed. Phenotypic methods are best suited for low-dose events and genic methods are best suited for high-dose events. When resistance has not yet been found, an F_2 screen is the most cost-effective method for high-dose events. When a suitable resistance has been found, an F_1 screen becomes more cost-effective for those same events.

Resistance risks are real and serious. However, they can be managed to preserve the usefulness of transgenic insecticidal crops into the future.

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