Final Report

Genetics and Mode of Action of Resistance to Bt Toxins in Heliothine Pests of Cotton

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Aim

To determine the genetic basis of resistance to Bt in *Helicoverpa armigera* using the newly developed genetic map for this species. This map will assist in analysing the mechanisms of resistance to Bt toxins and other proteins to be deployed in transgenic cotton for pest control. It can also be applied to insecticide resistance mechanisms.

Objectives

1. Establish lines of known parentage from available resistant strains of *H. armigera* (derived from VICRATS and RATS from Dr Neil Forrester, formally NSW Agriculture)
2. Establish the genetic mapping techniques, determine the contributions of different chromosomes to the resistance phenotype, compare this map to those available for Bt resistant *Heliothis virescens*
3. Provide the strains to Dr Ray Akhurst, CSIRO Entomology, for analysis of the mechanisms associated with resistance and to Dr Forrester for field studies.

Summary of achievements against objectives

1. Five resistant strains of *H. armigera* are now in culture through collaborative arrangements: three from field collections, (one strain, BX, developed by Dr Akhurst) and two selected in the laboratory after mutagenesis. Although lines of known parentage were established for the Bt resistant strains VICRATS and RATS, two other resistant strains, BX and one of the laboratory-developed strains, EMS21, were chosen for further work because both survive on Bt cotton plants.

   From our preliminary genetic crosses, we hypothesise that the BX strain is close to homozygosity for a major resistance gene and that putative heterozygotes (F_1) are intermediate in expression between the fully resistant and susceptible strains.

   Larvae from three strains (BX, susceptible and the F_1 cross) were tested on whole Bt cotton plants. The F_1 and resistant strains showed significant survival and growth compared with the susceptible strain. This result has implications for resistance management.

2. Genetic mapping of the resistance genes has commenced. Two of the three generations of genetic crosses have been completed. Mapping techniques have been established after modifications to utilise the newer, faster and safer technology of the DNA sequencer.

3. Bt resistant strains, showing significant levels of resistance, are being maintained by both Dr Daly and Dr Akhurst's groups for security of the strains. Strains are being used for research purposes by each group as needed. The two groups meet regularly to discuss results.

Background

Resistance is an ongoing concern with the management of *H. armigera* in the Australian cotton industry. Management strategies are in place to either prevent, or retard further development of resistance to either chemical insecticides or to the Cry1Ac protein in

transgenic plants. While these strategies have been successful at slowing down the rate at which resistance has developed, they have neither prevented the ultimate spread of resistance to most of the field populations nor the evolution of new mechanisms of resistance that make resistance increasingly difficult to manage. As a consequence, mixtures of insecticides are increasingly common and the industry now uses more insecticide to control *H. armigera* today than it did ten years ago. A number of studies need to be undertaken to understand why the situation continues to deteriorate with respect to resistance. Dr Jonathan Holloway, NSW Agriculture, is investigating a number of these issues with insecticides.

This project is about increasing our ability to gain a firm understanding of the genetic basis of resistance to Cry1Ac protein. This is needed if we are to accurately interpret the results of field experiments. In particular, we need to know how many different major genes, and therefore mechanisms, are involved in resistance and what is their relative contribution to changes in resistance status of populations. Only when we have this information, can we readily distinguish between competing views on why and how resistance is evolving e.g. the relative importance of immigration at diluting resistance, the impact of destroying pupae and the value of synergists.

A number of research groups (Forrester, Akhurst and Daly) have collaborated on the development of strains of *H. armigera* resistant to the *cry1Ac* toxin gene in Bt cotton. Each group has tried a number of complementary approaches. To date there are five Bt resistant strains in laboratory culture, three derived from the field and two induced by mutation. Two of these resistant strains have shown significant resistance to presquare Bt cotton plants in bioassays and on whole Bt plants. The most promising of these is a field-derived strain (BX), developed by Dr Akhurst, that now has 100 to 300-fold resistance to Bt. It is not known at this stage whether the different resistant strains have the same or different resistance mechanisms.

This project takes advantage of recent advances in genetic studies of *H. armigera,* in particular, the development of a genetic linkage map for it and the related heliothine species, *Heliothis virescens* (Heckel *et al.,* 1997). Genetic linkage maps are used routinely as part of genetic studies in model laboratory organisms. However, until recently they were not available for most pest species. Linkage maps provide a more rapid way of defining the genetic basis of resistance than more conventional methods. They also offer the first opportunity to determine how many different classes of mechanisms are involved. Pyrethroid resistance is an example of this. Despite more than 12 years of work done on resistance to these compounds in *H. armigera,* there is still controversy over which mechanisms of resistance are involved, esterases or mixed function oxidases and their relative contributions, nor is the role of penetration or target site insensitivity clear. The relative contributions of these mechanisms may also have changed over time.

Dr Dave Heckel, University of Melbourne, has developed a first-generation linkage map for *H. armigera* (CRDC funded project, CSE58C) using DNA markers. He is currently completing the mapping of the pyrethroid resistance mechanisms present in a field strain studied by Dr Daly (Heckel *et al*, 1998). Dr Heckel has extensive experience with mapping heliothine genomes as his group have mapped a number of Bt-resistance loci in *Heliothis virescens*, one of which conferred 10,000 fold resistance (Heckel *et al.,* 1997). The power of the genetic mapping approach to analyse resistance is illustrated by its ability to distinguish between competing views on resistance. For example, one strain of *Heliothis virescens* appeared to show cross-resistance between Cry1Ac and Cry2A type resistance. This was unexpected from observations on the mode of action of these two toxins. Dr Heckel was able to demonstrate that the strain had two independently derived resistance loci, each conferring resistance to only one of the toxins.

While the linkage maps can be applied to any of a number of cases of resistance in *H. armigera,* we have chosen to analyse newly derived Bt resistance. Delays in producing pyramided constructs in transgenic cotton increase the risk of resistance to the Cry1Ac toxin. The work will also facilitate advances in other Bt-related research, in particular studies on the mechanisms of resistance (Dr Akhurst) and of field-based resistance (Dr Holloway). Ultimately it will also enable the insecticide resistance mechanisms studied by Dr Holloway to be mapped.

Methodology

Rearing of resistant strains

The resistant strains were reared using the revised method for general stock that has continued to produce consistently high quality material for experiments. After discussions with Dr Heckel in late 1996, the resistant strains were out-crossed and the progeny divided into three to five sub-cultures. These were reared and selected separately. Survivors were sexed and males and females were rotated between sub-cultures for each strain. This method delays the decrease of fitness associated with inbreeding, resulting from repeated selection.

Selection of resistant strains

Selection was continued by the egg treatment method using the commercial formulations of MVP or DiPel (see Final Report CRDC project NCQ 1C). In addition, a line was established within each strain for selection by diet incorporation and later, surface treatment of diet, with Bt HD73, isolated by Dr Akhurst's group.

Bioassays

Strains have been screened using a variety of bioassay methods to ensure that the resistance levels recorded could be properly quantified and were valid for field situations. These methods included leaf mush, diet incorporation, leaf discs (Olsen and Daly, in press) and whole plants. A diet surface treatment method with Bt HD73, has recently been included, since this method uses less of the laboratory prepared toxin. It is also the preferred method used by Dr Akhurst's group and this allows both groups to directly compare bioassay results.

Genetic crosses

Three generation pedigrees were established for three strains, according to the techniques of Dr Heckel (CSE 58C). The aim was to produce six backcross or F_2 families for linkage analysis. Having three generations guarantees the gametic phase will be known for most loci. Analysis of both types of reciprocal backcross is advantageous in *H. armigera* because, in common with other Lepidoptera, there is no crossing-over in females.

Genetic mapping

From Dr Heckel's work, it appeared that the most promising approach was to use AFLPs to establish linkage relationships within *H. armigera.* AFLPs are more efficient than alternative approaches using RFLPs that have a lower level of polymorphism, or RAPDs that have a high polymorphism but require many more PCR primers . AFLP analysis methods were adapted for use on the ABI Prism 377 DNA Sequencer with Genescan and Genotyper software. This provides a faster and safer method, since primers are labelled with fluorescent dyes and not radioisotopes. The use of paper combs has recently been tested successfully by other users of the DNA Sequencer. This method will reduce the risk of OOI injuries and reduce the cost of fine pipette tips for loading on the conventional combs. We will now test the suitability of paper combs for AFLP analysis.

Analysis of data
The dose response of larvae to Bt toxin was analysed, and slopes and LD_{50} estimates were calculated, using the logit analysis of GLIM version 3.77 (Payne, 1985).

Results and Discussion

1. Establish lines of known parentage from available resistant strains
Lines of known parentage were established for two Bt resistant strains (RATS and VICRATS) by Dr Heckel during his sabbatical in CSIRO Entomology in 1997. However, it was decided to use a third strain, BX, selected by Dr Akhurst's group, for detailed genetic analysis because it showed a high level of resistance in bioassays and on Bt cotton plants. It is has shown resistance levels of over 300-fold to Cry1Ac toxin. This Bt resistant strain was selected from field collected material and then out crossed to a laboratory strain to improve fitness. It was considered that the strain needed further selection and preparation before genetic crosses and AFLP mapping could be undertaken.

To assess the genetic homogeneity of the resistant strain and for additional selection, almost 200 single pairs were set up. Only the most viable pairs, producing good numbers of fertile eggs, were retained, reared and screened by bioassay. Because of the significant amount of work involved in this method of assessing and selecting the strain, the work load was split between Dr Daly's and Dr Akhurst's research teams, with Dr Akhurst's staff undertaking the bioassays. Results were assessed by both teams at regular meetings. A total of 45 pairs were screened and assessed and 14 of these were retained, reared individually and re-tested because they showed the highest resistance. After the second round of bioassay screening, these selected single pair lines showed no increased resistance, but a small decline, when compared to the original resistant strain. Loss of fitness, because of inbreeding, may partly be the cause.

Two replicates of preliminary genetic crosses and bioassays were carried out by Dr Daly's staff at the same time as the single pair crosses to investigate the selection status of the strain and also to assist with the planning of the full genetic investigation. The Bt resistant BX strain was crossed to GR, a Bt susceptible strain that is used for general laboratory use. The resulting F_1 generation was backcrossed both to the susceptible and resistant strains. Bioassays used HD73 toxin in the diet incorporation method. These first crosses were based on 1 to 20 individuals from the resistant strain because of a shortage of numbers. The crosses and bioassays were repeated for a third time with a larger sample (40 to 100 individuals) as well as with single pairs, to substantiate the resistance and selection status of the strain. The bioassay method was changed to surface treatment with HD73 toxin because it required less of the toxin. Although fitness of the resistant strain deteriorated and the backcrosses to the resistant strain produced only infertile eggs, all successful crosses showed trends the same as those in the preliminary crosses (Table 1).

The LC_{50}s of the F_1 crosses in all replicates lie about mid-way between those of the parental strains, and those of the backcrosses fall between their respective sources, suggesting that the major gene involved is intermediate in expression (incompletely dominant).

Table 1: Statistical analysis of the dose response of susceptible and resistant parents, F_1 and backcrosses for the Bt resistant strain BX, in three set of bioassays.

Strain/genetic cross	LC_{50}	95% CIs (Lower)	(Upper)	Slope	se
Diet incorporation A (ug/ml)					
Resistant	830	510	1351	1.5	0.3
Susceptible	1.5	0.9	2.4	1.1	0.2
Resistant x susceptible (F_1)	56	31	102	0.6	0.2
(F_1) x resistant	167	115	243	0.6	0.2
(F_1) x susceptible	23	18	31	0.6	0.1
Diet incorporation B (ug/ml)					
Resistant	307	188	503	0.9	0.2
Susceptible	1.4	0.8	2.2	1.8	0.3
Resistant x susceptible (F_1)	15	11	21	1.3	0.2
(F_1) x resistant	37	27	52	1.1	0.1
(F_1) x susceptible	7	5	10	1.0	0.1
Surface treatment A (ng/cm^2)					
Resistant	approx. 950			One dose only	
Susceptible	7.61	5.73,	10.12	1.3	0.2
Resistant x susceptible (F_1)	139	115	167	1.1	0.1
(F_1) x resistant	No crosses successful				
(F_1) x susceptible	21	17	26	1.6	0.2

Both the parental and F_1 generations (outcrossed to the susceptible strain) were tested on whole Bt plants, Sicot 289i and conventional cotton as controls. The heterozygous F_1 individuals represent the way the gene would most commonly occur in the field. The results (Table 2) indicate that both the resistant and the F_1 strain (resistant x susceptible) could grow on and damage Bt cotton significantly more than the susceptible strain. The resistant strain, BX, seemed to grow more slowly than the susceptible strain and F_1 (also observed in bioassays) and damage to the plants was less than for the F_1 on both Bt and conventional plants, although the number of survivors was higher on the Bt cotton.

Table 2: Comparison of survival and growth of the parental and F_1 generations of the Bt resistant strain, BX, on Bt cotton compared to conventional cotton.

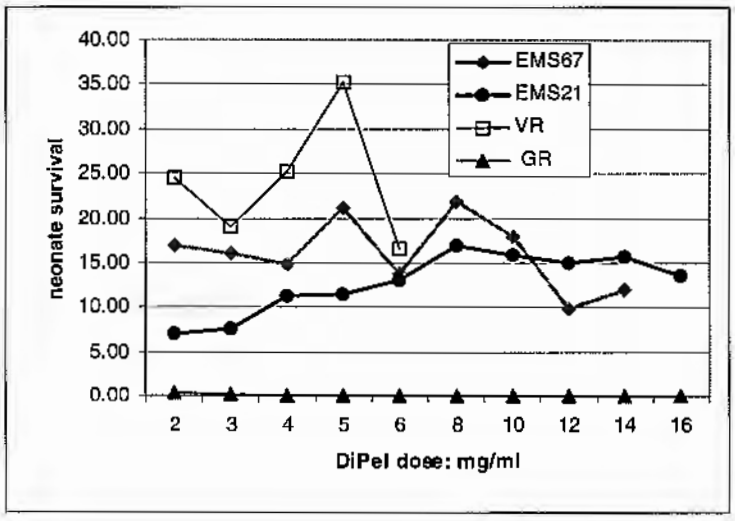
Insect strain	Ratio of larvae surviving on conventional/Bt cotton	Average weight of larvae (mg)
BX (resistant)	1.07	0.75
GR (susceptible)	0.25	0.61
F_1 (resistant x susceptible)	0.68	1.16

These results, together with those of the single pair selection, showing no improvement in resistance levels, suggest that the resistant strain could be close to homozygosity for the major resistance gene. Based on these results, the full genetic

investigation was planned by the two Canberra based CSIRO groups and Dr Heckel. Two generations of genetic crosses have been completed. Availability and fitness of the resistant strain, BX, has continued to be a problem, so the strain has been outcrossed to the susceptible laboratory strain, GR, and reselected at a discriminating dose for heterozygotes using surface treated diet. As with the egg treatment method, this allowed rapid selection of the strain with vigorous survivors. This reselected strain will be maintained using the sub-culture method. It was not routinely reared by this method previously.

Four other strains of *H. armigera,* showing varying degrees of resistance to Bt, have been maintained and tested in our laboratory. Dr Forrester derived two of these strains, VICRATS and RATS, from field populations. The VICRATS (VR) population was selected from individuals collected off maize crops near Bairnsdale in Victoria. They have not had extensive exposure to Bt in the field. The RATS (R) population is from survivors collected from transgenic Bt cotton in the Narrabri district. Two other strains, EMS21 and EMS67, have been derived in the laboratory by mutagenesis. Over the past two years these four strains have been selected for 15 to 18 generations, using the egg treatment and diet surface treatment methods with MVP and DiPel. The dose was increased as fitness and survival of the strain allowed. Surviving larvae continue to develop up to 3rd instar at 7 days after hatching, at the highest doses. Over the past two years EMS21 has shown a continual increase in resistance when compared with the susceptible strain, using the egg treatment selection method (Figure 1). Larvae of strains EMS67 and VR have been fairly consistent in their growth and survival with increasing doses of toxin. The susceptible strain had less than 1% survival at the 2 and 3 mg/ml dose of DiPel 2X. The RATS strain did not tolerate selection with DiPel so recently selection with Bt HD73 as surface treated diet has begun. Selection lines with surface treatment of HD73 toxin have also been started for the other resistant strains.

Figure 1: Percentage survival of two laboratory induced (EMS 67, EMS 21) and one field collected (VR) Bt resistant strains and one susceptible strain (GR), using the egg treatment selection method, with increasing doses of Bt toxin, as the commercial formulation DiPel 2X.



The resistance levels of the strains have been monitored with diet incorporation bioassays using MVP®, which contains only Cry1Ac toxin encapsulated in killed *Pseudomonas fluorescens* cells, DiPel® which contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2A

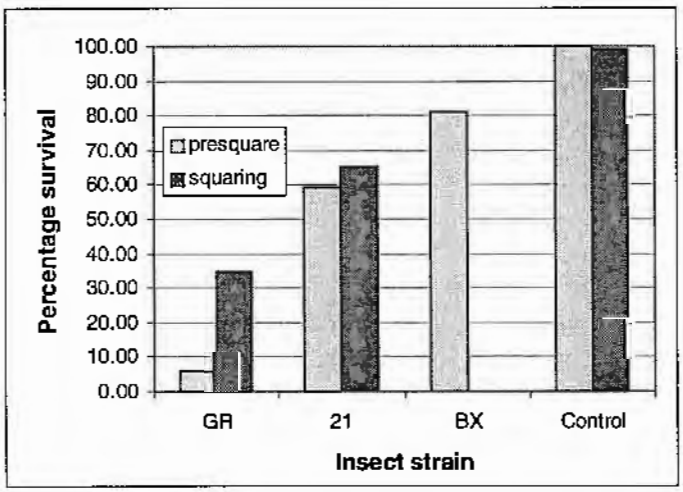
and Cry2B toxins and laboratory purified Cry1Ac crystals from the Bt strain HD73 with spores. In Bt cotton plant bioassays, the leaves of presquare Bt cotton plants were incorporated into ground leaf from normal presquare cotton plants. The results of bioassays are summarised in Table 3.

Table 3. The resistance ratios between the LC_{50} of the resistant strain and that of the susceptible laboratory strain for four Bt resistant strains in four bioassay methods.

Strain	Resistance Ratio			
	MVP in Diet	DiPel in Diet	HD73 in Diet	Bt Leaf in Leaf Mush
VR	21	10	15	28
R	101	-	-	24
EMS 21	8	4	9	9
EMS 67	19	5	5	5

Three Bt resistant strains were tested on whole Bt cotton plants producing Cry1Ac toxin, as this was considered a necessary step in assessing their resistance to Bt cotton. All plants were grown and the test conducted in growth rooms. A filter paper disc of eggs close to hatching was placed in the crown of each plant. The disc was removed at the end of the day and the number of hatched eggs was counted. Larvae surviving after eight to nine days were counted. BX was tested only on presquare Bt plants, VR and EMS21 were tested on presquare and squaring Bt cotton plants. All were tested against the Bt susceptible strain, GR, and also their own strain on conventional cotton. On conventional cotton, all strains showed a similar, high rate of survival. However in the VR test, squaring plants became stressed and dropped squares and some presquare plants started to produce squares which made the results difficult to assess because of the variation in efficacy of the plants. The results from BX and EMS21 are shown in Figure 2.

Figure 2: Survival of larvae of three *H. armigera* strains on whole Bt cotton plants, at two different growth stages.



The control represents survival of GR (Bt susceptible, general rearing laboratory strain) on conventional (non-Bt) cotton. Both BX and EMS21 showed considerably increased survival on presquare Bt cotton compared to the susceptible GR strain. In addition, 65% of

resistant BX larvae on Bt cotton reached 2nd instar after nine days while the susceptible larvae did not grow beyond 1st instar.

2. Establish genetic mapping techniques and undertake genetic analysis of existing resistant strains

Dr Heckel established lines of known parentage for two Bt resistant strains (RATS and VICRATS) during his sabbatical in 1997. On a return visit to Canberra in 1998, he instructed Ms Karen Olsen in the techniques for DNA isolation and quantification and preparation of *H. armigera* DNA for AFLP analysis. This included restriction digestion, ligation of adaptors, PCR and primer labelling. Material from the existing RATS crosses was used. During his visit, Entomology installed an ABI Prism 377 DNA Sequencer with Genescan and Genotyper software. This allows it to be used for AFLP analysis. It provides a faster and safer method, since primers are labelled with fluorescent dyes and not radioisotopes. Dr Wes Keys of CSIRO Plant Industry provided initial instruction in the use of the sequencer for AFLP analysis. In this pilot study, the prepared DNA run on the ABI DNA Sequencer produced results but the signal was weak. This problem was addressed by adapting the two PCR programs. An additional problem with contamination of samples was also overcome. We will now test the suitability of disposable paper combs for loading samples for AFLP analysis on the sequencer, using DNA samples prepared from the RATS strain.

3. Availability of resistant strains

Rearing and selection of three of the most resistant field strains is been carried out by both Dr Daly's and Dr Akhurst's research groups. This will ensure security of these strains as *H. armigera* is a notoriously difficult species to maintain in the laboratory. Each group also maintains and tests additional resistant strains and these are available to both groups.

Industry Significance/Commercial Impact

Characterisation of resistant strains, generated in a laboratory, will help us to develop management strategies that minimise selection for resistance in the field. Early characterisation of resistance will give the industry its best hope of managing that resistance. Bt resistant strains of *H. armigera,* currently in culture, will be used to determine the likely characteristics of future field-derived resistance. In this way, researchers will be able to investigate the likely success of synergists to control resistance and the role of insecticides in managing Bt resistant larvae.

The research is a very important contribution to the TIMS strategy for Bt cotton. In particular, the results question the assumption that Bt resistance will be recessive because at this stage they suggesting an incompletely dominant gene.

Dissemination of Research Results

These results have been presented at a CSD-sponsored review of CSIRO projects (June 2000), to cotton consultants and to a scientific meeting of the TIMS committee (March 2000).

Publications

Olsen, K.M. & Daly, J.C. Plant-Toxin Interactions in Transgenic Bt Cotton and their Effect on Mortality of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Journal of Economic Entomology. In press*

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Plain English Summary

Resistance to both insecticides and Cry1Ac in Bt cotton is an ongoing concern in the management of *Helicoverpa armigera* in the Australian cotton industry. While management strategies for insecticides have been successful in slowing down the rate at which resistance has developed, the industry uses more insecticide to control *H. armigera* today than it did ten years ago. This, combined with the reduced bioefficacy of mature Bt cotton plants, means that there is an ongoing need to consider the resilience of the resistance management strategy in place for Bt cotton.

This project has examined the genetics of Bt resistance in *H. armigera*. This involved selecting for a resistant strain of insects and then seeing how many genes are contributing significantly to resistance and the relative contribution of each gene. Our work is linked closely with Dr Akhurst's project on the mechanisms of resistance. Such combined knowledge allows interpretation of the outcomes of resistance management strategies imposed on the cotton industry and their subsequent refinement to improve their effectiveness while reducing the cost to the grower.

A number of approaches for selecting a resistant strain were tried by various research groups. Five strains showing varying levels of resistance were obtained. This work concentrated on only one of these, the Bt resistant strain (called BX) from Dr Akhurst, because it showed high levels of resistance in bioassays and on Bt cotton. This strain was derived originally from field-collected material.

Genetic studies involved traditional crosses of the different strains and their analysis using dose-response bioassays. Results to date suggest that the BX resistant strain seems to be fixed for a major resistance gene that in heterozygotes is intermediate in expression between the susceptible (SS) and fully resistant (RR) individuals. The heterozgyous individuals (RS) represent the way the gene would most commonly exist in the field. The heterozygous and resistant strains showed significant survival on Bt cotton when compared with the susceptible strain.

Results from such studies can be ambiguous, so work on the genetic mapping of the putative genes, using the techniques developed by Dr David Heckel (Melbourne University) in an earlier CRDC-funded project has begun. Together these approaches will allow careful analysis of resistance. Genetic mapping techniques have been established with the help of Dr Heckel and Dr Wes Keys (CSIRO Plant Industry) after modifications, to utilise the newer, faster and safer technology of the DNA sequencer. DNA has been extracted from a Bt resistant strain and is being used to fine tune the AFLP mapping method. Using Dr Heckel's approach, we have begun the mapping of Bt resistance in *H. armigera* and this work will continue in a new joint project with Dr Heckel.

The research is a very important contribution to the TIMS strategy for Bt cotton. In particular, the results call into question the assumption that Bt resistance will be recessive because at this stage they suggest an incompletely dominant gene, which means resistance to Bt could develop faster in the field than originally thought.