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Part 1 - Summary Details

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

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Part 2 – Contact Details

Administrator:	Linda Leavitt		
Organisation:	CSIRO Entomology		
Postal Address:	GPO Box 1700 Canberra ACT 2601		
Ph: 02 62464030	**Fax:** 02 62464094	**E-mail:**	Linda.leavitt@csiro.au
Principal Researcher:	Rod Mahon		
Organisation:	CSIRO Entomology		
Postal Address:	GPO Box 1700 Canberra ACT 2601		
Ph: 02 62464082	**Fax:** 02 62464042	**E-mail:**	Rod.Mahon@csiro.au
Supervisor:	Geoff Baker		
Organisation:	CSIRO Entomology		
Postal Address:	GPO Box 1700 Canberra ACT 2601		
Ph: 02 62464406	**Fax:** 02 62464042	**E-mail:**	Geoff.Baker@csiro.au

Signature of Research Provider Representative: _____

Part 3 – Final Report Guide (due 31 October 2008)

Background

Genetics of Bt Resistance in *Helicoverpa armigera*: Resistance to Cry2Ab

This report presents the results of a successful collaboration between CSIRO Entomology (Rod Mahon and Karen Olsen) and Dr David Heckel of the Max Planck Institute for Chemical Ecology in Jena. For the duration of this project David retained linkages to the University of Melbourne where a component of the work was performed.

Prior to the start of the project and as the results became available from this work, Cry2Ab resistance has become increasingly important to the Australian cotton industry. We planned to study a recently isolated Cry2Ab resistant colony (SP15) and also a laboratory strain (EMS21) that showed low-level resistance to the toxin. Both have been examined and the results are reported here, however, the SP15 study has received most attention. Work of the CSIRO Bt group (Rod Mahon, Karen Olsen, Sharon Downes) has shown that the SP15 form of resistance is present at a frequency of ca 0.004 in field populations of *H. armigera*. At that frequency, and depending on the characteristics of the resistance (many of which are addressed herein) the resistance may pose a threat to the long-term efficacy of Bollgard II®. The polymorphism for resistance clearly pre-dated man's use of the toxin in transgenic cotton and genetical tools offer the most effective means of detecting any increases in frequency that would imply that resistance was developing. Much of this project has been directed towards isolating the gene involved with resistance so those tools can be developed. Other important aspects of the resistance were also explored including dominance, cross resistance to Cry1Ac and population cages experiments to evaluate fitness cost associated with the SP15 form of resistance.

Objectives

The overall goal of this project was to evaluate the genetic potential for cotton bollworm to develop resistance to the Cry2Ab toxin in Bollgard II®. Genetic crosses, bioassays and molecular techniques were used to investigate the inheritance of Bt resistance. We proposed to examine the genetic basis of resistance to the Cry2Ab toxin, including the number of genes involved, their dominance and fitness. A major objective was to map the gene (or genes) conferring specific resistance to Cry2Ab.

Understanding the genetic basis of resistance, including the number of genes involved, their dominance and fitness is fundamental to the development of appropriate resistance management programs.

Methods

Characterisation of SP15

Toxins. Cry2Ab protein toxin was produced in the laboratory from a clone of the *cry2Ab* gene of *B. thuringiensis* var. kurstaki HD-1 in *B. thuringiensis*. The original clone was provided by L. Masson (National Research Council, Montreal, Canada). The Bt strain expressing the *cry2Ab* gene was grown in nutrient broth and the cells removed by centrifugation. The resulting pellet was washed 3 times, resuspended and sonicated. A sample of the final suspension was transferred into a SDS buffer and electrophoresed on a 10% polyacrylamide gel. The concentration of toxin was estimated by scanning the gel and

analyzing the density of the toxin band relative to a BSA standard using Scion Image 1.62 software (Scion Corporation, Frederick, MD).
Cry2Ab cotton leaf material was obtained from the cotton variety Sicala V-2 transformed with the *Bacillus thuringiensis* var. *kurstaki cry2Ab* gene construct. The cry2Ab construct is the property of Monsanto Company (St Louis, MO). The discriminating dose of 2 µg/cm2 Cry2Ab toxin as a diet surface contamination, was prepared by grinding laboratory-grown leaves of this variety. Toxin in the leaf was calibrated using an Enzyme-linked Immunosorbent Assay (ELISA) method on aliquots of leaf material after freeze drying and homogenisation. Cry2Aa protein toxin was supplied by W. J. Moar (Auburn University, AL) and was produced from a cloned gene of the *Bacillus thuringiensis* var. *kurstaki* NRD-12 strain in E. coli. The toxin was stabilised in a 0.01% sodium azide solution.

Transgenic Cry2Ab corn was used as the source of the screening toxin in the population cage experiments. It was kindly made available by Monsanto company (St Louis, MO) as a freeze-dried, milled powder.

Cry1Ac protein toxin was cultured by Genesearch (Brisbane, Australia). The HD-73 strain of *Bacillus thuringiensis* var. *kurstaki* (producing only the Cry1Ac toxin and spores) was mass produced by a fermentation process with a resulting spore/crystal mix. The pellets produced were resuspended and washed three times.

DiPel 2X® is a commercial, formulated compound produced from the *Bacillus thuringiensis* var. *kurstaki* HD-1 strain (32,000 IU toxin per mg, Abbott Australasia Pty Ltd). It was expected to contain a combination of Cry1Aa, Cry1Ab Cry1Ac, and Cry2Aa toxins and spores.

Insect Strains. Rearing methods were as described in Teakle and Jensen (1985) except that larvae were reared individually in 32-well plastic trays (Oliver Products Company, Grand Rapids, MI). The diet was modified by replacing soybean flour with chickpea flour and by substituting propionic acid (0.08%) for formalin.
The susceptible, general laboratory strain (GR) used in these analyses was established in the mid-1980s from a series of collections in cotton fields in the Namoi Valley, northern New South Wales (NSW), Australia. From 1990 to 2000, the colony was supplemented with collections of larvae from the same region when considered necessary to retain the vigour of the strain. GR is susceptible to all Cry toxins tested. Cry2Ab susceptibility was monitored regularly by evaluating the response to a discriminating dose of Cry2Ab (2µg/cm2 as diet surface contamination) that kills or severly stunts the growth of susceptible neonate larvae. GR was used as a susceptible control in all assays and also in crosses to the resistant strain. The resistant strain, SP15, was established from a single *H. armigera* pair collected as eggs on corn near Griffith, NSW, in December 2002. Progeny from the pair were subjected to an F_2 screen (Andow and Alstad 1998). The F_2 screens were performed with the specific intention to detect resistance to Cry toxins in *H. armigera*. SP15 initially possessed a very restricted gene pool as it originated from an isofemale line. Lepidopteran colonies in laboratories suffer severe inbreeding depression rapidly leading to a loss of vigour that strongly influences the outcome of bioassays. Consequently, SP15 was outcrossed to the susceptible strain, GR, three times, at generations 3, 9 and 11 (where the field collected insects were F_0) to maintain fitness and to produce a strain that was 87.5% isogenic with the susceptible strain. Following each outcross, the colony was maintained without selection for one generation and then re-selected with 1- 2µg/cm^2 Cry2Ab toxin as a diet surface contamination treatment. All subsequent generations were selected at this dose. The assays reported here were performed with individuals from the third to the sixteenth generation.

However, most of the analyses were conducted with assays on the near-isogenic 3rd outcross, (generations 15 and 16) in order to reduce the potentially misleading effects of hybrid vigour evident when crossing colonies of *H. armigera.*

Bioassays. First instar *H. armigera* larvae (1 to 20 h old) were used in all bioassays. Most bioassays used the diet surface contamination method. For these, rearing diet was dispensed into 45 well assay trays, with approximately 2 ml of diet per well. A constant volume (100 µl) of serially diluted Cry2Ab toxin was pipetted onto the 2.7 cm^2 surface of the cooled and set diet. The solution was dispersed over the entire diet surface and residual surface liquid was allowed to evaporate in a cool airflow. Doses were calculated as µg of toxin per cm^2 of diet surface. After the addition of one neonate larvae per well, trays were heat sealed and maintained at 25°C and 45-55% RH. Larvae were scored after seven days as either alive (exhibiting normal movement) or dead (dead, moribund, uncoordinated movement). Each bioassay consisted of a control (diet with no toxin), plus three to six test doses that based on prior knowledge were expected to induce from 1-99% mortality. Forty-five larvae (one tray) were tested per control and dose. With appropriate substitution of toxins, this method was also used to characterise the response of SP15 to Cry2Aa and Cry1Ac toxins and also to DiPel®, a commercial formulation of Cry toxins. Critical bioassays and crosses were repeated three times.

Data analysis. The dose response of larvae to Cry toxins were analysed and slopes and LC_{50} estimates were calculated using the logit transformation in GLIM version 3.77 (Payne 1985). Comparisons of LC_{50}'s were generally made using models with a common slope. However, where the slopes differed significantly ($p < 0.05$), LC_{50} estimates were calculated with independent slopes.

AFLP analyses

The SP15 strain was selected over two generations to increase its homozygosity, before amplified random fragment length polymorphisim (AFLP) analysis was initiated. Single pair crosses (one male, one female) between the resistant colony (assumed to be homozygous resistant) and a susceptible laboratory colony (GR) were initiated. Offspring (F_1) were either backcrossed to resistant individuals or selfed (mated among themselves). A portion of offspring from this cross (F_2s) were exposed to a dose of toxin that killed susceptible and heterozygous insects. An ALFP analysis was then conducted on the grandparents, parents, survivors of the assay and an unselected cohort. Differences in the frequency of an AFLP between selected and non-selected cohorts indicated an association with a gene conferring resistance. Once an association between an AFLP variant and resistance was established, association of the variant with known markers on individual chromosomes of Lepidoptera were established. Through this route, the resistance could be assigned to a particular linkage group.

For the EMS21 strain, AFLP analysis was performed in Canberra using an automatic sequencer. For the SP15 strain, AFLP analyses were conducted in Melbourne on polyacrylamide gels and DNA bands revealed by autoradiography. Analysis and interpretation of the results was undertaken using custom software to attempt to identify the linkage of both the Cry1Ac and Cry2Ab resistance present in this strain.

Population cage experiments

Population cages were established using the selected colony SP15 (presumed to be homozygous for the allele that confers resistance) and the susceptible laboratory colony, GR. SP15 males and GR females were crossed and their offspring set up in cages and thereafter maintained as a colony. Offspring from the reciprocal cross (SP15 females and GR males) were similarly maintained in a separate colony. At the F_2 generation, and selected subsequent

generations, a sample of neonates (800 - 900) were exposed to a discriminating dose of Cry2Ab corn powder and the frequency of survivors (presumed to be homozygous resistant) were scored. The initial frequency of the 'resistant allele' was assumed to be 0.5. Subsequently, the frequency was estimated as the square of the proportion of homozygous resistant individuals.
The experiment was repeated on two occasions. The independent lines were maintained for between 3 and 5 generations, and while the data are not presented here, for one of the experiments, the colonies are still extant and presently entering the 6th generation.

Results

1. Extract DNA from laboratory derived resistant strain EMS21, and conduct linkage mapping of Cry2Ab resistance using AFLPs.

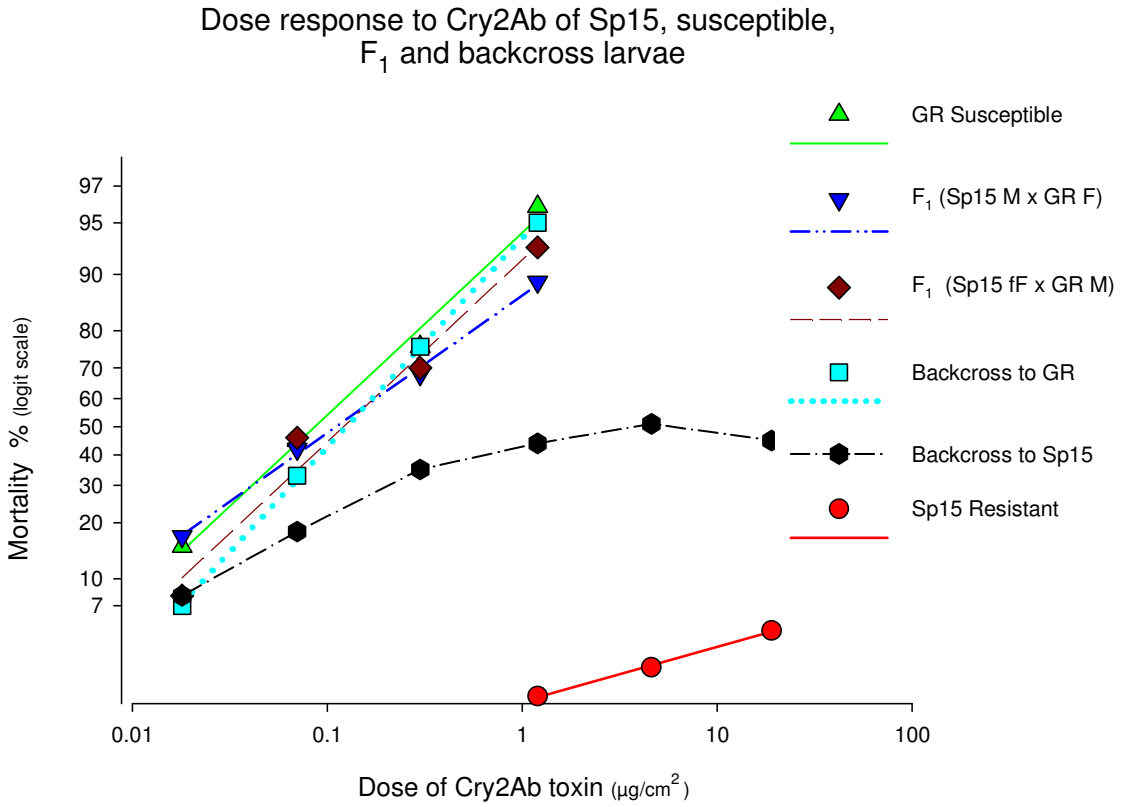
The Cry2Ab resistant strain EMS21 exhibits low level (53 fold) resistance to the Cry2Ab toxin. The resistance is incompletely dominant. The strain also exhibits a low level of resistance (65 fold) to Cry1Ac. The study was hampered by the absence of variability among ALFP's between the resistant and susceptible strain. The lack informative markers was likely due to the consequence of similarity in genetic backgrounds of the resistant strain and susceptible strain as they share a partially-common origin. No major associations between the AFLP variants that were found and resistance to either Cry1Ac or Cry2Ab were detected, indicating that the resistance probably resulted from cumulative effect of multiple genes on different linkage groups. Further, the Cry1Ac and Cry2Ab resistance did not appear to be linked. The conclusion drawn from this study was that the polygenic form of resistance was the consequence of sustained laboratory selection for resistance/tolerance/fitness genes rather than to the presence of a single major gene. One important conclusion from this study was that despite early advice to the contrary, the use of an automated sequencer for the broad analysis of linkage groups associated with resistance is in appropriate. Even small variations in the intensity and position of the bands which normally occur, induced errors into the analysis. This in turn made it difficult, if not impossible, to interpret accurately. Working with manual gels as performed for the SP15 analysis, produced a more controlled environment for screening the AFPLs.

2. Establish lines of known inheritance and assess possible modes of inheritance of field-derived Cry2Ab resistant strain, SP15.

Three replicates of mortality and development assays using the three genotypes (homozygote, heterozygote, susceptible) were conducted to characterise this strain after the first outcross. The results of both assay methods indicated that resistance was almost, if not entirely recessive. Although the heterozygotes consistently exhibited a small advantage over the susceptible strain (2.2 to 5.5-fold), the difference was not statistically significant (development assay: $F = 0.713$; $df = 1, 37$; $P = 0.404$, mortality assay: $F = 0.241$; $df = 1, 18$; $P = 0.629$).

Assays on reciprocal crosses between the resistant strain and a susceptible strain and the various backcrosses were repeated following a 3rd outcross to the susceptible strain and subsequent re-selection. The outcrosses increased the vigor of the resistant strain and dominance was negligible (Figure 1).

Figure 1



The homozygous resistant strain showed a high level of resistance and exhibited little or no mortality when exposed to either Cry2Ab toxin in cotton leaf or to toxin placed on the surface of diet. The resistance ratio for SP15 is very high, (>6,830). This estimate is conservative because the LC_{50} was calculated from a common slope generated by other genotypes (the susceptible strain GR and F1's produced by crossing SP15 and GR). It was necessary to make use of the common slope because mortality for SP15 was virtually unchanged at all concentrations of toxin leading to an assay line that was too flat to yield a LC_{50}. The assays reported here that were performed following outcrosses have led us to conclude that the resistance is recessive in the laboratory. This was an important finding as any measure of dominance would imply that this form of resistance would pose a far greater challenge to our resistance management strategy especially given the high frequency (0.004) of this gene in the field (data not shown).

The results from the bioassays also demonstrated that the form of Cry2Ab resistance present in SP15 is inherited as a single gene and that the locus is not sex-linked. SP15 possessed no cross resistance to Cry1Ac (Table 1) and its LC_{50} for this toxin did not differ significantly from the susceptible strain GR. The susceptibility of this strain to Cry1Ac is especially important given the presence of this toxin in Bollgard II® and the extensive adoption of this transgenic variety. Similarly, SP15 proved to be completely susceptible to the commercial product DiPel. As would be expected from the sequence similarity of Cry2Ab and Cry2Aa, SP15 was cross resistant to Cry2Aa.

Table 1. Bioassays of SP15, (3rd outcross), and GR challenged with Cry toxins and DiPel

Strain / Cross	Toxin	LC_{50}	95% CI	Slope	±SE	Resistance Ratio
GR	Cry1Ac	0.011 [a]	0.009, 0.018	1.225	0.1161	-
SP15		0.017 [a]	0.0128, 0.024	1.227	0.1172	1.56
GR	Cry2Aa	0.011 [b]	0.008, 0.017	1.259	0.1552	-
SP15		123.971 [c]	40.51, 379.39	0.431	0.0716	9,640
GR	DiPel	0.023 [d]	0.017, 0.033	0.672	0.0768	-
SP15		0.018 [d]	0.014, 0.026	0.954	0.09	0.78

[abcd] LC_{50} values followed by different letters are significantly different, ($P < 0.05$).

3. Extract DNA from the Cry2Ab resistant strain, SP15, and conduct linkage mapping for Cry2Ab resistance using AFLPs.

Analysis of this form of resistance was initiated on the original field-derived strain. This had two advantages. Firstly, the cross was performed before the strain deteriorated through a decline in fitness caused by inbreeding. Secondly, the early analysis maximised the between-strain differences in the suite of AFLP variants carried by the resistant (SP15) and susceptible (GR) strains. Thereby we avoided one of the difficulties encountered in the analysis of Cry1Ac resistance in EMS21 and BX (CRDC project CSE 89C), namely, the common genetic background of EMS21, BX and GR, that limited the inter-strain variation.

Genetic crosses, screening for resistance and DNA extraction were undertaken in the first year of the project. Obtaining reliable AFLP markers still proved difficult, however, four linked AFLP bands showed a strong association with Cry2Ab survival in one of the backcross families, and one of these was cloned and sequenced. The sequence showed no similarity to any known sequences in GenBank and probably represents an intergenic region or intron. When used as a probe on a Southern blot made from a different mapping family (not derived from SP15), this sequence proved to occur in a single copy in the *H. armigera* genome, and mapped to a chromosome with two previously-mapped ribosomal protein (Rp) genes. No other linkage groups in the SP15 backcross family showed any association with resistance; confirming our hypothesis that Cry2Ab resistance in SP15 is caused by a single gene. Using the independently-derived linkage relationships among the ~40 previously-mapped Rp genes in *H. armigera* and other species we can reject the following candidate Cry2Ab resistance genes because they map to different linkage groups:

- Cadherin (BtR-4 of *H. virescens*, linkage group 9; cadherin mutations cause Cry1A resistance in *H. virescens, Pectinophora gossypiella,* and *H. armigera* in China)
- All aminopeptidases cloned from *H. armigera* to date
- BtR-5 of *H. virescens*, linkage group 10, causes Cry1Ac resistance in CP73 strain
- BtR-1 of *Plutella xylostella*, linkage group 2, causes Cry1A resistance in NO-QA strain

Resistance maps to linkage group 5. A candidate gene, Bre-5, that has been shown to confer resistance to Bt toxin in *C. elegans* has been identified in that linkage group. Work is proceeding on analysis of Bre-5 gene in SP15 as well as two additional isolates expressing Cry2Ab resistance isolated from the field (SP566 and SP202). Complementation tests performed with SP15 and the resistance in both SP566 and SP202 showed the resistance in each strain to be the result of a single gene.

4. Analyse results of AFLPs from SP15 strain and compare with previous results.

The characteristics of the EMS21 strain (polygenic, low dual resistance, partially dominant) contrast with the high level, single locus, functionally recessive Cry2Ab resistance present in the strain, SP15. The form of resistance present in SP15 is far more relevant to the cotton industry because: a) it is due to a single gene rather than many genes that in the field would be less likely to be aggregated in the same individual insect and therefore cause problems, b) it was identified directly from the field through the use of an F_2 screen and therefore did not sustain long-term laboratory selection that might select for secondary or 'helper' genes or indeed additional resistance mechanisms, and c) the this form of resistance appears to be unexpectedly common in field populations of *H. armigera*.

5. Rearing and selection of strains and potential selection of 3rd strain for analysis.

Choice of strains for AFLP analysis.

Because of the 20% reduction in the budget requested by CRDC, we did not envisage being able to complete the genetic analysis of a 3rd strain resistant to Cry2Ab toxin (as indicated in the original proposal). However, we carefully chose which two strains to analyse.

We have maintained several Cry2Ab resistant strains in the laboratory and have investigated the characteristics and level of their resistance. These strains include EMS21, VicRATS, TABOC, SP15, SP202 and SP566. The Cry2Ab strain, SP15, identified in CRDC project, CSE 104C was substituted for VicRATS as the second Cry2Ab resistant strain to be analysed. This was done partly because the resistance expressed by the laboratory-selected Cry1Ac and Cry2Ab resistant VicRATS strain was low, incompletely dominant and might not be due to a single gene, and this would complicate AFLP analysis in a similar fashion to that described above for the laboratory-selected strain EMS21. We have concentrated additional effort on SP15 partly because its resistance is completely different to EMS21 and VicRATS but more because the reasons outlined above that makes this form of resistance a possible threat to Bollgard II® and therefore of major importance to the industry. Also, the two additional field derived strains, produced by F_2 screening (SP202 and SP566), have proved to be allelic to SP15 which adds to the probable importance of this form of resistance.

Another Cry2Ab resistant strain, TABOC, has been selected by Dr Akhurst's group from field material collected in the 2002/03 season. Initial testing showed that the gene that confers resistance in this strain is not allelic with that of SP15. The level of resistance of this strain is low (70 fold). Further, assays indicate that the resistance is incompletely dominant and that while a major gene is probably present, like EMS21, it has been selected in the laboratory for many generations. As a consequence, the resistance exhibited by the strain could be complex with tolerance/helper genes involved. These observations led us to consider that the resistance in TABOC did not appear to warrant AFLP investigation, especially as all field derived examples of Cry2Ab resistance so far analysed are 'SP15 -like'.

6. Transfer the BX Cry1Ac resistance gene into one of the Cry2Ab-resistant strains using marker-assisted introgression

A "super" resistant strain "SPOC" was constructed in the laboratory. SPOC combined both high level resistance to Cry1Ac (from BX) and high level resistance to Cry2Ab (from SP15). The construction of SPOC was useful for several reasons. As indicated above, 'SP15-like' resistance is reasonably common and while 'BX-like' resistance appears to be rare, it is the only form detected in Australian field populations of *H. armigera*. Therefore the combination of resistances in SPOC may be the most likely genotype to emerge following sustained selection on Bollgard in a "worst-case scenario". That we could form a viable

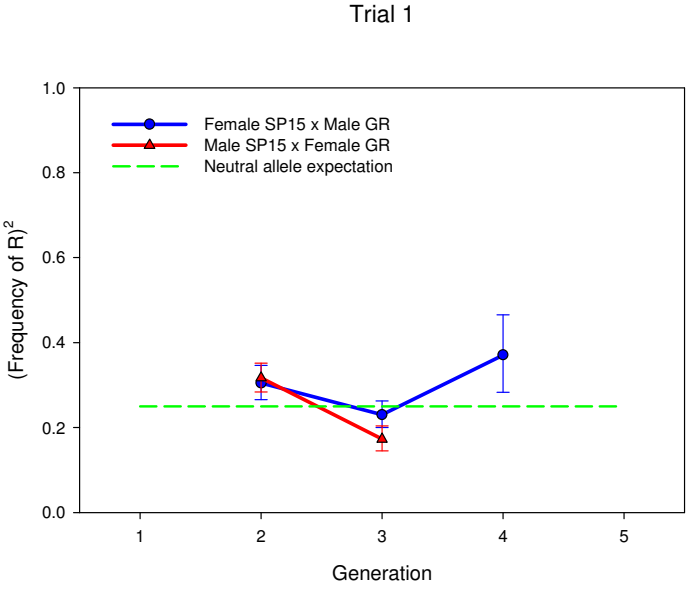
colony of SPOC indicates that the two unrelated forms of resistance can co-exist in the same insect.

While results to date are only preliminary, SPOC appears to carry fitness costs similar to BX but to date we have not identified obvious additional fitness costs due to SP15. Our initial objective was to use this strain to track and compare the inheritance and persistence of these two unrelated resistance genes in successive generations. However, after investigating a range of discriminating doses, it appears that the resistance in the BX strain may be more complex that originally believed and because so far a genetic marker to track the major gene in BX has eluded us, identification of genotypes (particularly differentiating between heterozygous and homozygous resistant individuals) is not reliable. Molecular markers to track SP15 resistance have become available only within the last month. Therefore, rather than using marker assisted introgression SPOC was created using discriminating doses of both Cry1Ac (to remove all individuals susceptible to Cry1Ac and most heterozygotes) and Cry2Ab (to remove all individuals not homozygous for SP15 form of resistance). The resultant colony (especially after several generations of selection) was highly resistant to both toxins.

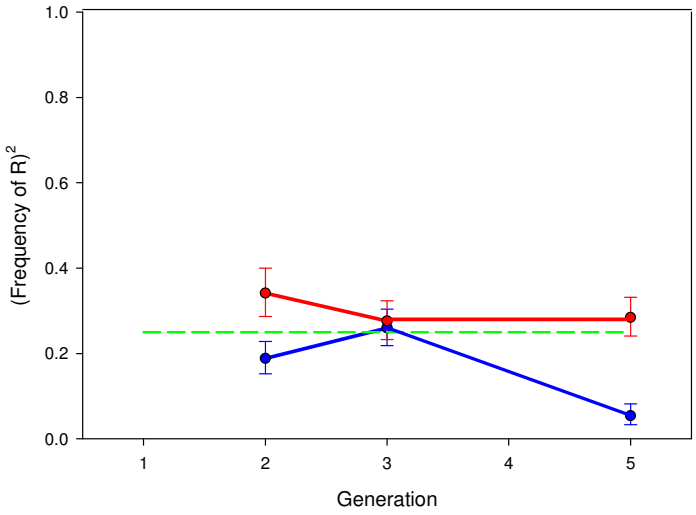
7. Measure the dynamics of selection and fitness cost of Cry1Ac and Cry2Ab resistance mechanisms in the introgressed strain.

As explained above, marker assisted tracking of Cry1Ac has not been possible, and for Cry2Ab has only recently become available. Therefore we began following the fate of Cry2Ab resistance in SP15 alone in population cages when the only means available to track the frequency of the allele that confers resistance was by bioassay.

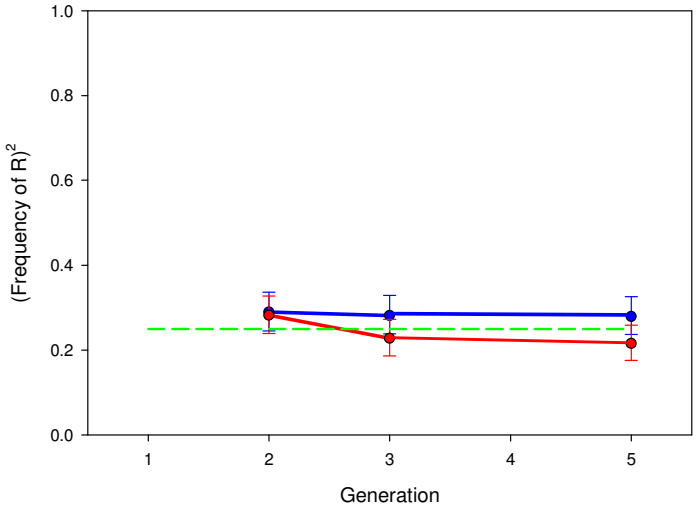
Figure 2. Population cages of SP15. In each trial the proportions and 95% CI of homozygous resistant insects are presented. Where the female SP15 were used to initiate the colony, data points are blue and the reciprocal crosses are red. The green line represents the expected proportion if the allele that confers resistance has no fitness costs.



Trial 2



Trial 3



The population cages results are presented in Figure 2. While the frequency of homozygous resistant individuals change a little each generation, rarely did the frequency deviate significantly from 0.25 frequency expected if the resistance allele was selectively neutral. On one occasion, (trial 2 generation 5 of the female line) the frequency of survivors declined dramatically. In reviewing our records, we realise that on that occasion our procedures varied from normal, and we thus tend to believe this is may be an aberrant result. We are performing further work to investigate this anomaly.

It is especially significant that the frequency of the resistant allele was maintained (in all but the one occasion) despite the fact that the vigour of the insects in the colonies generally

decreased. This decline was attributed to changes to in our standard rearing practise of selecting the 'best' pupae to initiate the next generation of a colony. For these lines, such selection was inappropriate, and even slow and small pupae were retained and permitted to contribute to the next generation.

These experiments imply that there is little or no fitness cost associated with the resistance under laboratory conditions. The caveat 'under laboratory conditions' is important as the flight ability, mating propensity, survival with different diets etc of each genotype may be quite different under field conditions, relative to the benign conditions in the laboratory. Nevertheless if there are indeed no fitness costs, particularly no dominant fitness costs, this finding has significant implications for the ability of this form of resistance to increase in frequency. From a resistance management perspective, it would be ideal if resistant alleles have a dominant fitness cost and thus heterozygotes would be less favoured when in non-Bt crops, including mandated refuges. Our cage trials offer no such expectations. Work is underway in the CRDC-funded project CSE 109 to more completely assess fitness costs. In that study we are exploring fitness of the genotypes on different host plants, during diapause, different temperature regimes etc.

8. Publication of results.

A manuscript on the characterisation the form of resistance in SP15 and the underlying genetics is under review by the Journal of Economic Entomology, "Resistance to the Bt toxin Cry2Ab in a strain of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in Australia" by R. J. Mahon, K. M. Olsen, K. A. Garsia and S. R. Young.
A second manuscript that will contain some of the information included in this report and will deal with the frequency of Cry1Ac and Cry2Ab resistance is in preparation for submission to the Journal of Economic Entomology.

Much of the work presented herein was presented orally and/or in posters by R. J. Mahon, Karen Olsen and Sharon Downes at a range of venues:

The Beltwide Cotton Conference. Texas, January 2006.
The Pacific Rim Conference on the impacts of *Bacillus thuringiensis* on the environment, Victoria, Canada. November 2005
Society of Invertebrate Pathology and VIII International Conference on *Bacillus thuringiensis* (ICBt). China, August 2006.
XXII International Congress of Entomology. Brisbane, August 2004
The Australian Cotton Conference. Gold Coast, August 2004.
The Australian Cotton Conference. Gold Coast, August 2006.
The Combined Invertebrates Conference. Canberra, December 2005.
The Cotton Trade Show. Moree, May 2006.
Bt resistance forum June 2005.
CSD Yearbook. 2006.
The Australian Cottongrower. December 2003.

The Bt Technical panel of TIMS has been kept informed on the findings and implications of this research.

Outcomes

Through better understanding of the genetic basis of resistance, including the number of genes involved, their dominance and fitness, strategies for resistance management of Bollgard II® can be fine-tuned. Since the project began, the importance of this work has

increased as we accumulated information on the frequency of Cry2Ab resistance in the field. The combined efforts of the Narrabri and Canberra based members of the CSIRO 'Bt group' have determined that the frequency of this form of resistance is unexpectedly common in field populations of *H. armigera*. Detection of any increase in that frequency has become very important. If, as is hoped, the gene in question has been identified, it may be possible to develop a DNA-based method to identify the resistance variant in the field-collected insects. This would be an invaluable tool to detect changes in frequency and would contribute substantially to the resistance management and therefore the long-term efficacy of Bollgard II®. Maintaining the usefulness of *Bt* varieties of cotton is beneficial to the cotton industry through a long-term reduction in the use of chemical insecticides. Such insecticides are of environmental concern and reduced spray costs also enhance profitability.

1. Extract DNA from laboratory derived resistant strain EMS21, and conduct linkage mapping of Cry2Ab resistance using AFLPs.

DNA was extracted from EMS21 and AFLP analysis was completed on the automatic sequencer. Evaluation of the AFLP analysis indicated that the resistance was the consequence of multiple genes, and, that the Cry1Ac and Cry2Ab resistance did not appear to be linked. It was concluded that this polygenic form of resistance was the consequence of sustained laboratory selection, for resistance /tolerance/fitness genes rather than to the presence of a single major gene.

2. Establish lines of known inheritance and assess possible modes of inheritance of field-derived Cry2Ab resistant strain, SP15.

The homozygous resistant strain showed a high level of resistance and exhibited little or no mortality when exposed to either Cry2Ab toxin in cotton leaf or to toxin placed on the surface of diet. The resistance ratio for SP15 is very high. Assays were carried out on reciprocal crosses between the resistant strain and a susceptible strain and various backcrosses. The results demonstrated that the Cry2Ab resistance present in SP15 is recessive in the laboratory, is inherited as a single gene and the locus is not sex-linked. There was no cross resistance to Cry1Ac. The susceptibility of this strain to Cry1Ac is especially important given the presence of this toxin in Bollgard II® and the extensive adoption of this transgenic variety.

3. Extract DNA from the Cry2Ab resistant strain, SP15, and conduct linkage mapping for Cry2Ab resistance using AFLPs.

DNA was extracted from the genetic crosses between the homozygous resistant strain, SP15, and a susceptible strain (GR). AFLP analysis was completed with some difficulty. Four linked AFLP bands showing a strong association with Cry2Ab survival in one of the backcross families were identified, and one of these was cloned and sequenced. Resistance mapped to linkage group 5. A candidate gene Bre-5, that has been shown to confer resistance to Bt toxin in *C. elegans* has been identified in that linkage group. Work is proceeding on analysis of Bre-5 gene in SP15 as well as two additional isolates expressing Cry2Ab resistance isolated from the field (SP566 and SP202). The AFLP analysis confirmed that Cry2Ab resistance in SP15 is caused by a single gene.

4. Analyse results of AFLPs from SP15 strain and compare with previous results.

The characteristics of the EMS21 strain (polygenic, low dual resistance, partially dominant) contrasted with the high level, single locus, functionally recessive Cry2Ab resistance present in the strain, SP15. The form of resistance present in SP15 is far more relevant to the cotton industry.

5. Rearing and selection of strains and potential selection of 3rd strain for analysis.
We maintained several Cry2Ab resistant strains in the laboratory and investigated the characteristics and level of their resistance. These strains included EMS21, VicRATS, TABOC, SP15, SP202 and SP566. The resistance in two additional field derived strains, SP202 and SP566, proved to be allelic to SP15. VicRats and TABOC did not warrant AFPL analysis because, like EMS21, their resistance was a result of prolonged selection in the laboratory and all field derived examples of Cry2Ab resistance so far analysed are 'SP15 - like'.

6. Transfer the BX Cry1Ac resistance gene into one of the Cry2Ab-resistant strains using marker-assisted introgression
A "super" resistant strain "SPOC" was constructed in the laboratory. SPOC combined both high level resistance to Cry1Ac (from BX) and high level resistance to Cry2Ab (from SP15).

7. Measure the dynamics of selection and fitness cost of Cry1Ac and Cry2Ab resistance mechanisms in the introgressed strain.

Our initial objective was to use "SPOC" to track the two resistance genes in successive generations. However, it appeared that the resistance in the BX strain was more complex than originally believed and because we had no genetic marker to track the major gene in BX, identification of genotypes was not reliable. Although a genetic marker for Cry2Ab resistance was not available at the time this work was undertaken, SP15 was used alone because its high level of resistance and absence of dominance in the laboratory allowed tracking of the gene with bioassays. The frequency of homozygous Cry2Ab resistant individuals deviated little from the expected frequency over three to five generations. This implies that there is little or no fitness cost associated with the resistance under laboratory conditions.

8. Publication of results.
A manuscript covering the characterisation of the resistance in SP15 is being reviewed by the Journal of Economic Entomology, "Resistance to the Bt toxin Cry2Ab in a strain of Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) in Australia" by R. J. Mahon, K. M. Olsen, K. A. Garsia and S. R. Young. Results of this work were also presented orally and as posters at several venues in Australia and overseas.

Conclusion
As anticipated, this work has provided important insights to the genetics of Cry2Ab resistance. The most important component was the characterisation of SP15. In the laboratory this form of resistance proved to be recessive, susceptible to Cry1Ac, and due to the influence of a single gene. We now have a much more complete understanding of the threat posed to Bollgard II® by this form of resistance. In addition, insights were gained at the end of the project (and further work is underway) about the possible identity of the gene responsible. This offers the possibility that a molecular technique may become available to detect this form of resistance far more readily than the current labour-intensive F_2 technique currently used in the Bt monitoring program.

Extension Opportunities
The research performed in this study has answered many questions about the likely significance of Cry2Ab resistance to the Australian cotton industry. The work was performed

under laboratory conditions using laboratory adapted insects and strains possessing laboratory selected genomes. Recent analysis of data collected during the F2 tests conducted by CSIRO indicate that Cry2Ab resistance when in a field derived genetic background may in important ways behave differently. This will be the focus of a further submission to CRDC.

Given the incidence of the Cry2Ab resistant allele in field populations of *Helicoverpa armigera*, it is important to maintain the efficacy of the monitoring program to detect as early as possible significant increases in frequency. Therefore the possibility of developing a DNA-based detection of resistant alleles is exciting and merits exploration. If such a technique became possible, it would enable far more individuals to be scored each season than is currently possible with the labour-intensive F2 technique currently employed. This would enable the detection of changes earlier, and thus allow more opportunities to respond in a manner that would limit additional changes in frequency before field-resistance became a problem. Further development of a DNA based diagnostic system will be a second focus of the submission to CRDC mentioned above.

Part 4 – Final Report Executive Summary

This report presents the results of a successful collaboration between Rod Mahon and Karen Olsen (CSIRO Entomology) and Dr David Heckel (Max Planck Institute for Chemical Ecology, Jena, Germany). For the duration of this project David retained linkages to the University of Melbourne where a significant component of the work was performed.

The project explored resistance to Cry2Ab in the cotton pest *Helicoverpa armigera.* This species has a remarkable track record of evolving resistance to conventional insecticides and is thus the most likely of the two Helicoverpa species found regularly in cotton (the other is the native *H. punctigera*) to evolve resistance to the toxins present in transgenic cotton. In other research funded by CRDC, we have found that forms of genes, (alleles) conferring resistance to Cry2Ab toxin in *H. armigera* are surprisingly common. Because we found the resistance prior to the widespread deployment of transgenic cotton that express this toxin (Bollgard II®), it is clear that the presence of these 'resistant alleles' pre-dates man's activities. Because these alleles are unexpectedly common, (approximately 4 in 100 alleles tested are the 'resistant' form) it is important to understand the characteristics of this resistance in order to assess the likelihood that it will become a threat to the long-term efficacy of cotton varieties that express the Cry2Ab toxin as well as a second toxin, Cry1Ac.

We have found that the resistance present in a colony of insects derived from field-collected *H. armigera* allele is due to a single gene. This fact was established by two quite distinct methods. Firstly, it was found that comparative bioassays of resistant, susceptible, F_1 offspring and various backcrosses to the parental (susceptible and resistant) colonies implied that resistance was due to a single autosomal gene. This was confirmed through the study of linkage relationships between genetic variants and resistance. Importantly, the resistance was recessive in the laboratory. If extended to field conditions, this makes this form of resistance less of a threat than would be the case if it was dominant (like most forms of resistance to conventional insecticides).

Of immediate significance to the current varieties of transgenic cotton grown in Australia was the finding that insects seemingly totally resistant to Cry2Ab toxin, are fully susceptible to

Cry1Ac, the second toxin in Bollgard II®. Thus it is only during the latter part of the cotton season when Cry 1Ac toxin is diminished in Bollgard II® (similar to the situation in Ingard varieties) that insects resistant to Cry2Ab possess an advantage over susceptible insects. Only under these conditions are the 'resistant' alleles likely to increase in frequency. Nevertheless, this window of opportunity is of concern, as if that advantage persists over time, it will lead to a loss of efficacy of this toxin. Clearly careful watch on the frequency of such resistance is important to enable the industry to enjoy the full benefit of any technology that involves Cry2Ab toxin.

CRDC funds an active program to monitor the frequency of resistance to Bt toxins. The most effective means to assess the frequency of resistance is by the use of a simple, but labour intensive genetic system, the F_2 screen. Work in this project has identified a likely candidate gene 'Bre-5', mutations at which may result in the resistance we have studied. If this information proves to be correct, and can be exploited to develop a DNA means to detect these mutations, this would enable the detection of changes in frequency of the 'resistant alleles' earlier, and thus allow more opportunities to respond in a manner that would limit additional changes in frequency before field-resistance became a problem.

For further information contact Dr Rod Mahon: Rod.Mahon@csiro.au