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*Review*

# Gene pyramiding as a *Bt* resistance management strategy: How sustainable is this strategy?

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**Reports on the emergence of insect resistance to *Bacillus thuringiensis* delta endotoxins have raised doubts on the sustainability of Bt-toxin based pest management technologies. Corporate industry has responded to this challenge with innovations that include gene pyramiding among others. Pyramiding entails stacking multiple genes leading to the simultaneous expression of more than one toxin in a transgenic variety. Questions have been raised on the sustainability of gene pyramiding since the use of insecticide mixtures has shown that cross resistance and/or multiple resistance can render such strategies to be less effective in the long term. Current theoretical and practical evidence in insect population genetics suggest that gene pyramiding cannot be sustained as a resistance management strategy *per se*. Pyramiding is useful as a strategy to broaden the range of insect pests controlled in each transgenic variety, and it still has to be deployed in tandem with Bt resistance management strategies such as crop refugia, biological pest control, temporal and spatial crop rotations among others.**

**Key words**: Gene pyramiding, *Bacillus thuringiensis*, resistance management.

## INTRODUCTION

Insect Pest Management took a new dimension with the development and deployment of transgenic *Bacillus thuringiensis* (Bt) varieties in cotton, corn and potatoes (Shelton et al., 2002; Ferry et al., 2004). Numerous Bt strains producing various endotoxins are in commercial use as conventional sprayable formulations, and Bt transgenic crops are now being widely grown in a number of developed and developing countries (Schnepf et al., 1998; Shelton et al., 2000; Ferry et al., 2004; Cohen, 2005; Ferre and Van Rie, 2002; Wu and Guo, 2005).

The development of insect resistance to transgenic crops producing Bt toxins poses a major threat to their sustainable use in agriculture (Ferre and Van Rie, 2002; Ru et al., 2002; Gahan et al., 2005). Due to the urgent need for a more complete understanding of the parameters of effective Bt resistance management, companies developing *Bt* bio-pesticidal sprays and transgenic crops formed the *Bacillus thuringiensis* Management Working Group in 1988 to promote research on the judicious use of Bt products (Schnepf et al., 1998).

At present, it is only the diamondback moth *Plutella xylostella* that has developed field resistance to Bt and this is due to a reduction in toxin binding to gut receptors (Shelton et al., 2002; Ferre and Van Rie, 2002; Kain et al., 2004). Corporate industry and the academia have responded to this emerging threat with an ingenious technique of gene pyramiding, particularly in transgenic cotton varieties. Essentially gene pyramiding entails the simultaneous expression of more than one toxin in a transgenic plant (Shelton et al., 2002).

The rationale behind gene pyramiding stems from the age old philosophy of the use of insecticide mixtures to broaden the spectrum of insects controlled in one spray event. However, lessons drawn from the use of insecticide mixtures have shown that the strategy has at times led to faster development of resistance in insect species as insects are exposed to multiple toxins at the same time (Metcalf, 1994). Such a scenario induces intense resistance selection pressure and even in situations where strategies such as temporal and spatial

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rotation of active ingredients are employed, resistance still builds up to unsustainable levels, albeit at a slower pace (ffrench-Constant et al., 2000). Gahan et al. (2005) reinforce this position when they argue that the Bt pyramiding strategy could fail if a single gene in a pest confers resistance to both toxins. The question being asked, therefore, is whether or not gene pyramiding will succeed where insecticides have failed.

In the endeavor to answer this question, this review briefly outlines the mode of action of Bt toxins, discusses the philosophy behind gene pyramiding- its practical merits and limitations, looks into alternative Bt resistance management strategies and finally evaluates the worth of gene pyramiding as Bt resistance management strategy.

## MODE OF ACTION OF *BACILLUS THURINGIENSIS* δ-ENDOTOXINS

*Bacillus thuringiensis* is a gram positive bacterium that produces crystalline inclusions during sporulation (Maddox, 1994). These inclusions consist of either one or a subset of related insecticidal crystal (Cry) proteins (Maddox, 1994; Ferry et al., 2004). The crystal inclusion is dissolved by the alkaline larval midgut of a susceptible insect species to release a 130 kDa protoxin that is cleaved and activated by midgut proteases. Once activated, the toxin interacts with an appropriate midgut epithelial cell receptor and inserts into the gut membrane, producing pores that disturb the osmotic balance causing cells to swell and eventually lyse and as a result the larvae stop feeding and die (Schnepf et al. 1998, Ferre and Van Rie, 2002; Shelton et al., 2002). The highly specific insecticidal activity exhibited by Bt Cry proteins is affected by differences in the larval gut that alter the solubilization and/or processing efficiency of the protoxin and by the presence of specific high affinity toxin binding receptors in the guts of different insects (Maddox, 1994; Knight et al., 2004).

## RATIONALE BEHIND GENE PYRAMIDING

Gene pyramiding has been hailed as a lasting Bt resistance management strategy (Jackson et al., 2003, Shelton et al., 2002). However, a closer look at the strategy reveals that pyramiding was developed as a practical strategy to broaden the range of insect species that were not being adequately controlled by a single toxin as in the case of the single gene Bollgard® Bt cotton variety.

The strategy of Bt gene pyramiding rests on three core assumptions (Gahan et al., 2005). The first assumption is that insects resistant to only one toxin can be effectively controlled by a second toxin produced in the same plant. This assumption forms the basis for the Bollgard® II cotton variety which has two toxins namely, Cry 1Ac and Cry 2Ac. The Cry 1Ac toxin controls tobacco budworm and pink bollworm while the Cry 2Ac toxin controls corn earworm (Jackson et al., 2003; Ferry et al., 2004; Purcell et al., 2004).

The second assumption is that strains resistant to two toxins with independent actions cannot emerge through selection pressure with one toxin alone. Karim et al. (2000) contend that the use of multiple toxins to impede evolution of resistance is premised on the idea that if insects homozygous for one resistance gene are rare, insects homozygous for multiple resistance genes are extremely rare. When using multiple crystal proteins, even insects homozygous for one or two resistance genes but heterozygous for another resistance gene would still be controlled by crops expressing multiple Bt toxins (Schnepf et al., 1998; Sisterson et al., 2004).

The third assumption underlying the strategy of Bt gene pyramiding is that a single gene will not confer resistance to two toxins that are immunologically distinct and that have different binding targets (Gahan et al., 2005).

### Practical merits of gene pyramiding

Transgenic cotton expressing a truncated version of the Cry 1Ac gene has been commercially available in the United States since 1996 (Ferry et al., 2004; Bates et al., 2005). The technology has been noted to provide excellent control of the tobacco budworm (*H. virescens*), although control of the bollworm (*H. zea*) has been somewhat less than had initially been suggested (Harris et al., 1998; Jackson et al., 2003;Bird and Akhurst, 2005).

Second generation dual- Bt gene cottons Bollgard II® (Cry 1Ac + Cry 2Ab) and WideStrike™ (Cry 1Ac + Cry 1F) express two *Bt* endotoxins and were introduced in order to raise the level of control for *H. zea*, which was not satisfactorily controlled by the Cry 1Ac toxin alone (Jackson et al., 2003; Ferry et al., 2004; Bates et al., 2005; Gahan et al., 2005). The Cry 1Ac and 2Ab toxins have different binding sites in the larval midgut and are considered to be a good combination to deploy in delaying resistance evolution. This is due to the fact that a species cannot easily evolve resistance to both toxins because that would require two simultaneous, independent mutations in genes encoding the receptors (Jackson et al., 2003). Bird and Akhurst (2005) reinforce this argument when they reported on insects that developed resistance to one crystal protein group by changing particular receptor sites but were still fully susceptible to other crystal proteins.

### Limitations of the Bt gene pyramiding strategy

The assumptions upon which the strategy of Bt gene pyramiding are founded, also tend to be the weakest points in this seemingly solid foundation.

**Diversity and plasticity of resistance genes:** The assumption that insects resistant to only one toxin can be effectively controlled by the second toxin produced by the plant fails to take full consideration of the enormous genetic plasticity in insect populations. The assumption also fails to account for the argument that in many insect populations there may be a proportion of resistant alleles that can increase over time and space as the proportion of homozygous individuals increases (ffrench-Constant et al., 2002; Ferre and Van Rie, 2002).

Shelton et al. (2002) argue that in order to have an effective resistance management strategy using the high dose/refuge strategy, the frequency of resistant alleles and the survival of individuals heterozygous for resistance must be low. The high dose/refuge strategy advocates for the use of highly effective insecticides that eliminate most of the tolerant individuals in the insect population. However, due the dynamic and fluid nature of insect/crop interactions and the specificity of such interactions to a given locality, it is difficult to define levels of heterozygous resistant individuals that can be classified as being low, medium or high (Ru et al., 2002).

**Continuous exposure of insects to the active toxin:** The Bt toxin expressed in transgenic plants is driven by a constitutive promoter meaning that in theory the toxin is continuously produced at high levels for the duration of the growing season. This creates some potential for cross resistance to pyramided genes, as it confers intense and uninterrupted selection pressure on the insects, unlike in a conventional spray treatment where selection pressure is maintained for a few days as the active material dissipates (Harris et al., 1998). The development of cross and multiple resistance to synthetic insecticides came about partly as a result of the widespread use of insecticide mixtures where insects were exposed to multiple toxins at the same time (Metcalf, 1994). This suggests that insect resistance to Bt toxins might also arise in a similar manner.

**Multiplicity of binding sites:** The assumption that strains resistant to two toxins with independent modes of action cannot evolve through selection with one toxin alone fails to account for the fact that one toxin can bind to several sites. Such a scenario can lead to the development of cross resistance or multiple resistance of an insect in cases where it was never exposed to the original toxin (ffrench-Constant et al., 2000). As an example, genetic resistance studies have identified five separate loci or groups of loci as conferring resistance to Cry 1Ac toxin in *H. virescens* (Schnepf et al., 1998).

In 1985, the first report on insect resistance to Bt was published (Harris et al., 1998). The diamondback moth, *Plutella xyllostella*, evolved high levels of resistance in the field as a result of repeated use of Bt (Schnepf et al., 1998; Shelton et al., 2000; Ferre and Van Rie, 2002). In the field, *P. xylostella* evolved resistance against HD-1 (*B. thuringiensis* var. *kurstaki*), which is composed of 13.6% Cry 1Aa, 54.2% Cry 1Ab and 32.2% Cry 1Ac (Karim et al., 2000). In *Plodia interpunctella*, *P. xylostella* and *H. virescens*, the resistance is due to a change in binding affinity of receptors and binding sites on the brush border membrane vesicles (BBMVs) of the insect midgut (Karim et al., 2000). A strain of *H. virescens* selected for resistance to Cry 1Ac under laboratory conditions, was cross resistant to Cry 1Aa, Cry 1Ab. Cry 1Ba, Cry 1Ca and Cry 2Aa toxins (Karim et al., 2000).

Karim et al. (2000) noted that *P. gossypiella* was susceptible to Cry 1Aa, Cry 1Ab, Cry 1Ac and Cry 2Aa toxins. The Cry 1Ac and Cry 1Ab were more potent on *H. zea* than Cry 1Aa and Cry 2Aa. The Cry 1Ba, Cry 1Ca, Cry 1Da, Cry 1Ea, Cry 1Fa, Cry 1Ga, Cry 1Ha and Cry 2Ba were not potent against both pests. In heterologous competitive binding assays to investigate the binding site cross reactivity, it was shown that Cry 1Aa, Cry 1Ab and Cry 1Ac recognize the same binding site which is different from Cry 2Aa.

Li et al. (2005) demonstrated that laboratory selected *Ostrinia nubularis* strains developed resistance to several individual Bt protoxins after repeated exposure to Dipel (*B. thuringiensis* var. *kurstaki*). In line with this evidence, the authors argued that if Dipel or other protoxin based Bt formulations become popular and are used extensively against *O. nubilaris*, resistance may develop to one or more of the individual Bt toxins.

## MULTIPLICITY OF RESISTANCE MECHANISMS

The assumption that strains resistant to two Bt toxins with independent binding properties cannot result through selection with one toxin alone does not account for the possibility of resistance related to disruption of other steps in the mode of action of Bt toxins. Steps that can be disrupted include ingestion, solubilization, proteolytic processing, membrane insertion, activation of protoxin to toxin, crossing from peritrophic membrane, binding to receptors, pore formation and lysis of midgut cells (Karim et al., 2000; Ferre and Van Rie, 2002). The bulk of the cases of resistance are related to changes in receptor binding properties on brush border membrane vesicles (BBMVs) of the insect midgut (Karim et al., 2000; Ferre and Van Rie, 2002), but there are real chances that resistance can develop from alterations in other biochemical pathways in Bt toxin metabolism in insects.

**The yield versus resistance trade-off:** It has been argued that the greater the number of genes that are engineered into a transgenic plant, the more plant protein will be diverted away from creating useful yield and into manufacturing the foreign substance instead. This scenario sets the risk of significant agronomic and yield penalties which may make the variety unattractive to the grower (Shelton et al., 2000).

**Gene silencing:** Gene silencing is a plant response that can recognize and methylate foreign gene promoters rendering them inoperative. This response can have a major impact on the potential of transgenic crops to carry functional stacked genes. If gene silencing occurs during the seed production cycle, the whole seed lot could spontaneously lose the desired gene effect (Harris et al., 1998). Gene silencing is therefore a potential limiting factor as to the number and types of genes that can be inserted into a particular crop variety.

## Alternative bt resistance management strategies

Some workers have argued that insect resistance to transgenic toxins is inevitable and it is just a question of how rapidly it occurs (Harris et al., 1998). However, there is reason to be optimistic as evidence shows that some of the measures have helped in slowing down the emergence of resistance and some of these are discussed below.

**Refugia for susceptible populations:** The foundation of resistance management strategy was the adoption of refugia, which are adjacent areas of non-transgenic cotton which would produce high numbers of Bt susceptible insect that might dilute any potential resistance by cross-breeding with the small number of survivors from the Bt cotton. The concept relies on very high and constant expression of the insecticidal protein, sufficient to kill both susceptible and a very high proportion of heterozygous resistant individuals (Gould, 1998; Shelton et al., 2000; Bates et al., 2005).

Ru et al., (2002), used a model which predicted that in north China the expected life of Bt cotton will be about seven years if all farmers grow Bt cotton but can be extended to ten years if only the cotton planted in spring is Bt (about 70% of the total cotton area). In the use of refugia, care must be taken to ensure that refuges, particularly those sprayed with efficacious insecticides, produce adequate numbers of susceptible alleles (Wu and Guo, 2005).

**Differential toxin expression-dosage control:** Selection pressure in transgenic plants can be reduced by restricting the expression of the crystal protein genes to certain tissues of the crop, the remainder providing a form of spatial refuge (Schnepf et al., 1998; Shelton et al., 2000). This strategy is based on differential toxin expression across the crop's phenology. Material from the extremes of the plant (the tips, bolls and leaves adjacent to the bolls) results in high mortality to insects whilst lower mortality was observed from feeding on leaf tissue in the $2^{nd}$ to $4^{th}$ node and in the flowers (Harris et al., 1998).

**Temporal and spatial rotations of Bt products:** Rotation over time and space of transgenic varieties or sprays of a particular Bt toxin with those of another type that bind to a different receptor has potential value when a fitness cost is associated with resistance (Bates et al., 2005; Bird and Akhurst, 2005).

## Is gene pyramiding a sustainable resistance management strategy in the long term?

A thorough understanding of the biochemical and genetic basis of resistance to Bt is the cornerstone in the design and deployment of appropriate management tactics to delay or reduce the evolution of resistance in insect populations (Karim et al., 2000; Ferre and Van Rie, 2002). There is no doubt that Bt resistance management cannot succeed through a single measure and hence the need for an integrated approach (Bates et al., 2005).

Each pest-crop complex may require site specific resistance management strategies that have to address the use of both Bt sprays and transgenic varieties. The farmers' experiences with transgenic varieties grown under different agronomic conditions are vital to define the area specifics of resistance management (Schnepf et al., 1998; Shelton et al., 2000). A resistance management strategy should be as broadly encompassing as is feasible and should be acceptable to stakeholders involved, including the technology suppliers, seed companies, extension workers, crop consultants, regulators and most importantly, the farmers (Gould, 1998).

Future pest management practices will have to rely on the introduction of transgenic cottons that express other insecticidal toxins in addition to the Cry toxins (Ferry et al., 2004; Wu and Guo, 2005). Recently, transgenic cottons were engineered to produce a vegetative insecticidal Bt protein (Vip3A) during the vegetative stage (Shelton et al., 2000; Jackson et al., 2003). Such broadening in the scope and mode of action of toxins gives growers more options in their overall resistance management efforts.

## CONCLUDING REMARKS

From current theoretical evidence and practical experiences, it is clear that gene pyramiding is useful in broadening the range of insect species controlled in one transgenic variety but it is not a panacea to Bt resistance be seen as one of the many strategies that can be problems that may emerge in future. Pyramiding should deployed in an integrated approach to delay the emergence of Bt resistance in pest species. Biological pest control using parasitoids and predators, cultural practices and other pest management tactics are all essential tactics in preserving the efficacy of Bt based

products. In the case of cotton, it is prudent in the meantime to continue with the refugia set aside strategy to delay insect resistance in transgenic Bt cotton.

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