C R D C

FINAL REPORT

Genetics of Bt Resistance in *Helicoverpa armigera*: Understanding Bt Resistance

CSE 89C

September 2003

Rodney Mahon[1] and David Heckel[2].
[1]CSIRO Entomology, Canberra, ACT
[2]University of Melbourne, VIC.

Cotton
RESEARCH & DEVELOPMENT

Annual, Progress and Final Reports

Part 1 - Summary Details

REPORTS

Please use your TAB key to complete part 1 & 2.

CRDC Project Number: **CSE89C**

Annual Report: ☐ Due 30-Sep-03

Progress Report: ☐ Due 29-Jan-03

Final Report: ☒ Due 30-Sep-03
(or within 3 months of completion of project)

Project Title: **Genetics of Bt Resistance in Helicoverpa armigera: Understanding Bt Resistance**

Project Commencement Date: 1/7/00 **Project Completion Date:** 30/6/03

Research Program: Insect Management

Part 2 – Contact Details

Administrator: Mr Mark Hardwick, Finance Manager.
Organisation: CSIRO Entomology.
Postal Address: GPO Box 1700, Canberra, ACT 2601.
Ph: 02-62464406 **Fx:** 02-62464095 **E-mail:** Mark.Hardwick@csiro.au

Principal Researcher: Dr Rod Mahon.
Organisation: CSIRO Entomology.
Postal Address: GPO Box 1700, Canberra, ACT 2601.
Ph: 02-62464082 **Fx:** 02-62464000 **E-mail:** Rod.Mahon@csiro.au

Supervisor: Dr Geoff Baker.
Organisation: CSIRO Entomology.
Postal Address: GPO Box 1700, Canberra, ACT 2601.
Ph: 02-62464406 **Fx:** 02-62464000 **E-mail:** Geoff.Baker@csiro.au

Researcher 2 Dr David Heckel, Senior Lecturer
Organisation: University of Melbourne
Postal Address: Department of genetics, Parkville, Vic 3052
Ph: 03-9344-9877 **Fx:** 03-9344-5139 **E-mail:** d.heckel@genetics.melbourne.edu.au

Signature of Research Provider Representative: 

Background to the Project

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. However, these strategies, particularly those concerning the Cry proteins, are based on models of resistance, as information is lacking regarding putative resistance genes in *H. armigera.* This project aimed to increase our understanding of the genetic basis of resistance, ideas about which underlie the basis of the resistance management strategies. The focus was on Bt resistance, but the work is also directly applicable to conventional chemistry insecticide resistance.

While it is possible to design strategies in the absence of information about the genetics of the forms of field resistance, there is a risk that the industry will be burdened by management practices that are inefficient, costly, and perhaps do little to achieve the desired outcome. Genetic studies can reveal how many genes are involved in resistance, how each gene contributes to the final level of resistance in each insect, and assist our understanding of the mechanisms of resistance. It is only with this knowledge that we can anticipate the outcomes of resistance management strategies imposed on the cotton industry, and perhaps, refine such strategies.

This project was a collaborative one between the CSIRO genetics group in Canberra and Dr Heckel's group at the University of Melbourne. It made use of recent advances in genetic studies of *H. armigera*, in particular, the development of a genetic linkage map by Dr Heckel for this species, developed through the use of AFLPs. Linkage maps enable a speedier means to define the genetic basis of resistance than is available through more conventional approaches. They also offer an opportunity to determine how many different classes of mechanisms are involved. Pyrethroid resistance provides an illustration of these points. Despite over twelve years of work done on this resistance in *H. armigera*, there is still controversy about which mechanisms of resistance are involved: esterases, mixed function oxidases and their relative contributions, nor is the role of penetration or target site insensitivity clear. The linkage-mapping approach has begun to resolve these uncertainties in the case of pyrethroid resistance, and the same approach will be also useful in sorting out mechanisms in existing Bt resistant strains, and also those that might be established in the future. This project aimed to analyse the genetic inheritance of Bt resistance to Cry1Ac toxin in resistant strains of *H. armigera* using genetic crosses, bioassays and molecular techniques by: (a) selecting and assessing genetic homogeneity of resistant strains; (b) undertaking crosses and bioassays to ascertain possible modes of inheritance; (c) determining the contributions of different chromosomes to the resistance phenotype using molecular mapping techniques; (d) determining the impact of resistance on the structure of resistance management strategies for Bt cotton.

At the beginning of this project, there were five strains of *H. armigera* available that exhibited levels of resistance to transgenic Bt cotton plants. Dr N.W. Forrester derived two of these strains from field-collected insects. One strain, established from field-collected material, was selected in the laboratory using laboratory produced Cry1Ac toxin (Bill James in Dr R. Akhurst's laboratory). The other two were produced by K. Olsen in Dr J. Daly's laboratory,

by mutagenesis of a laboratory strain followed by selection using the commercially available Bt product, DiPel.

Summary of Achievements against Objectives

1. **Extract and prepare DNA for linkage mapping, using two existing resistant strains (RATS and BX), extract DNA from the frozen material developed in CSE 58C and CSE 73C.**
   DNA was extracted from more than 200 insects (moths and pupae) of the BX strain to provide the basis for the genetic analysis. DNA from the resistant strain, RATS, was extracted prior to the start of this project.
2. **Screen AFLPs for linkage to Bt resistance in both resistant strains using manual and automated sequencing gels.**
   In BX, 149 polymorphic AFLPs were scored and assigned to 26 linkage groups. Three unassigned "singletons" showed a non-random association with resistance. One of these, AA-CTA_330.14 showed a highly significant association with resistance.
3. **Localise Bt-linked AFLPs on the AFLP map previously made for pyrethroid resistance study.**
   AFLP AA-CAT_330.14 was assigned to linkage group A14 in the previously constructed AFLP map of *H. armigera*.
4. **Isolate and subclone AFLPs most tightly linked to Bt resistance genes of largest effect in RATS and BX.**
   The AFLP AA-CTA_330.14 was isolated, subcloned and sequenced. It exhibited no correspondence to any known gene in GenBank, and therefore presented difficulties when 'mapping' to other species. To confirm the mapping result in BX, a different technique (D-HPLC) was used with success. In a separate, non-resistant family, the AFLP proved to be linked to a known gene, RpS15 (a ribosomal protein), and also to the voltage-gated sodium channel. This enables comparison to other species with Bt resistance such as *Heliothis virescens* and *Plutella xylostella*.
5. **Rearing and selection of other strains to derive a genetic homogeneous strain.**
   Bt resistant strains EMS21, VICRATS and BX were maintained. Strains EMS21 and VICRATS exhibited significantly lower mortality than the susceptible strain when exposed to Cry1Ac and/or Cry2Ab toxin. A new resistant strain established in July, 2003, SP15, that exhibits a 'high' level of resistance to Cry2Ab toxin, has been identified and established in the laboratory. Appropriate crosses are currently being performed that will enable AFLP analysis of this form of resistance.
6. **Establish lines of known parentage, through single pair crosses, of one of the remaining resistant strains, preferably one thought to have a different resistance mechanism to BX or RATS.**
   Assay data for BX and EMS21 suggest that there are different resistance mechanisms involved in the two strains. EMS21 has shown resistance to both Cry1Ac and Cry2Ab toxins. The three-generation crosses required for ALP analysis were conducted for the EMS21 line and insects were frozen for AFLP analysis. A strain, "Silver", originating from the Resistance Monitoring Program in Narrabri (Dr Ho), was found to exhibit no resistance to Cry1Ac when tested in our laboratory.
7. **Extract and prepare DNA of this 3rd strain for linkage mapping.**
   DNA from insects (from 6 above) was extracted from 100 pupae and adults in preparation for AFLP analysis
8. **Use AFLPs in a linkage analysis of the 3rd resistant strain.**

AFLPs from 22 primer pairs have been screened and are presently undergoing analysis.

9. **Integrate all Bt-linked AFLPs into a single linkage map.**
   Although AA-CTA_330.14 is now integrated with the pyrethroid-resistance map of *H. armigera*, a more useful result is the homology to Linkage Group 10 of *Heliothis virescens*. Linkage studies comparing the two species at Melbourne and Clemson Universities show a good correspondence between the chromosomes of these two species.
10. **Identify possible different resistance mechanisms in the different strains based on linkage results.**
    In BX, the midgut cadherin responsible for Bt resistance in *H. virescens* and *Pectinophora gossypiella* is on a different linkage group than AA-CTA_330.14, thus excluding cadherin mutations as the mechanism of resistance exhibited by BX. One aminopeptidase was also excluded by a similar linkage result in BX. By correspondence to *H. virescens* Linkage Group 10, four additional aminopeptidases are excluded. However, a Bt resistance gene from the CP73 strain in *H. virescens* (BtR-5) also maps to Linkage Group 10. This suggests that the BX resistance mechanism of *H. armigera* may be similar to Cry1Ac resistance in CP73 of *H. virescens*. The most likely mechanism in BX and CP73 is a gene that affects post-translational modification of the Bt-toxin receptor, reducing but not abolishing binding in the resistant strain.
11. **Preparation of results for publication in international scientific journal.**
    The results of this project were communicated to researchers, collaborators in similar fields through informal meetings, correspondence and conferences. The results will be published in an international scientific journal.

Methodology

The methods used in this project were a continuation of those developed and used in project CSE 73C and are summarised here.

Rearing of resistant strains.

The resistant strains were reared using standard laboratory rearing protocols that produce consistently high quality material for experiments. This method delays the decrease of fitness associated with the inbreeding resulting from repeated selection regimes.

Selection of resistant strains

Laboratory selection for resistance proceeded by exposing eggs to a dose of the commercially formulated MVP or DiPel (see Final Report CRDC project NCQ 1C). In addition, lines have been established within each strain that have been selected by exposure of larvae to diet, the surface of which has been 'contaminated' with either Cry1Ac or Cry2Ab.

Bioassays

Strains have been screened using a variety of bioassay methods to ensure that resistance levels encountered could be quantified and were likely to be valid for field situations. These methods included leaf mush, diet incorporation and leaf disc assays (Olsen and Daly 2000), and also assays that involved the surface contamination of the diet by Bt toxins.

Genetic crosses

Three-generation pedigrees were established for two strains according to the techniques of Heckel (CSE 58C). The aim was to produce six backcross or F_2 families for linkage analysis. Analysis of both reciprocal backcrosses is advantageous in *H. armigera* because, in common with other Lepidoptera, there is no crossing-over in females.

Genetic mapping

AFLPs were used to establish linkage relationships within *H. armigera* by two procedures. In Canberra the automated ABI Prism 377 DNA Sequencer with Genescan software was employed while in Melbourne, manual sequencing gels were used.

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). A Student's t-test on the parameter estimates, calculated from the log-transformed data using GLIM, was used to determine relationships between bioassay results.

Preliminary analysis of AFLP data produced by the automated sequencer was performed using the GeneScan software for the ABI DNA Sequencer. A Java computer program, custom written by CSIRO IT staff and a Pascal computer programs custom written for the analysis of Lepidopteran linkage data by David G. Heckel, was used to group AFLPs into linkage groups and test for association with resistance by collating AFLP and bioassay data.

Results and Discussion

1. Extract and prepare DNA for linkage mapping, using two existing resistant strains (RATS and BX), extract DNA from the frozen material developed in CSE 58C and CSE 73C.

The required genetic crosses and associated assays on the Cry1Ac resistant strain BX, were successfully completed. Material from these crosses (over 500 individual moths or pupae) was stored at -80^0C until analysed. DNA was extracted from more than 200 of these insects (moths and pupae) for genetic analysis using the AFLP approach. DNA extracts were further analysed by Dr Heckel. DNA previously extracted from the resistant strain RATS was also available for analysis.

2. Screen AFLPs for linkage to Bt resistance in both resistant strains using manual and automated sequencing gels.

In the BX backcross, 149 polymorphic AFLPs were scored, using the manual sequencing approach. These polymorphisms 'covered' a total of 26 of the known 31 linkage groups of *H. armigera*. None of these linkage groups were significantly associated with resistance. However, three "singleton" AFLPs (each of which was unlinked to all other AFLPs) did show an association with resistance. For two AFLPs, this association was subsequently shown to be spurious, as the association occurred in the opposite direction predicted from a real resistance gene. However, the most significant of these, (AA-CTA_330.14) was confirmed to be markedly associated with resistance phenotypes ($\chi^2 = 11.29$, $P < 0.001$). In earlier studies by Heckel, marking all 31 linkage groups with 2 or more AFLPs has been routinely achieved. However, since the BX strain had been previously intercrossed with the susceptible strain before the mapping, the level of AFLP polymorphism was considerably lower than usual. Thus although suggestive, association between the single AFLP and resistant phenotypes did not provide the level of significance achieved in previous studies. This eventually required isolation and sequencing of the DNA fragment to confirm that the mapping result was not an artefact.

3. Localise Bt-linked AFLPs on the AFLP map previously made for pyrethroid resistance study.

The recent identification of the gene conferring resistance to Cry1Ac in *H. virescens* was expected to enable us to establish if a homologous gene is involved in the form of resistance to this toxin found in the BX strain of *H. amigera*. However, initial analysis of the sequence of AFLP 330.14 revealed that it did not correspond to any known gene in GenBank, and therefore presented difficulties when attempting to map in other species. Attempts to map AA-CAT_330.14 in *H. virescens* by Southern hybridization failed.

Previous work had produced an AFLP map of *H. armigera* that localised pyrethroid resistance in the AN02 strain.. However, it was found that the AA-CTA_330.14 AFLP identified in the current study, did not segregate in the AN02 cross. Indirect means were thus required to assign it to a linkage group. As described in the next section, AA-CTA_330.14 was eventually found to be linked to the voltage gated sodium channel, which occurs on linkage group A14 of the AN02 map.

4. Isolate and subclone AFLPs most tightly linked to Bt resistance genes of largest effect in RATS and BX.

The AFLP band AA-CTA_330.14 was excised from the gel, reamplified, and cloned. This procedure was complicated by the presence of another co-migrating band in the gel. Sequences of both bands were obtained and the unlinked co-migrating band was excluded. To confirm the segregation of AA-CTA_330.14 within *H. armigera*, an alternative technique was used. Specific primers were made to amplify a smaller fragment and a polymorphism was resolved using D-HPLC (denaturing high-pressure liquid chromatography). Because of the complexity of the D-HPLC pattern, fragments from grandparents and parents in the BX crosses used in the analyses were sequenced, and the genotypes of the progeny reconstructed from these patterns. This succeeded in confirming the segregation pattern of AA-CTA_330.14, but in the process, revealed that some of the original AFLP scores had been confounded by the co-migrating band. Correction of the scores resulted in lower significance of the Bt-resistance linkage result ($\chi^2 = 3.8$, $P < 0.05$). However, this is still the most significantly associated marker with Bt resistance in BX.

In a separate, concurrent study funded by the ARC in Heckel's lab in Melbourne, linkage relationships among ribosomal protein genes were being investigatigated. When the DNA fragment from AA-CTA_330.14 was used to probe Southern blots made from an *H. armigera* family (unrelated to the BX strain and fully Bt-susceptible), linkage to the RpS15 ribosomal protein gene was found. Most of the RpS15 gene was cloned and sequenced from the parents of the BX cross. However, no sequence variation was found, and thus RpS15 could not be mapped in BX.

In another concurrent study in the Gahan laboratory at Clemson University, RpS15 was found to be linked to the voltage-gated sodium channel Hscp in *H. virescens*. In Melbourne, an intron-containing a fragment of the sodium channel gene was cloned and sequenced from the parents of the BX cross. In this case, polymorphism was found, and by use of the D-HPLC technique, Hscp was shown to be linked to AA-CTA_330.14 in the BX cross. It would appear that the crossover rate between Hscp and AA-CTA_330.14 is approximately 25%.

The sodium channel Hscp is known to be linked to a cytochrome p450 gene in *H. virescens*, according to a published report from the Brown laboratory at Clemson (Park & Brown 2002). The homologue of this p450 gene has been assigned to AFLP group A14 in the AN02 cross in Melbourne. This chain of evidence implies that AA-CTA_330.14 also occurs in group A14 on the AN02 map.

Thus, the availability of the AFLP linked to Bt resistance in BX, combined with other linkage studies on *H. armigera* and *H. virescens*, has identified Hscp as a reliable marker gene for the chromosome harbouring the major Bt resistance gene in BX. The voltage-gated sodium channel is ubiquitous in insects, and DNA polymorphism within introns of that gene can be used for linkage tests for Bt resistance in other Lepidopteran species such as *Plutella xylostella*. Although useful in establishing which linkage group harbours Bt resistance in

such an analysis (because of the absence of crossing-over in female Lepidoptera), unfortunately, Hscp is not close enough to the Bt resistance gene to be used as a marker in field populations. In field populations, extensive recombination would soon break down any association between resistance and Hscp alleles.

5. Rearing and selection of other strains to derive a genetic homogeneous strain.
Bt resistant strains EMS21, VICRATS and BX are being maintained, selected and periodically monitored for levels of resistance. The resistant strain, EMS21, continues to be of most interest. This strain was generated using the mutagen EMS (Ethyl Methanesulfonate) and subsequently selected through exposure to Dipel, that contains several Bt proteins. Through the selection process, the EMS21 strain survived increasingly higher doses of Dipel. In assays with Cry1Ac toxin, resistance levels have increased from 15.5-fold in late 2000 to 65-fold in late 2002. A better understanding of the nature of the resistance exhibited by this strain was sought by challenging it with several toxins presented in bioassays where the diet was surface-treated with appropriate toxins. The results of the survival assays on Cry2Aa toxin or Cry1Ac toxin (two assays), and Cry2Ab toxin or DiPel (one assay each) are summarised in Table 1. When challenged with different toxins, EMS21 exhibited a moderate level of resistance to Cry1Ac (65-fold), a similar level of resistance to Cry2Ab (53-fold) and a low level of resistance to both Cry2Aa (13-fold) and Dipel (22-fold).

Table 1. LC_{50}s and statistical analysis of surface treated diet bioassays, using four Bt toxins against a susceptible (GR) and resistant strain (EMS21) of *H. armigera*.

Toxin (year of assay)	*H.armigera* strain	LC_{50} ug/cm^2	95% CIs	F statistic (res vs sus)	P value	Resistance level
Cry1Ac (2002)	Susceptible	0.003	0.002, 0.004	$F_{1,13} = 60.48$	< 0.001	64.8-fold
	Resistant	0.17	0.12, 0.22			
Cry2Aa (2001)	Susceptible	0.22	0.17, 0.3	$F_{1,16} = 34.38$	< 0.001	13-fold
	Resistant	2.88	1.86, 4.45			
Cry2Ab (2003)	Susceptible	0.11	0.08, 0.15	$F_{1,5} = 36.75$	< 0.01	53.4 -fold
	Resistant	5.94	3.23, 10.94			
DiPel (2001)	Susceptible	0.07	0.04, 0.09	$F_{1,5} = 40.09$	< 0.01	22-fold
	Resistant	1.12	0.76, 1.67			

Experiments were also performed to challenge EMS21 larvae with leaf discs of late presquare Bt cotton. Plants tested expressed the *cry1Ac* (Sicala V-2i), the *cry2Ab* (Sicala V-2X) toxin genes, and also Sicala V-3B (Bollgard II), which has both the *cry1Ac* and *cry2Ab* genes. The plants were at the 7 to 9 node stage and had been grown at a temperature range of 19–29°C. The results are summarised in Table 2.

Table 2. Mortality of neonate larvae in a leaf disc bioassay and statistical analysis of results, using three Bt cotton varieties against a susceptible (GR) and resistant strain (EMS21) of *H. armigera*. Sixty six to 96 neonates were used per strain/plant variety.

Cotton Variety	Bt Toxin Expressed	Mortality of Susceptible Strain	Mortality of Resistant Strain	t statistic and df (res vs sus)	P value
Sicala V-2i	Cry1Ac	58%	28%	t = 2.2, 9 df	< 0.05
Sicala V-2X	Cry2Ab	82%	35%	t = 4.3, 10 df	< 0.001
Sicala V-3B (Bollgard II)	Cry1Ac + Cry2Ab	73%	29%	t = 5.1, 11 df	< 0.001

In these leaf disc assays, larvae from the EMS21 strain exhibited significantly greater survival than the susceptible strain when fed leaves from plants producing either Cry1Ac, and/or Cry 2Ab toxin.

Leaf disc bioassays were repeated on four resistant strains and the susceptible strain, to investigate cross-resistance between Cry1Ac, Cry2Aa and Cry2Ab. Table 3 summarises the results of two experiments.

Table 3. Mean mortality (percentage) and standard errors for neonate larvae of five *H. armigera* strains on leaf discs from three varieties of Bt plants, producing either Cry1Ac or Cry2Ab, or, both toxins together (Bollgard II). Sixty four to 128 neonates were exposed per strain/plant variety. All plants were late presquare or early fruiting stage at the time they were sampled.

	% mortality ± SE for toxin(s) produced by Bt plant variety		
***H. armigera* Strain**	**Cry1Ac**	**Cry2Ab**	**Cry1Ac and Cry2Ab**
GR (susceptible)	37% ± 9.6	76% ± 6.2	79% ± 5.5
EMS21	13% ± 4.2*	46% ± 6.1*	35% ± 3.1***
VIC RATS	11% ± 4.4*	24% ± 4.9***	20% ± 3.9***
BX	5% ± 4.7*	62% ± 10 ^{ns}	22% ± 9**
EMS67	11% ± 1.4*	53% ± 19.5 ^{ns}	54% ± 16.6 ^{ns}

* Mean mortality is significantly different to mean of susceptible, P< 0.05; ** P<0.01; *** P< 0.001; ^{ns} mean is not significantly different to susceptible, P> 0.05.

Strains EMS21 and VIC RATS exhibited significantly lower mortality than the susceptible strain on leaf discs from plants producing Cry1Ac and/or Cry2Ab toxin. EMS67, derived in the laboratory through mutagenesis by exposure to EMS, was later discarded because it failed to show sustained increase in levels of resistance to the toxins following selection, and generally performed more poorly in assays than EMS21 or VICRATS.

Both the diet and leaf disc bioassays indicated that larvae from the resistant strain EMS21 consistently survive better than the susceptible strain on Cry1Ac and Cry2Ab toxins, and also when exposed to the combination of both toxins presented by Bollgard II plants.

Recently a new resistant strain, SP15 that exhibits a high level of resistance to Cry2Ab toxin, has been included in the line-up of resistance strains. It exhibits no cross-resistance to Cry1Ac, and was identified through the F_2 screening method of Andow and Alstad, (1998) from field-collected insects. It is presently being reselected following outcrossing.

During the course of this project, we received material from Dr Dang Ho of NSW Department of Agriculture in Narrabri. This "Silver Strain" (SS) of *H. armigera*, was made up of survivors from the discriminating dose of MVP (3 ul/ml diet) of the Resistance Monitoring Program. Dr Ho had found that the LD_{50}, of the SS F_2 generation was ca 48-fold that of GR, a susceptible strain. We assayed neonates of this strain against of Cry1Ac, Cry2Aa and Cry2Ab toxins in surface treatment bioassays and MVP in a diet incorporation assay. In the Cry1Ac assays, the LD_{50} of SS was only 2-fold that of the susceptible strain in one assay and it was less tolerant than the susceptible strain when the assay was repeated. SS was also less tolerant of Cry2Ab, Cry2Aa and MVP than the susceptible strain, thus there was no evidence of enhanced vigour in the strain, which was suggested as a possible source of the "resistance" in SS. Finally we replicated the assay exactly as conducted in Dr Ho's laboratory, with early 3rd instar larvae, assayed with MVP in diet and then scored after 10 days, not 7 days as in our earlier assays. Again there was no significant difference between the LD_{50}s of the strains ($F_{3,11} = 0.77$, $P > 0.05$) and SS was actually less tolerant than the susceptible strain.

These results indicated that the SS colony we examined exhibited no evidence of resistance to either Cry1Ac or to MVP. We are largely at a loss to explain the discrepancy between our results and those obtained in Narrabri, particularly the MVP results. Perhaps the resistance gene (s) have been lost from the culture. It should be pointed out that SS proved difficult to rear in our laboratories through poor fitness. This loss of fitness is not necessarily a function of SS itself, but rather a result of the sensitivity of colonies of this species to a change of environment. Lack of fitness cannot, however, totally account for the poor performance of SS in assays.

6. Establish lines of known parentage, through single pair crosses, of one of the remaining resistant strains, preferably one thought to have a different resistance mechanism to BX or RATS.

The strain EMS21, was selected for resistance mapping because it has shown modest resistance to both Cry1Ac and Cry2Ab toxins in bioassays and also on cotton varieties expressing one or both toxins. In contrast, the first resistant strain analysed, BX, exhibits high level resistance to Cry1Ac and has not shown significant cross-resistance to Cry2Ab. This would suggest that there are different mechanisms involved in the two strains.

Suitable discriminating doses for susceptible, resistant and heterozygous individuals were established prior to making a series of crosses between EMS21 and a susceptible strain (GR) to produce F_2's. Genetic crosses and bioassays using both Cry1Ac and Cry2Ab toxins were performed on the F_2's and parent moths and survivors were frozen in preparation for AFLP analysis.

7. Extract and prepare DNA of this 3rd strain for linkage mapping.

In order to find a safer and more efficient method of DNA extraction than the phenol/chloroform method that was used initially, various techniques were compared and assessed on the basis of the quality of AFLPs generated. The DNA extractions from 100 pupae from the EMS21 x GR crosses for the initial round of AFLP screening was completed using the best of the methods tested. Techniques for DNA preparation were again fine-tuned following the purchase of a new thermal cycler prior to processing the samples.

8. Use AFLPs in a linkage analysis of the 3rd resistant strain.

Analyses techniques in the two laboratories, Melbourne and Canberra, were standardised prior to analysis of insects associated with the EMS21 crosses. To date, AFLPs from 22 primer pairs have been generated and are currently undergoing analysis to determine linkage relationships between specific polymorphisms and resistance to both Cry1Ac and Cry2Ab toxins.

9. Integrate all Bt-linked AFLPs into a single linkage map.

As discussed above, the one Bt-resistance linked AFLP found (AA-CTA_330.14) has been localised to group A14 in the *H. armigera* AFLP linkage map based on crosses with the pyrethroid-resistant strain AN02. This achievement however is less useful than originally anticipated, for two reasons. First, the AN02 strain no longer exists as a separate strain, so the mapping cross cannot be replicated. Second, the identification of the sodium channel gene Hscp as a linked marker removes the requirement to use the AN02 map as a reference, because Hscp occurs and is highly conserved in all insect species. Thus in any new AFLP maps based on future crosses with new Cry1Ac and Cry2Ab resistant strains of *H. armigera*, Hscp can be readily mapped.

10. Identify possible different resistance mechanisms in the different strains based on linkage results.

Dr Heckel and colleagues found in related research on Bt resistance in the American cotton pest, *Heliothis virescens* (Gahan et al. 2001), that high levels of Cry1Ac resistance are conferred by a "knockout" of a gene encoding a cadherin-like protein. More recently, three separate mutations in the cadherin gene of another American cotton pest, pink bollworm *Pectinophora gossypiella*, have been shown to confer high resistance to Cry1Ac-expressing cotton (Morin et al. 2003). We sought to test whether the cadherin gene was also related to resistance in *H. armigera*. Because of the high degree of relatedness of the two species, it was possible to obtain partial sequence information from *H. armigera*. An intron-size polymorphism was used to map the cadherin to AFLP Linkage Group 14 in the BX backcross. Since that linkage group has no significant association with resistance in BX, we can rule out the occurrence of mutations in the cadherin itself as causing resistance in this case.

Other studies have implicated midgut aminopeptidases (APNs) in binding Bt toxins, although there is still no published evidence linking modified APNs with resistance. One aminopeptidase from *H. armigera*, APN4 (GenBank AF535165, Connie Angelucci, pers. comm) was mapped in BX and found to occur on a separate linkage group to the 26 AFLP linkage groups identified. It was also unlinked to AA-CTA_330.14 and showed no association with resistance in BX.

More recently, additional (unpublished) studies at Clemson University have shown that the main Cry1Ac resistance gene (BtR-5) in the CP73 strain of *H. virescens* occurs on Linkage Group 10 and is linked to the Hscp sodium channel. The CP73 strain is of particular interest

because it is about 60-fold cross-resistant to Cry2B (Gould et al. 1992). However no gene for Cry2B resistance was found on Linkage Group 10, so the cross-resistance cannot be due to BtR-5 and must be due to other genes on other linkage groups.

The occurrence of Cry1Ac resistance in the two species of heliothines, linked to the same marker gene Hscp, suggests but does not prove that the genetic basis is the same in *H. virescens* and *H. armigera*. A growing number of studies have implicated the midgut cadherin protein as the main receptor for Bt toxin. Mutations in the cadherin gene have been ruled out by linkage analysis from being the cause of Cry1Ac resistance in the CP73 strain of *H. virescens* and the BX strain of *H. armigera*. The most likely mechanism at this point is a post-translational modification of the cadherin and/or other Bt receptors that reduces but does not completely eliminate toxin binding and confers moderate levels of resistance.

11. Preparation of results for publication in international scientific journal.
In August, 2000 Dr Daly delivered an invited paper 'Genetics of Insecticide Resistance' in the International Symposium on Resistance at the International Congress of Entomology in Brazil. This presentation included results from this research.
In June 2002, Dr Mahon presented a paper that included aspects of this work to the AGM of Cotton Consultants Australia in Dalby, Qld.

Dr Heckel delivered an invited talk 'Mechanisms of defence against and resistance to *Bacillus thuringiensis* toxins' at the 4th Annual Pacific Rim Conference on *Bacillus thuringiensis*, in Canberra in November 2002.

Conclusion

This project was a collaborative one between the CSIRO genetics group in Canberra and Dr Heckel's group at the University of Melbourne. It made use of recent advances in genetic studies of *H. armigera*, in particular, the development of a genetic linkage map by Dr Heckel for this species, using AFLPs.

The BX strain received the most attention in this project, as it is resistant to high levels of Cry1Ac. The linkage analysis confirmed earlier evidence that suggested that the resistance results from a single gene or closely linked genes, as its effect is confined to a single linkage group. Comparison to other species with Bt resistance such as *Heliothis virescens* suggested that the BX resistance mechanism of *H. armigera* may be similar to Cry1Ac resistance in the strain CP73 of *H. virescens*. The most likely mechanism in BX and CP73 is a reduction of binding in the resistant strain.

Since the BX type of Cry1Ac resistance is not target-mediated (by the cadherin), the BX mechanism may have the potential to interact with and potentially enhance Cry2Ab resistance mechanisms. In this case, resistance to Bollgard II would be higher than expected by the current widespread assumption that cross-resistance cannot evolve.

Another exciting development during this project was the identification of a strain of *H. armigera,* EMS21, which is in fact, resistant to both Cry1A and Cry2A Bt toxins. Although the levels of resistance are moderate, any strain exhibiting resistance to both toxins is of great interest. The genetic linkage of this resistance is undergoing analysis. A new resistant strain, SP15, that exhibits a high level of resistance to Cry2Ab toxin, has been identified from the

field and established in the laboratory. Appropriate crosses are currently being performed that will also enable AFLP analysis of this form of resistance.

With the information from this project and the resistant strains currently in culture in our laboratories, we are in a very strong position to explore the issues surrounding resistance to the toxins in Bollgard II.

Relevance to Corporation's three Outputs

Understanding the genetic basis of resistance to Bt toxins used in transgenic varieties of cotton can provide important input to resistance management strategies for major pests. Retention of the susceptibility of *H. armigera* populations to Bt toxins will be beneficial to the cotton industry through reduced use of chemical insecticides that are of environmental concern, improved safety and enhanced profitability.

The identification of a strain of *H. armigera* which exhibits a level of resistance to both Cry1A and Cry2A, is of great interest. The genetic linkage of this resistance, in EMS21, is currently being analysed and the information will be incorporated into models used to evaluate the potential of the development of resistance to Ingard and Bollgard II varieties containing the *cry1Ac* and/ or *cry2Ab* gene and so assist in resistance management programs. There are obvious benefits if such a program can be fine-tuned in a manner that delays the development of resistance in the field. Similar benefits should accrue through knowledge gained from genetic mapping of the strain "BX", which is highly resistant to Cry1Ac toxin and the new strain, SP15, which is highly resistant to Cry2Ab.

Future research

This project examined the genetics of resistance in *H. armigera* to Cry1Ac. The BX strain received the most attention, as it is resistant to high levels of Cry1Ac. Beginning in 2003-2004, INGARD varieties will be replaced with Bollgard II varieties that express both Cry1Ac and Cry2Ab toxins. Thus the focus of resistance management will shift from a focus on Cry1Ac to the maintenance of susceptibility to Cry2Ab.

We plan to evaluate the genetic potential for cotton bollworm to develop resistance to Bollgard II. Genetic crosses, bioassays and molecular techniques will be used to investigate the inheritance of Bt resistance in resistant strains that are available through selection in the laboratory or detection in the field. EMS21 shows resistance to Cry2Ab and Cry1Ac toxins. Linkage analysis of this resistance has begun. A new resistant strain, SP15, with a high level of resistance to Cry2Ab toxin, has been identified from the field and established in the laboratory. Appropriate crosses are currently being performed that will enable AFLP analysis of this form of resistance.

Our finding that the BX type of Cry1Ac resistance is not target-mediated (by the cadherin) underscores the importance of exploring its interaction with Cry2Ab resistance mechanisms. If BX had exhibited target-site resistance to Cry1Ac, we would expect no effect on Cry2Ab sensitivity because of the difference in targets. Therefore, the BX mechanism may have the potential to interact with and potentially enhance Cry2Ab resistance mechanisms. In this case, resistance to Bollgard II would be higher than expected by the current widespread

assumption that cross-resistance cannot evolve. With the linked ribosomal protein gene as a marker, we can now investigate this issue in detail.

Understanding the genetic basis of resistance, including the number of genes involved, their dominance, fitness and interaction is fundamental to the development of appropriate resistance management programs. We propose to further analyse the nature of one form of resistance to Cry1Ac, expressed by the resistant strain BX. In addition, we propose to investigate the interaction of separate Cry1Ac and Cry2Ab resistance genes.

Publications / publication plan

The results of this project have been and will continue to be communicated to researchers, collaborators in similar fields through informal meetings, correspondence and conferences. Communications to date are listed under Objective 8. The results will also be published in an international scientific journal.

Assessment of Impact on the Cotton Industry

Ultimately, the project will support more robust resistance management strategies for the cotton industry. Current strategies make assumptions about how and why resistance is evolving. This project tested these assumptions, about the nature of resistance, the number of genes and the underlying mechanisms.

Characterisation of resistant strains will help us to develop strategies that minimise selection for field resistance. Early characterisation of resistance will give the industry its best hope of managing that resistance. Strains of *H. armigera* currently in culture and resistant to Bt, 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 or the role of insecticides to manage Bt resistant larvae.

References

Andow, D. A., and D. N. Alstad (1998) F_2 screen for rare resistance alleles. J. Econ. Entomol. 91: 572-578.

Gahan, Linda J., Fred Gould & David G. Heckel (2001) Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science 293: 857-860.

Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferré, F. J. Silva, and W. J. Moar (1992) Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. PNAS USA 89: 7986-7988.

Heckel, D. G. (2002) Mechanisms of defence against and resistance to *Bacillus thuringiensis* toxin. pp 52-66 in Biotechnology of Bacillus thuringiensis and its environmental impacts. Proceedings of the 4th Pacific Rim Conference. R.J. Akhurst, C.E.Beard, & P.A. Hughes, eds. CSIRO.

Morin, S, R. W. Biggs, M. S. Sisterson, L. Shriver, C. Ellers-Kirk, D. Higginson, D. Holley, Li. J. Gahan, D. G. Heckel, Y.Carriere, T.J. Dennehy, J. K. Brown, and B. E. Tabashnik (2003) Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proceedings of the National Academy of Sciences of the USA 100: 5004-5009.

Olsen, K. M., and J. C. Daly (2000) Plant-Toxin Interactions in Transgenic Bt Cotton and their Effect on Mortality of *Helicoverpa armigera* (Lepidoptera: Noctuidae). J. Econ. Entomol. 93 (4): 1293-1299.

Park, S., and T. M. Brown (2002) Linkage of genes for sodium channel and cytochrome p450 (Cyp6B10) in *Heliothis virescens*. Pest Management Sci. 28: 209-212.

Payne, C. D. (1985) The GLIM system release 3.77 manual. Numerical Algorithms Group, Oxford, UK.

Executive summary

The development of resistance to pesticides is an ongoing concern, in many agricultural systems. The management of *H. armigera* in the Australian cotton industry is of particular concern, as this species has demonstrated a remarkable ability to evolve resistance to many insecticides. Management strategies are based on models of resistance as information is lacking regarding putative resistance genes. This project aimed to increase our understanding of the genetic basis of resistance, ideas which underlie the structure of the resistance management strategies. The focus was on Bt resistance but the work is also directly applicable to resistance to conventional insecticides.

This project was a collaborative one between the CSIRO genetics group in Canberra and Dr Heckel's group at the University of Melbourne. It made use of recent advances in genetic studies of *H. armigera*, in particular, the development of a genetic linkage map by Dr Heckel for this species, using AFLPs. The linkage-mapping approach has begun to resolve questions relating to pyrethroid resistance, and the same approach has also been useful in sorting out mechanisms in different Bt resistant strains.

The BX strain received the most attention in this project, as it is resistant to high levels of Cry1Ac. The linkage analysis confirmed earlier evidence that suggested that the resistance results from a single gene or closely linked genes as its effect is confined to a single linkage group (chromosome). An AFLP, AA-CTA_330.14, was found to show a highly significant association with Bt resistance and was assigned to linkage group A14 in the previously constructed AFLP map of *H. armigera*. This AFLP was shown to be linked to a known gene, RpS15 (a ribosomal protein) and also to the voltage-gated sodium channel. This enabled comparison to other species with Bt resistance such as *Heliothis virescens* and *Plutella xylostella*. A Bt resistance gene from the CP73 strain in *H. virescens* (BtR-5) also maps to the same linkage group as the Hscp sodium channel in that species. This suggests that the BX resistance mechanism of *H. armigera* may be similar to Cry1Ac resistance in CP73 of *H. virescens*. The most likely mechanism in BX and CP73 is a reduction of binding in the resistant strain.

An exciting development during this project was the identification of a strain of *H. armigera,* EMS21, which is resistant to both Cry1A and Cry2A Bt toxins, although the levels of resistance are moderate. The genetic linkage of this resistance is undergoing analysis. This information will be incorporated into models used to evaluate the potential of the development of resistance to cotton varieties containing the *cry1Ac* and/ or *cry2Ab* gene and so assist in resistance management programs of both Ingard and Bollgard II varieties. There are obvious benefits if such a program can be fine-tuned in a manner that delays the development of resistance in the field. Similar benefits should accrue through knowledge gained from genetic mapping of the strain "BX", which is highly resistant to Cry1Ac toxin. A new resistant strain, SP15, that exhibits a high level of resistance to Cry2Ab toxin, has been identified and established in the laboratory. Appropriate crosses are currently being performed that will also enable AFLP analysis of this form of resistance.

Ultimately, the information provided by this project will support more robust resistance management strategies for the cotton industry. Early characterisation of resistance gives the industry its best hope of managing that resistance.