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Genetic Diversity of *Bt* Resistance: Implications for Resistance Management

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Abstract: The bacterium *Bacillus thuringiensis* is the main source of insecticidal proteins in insect resistant plants. However, biochemical and genetic studies have shown that insect resistance to *B. thuringiensis* (*Bt*) toxins can occur and with the advent of *Bt* transgenic crops this is a major concern. Several insect species have shown resistance to these toxins in the laboratory but the diamondback moth, *Plutella xylostella* is the only species which has evolved resistance under field conditions to date. Many studies have been done to elucidate the mode of action of the toxins and the mechanisms and genetics of resistance. In this article *Bt* toxins, their mode of action, mechanisms and genetics of resistance and management strategies for delaying resistance are reviewed. The emphasis is placed on examining the presently recommended high dose/refuge strategy.

Key words: *Bacillus thuringiensis*, cry toxins, high dose/refuge, insect resistance, resistance management, transgenic plants

Introduction

Since the widespread introduction of synthetic insecticides in the late 1940s, a steady stream of new compounds has been developed. Due to their efficacy and specificity these insecticides are much more effective than the control methods that preceded them. However, due to the specific mode of action and ease of metabolism of many of these pesticides, pest populations can negate them by utilizing alleles which provide resistance and pesticide resistance has doggedly followed the introduction of new pesticides (Roush and Tabashnik, 1990).

Since the first commercial *Bacillus thuringiensis* (*Bt*) formulations appeared in the late 1930's, they have been used widely as niche products for pest control. It had been presumed that resistance to *Bt* toxins was unlikely because of its unique mode of action (Bauer, 1995). However, this was subsequently shown not to be the case and the Indian mealmoth, *Plodia interpunctella* Hüb. (Lepidoptera:Pyralidae) was the first insect reported to have developed resistance to *Bt* toxins, albeit under laboratory conditions. While various other insect species have been subsequently shown to develop resistance under laboratory conditions (Table 1), the diamondback moth, *Plutella xylostella* L. (Lepidoptera:Plutellidae) remains the only insect species which has developed resistance in the field. The development of resistance to *Bt* toxins is seriously threatening their life expectancy as pest control agents, particularly with the introduction of commercially grown Transgenic crops expressing insecticidal *Bt* proteins which increase the risk of resistance by providing a constant selection pressure (Wright *et al*., 1997; Tabashnik *et al*., 1998). As many major pests have already developed resistance to chemicals, the fact that *Bt* makes up to 98% of all biopesticides and that the demand for *Bt* crops is increasing makes the impact of resistance potentially dramatic.

100 years of *Bacillus thuringiensis* discovery: *Bacillus thuringiensis* represents a major group of microbes used for insect biocontrol (Macintosh *et al*., 1991). It is a gram-positive soil bacterium distinguished from other bacilli by its production of parasporal crystal proteins (Yamamoto and Powell, 1993). *Bacillus thuringiensis* is quite closely related to *B. cereus* (Carlson *et al*., 1994) and classical biochemical and morphological methods of classifying bacteria have consistently failed in distinguish *B. thuringiensis* from *B. cereus*. Chromosomal DNA hybridization, phospholipids and fatty acid analysis and genomic restriction digest analysis support a single-species hypothesis (Schnepf *et al*., 1998). Cry toxin synthesis is also transmissible from *B. thuringiensis* to *B. cereus* via conjugation (Gonzalez *et al*., 1982). *Bacillus thuringiensis* was first discovered as a potential microbial agent for insect control in 1901 when the Japanese bacteriologist Ishiwata (1901) isolated this bacterium from diseased larvae of Silkworm, *Bombyx mori* L. (Lepidoptera:Bombycidae) and named it "Sottokin" mean "sudden death *Bacillus*". A decade later, Ernst Berliner isolated a similar organism from a diseased granary population of the flour moth *Anagasta kuehniella* Zeller (Lepidoptera: Pyralidae) from Thuringia, Germany. Aoki and Chigasaki (1960) reported that its activity was due to a toxin present in sporulated cultures and that the toxin was not an exotoxin. In 1927, Matte re-isolated the organism from the same host, as did Berliner (Heimpel and Angus, 1960) and both Berliner and Matte observed, in addition to the spore, a second body which they called a "Restkorper" in the developing sporangia (Beegle and Yamamoto, 1992).

The first attempts to use *Bt* as a biopesticide using *B. thuringiensis* var. *kurstaki* (*Btk*), were carried out in the late 1920s and early 1930s against the European corn borer, *Ostrinia nubilalis* Hüb. (Lepidoptera: Pyralidae) in South East Europe (Van Frankenhuyzen, 1993). The first commercial product, Sporein®, became available in 1938 in France, primarily for control of *P. interpunctella* (Weiser, 1986). Work by Steinhaus (1951) restimulated interest in the commercial exploitation of *Bt* for control of lepidopteran pests of field crops and by 1957 the product Thuricide® (*Btk*) was available (Beegle and Yamamoto, 1992). The availability of commercial products initiated a period of "intermittent field testing" throughout the 1960s with inconsistent results (Mott *et al*., 1961). This resulted in two significant developments. First, the discovery of HD-1 a *Btk* isolate, which proved to be more potent than the isolates in existing commercial *Bt* products (Dulmage, 1970). The use of HD-1 population was also encouraged by its broad spectrum of activity against more than 100 lepidopteran species (Navon, 1993). Second, the establishment of an international system for standardizing the potency of commercial products. The first generally accepted standard was prepared in France from a fermentation of H-type *Bt* and called "E-61" (Burges, 1967). This was later replaced by HD-1 as the North American standard based on larvae of the cabbage looper, *Trichoplusia ni* Hüb (Lepidoptera:Noctuidae) (Beegle *et al*., 1986). The activity of *Bt* products is now routinely expressed as the number of International Units (IU) per unit product. Bonnefoi and Debarjac (1963) named as isolate as *B. thuringiensis* var. *aizawai* (*Bta*), which was particularly active against larvae of the wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) and *Spodoptera* spp. (Lepidoptera:Noctuidae). Since then several *Bt* vars. have been found and used against different pests (Beegle and Yamamoto, 1992).

Insecticidal toxins produced by the *Bacillus thuringiensis*: *Bacillus thuringiensis* typically produces several types of toxin, four of which are significant (Dulmage, 1970) α-exotoxins (heat-labile exotoxins), β-exotoxins (fly-factor or heat-stable exotoxins), δ-endotoxins (Crystal toxins) (Heimpel, 1August 16, 2002967) and "louse-factor" (Gingrich *et al*., 1974).

α-exotoxins: Toumanoff (1954) was the first to report that *Bt* produced a heat-labile insecticidal exotoxin which was toxic to *G. mellonella* larvae. While Smirnoff (1964) found a similar substance in Thuricide® filtrates, which was toxic to Lepidoptera, Coleoptera,

Diptera, Orthoptera and Hymenoptera. Heimpel (1967) coined the term α-exotoxin for such *Bt* heat-labile toxins. Krieg (1986) estimated the size of α-exotoxins at 45-50 KDa by gel filtration.

β-exotoxins: These were first discovered by Connel and Richardds (1959) and named "β-exotoxin" by Heimpel (1967) and since then defined as an adenine-nucleotide and ATP analogue and given the name "thuringiensin". These low molecular weight toxins are thermostable and have a broad spectrum of activity killing various lepidopteran dipteran, hymenopterans isopteran nematodes and mites. Gingrich *et al.* (1992) reported the existence of more than one type of heat tolerant exotoxin. Levinson *et al.* (1990) confirmed the existence of a second heat tolerant exotoxin and named II β-exotoxin, which is more specific than the type I β-exotoxin and very active against the Colorado potato beetle, *Leptinotarsa decemlineata* Say. (Coleoptera:Chrysomelidae). The β-exotoxin preferentially inhibits biosynthesis of RNA. (Beegle and Yamamoto, 1992). The products based on these β-exotoxins are also used effectively against several species of red spider mites as well as against larvae of houseflies and blowflies (Beegle and Yamamoto, 1992). The β-exotoxin can cause teratogenic effects and disrupt larval or pupal moulting. In mites, exotoxin has been reported as having a gonadotropic and morphogenetic action similar to that of juvenoids (Petrova, 1987).
A new variant of β-exotoxin has been described from *B. thuringiensis* var. *israelensis* by Weiser *et al.* (1992) and Horak *et al.* (1996). They found activity of water-soluble metabolites of *B. thuringiensis* var. *israelensis* toxic to aquatic molluscs and Trematoda. The toxin was termed "M-exotoxin" for molluscs-active exotoxin.

Enterotoxins: *Bacillus thuringiensis* isolates have been found to produce *B cereus*-diarrhoeal-type enterotoxins (Carlson *et al.*, 1994). *Bacillus cereus* enterotoxins are responsible for symptoms of food poisoning following ingestion of *B. cereus*. Damgaard *et al.* (1996) isolated enterotoxin producing strains of *B. thuringiensis* from various foods. Similarly Te Gazit *et al.* (1997) reported two enterotoxins producing strains that has previously identified as *B. cereus* and implicated in incidents of food poisoning.

Vegetative insecticidal proteins: A new class of insecticidal toxins, vegetative insecticidal proteins have been isolated from *B. thuringiensis*. Vip3A is a noval protein with a wide spectrum of activities against lepidopteran insects and was first reported by Estruch *et al.* (1996). For example Donovan *et al.* (2001) demonstrated that an important component of *B. thuringiensis* insecticidal activity against *Spodoptera exigua* (Lepidoptera: Noctuidae) is the synthesis of Vip3A protein by *B. thuringiensis* cells after ingestion of spores and crystal proteins by insect larvae. These proteins had no homology to known proteins and were expressed in vegetative growth stage (Estruch *et al.*, 1996). The midgut epithelium cells of the susceptible insects are the primary target for the Vip3A insecticidal proteins and their subsequent lysis appears to be the primary mechanism of lethality (Yu *et al.*, 1997).

δ-endotoxins (crystal proteins): During sporulation *Bt* produces parasporal crystalline inclusions bodies of a wide range of morphological types (Meadows *et al.*, 1992). The proteins comprising these crystals account for 20-30% of the total bacterial protein at sporulation (Boucias and Pendland, 1998). These can be classified into six general types: bipyramidal, rhomboid, spherical, rectangular, irregular pointed and irregular spherical (Chilcott and Wigley, 1994). The bipyramidal crystals show a greater frequency of toxicity than all other types and the majority of isolates with lepidopteran activity contain such inclusions (Attathom *et al.*, 1995). The crystal (Cry) proteins that form bipyramidal crystals are typically 130 KDA in size and several closely related proteins may be present in a single crystal (Yamamoto and Powell, 1993). Bipyramidal crystals synthesized in the host cells are typically about 1.1 µm long and 0.5 µm wide (Oeda *et al.*, 1989). Irregular spherical crystals can be mosquitocidal (Ohba *et al.*, 1995), while rhomboid crystals are active against some coleopteran species (Burtseva *et al.*, 1995). In addition to producing Cry toxins, several *Bt* strains also produce cytolytic endotoxins. These include the Cyt1A toxins from *Bt israelensis* and *Bt morrisoni* PG14 and Cyt2A from *Bt kyushuensis*. Unlike the Cry endotoxins, the Cyt endotoxins display broad unspecific activity *in vitro* and *vivo*. Crickmore *et al.* (1998) defined Cry proteins as "a parasporal inclusion (Crystal) protein from *Bt* that exhibit some experimentally verifiable toxic effect to a target organism, or any protein that has obvious sequence similarity to a known Cry protein".
The inclusion bodies act as gut poisons to species belonging to six orders of insect: Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera and Mallophaga (Höfte and Whiteley, 1989; Feitelson *et al.*, 1992) as well as to Nematoda and Protozoa (Feitelson *et al.*, 1992). A total of 89 different crystal proteins have been cloned (De Maagd *et al.*, 2001). The full list of delta-endotoxin is maintained by Dr N. Crickmore, University of Sussex UK and can be found at http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/.

Mode of action of Cry proteins: *Bacillus thuringiensis* crystals ingested by a susceptible insect larva liberate 130-140 KDa protoxin molecules that are solubilized in the high pH (8.0-10.0) environment of the lepidopteran and dipteran midgut (Koller *et al.*, 1992), or below pH 4 and above pH 10 in the case of the coleopteran-active toxin, Cry3A (Koller *et al.*, 1992). Insecticidal activity decreases rapidly following exposure of protoxins to conditions below pH 2 or above pH 11 (Tran *et al.*, 2001). By the action of midgut proteases these protoxins are processed into active, toxic fragments (approximately 55-70 KDa) (Schnepf *et al.*, 1998), which either act at the midgut membrane or pass through the gut into the haemocoel where it exerts its effect (Fast, 1981). The major proteases in the lepidopteran midgut are trypsin-like (Milne and Kaplan, 1993) or chymotrypsin-like (Novillo *et al.*, 1997). The conversion of Cry1A-type protoxin involves the removal of approximately 600 amino acid residues from the C-terminal end of the molecule, producing an active 67 KDa toxin (Aronson *et al.*, 1991; Visser *et al.*, 1993; Bravo *et al.*, 2002). The earliest physiological symptom of protoxin is an increase in glucose uptake by gut epithelial cells due to a stimulation of respiration (Faust *et al.*, 1974).
Differences in the extent of solubilization of protoxin can sometimes explain differences in the degree of the toxicity between Cry proteins (Du *et al.*, 1994). A reduction in solubility is also thought to be one potential mechanism for insect resistance (McGaughey and Whalon, 1992). However, the host specificity of *Bt* toxins depends largely upon their ability to bind to a specific midgut receptors (Estada and Ferre, 1994; Feldmann *et al.*, 1995). Rie *et al.* (1989) reported the occurrence of multiple receptors for Cry1A toxins. For example, receptor 1 binds all Cry1A toxins, receptor 2 binds Cry1Ab and Cry1Ac, while receptor 3 binds Cry1Ac only (Yamamoto and Powell, 1993). Cry1Ab and Cry1Ac share the same binding site in *T. ni* and *O. nubilalis*, whereas the Cry1Aa binds to a different site (Estada and Ferré, 1994).
In *Lymantria dispar* (Lepidoptera:Lymantriidae), Cry1Aa shares a binding site with Cry1Ac and in *Manduca sexta* (Lepidoptera: Sphingidae) it also share binding site with Cry1Ab (Hofmann *et al.*, 1988). However, Cry1Ab shares a common site with Cry1Aa, Cry1Ac and Cry1F in *P. xylostella* (Ballester *et al.*, 1994). Such heterogeneity, combined with differences in affinity and concentration of binding sites, may to a large extent account for the observed diversity in toxicity spectra. Additional factors which can play a role in determining specificity are protoxin stability (Arvidson *et al.*, 1989), differential solubilization of crystals (Aronson *et al.*, 1991) and subsequent proteolytic processing (Milne *et al.*, 1995).
Different domains of the Cry protein molecule are responsible for the steps of receptor recognition and pore formation (Van Frankenhuyzen, 1993). Elucidation of the three dimensional structure of Cry3A (coleopteran-specific), Cry1Aa (lepidopteran-specific) and Cyt2A (mosquito specific) showed that the toxic fragments of Cry proteins are composed of three distinct

structural domains. Domain I (a 7-α-helical bundle) has the ability to form pores or channels in the insect epithelial membrane (Thompson *et al.*, 1995). Domain-II (a triple-β-sheet structure) may be responsible for receptor recognition, which has a fold similar to that of the plant lectin jacalin and jacalin is known to bind carbohydrates via exposed loops at the apex of its β-prism fold. (Schnepf *et al.*, 1998). Domain III (a β-sandwich) in which the C-terminal end is buried, may protect the toxic fragment from further degradation during proteolytic processing (Smith and Ellar, 1994). The "Jelly-roll" configuration of Domain III is a relatively common structural feature that has been observed in a number of other proteins (Yamamoto and Powell, 1993). The β-sandwich structure of domain III is thought to play a key role in the biochemistry of the protoxin molecule, protecting the molecule from proteolysis within the gut (Li *et al.*, 1991). Also, the domain-exchange studies have found that the toxicity of a toxin to the insect host followed the movement of domain III, which is prerequisite step in receptor binding (Li *et al.*, 2001).

In contrast to Cry3A and Cry1Aa, Cyt2A consists of a single domain in which two outer layers of α-helix wrap around a mixed β-sheet. Cyt1A is believed to have a similar structure (Schnepf *et al.*, 1998). The β-sheet structure of Cyt2A suggests a pore based on a β-barrel (Li *et al.*, 1996). Chow *et al.* (1989) have observed that Cyt1A aggregates on the surface of the target cell. While Gazit *et al.* (1997) found that Cyt1A self-assembles within the membrane and also identified two α-helices (A and C), which are involved in both membrane interaction and intermolecular assembly.

The membrane bound Cry protein toxic fragment induces the formation of a pore, either aspecific or K^+ - specific, in the midgut epithelial cell membrane. Pores in the plasma membrane disrupt the actively maintained osmotic balance, causing the cells to swell and burst by colloid-osmotic-lysis (Knowles and Ellar, 1987; Visser *et al.*, 1993) due to an alteration in ion flux and inhibition of amino acid transport across the brush border membrane (BBM) (Giordana *et al.*, 1993). Direct permeability assays on cell lines (Knowles and Ellar, 1987) and BBM vesicles (BBMV) (Carrol and Ellar, 1993) showed that a non-selective Cry protein-induced pore was formed of about 0.6 mm radius, permeable to cations, anions and uncharged molecules up to the size of sucrose. A three-dimensional model of the Cry3A protein supports the hypothesis that the toxin causes the formation of pores or channels in the lipid bilayer (Li *et al.*, 1991).

Binding of the toxin is a two stage process involving reversible (Hofmann *et al.*, 1988) and irreversible phases (Mohan *et al.*, 1995). Irreversible binding is thought to involve tight binding between the Cry protein and receptor and insertion of the former into the apical membrane or both. For example, a truncated Cry1Ab molecule containing only Domain II and Domain III can still bind to midgut receptors but only reversibly, supporting the notion that irreversible binding requires the insertion of Domain I (Flores *et al.*, 1997). The changes in the gut result in vegetative propagation of normal gut microbial flora or of introduced microorganisms, which cause the larvae to stop feeding due to "extreme digestive discomfort" within as little as 2 minutes. This eventually results in a septicaemia which can contribute to or cause death of the larvae (Fast, 1981). Death caused by the bacterial septicemia usually occurs 2-3 days post-ingestion (Bauer, 1995). A proposed mode of action of *Bt* toxins has been shown (Fig. 1).

***Bacillus thuringiensis* and transgenic crops:** Since the first transgenic plants appeared in the early 1980s (Horsch *et al.*, 1984), there has been very rapid progress directed at using this new technology for the practical ends of crop improvement. Protection of crops from insect pests was quickly seized upon as a major goal of plant genetic engineering (Hilder and Boulter, 1999).

The development of transgenic crop plants expressing Cry proteins (toxins) from *Bt* has provided new options in the integrated pest management of a wide range of insect pest species, being compatible with biological methods of pest control due to the selective toxicity of the *Bt* toxins and their methods of delivery to the target pest (Wearing and Hokkanen, 1994). Cotton, maize and potato varieties engineered with Cry genes are fully approved for commercial use in various countries. The number of countries growing transgenic crops commercially has increased from 1 in 1992 to 13 in 1999 (Shelton *et al.*, 2002).

The more obvious advantages of engineered insect resistance in plants are protection of target tissue, weather independent protection, greater stability of Cry toxins, compared with conventional spray application of *Bt* products and, thus, lack of dependence on application timing for treatment success and protection is possible for the entire season and only those insects which feed on transgenic crops will be exposed (Ely, 1993).

The fast increase in the prevailing acreage of transgenic plants by more than 25 fold from 1.7 million ha in 1996 to 44.2 million ha in 2000 is expected to increase and global market is projected to move from less than US $ 500 million in 1996 to US $25 billion in 2010 (James, 1997; Frutos *et al.*, 1999; Shelton *et al.*, 2002).

However, a major concern is the potential vulnerability of *Bt* crops to the evolution of resistance by insect pests (Tabashnik *et al.*, 1997a; Wolfenbarger and Phifer, 2000). Large-scale cultivation of *Bt* transgenic crops will certainly impose selection pressure for pre-existing *Bt*-resistant insects to increase their numbers. Transgenic plant resistance with *Bt* genes may thus be a short-lived phenomenon rapidly overcome by insects in much the same way that insecticide toxicity has often been overcome (Van Emden, 1999).

***Bacillus thuringiensis* and non-target insects:** Short term risks to natural enemies is a function of the intrinsic susceptibility of the organism and the level of exposure to the toxin (Jepson *et al.*, 1994). Laboratory tests against various invertebrates indicate that *Bt* has limited impacts on non-target organisms. However, given the wide host range of many *Bt* strains, non-target impacts of *Bt* application can occur. For example larvae of the monarch butterfly, *Danaus plexippus* are susceptible to Cry1Ab (Losey *et al.*, 1999; Jesse and Obrycki, 2000; Hellmich *et al.*, 2001). However, experiments investigating the effects of conventional *Bt* sprays on other non-target lepidopterans have shown that it is difficult to generalize about susceptibility to *Bt* and susceptibility must be dealt with on a species by species basis (Peacock *et al.*, 1998).

Insect natural enemies are important control agents of insect pests. They are often generalists and will attack several insect pests. Control measure which reduce natural enemy populations may, therefore, result in outbreaks in non-target pests. *Bacillus thuringiensis* has rarely been found to be toxic to natural enemies (Johnson and Gould, 1992, Hoy, 1998). However some laboratory studies have reported negative effects of *Bt* toxins on natural enemies. For example Cry1Ab increased mortality of the predatory Lacewing, *Chrysoperal carnea* Stephens (Neuroptera: Chrysopidae) when its larvae were provided with Cry1Ab fed prey (Hilbeck *et al.*, 1998a) as well as Cry1Ac directly in an artificial diet (Hilbeck *et al.*, 1998b). However other studies with *Chrysopa* spp., including use of exotoxin-containing products (Zuo *et al.*, 1994), *Bt kurstaki* (Salama and Zaki, 1984) and *Bt tenebrionis* (Langenbruch, 1992) did not report significant effects. Some examples of effects of *Bt* on insect predators are given in Table 1.

As with predators a range of effects on parasitoids have been recorded, with several studies finding no impact. For example Schuler *et al.* (1999, 2001) found no detrimental effects of a *Bt* transgenic oilseed rape line expressing Cry1Ac on the ability of the parasitoid *Cotesia plutellae* (Hymenoptera:Braconidae) to control *P. xylostella*. However, treatment of hosts with *Bt* may adversely affect larval parasitoids by increasing mortality of larval parasitoids within hosts which survived the treatment and by reducing the body size of adult parasitoids (Monnerat and Bordat, 1998). If *Bt* kills the host too quickly the parasitoid does not have sufficient resources to develop. For example, in a parasitoid of spruce budworm, *Apanteles fumiferanae* (Hymenoptera:Braconidae) populations were reduced by 50-60% because of lack of parasitoid emergence before host death (Nealis and Frankenhuyzen, 1990). Similarly, *A. glomeratus, A. melanoscelus* and *A. litae* showed

reduced adult life-span after *Bt kurstaki* application against the host (Salama *et al.*, 1996). The effects of *Bt* on parasitoids are summarized in Table 2.

Effect of *Bacillus thuringiensis* on humans: *Bacillus thuringiensis* has been used for over 60 years for insect pest control but there have been very few reports of clinical infection caused by the organism (Siegel, 2001). However, it has been suggested that the low number of reported cases may be an underestimate due to inadequate diagnostic laboratory facilities, failure to identify *Bacillus* isolates to species, the mixed microbiological nature of some clinical specimens and the rejection of clinically significant isolates as contaminants. For example, Jackson *et al.* (1995) reported that *Bt* isolates recovered from patients with burn wounds were initially incorrectly identified as *B. cereus.* There are two incidence of reported allergic reaction have been found (McClintock *et al.*, 1995). In the first case it was concluded the individual was most likely, suffering from a previously diagnosed disease while the second case involved an individual with a previous history of life threatening food allergies.

Proven cases that *Bt* causes clinical disease in mammals remain extremely rare and the risk to public health from *Bt* is considered to be extremely small (Drobniewski, 1993). Similarly, all mammalian toxicity testing of individual Cry toxins expressed in *Bt* plants has proved negative. Since *Bt* affects insects through unique receptor sites in the cell membranes of the insect gut, there are no known receptor sites in mammalian species which could be affected. Thus, there is a reasonable certainty that no harm will result to mammals from dietary exposure to residues of *Bt* (Anonymous, 1997). It has also been shown that Cry proteins rapidly degrade *in vitro*, usually within 30 s. Cry proteins range in size from approximately 60 to 120 KDa (Schnepf *et al.*, 1998). These proteins degrade in simulated digestion models to polypeptides of less than 2 KDa (less than 10 amino acids in length). These *in vitro* models are significantly less robust than the gastrointestinal systems of humans or other animals, which suggests that the Cry proteins will be rapidly and extensively degraded upon consumption (Betz *et al.*, 2000).

Resistance to *Bacillus thuringiensis*: Resistance is a complex genetic, evolutionary and ecological phenomenon (Metcalfe, 1989) and generally occurs by selection of rare individuals in a population that can survive the insecticide. It is a function of survival of the fittest. Homozygous resistant genes are rare in untreated populations compared with heterozygotes and homozygous susceptible (Dent, 2000). Widespread application of pesticides propagates resistant alleles through preferential survival either by selection or random mutation. Continued selection pressure in the same direction will lead to these alleles being essential in the population for offspring production (Sutherst and Comins, 1979). Commercial *Bt* formulations had been used in the field for more than three decades before resistance appeared (Tabashnik, 1994). The eventual development of *Bt* resistance in populations of *P. xylostella* was perhaps inevitable, however, given its biology, its history of insecticide resistance and the very high frequency of *Bt* applications applied to high value crucifer crops to control this pest in the late 1980s. In the laboratory, a total of 14 species have developed resistance to *Bt* Cry toxins (Table 3) (Ferré and Van-Rie, 2002).

Insects with a large number of generations per annum will tend to show a more rapid development of resistance than insects with only one generation in a growing season (Georghiou and Taylor, 1986). In a population with a low influx of genes resistance may become rapidly fixed while in populations with a continuous influx of novel genes resistance may be continuously diluted (Peferoen, 1997). The way insecticides are applied also has a dramatic effect on the rate of resistance development. Topical application of *Bt* compared with expression of a *Bt* Cry protein in a plant imposes different selection pressure on an insect population.

***Bacillus thuringiensis* resistance mechanisms:** The mechanism by which an insect evolves resistance to a particular toxin is unavoidably related to the toxin's mode of action (Gill *et al.*, 1992; Marrone and MacIntosh, 1993). Although several different resistance mechanisms have been proposed (Gill *et al.*, 1992), the most likely ones to date involve changes in *Bt* receptors or solubilization-activation of the crystal proteins (Ferré and Van-Rie, 2002). Receptor mediated mechanism may include loss of Cry toxin binding sites, increase in non-specific binding not related to toxicity. While solubilization and proteinase mediated resistance mechanism could involve changes in gut pH or in proteinases involved in protoxin activation.

Receptor-mediated mechanisms: Studies on various lepidopteran species have suggested that toxin binding to midgut receptors is responsible for toxin sensitivity or specificity among several different species (Ballester *et al.*, 1994). In this type of resistance, the protoxin is solubilized and activated in the resistant population as it is in susceptible insects. However, the activated toxin can no longer recognize a suitable binding site or epitope on the receptor (Rie *et al.*, 1990; Ferré *et al.*, 1991). The first case of resistance associated with altered midgut binding of a toxin was reported in *P. interpunctella* (Rie *et al.*, 1990). The resistant strain of *P. interpunctella* showed reduction in binding affinity but no change in the number of binding sites of Cry1Ab. This indicated that alteration in the binding site for Cry1Ab was preventing effective binding of the toxin.

The examination of Cry toxin binding in *P. xylostella* revealed loss in specific binding to Cry1Ab, suggesting that resistance was also due to a change in the Cry1Ab binding site (Ferré *et al.*, 1991). A Hawaiian colony of *P. xylostella* that was resistant to all three Cry1A toxins was found to exhibit reduced binding of Cry1Ac in BBMV assays (Tabashnik *et al.*, 1994a) and rapid reversal of resistance was associated with the restoration of the binding properties of Cry1Ac (Tabashnik *et al.*, 1994b). Similarly, a population of *P. xylostella* from Malaysia highly resistant to Cry1Ac and moderately to Cry1Ab exhibited reduced binding to both toxins in BBMV assays and reversal of resistance was associated with the restoration of binding properties of the toxins (Sayyed *et al.*, 2000a).

Solubilization/proteinase-mediated mechanisms: Proteolytic activation of Cry1 toxins involves the removal of peptide sequences from both the N- and C- termini of the protoxin (Bravo *et al.*, 2002). Resistance could thus involve a decrease in proteolytic properties of the insect midgut (Marrone and MacIntosh, 1993).

Since proteolytic enzymes are involved in the dissolution and activation of *Bt* protoxins (Dai and Gill, 1993; Milne and Kaplan, 1993), the first evidence for protease involvement in *Bt* resistance was found in a *Bt* resistant strain of *P. interpunctella* that displayed a slower processing of protoxins than in the susceptible strain and activation of Cry1 protoxins with midgut enzymes resulted in proteins of intermediate size of 60 KDa proteins (Oppert *et al.*, 1994). In this strain of *P. interpunctella*, the major serine proteinase was absent. Since these proteinases are involved in the activation of *Bt* protoxin (Oppert *et al.*, 1996), lack of critical *Bt* activating enzymes could contribute to toxin resistance. Similarly, two other resistant strains of *P. interpunctella* were found to have a genetically-linked reduced protoxin activation compared with a susceptible strain due to the absence of major trypsin-like gut enzyme (Oppert *et al.*, 1997). In the CP73-3 colony of *Heliothis virescens* (Lepidoptera:Noctuidae) slower activation of Cry1Ab protoxin and faster degradation of Cry1Ab toxin in midgut extract was observed by Forcada *et al.* (1996). While Inagaki *et al.* (1992) found that complete degradation of Cry1Ab by proteases derived from the resistant *Spodoptera litura* (Lepidoptera:Noctuidae) was the likely cause of resistance. Similarly, Keller *et al.* (1996) suggested that reduced sensitivity of fifth-instar larvae of *S. littoralis* to Cry1C could be attributed to increased degradation of the toxin by proteases in the less susceptible larvae. Milne *et al.* (1995) reported that a protein complex present in the midgut of spruce budworm, *Choristoneura*

Table 1: The effects of *Bacillus thuringiensis* on parasitoids of different insect pest species

Parasitoids	Target Pest	*Bt* products	Effect of *Bt* on parasitism	References
Encarsia formosa	*Trialeurodes vaporariorum*	*Bt* var. *aizawai*	Not toxic	Hayashi (1996)
Apanteles plutellae	*Plutella xylostella*	*Bt* var. *kurstaki*	Not toxic	Talekar and Yang (1991)
Cotesia marginiventris	*Heliothis virescens*	*Bt* var. *kurstaki*	Not toxic	Atwood *et al.* (1998)
Cotesia plutellae	*Plutella xylostella*	*Bt* var. *kurstaki*	Negative effect on parasitoid	Chilcutt and Tabashnik (1997)
Cotesia Plutellae	*Plutella xylostella*	*Bt Canola*	No effect	Schuler *et al.* (1999) ; (2001)
Meteorus leviventris	*Agrotis ypsilon*	*B. thuringiensis*	Retarded development/emergence	Hafez *et al.* (1997)
Pteromalus puparum	*Pieris brassicae*	*B. thuringiensis*	No effect on adults	Mushtaque *et al.* (1993)
Diadegma eurocerophaga	*Plutella xylostella*	*Bt* var. *kurstaki*	Not toxic	Talekar and Yang (1991)
Diadegma insulare	*Plutella xylostella*	*Bt* var. *kurstaki*	No effect in the field	Caballo *et al.* (1989)
Diadegma pierisae	*Pieris brassicae*	*Bt* var. *kurstaki*	No effect on adults	Mushtaque *et al.* (1993)
Diadegma semiclausum	*Plutella xylostella*	*Bt* var. *kurstaki*	Least toxic of insecticides tested	Obra and Rejesus (1997)
Diadegma sp.	*Plutella xylostella*	*Bt* var. *kurstaki*	Not toxic	Monnerat and Bordat (1998)
Telenomus remus	*Spodoptera litura*	*Bt* var. *kurstaki*	No effect	Chari *et al.* (1996)
Trichogramma cacoeciae	*Sitrotraga cerealella*	*Bt* var. *kurstaki*	High concentration repellent	Hassan and Krieg (1975)
Trichogramma evanescens	*Ostrinia furnacalis* *Helicoverpa armigera*	*Bt* var. *kurstaki*	No effect on parasitoid abundance	Tandan and Nillama (1987)
Trichogramma evanescens	*Spodoptera lituralis*	*Bt* var. *galleriae*	*Bt* treated eggs parasitism decreased	Salama and Zaki (1985)
Trichogramma exiguum	*Helicoverpa zea, Manduca spp.* and *Trichoplusia ni*	*Bt* var. *kurstaki*	No effect	Campbell *et al.* (1991)

Table 2: Effect of *Bacillus thuringiensis* on predators of different insect pest species

Predators	Target Pest	*Bt* products	Effect of *Bt* on parasitism	References
Chauliognathus lugubris	*Chrysoptharta bimaculata*	*Bt* var. *tenebrionis*	No toxicity	Beveridge and Elek (1999)
Pterostichus spp.	*Cydia pomonella*	*Bt* var. *kurstaki*	No effect	Riddick and Mills (1995)
Coccinella septempunctata	*Helicoverpa armigera*	*Bt* var. *kurstaki*	No effect	Manjula and Padmavathamma (1996)
Coccinella undecimpunctata	*Spodoptera littoralis*	*Bt* (Bactospeine)	Harmless	El Husseini (1984)
Chrysopa carnea	*Spodoptera littoralis*	*Bt* var. *kurstaki*	Slightly affected predator population	Salama and Zaki (1984)
Chrysopera carnea	*Ephestia kuehniella*	Cry1Ab	Toxic to *C. carnea*	Hilbeck *et al.* (1998a)

Table 3: Species of insects selected in the laboratory or field[1] for resistance to *Bacillus thuringiensis*

Insect species	Common Names	Family	References
Aedes aegypti	Yellow fever mosquito	Culicidae	Goldman *et al.* (1986)
Cadra cautella	Almond moth	Pyralidae	McGaughey and Beeman (1988)
Choristoneura fumiferana	Spurce budworm	Tortricidae	Van Frankenhuyzen *et al.* (1995)
Chrysomela scripta	Cottonwood leaf beetle	Chrysomelidae	Bauer *et al.* (1995)
Culex quinquefasciatus	House mosquito	Culicidae	Georghiou and Taylor (1986)
Heliothis virescens	Tobacco budworm	Noctuidae	Stone *et al.* (1989)
Homoeosoma electellum	Sunflower moth	Pyralidae	Brewer (1991)
Leptinotarsa decemlineata	Colorado potato beetle	Chrysomelidae	Whalon *et al.* (1993)
Ostrina nubilalis	European corn borer	Pyralidae	Bolin *et al.* (1998)
Plodia interpunctella	Indian meal moth	Pyralidae	McGaughey (1985)
Plutella xylostella[1]	Diamondback moth	Plutellidae	Kirsch and Schmutterer (1988)
Spodoptera littoralis	Cotton leafworm	Noctuidae	Müller-Cohn *et al.* (1996)
Spodoptera exigua	Beet armyworm	Noctuidae	Moar *et al.* (1995)
Trichoplusia ni	Cabbage looper	Noctuidae	Estada and Ferré (1994)

Table 4: Cross-resistance pattern to *Bacillus thuringiensis* and its toxins in different insect pest species

Insect species	*Bt* products	Cross-resistance	References
Plodia interpunctella	*Bt* var. *kurstaki*	*Bt* var. *galleriae*	McGaughey and Johnson (1994)
Plodia interpunctella	*Bt* var. *aizawa*	*Bt* var. *kurstaki*	McGaughey and Johnson (1994)
Trichoplusia ni	Cry1Ab	Not resistant to Cry1Aa or Cry1Ac	Estada and Ferre (1994)
Spdoptera littoralis	Cry1C	partial to Cry1D, Cry1E and Cry1Ab	Muller-Cohn *et al.* (1996)
Spodoptera exigua	*Bt* var. *kurstaki*	Cry1Ab, Cry1C, Cry1E, Cry1H and Cry2A	Chaufaux *et al.* (1997) Moar *et al.* (1995)
Plutella xylostella	*Bt* var. *kurstaki*	Cry1F, Cry1J	Tabashnik *et al.* (1997b)
Plutella xylostella	*Bt* var. *aizawa*	*Bt* var. *kurstaki*, Cry1Ac	Sayyed *et al.* (2000a); Sayyed and Wright (2001a)
Plutella xylostella	Cry1C	Cry1F, Cry1J	Zhao *et al.* (2001)
Chrysomela scripta	Cry3Aa	Cry1Ba	Federici and Bauer (1998)
Culex quinquefasciatus	*Bt* var. *israelensis*	*Bt* var. *jegathesan*	Wirth *et al.* (1998)
Culex quinquefasciatus	Cry11A	marginally to *Bt* var. *israelensis*, *jegathesan* and *kyushuensis*	Cheong *et al.* (1997)

fumiferana (Lepidoptera:Tortricide) could inactivate Cry1Aa by precipitation followed by proteolysis, thus accounting for resistance to this toxin. However administration of serine protease inhibitors with protoxin in a susceptible strain of *P. xylostella* did not lead to a decrease in toxicity, which suggested that the proteolytic enzymes involved in the activation of endotoxins in this population belonged to a different group of proteases (Tabashnik *et al.*, 1992a).

Cross-resistance patterns: Cross-resistance patterns can help to identify resistance mechanisms (Roush and Tabashnik, 1990). reported for a number of toxins (Table 4). Some insect species Generally the incidence of cross-resistance is low but it has been can be readily selected for resistance to several different *Bt* toxins (McGaughey and Johnson, 1994). For example, it has been shown that *P. interpunctella* can be selected for resistance to Cry1Aa, Cry1Ab, Cry1Ca and Cry1Da and possibly other Cry toxins contained in *B. thuringiensis* var. *aizawai* (*Bta*) (McGaughey and Johnson, 1994). Cross-resistance between *Bt* toxins has also been reported in *H. virescens* (Gould *et al.*, 1992; Gould *et al.*, 1995) and *P. xylostella* (Tabashnik *et al.*, 1994c; Wirth *et al.*, 1997; Sayyed *et al.*, 2000a, Sayyed and Wright, 2001a). Cross-resistance among Cry1A toxins is not surprising, owing to their structural and functional similarities (Shelton *et al.*, 1993) and

studies have shown that these toxins may bind to the same receptor in most of the insect species tested (Ballester *et al.*, 1999).

Several studies have reported an absence of cross-resistance between synthetic insecticides and *Bt*. For example *Bt* resistant *P. xylostella* showed no cross-resistance to phenthoate, fenvalerate, chlrofluazuron or abamectin (Iqbal *et al.*, 1996; Sarnthoy *et al.*, 1997). However, resistance to *Btk* in the sheep lice, *Bovicola* (*Damalinia*) *ovis* Schrank (Phthiraptera: Trichodectidae) was shown to be inversely related to pyrethroid resistance (Drummond *et al.*, 1995).

Cross-resistance patterns and their underlying physiological mechanism are very complex and somewhat unpredictable, even in closely related groups of toxins and insects (Table 4; Tabashnik, 1994). Where resistance is due to reduced binding, then cross-resistance can be predicted from competitive binding studies with susceptible insects, however when the resistance is unrelated to binding, then this can produce unexpected results such as the broad cross-resistance as in *H. virescens* (Gould *et al.*, 1995) and *P. xylostella* (Sayyed *et al.*, 2000a, Sayyed and Wright, 2001a).

Multiple mechanisms of resistance: These evolve as a consequence of serial exposure to different toxins or combinations of toxins (Wright *et al.*, 1997; Frutos *et al.*, 1999). For example, Gould *et al.* (1992) reported that a strain of *H. virescens* selected for Cry1Ac also showed resistance to Cry1Ab and Cry2Aa. Binding analyses showed that there was no change in the binding characteristics of Cry1Ab and Cry1Ac and although the resistant strain seemed to have fewer receptors for Cry1Ac, these were of high affinity. There was no significant decrease in the affinity or number of binding sites which could explain the level of resistance which was therefore considered to occur post-binding (Moar *et al.*, 1995). In a *S. littoralis* population, experiments suggested the low level of activity of Cry1A toxins was due to an inability to carry out the pore formation step (Escriche *et al.*, 1998). Similarly, a *P. xylostella* population (SERD3) from Malaysia resistant to both *Btk* and *Bta* showed decreased binding to Cry1Ab but not to Cry1Aa, Cry1Ac or Cry1Ca, suggesting that reduced toxin binding alone could not account for the resistance observed (Wright *et al.*, 1997). In another population (SERD5), collected from the same location, reduced activation of protoxin is thought to be a major mechanism (Sayyed and Wright, 2001b).

Genetics of *Bacillus thuringiensis* resistance: The genetic basis of resistance is complex (Heckel, 1994). It includes the initial frequency of resistance alleles, natural variability, mode of inheritance (degree of dominance, sex linkage) and fitness costs associated with resistance.

Initial frequency of resistance alleles: Although such information is useful for assessing the evolution of resistance, it is not easily available (Frutos *et al.*, 1999) and the assessment of the initial frequency of resistance alleles is almost never conducted prior to development of resistance (Tabashnik *et al.*, 1990; Gould *et al.*, 1997).

The initial frequency of resistance alleles influences the rate at which resistance will evolve (Alstad and Andow, 1995; Gould *et al.*, 1997). Change in allele frequency is the key indicator of the effectiveness of an insecticide. The initial allelic frequency is generally assumed to range from 10^{-6} to 10^{-3} (Roush and McKenzie, 1987; Gould, 1998). A direct approach, making use of a homozygous recessive resistant strain, has estimated the frequency of a major *Bt* resistance allele in a field population of *H. virescens* to be 1.5×10^{-3} (Gould *et al.*, 1997). By applying the same approach in *P. xylostella,* Tabashnik *et al.* (1997a) estimated the frequency of a recessive *Bt* resistance allele in a susceptible laboratory population to be 1.2×10^{-1}. While Alstad and Andow (1996) used a F_2 screening procedure to estimate the frequency of Cry1Ab resistance alleles as $< 1.3 \times 10^{-2}$ in a laboratory population of *O. nubilis*. Using a slightly modified procedure Andow *et al.* (2000) and Bentur *et al.* (2000) estimated the initial frequency of resistance in an Iowa population of *Ostrinia nubilalis* (Lepidoptera:Pyrallidae) to *Bt* corn $< 3.9 \times 10^{-3}$ and $< 3.6 \times 10^{-3}$ respectively. Using a new approach, Tabashnik *et al.* (2000) estimated initial frequency of resistance in a field population of *P. gossypiella* in Arizona to be 1.6×10^{-1}.

Natural variation in susceptibility to *Bt*: Intra specific variation in susceptibility to *Bt* between different geographical populations has been reported for various insect species. For example, a population of rice stripped stemborer, *Chilo suppressalis* Walk. (Lepidoptera: Pyralidae) from Southeast Asia was susceptible to Cry1Ca (Lee *et al.*, 1997) whereas a population from Southern France was not (Fiuza *et al.*, 1996). Similar results were reported for *O. nubilalis* collected from different places of USA (Huang *et al.*, 1997). However, the spruce budworm, *Choristoneura fumiferana* Clem (Lepidoptera: Tortricidae) showed limited geographical variation in susceptibility to *Bt* although intra-population variability showed a 2 to 30 fold difference between the highest and lowest percent mortalities at a single dose (Van Frankenhuyzen *et al.*, 1995). In general, the susceptibility of Cry proteins can vary among different populations of a given insect species (Cabrera *et al.*, 2001). This could affect the standardisation of potency of *B. thuringiensis* based products and make estimation of resistance levels in populations exposed to Cry toxins more difficult.

Another way to estimate the variability in resistance genes is to measure heritability (h^2). Tabashnik *et al.* (1994a) estimated heritability of resistance to *Btk* and Cry1A toxins and showed that compared with eight other insects species, *P. interpunctella* had relatively high h^2 values, indicating low phenotypic variation. However, relatively higher h^2 values were found in two populations of *P. xylostella* from Malaysia (Iqbal *et al.*, 1996; Wright *et al.*, 1997; Sayyed *et al.*, 2000a reflecting a low phenotypic variation probably resulting from continuous exposure to *Bt*.

Mode of inheritance of resistance: Information on the mode of inheritance can improve resistance monitoring, risk assessment, modelling and resistance management (Tabashnik *et al.*, 1992b). The inheritance of *Bt* resistance in insects has commonly been assumed to be recessive in nature. The mode of inheritance of resistance in various insect populations is summarised in Table 5. The single backcross method has been used to determine the number of loci involved in resistance. This has shown that the backcross data fitted fairly well to a single locus model (Ferré and Van-Rie, 2002). However resistance to Cry1Ca and Cry1Ac in Florida and Melaka populations of *P. xylostella* (Zhao *et al.*, 2000; Sayyed *et al.*, 2000a, Sayyed and Wright, 2001a), to Cry1Ab in a North Carolina population of *H. virescens* (Sims and Stone, 1991), to Cry1Ca in a population of *S. littoralis* from Israel (Chaufaux *et al.*, 1997) and to Cry3Aa in a Cry3A-selected laboratory population of *L. decemlineata* (Rahardja and Whalon, 1995) was found to be controlled by the combined interaction of resistance alleles from the various loci.

It has been shown consistently that the resistance to *B. thuringiensis* is autosomally inherited. However, in some cases the sex of the resistant parent has a significant influence on inheritance of resistance. For example, resistance to Cry1C in a *S. littoralis* population (Chaufaux *et al.*, 1997), to Cry1Ab in BL and to Cry1Ac in SERD4 populations *P. xylostella* had some maternal influence (Real *et al.*, 1995; Sayyed and Wright, 2001a). In contrast, a Cry1Ab-selected population of *P. xylostella* exhibited some paternal influence.

At present, the evidence suggests that partial or completely recessive modes of inheritance (Table 5) are consistently associated with modification of binding sites and therefore altered specific binding (Liu and Tabashnik, 1997a; Gould *et al.*, 1997). Whereas more dominant alleles seem to be associated with other resistance mechanisms, conferring more broad spectrum resistance (Tabashnik *et al.*, 1998). For example, in the SERD5 population of *P. xylostella* resistance was incompletely dominant and reduced protoxin activation is suggested to be the major mechanism of resistance to Cry1Ac (Sayyed *et al.*, 2001b).

Table 5: Inheritance of resistance to *Bt* products and Cry toxins in different insect pest populations

Species	Origin	Population	*Bt* product/toxin	Mode of inheritance	Reference
Plodia interpunctella	Oklahoma	343-R	*Btk*	Recessive	McGaughey (1985)
Plutella xylostella	Hawaii	NO-Q	*Btk*	Recessive	Tabashnik *et al.* (1992a)
	Japan	ROO	*Btk*	Recessive	Hama *et al.* (1992)
	Florida	Loxa A	*Btk*	Recessive	Tang *et al.* (1997)
	Pennsylvania	PEN	Cry1Aa	Recessive	Tabashnik *et al.* (1997a)
	Philippines	PHI	Cry1Ab	Recessive	Tabashnik *et al.* (1997b)
	Hawaii	NO-95C	Cry1C	Incomplete dominant	Liu and Tabashnik (1997b)
	Thailand	BS	*Btk*	Recessive	Imai and Mori (1999)
	Malaysia	MEL	Cry1Ac	Incomplete dominant	Sayyed *et al.* (2000a)
	Malaysia	MEL	*Btk*	Recessive	Sayyed *et al.* (2000b)
	South Carolina	Cry1C SEL	Cry1C	Recessive	Zhao *et al.* (2000)
	Malaysia	SERD4	Cry1Ac, Cry1Ab	Incompletely dominant	Sayyed and Wright (2001a); A.H. Sayyed and D.J. Wright, Unpublished
	Malaysia	SERD5	Cry1Ac, Cry1Ac Canola	Incompletely dominant	A.H. Sayyed and D.J. Wright, Unpublished
	Philippines	PHI	Cry1Ab	Incompletely dominant	Cabrera *et al.* (2001)
Heliothis virescens	North Carolina	SEL	Cry1Ab	Incompletely dominant	Sims and Stone (1991)
	North Carolina	CP-73-3	Cry1Ac	Incompletely dominant	Gould *et al.* (1995)
	North Carolina	YHD2	Cry2A	Incompletely dominant	Gould *et al.* (1995)
Spodoptera littoralis	Israel		Cry1Ca	Incompletely dominant	Chaufaux *et al.* (1997)
Ostrinia nubilalis	Kansas	KS-SC-R	*Btk*	Incompletely dominant	Huang *et al.* (1999)
Leptinotarsa decemlineata	Michigan		Cry3A	Incompletely dominant	Rahardja andWhalon (1995)

Table 6: Mechanism of resistance to *Bt* products and Cry toxins in different insect pest populations

Insects	Origin	*Bt* product/ Cry toxins	Number of Allele	Mechanism of resistance	Reference
Heliothis virescens					
Strain CP73-3	North Carolina	Cry1Ac	Monogenic	Broad-resistance	Gould *et al.* (1992)
				Reduced activation	Forcada *et al.* (1996)
Strain YHD2	North Carolina	Cry1Ac	Monogenic	Reduced binding	Gould *et al.* (1995)
					Heckel *et al.* (1997)
Leptinotarsa decemlineata	Michigan	Cry3A	Polygenic	N.A.	Rahardja and Whalon (1995)
Plodia interpunctella					
Strain 343	Oklahoma	*Btk*	Monogenic	Reduced binding	Rie *et al.* (1990)
Strain 133r	Kansas	*Btk*	Monogenic	Reduced activation	Oppert *et al.* (1997)
Strain 198r	Kansas	*Btk*	Monogenic	Reduced activation	Oppert *et al.* (1997)
Plutella xylostella					
Strain NO-Q	Hawaii	*Btk*	Monogenic	Reduced binding	Tabashnik *et al.* (1994a)
Strain NO-QA	Hawaii	*Btk*	Monogenic	Reduced binding	Tabashnik *et al.* (1997a)
Strain NO-95C	Hawaii	Cry1C	Polygenic	Reduced binding	Liu and Tabashnik (1997a)
Strain SERD3	Serdang	*Btk*	Polygenic	Reduced binding	Wright *et al.* (1997)
Strain Loxa A	Florida	*Btk*, Cry1C	Monogenic	Reduced binding	Tang *et al.* (1997); Zhao *et al.* (2000)
Strain MEL	Melaka	*Btk*	Monogenic	N.A.	Sayyed *et al.* (2000b)
Strain MEL	Melaka	Cry1Ac	Polygenic	Reduced binding	Sayyed *et al.* (2000a)
Strain SERD4	Serdang	Cry1Ac, Cry1Ab	Polygenic	Reduced binding	Sayyed and Wright (2001a)
				Reduced activation	A.H. Sayyed and D.J. Wright Unpublished
Strain SERD5	Serdang	Cry1Ac	Polygenic	Reduced activation	Sayyed *et al.* (2001b)
Ostrinia nubilalis		*Btk*	Monogenic	N.A.	Huang *et al.* (1999)

Table 7: Fitness costs and reversion of resistance to *Bt* products and Cry toxins in different insect pest populations

Insect population	Origin	*Bt* products	Fitness costs	Reversion	Number of generations	References
Spodoptera littoralis	France	Cry1C	N.A.	> 500 folds	1	Müller-Cohn *et al.* (1996)
Heliothis virescens	North Carolina	*Btk*	No fitness cost	56 fold	5	Gould and Anderson (1991)
Leptinotarsa decemlineata	Michigan	Cry3A	Fitness costs	80 folds	8	Rahardja and Whalon (1995); Alyokhin and Ferro (1999)
Pectinophore gossypiella	Arizona	*Bt* cotton	Fitness costs	N.A.	N.A.	Liu *et al.* (1999)
Ostrinia nubilalis	Mennesota	Cry1Ac				Bolin *et al.* (1999)
Plutella xylostella						
NO-QA	Hawaii	*Btk*	High fitness costs	3200 folds	5	Groeters *et al.* (1994); Tabashnik *et al.*(1994b)
ROO	Japan	*Btk*	N.A.			Hama *et al.* (1992)
Loxa A	Florida	*Btk*	Lack of fitness costs			Tang *et al.* (1996); (2001)
NO-95	Hawaii	Cry1Ab	N.A.	Stable		Liu *et al.* (1996)
Melaka	Melaka	*Btk*, Cry1Ac	N.A.			Sayyed *et al.* (2000a); (2000b)
SERD4	Serdang	Cry1Ac	Lack of fitness			Sayyed and Wright (2001b)

Number of resistance alleles: Theoretical models describing the evolution of resistance in various insect population have been developed based on a monogenic mode of inheritance (Ferré and Van-Rie, 2002). However, results obtained by using different methods indicate that insects may have more than one factors conferring resistance to Cry toxins. For example, in backcross experiments results showed that *Bt* resistance populations did not fit a monofactorial model (Rahardja and Whalon, 1995; Liu and Tabashnik, 1997a; Sayyed *et al.*, 2000a; Sayyed and Wright, 2001a) (Table 6). The results of different approaches used to determine the number of resistance alleles are summarised (Table 6).

Genetic analysis using isozyme polymorphism has also been used to identify the number of factors conferring resistance. For example, a backcross design with 10 marker loci in the YHD2 strain of *H. virescens* revealed the existence of a major locus named *BtR*-4 on linkage group 9, responsible for as much as 80% of the total resistance to Cry1Ac (Heckel *et al.*, 1997). Using the

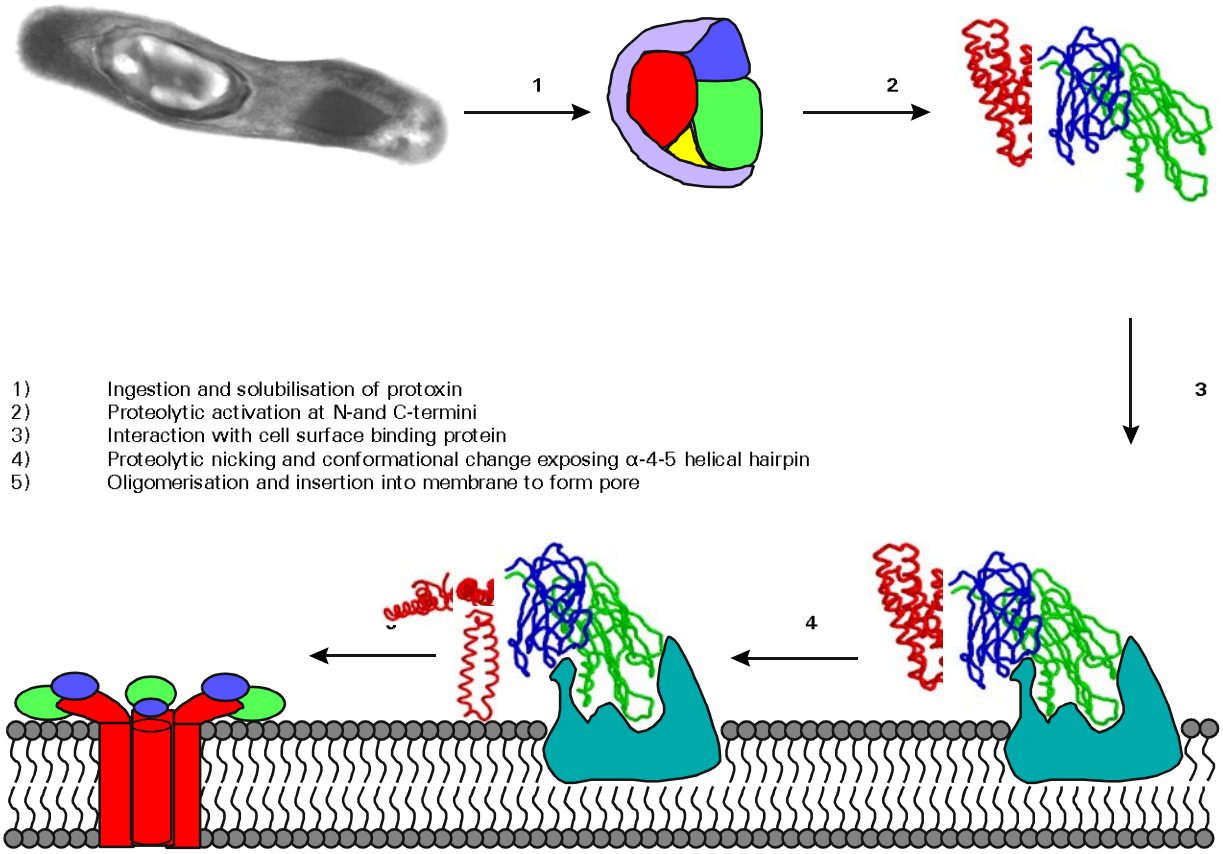

Fig. 1: Proposed mechanism of action of *Bt* toxin (After De Maagd *et al.*, 2001)

isozyme-linkage method, a strong correlation was found between Cry1A resistance and two mannose-6-phosphate isomerase isozymes in the PHI strain of *P. xylostella* (Herrero *et al.*, 2001).

Reversion of resistance and fitness costs: Instability of resistance is the tendency for the frequency of resistance genotypes to decrease in a population beyond effects directly attributable immigration or emigration (Tabashnik *et al.*, 1994a). Resistance to insecticides is often accompanied by fitness costs, such as a decreased rate of development, fecundity, survival or mating competitiveness relative to susceptible insects (Roush and McKenzie, 1987). In the majority of cases, resistance to *Bt* and individual Cry toxins has been found to be unstable. A rapid decline in resistance has been found in three selected strains of *P. xylostella* from Hawaii with 2800 to 90-fold resistance (Tabashnik *et al.*, 1994c). A fast rate of decline of resistance has also been reported for *Btk* resistant *P. xylostella* populations from Japan and Malaysia (Hama *et al.*, 1992; Sayyed *et al.*, 2000b). Such declines in resistance are most likely caused by fitness costs associated with resistance. However, *P. interpunctella* resistance did not decline in one strain (343-R) even after 29 generations on untreated diet. Similarly, in a *P. xylostella* population from Hawaii resistance in one isofemale line of the selected strain NO-Y remained stable after 32 generations without exposure (Tabashnik *et al.*, 1995). It has been shown that the decline in resistance in moderately resistant strains of *P; xylostella* (Tabashnik *et al.*, 1994a), *P. interpunctella* (McGaughey and Beeman, 1988) and *H. virescens* (Sims and Stone, 1991) was much slower than in populations with higher levels of resistance. The results of reversion of resistance studies are summarized in Table 7.

High fitness costs associated with evolution of *Bt* resistance have been reported (Tabashnik *et al.*, 1994a; Groeters *et al.*, 1994). However, in other cases few if any fitness costs have been found (McGaughey, 1985; Liu *et al.*, 1996; Tang *et al.*, 1997). For example, there were no differences in fitness between *Bt*-susceptible and resistant population of *H. virescens*, when larvae were fed diets that did not contain *Bt* (Gould and Anderson, 1991). However, there were increases in both development time and mortality for *Bt*-resistant *H. virescens* larvae forced to consume *Btk*. Relative to *Bt*-susceptible *L. decemlineata*, larval development was delayed and egg production was decreased in a *Bt*-resistant population feeding on potato plants not treated with *Bt* var. *tenebrionis* (Trisyono and Whalon, 1997; Alyokhin and Ferro, 1999). Also, more *Bt*-susceptible than *Bt*-resistant *L. decemlineata* survived after overwintering in diapause (Alyokhin and Ferro, 1999). While a resistant population of *P. gossypiella* feeding on transgenic *Bt*-cotton showed a longer development time than *Bt*-susceptible bollworms (Liu *et al.*, 1999).

In contrast, two populations of *P. xylostella* from Malaysia showed shorter development times and greater pupal weight compared with unselected sub-populations in the presence of Cry1Ac (Sayyed and Wright, 2001b). Likewise, *H. virescens* was reported to show enhanced growth in the presence of Cy1Ab toxin (Gould *et al.*, 1995). Liu *et al.* (1996) suggested that prolonged selection in the field reduced fitness costs either in substitution of alternate alleles conferring resistance or by selection of fitness modifiers at loci not directly related to resistance. Fitness costs are often difficult to describe owing to their compost nature and dependence on various physiological aspects as well as mechanisms of resistance (Frutos *et al.*,1999; Sayyed and Wright, 2001b). In addition, genetic drift or mutation

can cause instability (Tabashnik *et al.*, 1994a). However, these mechanisms may not be expressed in larger field populations. In addition, diverging opinions exist regarding the impact of fitness cost on delay of resistance. Some consider that fitness costs directly caused by resistance alleles will have important effect in the field (Tabashnik *et al.*, 1994b), whereas other consider that even strong fitness costs will have a minimal impact on evolution of resistance (Roush, 1997). Liu *et al.* (1996) suggested that stability is not necessarily a fixed trait even for a particular population.

Implications of genetic diversity for resistance management: The main purpose of resistance management is to diminish the selection of initially rare individuals carrying resistance alleles and hence to keep the frequency of resistance alleles sufficiently low for insect control (Schnepf *et al.*, 1998). Strategy development generally relies on theoretical assumptions and on computer models (Tabashnik *et al.*, 1994c; Roush, 1997, 1998). However, a thorough understanding of the genetics of resistance to Cry toxins is essential for the development and maintenance of resistance management strategies (Tabashnik *et al.*, 1991). It had been widely assumed that resistance to *Bt* in insects is inherited as a recessive trait (McGaughey, 1985; Tabashnik *et al.*, 1992a; Ferré *et al.*, 1995). However, as we have seen incompletely dominant, polygenic modes of inheritance of resistance to Cry1Ac were present in three field populations of *P. xylostella* from Malaysia. Some degree of dominance has also been reported in two other field-derived Cry toxin resistant populations of *P. xylostella* from the Philippines and Hawaii and in laboratory selected populations of at least five other insects species (Table 5). Thus, the monogenic recessive model of *Bt* resistance in *P. xylostella* (Tabashnik *et al.*, 1992a) may not necessarily be the most common in the field, with many resistant insect populations having more complex genetics of resistance. There is also evidence that a Cry toxin resistance mechanism (reduced activation) other than reduced binding is of major importance in *P. xylostella* (Sayyed *et al.*, 2001b).

Various strategies to manage resistance to *Bt* toxins have been proposed (Tabashnik *et al.*, 1994a; Gould, 1998; Frutos *et al.*, 1999), all of which rely to a greater or lesser extent on resistance being recessive, that the frequency of resistance alleles is low in the field compared with susceptible alleles and that the frequency of resistance alleles will decrease when the selection pressure is released. For example, if resistance is recessive, F_1 offspring produced by mating between susceptible and resistant adults are killed by eating *Bt* plants. If mating is random, initially rare homozygous resistant adults emerging from *Bt* plants are likely to mate with the more abundant homozygous susceptible adults emerging from non-*Bt* plants, producing F_1 progeny that cannot survive on *Bt* plants expressing high doses of toxin (Fishhoff, 1996). Mathematical models and data from laboratory and greenhouse studies indicate that resistance can be delayed substantially when these assumptions are valid (Gould, 1998). However, it is difficult to predict the exact concentration of Cry toxin for incomplete mode of inheritance of resistance. Whereas, Gould *et al.* (1994) proposed 25 times the concentration needed to kill 99 percent of susceptible insects. Population genetics theory (Tabashnik and Croft, 1982; Gould, 1986; Mallet and Porter, 1992; Alstad and Andow, 1996) and laboratory experiments (Liu and Tabashnik, 1997; Roush, 1998) predict that this approach will substantially delay evolution of resistance, if it is appropriately implemented and its assumptions are met.

The principal methods proposed for *Bt* resistance management are: (a) mixtures, mosaics or rotations of transgenic plants; (b) time or tissue-specific expression of toxin; (c) low doses of toxin in combination with natural enemies; (d) co-expression of different *cry* genes; and (e) high expression (dose) with refugia, which is the strategy recommended currently (Shelton *et al.*, 2000; Tang *et al.*, 2001). However, if non-recessive inheritance and multiple mechanisms of resistance to Cry toxins are, in fact, relatively common in field populations of insects, rethinking of resistance management strategies may be required (Tabashnik *et al.*, 1998).

The refuge/high-dose strategy entails high risk because it could greatly accelerate resistance if certain assumptions are not valid. For example, non-random (assortative) mating and movement of adults may lead to failure of this strategy. Resistant larvae of *P. gossypiella* on *Bt* cotton have been observed to take long duration to develop, compared with susceptible larvae on non-*Bt* cotton (Table 7). While Cry1Ac-SEL SERD4 and Cry1Ac-SEL MEL sub-populations of *P. xylostella* had a shorter development time on Cry1Ac-treated leaves compared with an unselected population on untreated leaves (Table 7). In the above examples assortative mating could occur if local populations of insects were sufficiently synchronised and mating occurred prior to dispersal. While this is perhaps unlikely to be the case with *P. xylostella*, where overlapping generations is common, such developmental asynchrony could be important in cotton crop pests with non-overlapping generations. If dispersal occurs after mating, immigration of resistant individuals could increase the frequency of heterozygotes in the refugia (Caprio and Tabashnik, 1992).

The refuge/high-dose strategy will also fail if the plants do not produce, or fail to maintain, a dose sufficiently high to kill most heterozygotes. For example in *Bt* cotton fields in Australia, the damage due to *H. armigera* was similar to that in refugia (Forrester and Pyke, 1997). Efficacy against *Helicoverpa* spp. typically declines through the boll maturation period, to the point where survival of larvae is little different to that in refugia although growth rates of survivors on the INGARD *Bt* crops are still dramatically reduced (Fitt, 2000). Clearly this pattern is not consistent with a high dose strategy and the changing efficacy of *Bt* cotton imposes additional risks for resistance management. In Australia, *Bt* cotton expressing two independent Cry toxins (Cry1 Ac and Cry2A) shows much more consistent efficacy compared with *Bt* cotton expressing Cry1Ac alone and will greatly enhance the sustainability of resistance management (Roush, 1996). Other possibilities for Cry toxins are also being investigated (Llewellyn and Higgins, 1998, Hanzlik and Gordon, 1998).

The refuge in the refuge/high-dose strategy should ideally be maintained free of any treatment with pesticides to ensure the presence of a sufficient number of susceptible adults (Gould, 1998). In fact, it is recommend the planting of 20% non-*Bt* plants of cotton that can be treated with a non-*Bt* foliar insecticide, or a 4% refuge of non-*Bt* plants that is left untreated. However, Shelton *et al.* (2000) have shown that the insect population in the sprayed refuge had a significantly lower average mortality at the diagnostic dose for resistance compared with the insects in the unsprayed refuge. Insects collected from the *Bt* plants would have a resistant genotype for *Bt* resistance and significantly greater numbers of *Bt*-resistant larvae were found on the *Bt* plants when the refuge was sprayed compared with when it was not sprayed. However, if the 20% non-*Bt* refuge is managed using an IPM strategy in such way that the population of beneficial insects increases this could result in a significant decrease in the pest population (Roush, 1996; Riggin-Bucci and Gould, 1997). Therefore, the use of chemical sprays can be reduced, increasing the effectiveness of the refuge (Gould, 1998).

The multiple toxin strategy is usually considered as an adjunct to the high-dose strategy. Instead of having a high concentration of one toxin, plants express high concentration of two or more toxins. The multiple toxin strategy coupled with a refugia could provide benefits even if a high dose (25 times the LD_{99} of susceptible insects) is not reached (Gould *et al.*, 1994). However, if the two toxins are each expressed at levels that only kill 50-80% of the insects, this strategy may not be highly effective in slowing the evolution of resistance (Gould *et al.*, 1994).

Cross-resistance between Cry toxins (McGaughey, 1994) and multiple resistance have important implications for *Bt* resistance management. In the presence of cross-resistance, a two-toxin resistance management strategy (*Bt* plants expressing two Cry toxins) can fail quickly. However, in the absence of cross-resistance there is a ten-fold advantage of using this strategy

(Roush, 1998). The potential of Cyt1A in preventing evolution of resistance in mosquito and *P. xylostella* larvae exposed to *Bti* and *Btk* (Wirth *et al.*, 1997; Sayyed *et al.*, 2001a) and resistance to Cry3A in *C. scripta* (Federici and Baur, 1998) has also been reported.

In crops such as cotton, with multiple pests, because of unique biology of each pest it is hard to design a resistance management strategy that is appropriate for all the pest species. A toxin may be highly active against a given pest (e.g., Cry1Ac *vs H. virescens*) but less active against another present on the same crop (e.g., Cry1Ac *vs H. zea* or *H. armigera*). In such a case, the second pest may be exposed to a moderate or weak dose on a *Bt* crop and may evolve resistance. This leads to use of *Bt* plants as a component of a comprehensive IPM approach (Hoy, 1998). The use of *Bt*-based formulations within an IPM program was shown to be effective for controlling pests (Trumble *et al.*, 1994; Meade and Hare, 1995). IPM can delay resistance by providing multiple sources of mortality (Denoholm and Rowland, 1992). For example, the use resistant cabbage cultivars will not only slower the development of *P. xylostella* but the synergistic interaction can also lead to more successful control by *Bt* as well as an improved efficacy of natural enemies (Schuler and Emden, 2000). As there is no single answer or strategy to delay resistance, only sound and flexible methods of pest control will provide sustainability. The refuge/high-dose strategy is currently the most promising approach, however the strategy is based on assumptions that as we have discussed may not always apply. Continued studies on the genetic diversity of *Bt* resistance in different insect populations are required and the susceptibility of pest populations must continue to be monitored to evaluate the success of resistance management plans.

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