On Farm

Research

On Farm Series | Cotton Research & Development Corporation

Part 1 - Summary Details

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Part 2 – Contact Details

Administrator: Linda Leavitt
Organisation: CSIRO Entomology
Postal Address: GOP Box 1700 Canberra ACT 2902
Ph: 02 6246 4030 **Fax:** 02 6246 4042 **E-mail:** Linda.Leavitt@csiro.au

Principal Researcher: Dr Rod Mahon
Organisation: CSIRO Entomology
Postal Address: GPO Box 1700, Canberra, ACT 2601
Ph: 02 6246 4082 **Fax:** 02 6246 4042 **E-mail:** Rod.Mahon@csiro.au

Supervisor: (Name & position of senior scientist overseeing the project).
Organisation:
Postal Address:
Ph: **Fax:** **E-mail:**

Signature of Research Provider Representative: ______________________________

Part 3 – Final Report Guide (due 31 October 2007)

Background

This project examined important components of the resistance to Cry2Ab toxin present in two newly-established colonies of *H. armigera.* This information is pertinent to an assessment of the potential threat posed by these forms of resistance. The colonies have different origins. SP15 and related strains were identified directly from field-collected samples in 'F_2 tests'. The other (TABOC) was selected by exposing a colony of *H. armigera* to low doses of Cry2Ab toxin. Initially the resistance in each strain was presumed to be due to a form of a single gene (an allele) that confers resistance to Cry2Ab toxin. That assumption was tested through genetic analysis of SP15 in another project (CSE 108C) and four other field-derived strains were examined during the course of this project.

In theory, Cry2Ab resistant genotypes should not represent a threat to Bollgard II because such insects are unlikely to be resistant to the second toxin (Cry1Ac) in BGII plants. That in essence captures the logic behind pyramids (where in our case, 'pyramids' are cotton plants that produce two Cry toxins). Because an assumption is made that resistant genotypes are rare, doubly resistant genotypes should be extremely rare to the point that they are almost non-existent.

Bollgard II is an imperfect pyramid. Because the same genetic components (the position of the inserted genes, the promoter and the gene itself) that cause Ingard plants to produce Cry1Ac are also present in Bollgard II, levels of Cry1Ac in both varieties decline post-squaring. This decline allows susceptible insects to survive on older Ingard cotton. It is thus probable that late in the season, *H. armigera* individuals feeding on Bollgard II are susceptible to Cry1Ac but resistant to Cry2Ab, and may thus survive and increase in frequency. As the Cry2Ab resistance allele increases in frequency there will be more numerous opportunities for such genotypes to combine with Cry1Ac resistance alleles, with the worst-case scenario resulting in a combined resistant genotype that would be capable of feeding on Bollgard II throughout the season.

Cry2Ab resistance in *H. armigera* is of considerable importance both because resistant alleles appear to be unexpectedly common and because resistance appears to be readily selected in the laboratory. In previous (eg CSE 104C) and current projects (eg CSE 112C) CSIRO Entomology have actively examined field-derived alleles isolated through the F_2 screening procedure since the 2002/03 season. Colleagues in Monsanto Australia have also adopted our methods and to date (September 2007) after combining both data sets, 17 out of 4316 alleles tested proved to be able to tolerate high levels of Cry2Ab toxin. Thus the frequency of the 'resistant allele' is approximately 4 in every thousand. Given what geneticists believed to be reasonable assumptions about mutation rates and fitness costs of mutations, resistance alleles were expected to be several orders of magnitude less common than observed, especially as the first instances were found prior to exposure of *H. armigera* populations to varieties of cotton that produce this toxin.

There are several features of any form of resistance that determine if it is likely to increase in frequency (and thus eventually cause field-failures) and in this project those features are evaluated. The dominance of the allele is perhaps the most important and of particular interest was to determine if heterozygotes possessed advantages when exposed to field-grown Bollgard II. Another important aspect of this project was to determine if the resistance in the various isolates from the field are all due to changes to the same gene (are allelic). If so,

resistance poses a greater threat to the longevity of Bollgard II than if resistance in each was caused by different genes. Another feature of the resistance addressed was to determine the extent of fitness of Cry2Ab resistant genotypes, as their overall fitness will be extremely important in determining the rate of evolution of resistance to Cry2Ab. Fitness of resistant genotypes in refuge crops will be particularly important, as this is one component of the Resistance Management Strategy that is within the control of the industry.

There were two distinct aims of the research performed in this project; 1) to examine various characteristics of the resistant colonies with particular attention being paid to aspects that affect the ability of Cry2Ab resistant individuals to increase in frequency on Bollgard II; 2) to examine the mechanism(s) underlying the resistance expressed by the various Cry2Ab colonies.

Objectives and summary of achievements

1. Investigation of selection and preparation of Cry2Ab resistant strains for experiments.

- *Prepare by selection with toxin, Cry2Ab-resistant lines SP15, SP202 and TABOC to be homozygous for resistant alleles (if possible). Determine LC_{50} and degree of dominance of the alleles that confer resistance in the two lines with bioassays of the appropriate crosses and backcrosses to a susceptible strain.*

Resistant strains were outcrossed and then re-selected to improve vigour. SP15 was fully characterised; the level of resistance is high (> 6,800 fold), it is inherited as a single major gene and is completely recessive in the laboratory. Resistance is not sex-linked and there is no cross resistance to Cry1Ac.

TABOC was also characterised. It exhibits a measure of dominance; the level of resistance is comparatively low (70 fold) relative to the resistance present in SP15 and it also exhibits resistance to Cry1Ac (106 fold) which is allelic with the Cry1Ac resistance present in the BX strain.

- *Determine through complementation tests if the Cry2Ab strains established from F_2s (ie SP15 and SP202) are allelic, and when available, perform similar tests with TABOC.*

SP15 proved to be allelic with four other isofemale lines derived from F_2 tests (SP202, SP566, Ha405 and HA738). A laboratory selected Cry2Ab resistant strain (IS2Ab) derived from the Cry1Ac resistant strain, BX, was also shown to share the same resistant locus as SP15. The Cry2Ab resistance in the laboratory selected strain (TABOC) showed no complementation when crossed to SP15 and is thus due a different gene or constellation of genes.

- *Establish near-isogenic lines of the resistant strains, (SP15, and depending on the outcome of complementation tests, SP202 and TABOC) and a susceptible strain through several generations of outcross to the susceptible strain and re-selection of the resistance, in preparation for fitness experiments.*

All our experiments have concentrated on the Cry2Ab resistant strain, SP15, since the other field derived resistant strains tested proved to be allelic to SP15. Investigation of TABOC was limited as the low level of Cry2Ab resistance together with the presence of Cry1Ac resistance, made it unsuitable for fitness studies.

SP15 was outcrossed and reselected 4-6 times; therefore 93.75 to 98.44% of its genome was shared with the susceptible strain at the time of comparison of fitness traits of the genotypes. This component of the project was of pivotal importance to subsequent work on fitness costs because *H. armigera* exhibits hybrid vigour when two colonies are crossed. We wished to avoid such vigour variability on crossing our resistant (RR) and susceptible (SS) colonies (to produce heterozygotes, RS) as we wanted to compare fitness of the three genotypes RR, RS and SS without confounding hybrid vigour effects. By making the genetic backgrounds between GR and SP15 essentially the same, minimal hybrid vigour would be generated from the RR X SS cross.

- *Establish an F_3 generation of an outcross between the homozygous resistant strains and a susceptible strain. Using progeny testing and discriminating doses to differentiate genotypes, ascertain the frequency of genotypes among F_3 prior to commencing a pupal diapause experiment.*

Preliminary diapause experiments were conducted, and a method to differentiate the genotypes was developed.

2. Experiments on performance on Bollgard II cotton and the relative fitness of the three genotypes, homozygous resistant (SP15), homozygous susceptible (GR) and heterozygotes (F_1 progeny of SP15 x GR)

- *Investigate the fitness of SP15 and if unique and if the resistance observed is not due to multiple loci, SP202 and TABOC through measurements on survival growth and development of the resistant strain compared to a susceptible strain, on conventional cotton, Bollgard II and selected refuge crops.*

SP15 proved to be as fit as or fitter than the susceptible strain under a wide range of conditions. There was no indication of either a recessive or dominant fitness cost associated with the allele that confers resistance to Cry2Ab toxin.

- *Undertake an experiment to investigate the effect of diapause on the homozygous susceptible, homozygous resistant and heterozygotes for SP15 and TABOC, by inducing diapause in an outcrossed line of the resistant strain, which contains all three genotypes at known frequencies.*

Initially diapause was induced in separate lines of the three genotypes as well as two mixed populations. Two further experiments were later conducted with separate homozygote resistant, heterozygote and susceptible lines. The results of these experiments showed no fitness costs associated with resistance during and following a six month period in diapause.

We also investigated the incidence of summer diapause *H. armigera*. Only a small proportion of insects entered diapause under the conditions employed.

- *(Objective added 2005) Assess possible fitness costs associated with Cry2Ab resistance in the field derived strain (SP15) when exposed to temperature stress. The survival and development of individuals of each genotype (susceptible, heterozygote and homozygote) will be assessed at each of three temperature regimes (low, medium, high). Large numbers of insects will be assayed in order to provide the power to detect slight*

differences in fitness and allow robust statistical analysis of the data. The experiment will be repeated once.

These experiments yielded robust data sets to compare the fitness of the genotypes under differing environmental conditions. Detailed comparison of growth rate and survival again yielded no evidence of fitness costs associated with resistance.

- *Measure the survival and rate of development of homozygous and heterozygous larvae of the resistant strains relative to a near-isogenic susceptible line grown on conventional cotton. Rear larvae through to adults, collecting additional data such as pupal weights, numbers of adults emerging and their fertility and fecundity. Repeat experiment.*
- *Measure the survival and rate of development of homozygous and heterozygous larvae of the resistant strains relative to a near-isogenic susceptible line grown on Bollgard II as a food source. Rear larvae through to adults, collecting additional data such as pupal weights, numbers of adults emerging and their fertility and fecundity. Repeat this experiment.*

Discussion of results from experiments addressing the above two objectives and in part the one below, have been combined because comparisons between them are informative. In summary, when fed presquare or early squaring Bollgard II, no homozygous susceptible or heterozygotes survived to pupation and survival of homozygous resistant individuals was very low. When fed fruiting Bollgard II, survival remained low for all genotypes, however homozygous resistant larvae performed better than other genotypes. Larvae grew more slowly on Bollgard II than conventional cotton, irrespective of genotype, and pupae were smaller and females laid fewer eggs. The performance of the resistant strain compared to the susceptible larvae when fed conventional cotton was comparable, thus neither homozygous resistant larvae nor heterozygotes exhibited detectable fitness costs.

- *Measure the survival and rate of development of homozygous and heterozygous larvae of the resistant strains relative to a near-isogenic susceptible line grown on selected refuge crops as the food source. Rear larvae through to adults, collecting additional data such as pupal weights, numbers of adults emerging and their fertility and fecundity. Repeat this experiment.*

Two refuge crops were selected as a food supply. Pigeon pea is the most commonly used refuge by growers and this choice was obvious. The second most frequently chosen refuge is cotton, with sprayed cotton and unsprayed cotton ‘coverage’ of Bollgard II being similar, although the actual area of sprayed cotton was ten fold that of unsprayed cotton. For our purposes sprayed cotton was not manageable, as depending on insecticide used and time since application, the capacity of sprayed cotton to support larvae would vary. Thus fitness of the three genotypes was assessed on unsprayed cotton and on pigeon pea. Like the observations on conventional cotton (above), no significant differences between the performances of the three genotypes were detected when pigeon pea was used as a food source.

- *(Objective added 2006) Assess the extent of dominance of a field-derived allele that confers resistance to Cry2Ab toxin when in a field genetic background. This involves the*

progressive replacement of laboratory genome (where the allele has proven to be recessive) with that from field collected H. armigera where the allele may be only partially recessive.

The first experiment to investigate this hypothesis was completed. The results were inconclusive; however more variation was seen in the iso-female field lines than among the laboratory-based lines or mass-mated lines.

Molecular and biochemical investigation of Cry2Ab resistance.

- *Determine if Cry2Ab binds specifically or non-specifically to H. armigera midgut tissues.*

The Cry2Ab protein proved to be 'sticky' and adhered to cellular material and also readily precipitated from solution. Considerable effort was devoted to determine the correct conditions to retain the protein in solution as this aspect of the project was expected to be highly informative. However this was not achieved.

- *Compare the gut protease profiles of resistant and susceptible strains and their effect on the Cry2Ab toxin.*

Proteases present in resistant and susceptible strains proved to be similar but a faint protease band present in the susceptible larvae (GR) was not evident in resistant larvae. However the difference was at the limit of resolution of the technique and no firm conclusion could be reached.

Gut extracts of resistant and susceptible insects were both capable of digesting Cry2Ab toxin. There was an indication that SP15 extract degraded toxin more quickly than that from a GR extract.

- *Identify proteins expressed at different levels in Cry2Ab-resistant and -susceptible strains by the use of proteomics (replaced an objective which would have further investigated Cry2Ab binding).*

Several differences in proteins expressed in midguts of resistant and susceptible insects were detected in two dimensional gels and an attempt to identify the sorts of proteins represented will be pursued when resources are available.

- *Investigate a possible association of the Bre-5 gene with Cry2Ab resistance in H. armigera. (This objective replaced objective which would have investigated the existence of an enhanced repair mechanism).*

Studies by our colleague David Heckle continuing research initiated during (CSE 108C) identified close association of the Bre-5 gene with the Cry2Ab resistance in SP15. This was investigated by sequencing sections of the Bre-5 gene in a range of *H. armigera* including additional GR and SP15 individuals and also wild caught moths. Unfortunately, the variation detected in the sequences eliminated the possibility that variants of the Bre-5 gene confer resistance.

- *Prepare the results of the experiments for publication.*

Several papers, that include the work performed in this project, have been accepted for publication in international scientific journals, presented at conferences or published in the Australian Cotton Grower. One further draft manuscript is presently under consideration by

J. Economic Entomology and several more papers are at various stages of preparation. Recent publications by the 'Bt group' are listed below.

Methods

Insect bioassays

The dominance, resistance and allelism of the resistant colonies were evaluated using our standard laboratory assays. While a brief summary of the rearing methods and assays are given here, further details of the techniques are presented in two recent publications and in a paper presently under review. A copy of the papers are attached to this report.

This study focused largely on the fitness of homozygous Cry2Ab-resistant individuals of the SP15 strain and heterozygotes generated by crossing a susceptible strain (GR) to the resistant strain. Outcrossed and re-selected SP15 was used in experiments to ensure that it was near-isogenic with the susceptible strain GR. Fitness of the genotypes was assayed on conventional cotton and refuge crops as well as during diapause and when challenged with different temperature regimes. We also investigated the performance of the genotypes on Bollgard II cotton to ascertain if there was any advantage gained by individuals carrying the resistance gene as either homozygotes or heterozygotes. Rates of survival at larval, pupal and adults stages were measured as well as determination of the duration of the life stages, fecundity and fertility where there were sufficient numbers of survivors/adults.

Diapause

Larva of the three genotypes were reared normally until late 3rd/4th instar then transferred to diapause inducing conditions. (18°C with a light : dark regime of 11: 13 hours). After pupation and assessment for induced diapause, the pupae were given a cold shock treatment (6 - 9°C) for two weeks. Pupa were then stored at 18°C for six months. Diapause was 'broken' by returning the pupae to normal rearing conditions at 25°C. The experiment was performed on two occasions.

Fitness when exposed to different temperature regimes

Preliminary data from winter and summer diapause experiments suggested the presence of fitness costs associated with larval development at high or low temperatures that could be attributed to the resistant gene. To explore this in more controlled conditions, we conducted two growth and mortality experiments where all three genotypes were exposed to three sets of temperature regimes. Large numbers insects were assayed in order that we could detect even subtle differences. Approximately 320 larvae per genotype in the first experiment and 220 in the second experiment, were reared from neonate to adult on insect diet at warm (30-32ºC), medium/control (24-26ºC) or cool (20-22ºC) temperature regimes.

Plant experiments

Bollgard II. Insects of each genotype were exposed to plants of Bollgard II cotton (Siokra V-16B) in the plant room experiments with conventional cotton plants (Siokra V-16) used as controls. Individual insects were positioned on plants and contained within polyester organza bags until close to pupation. Prepupae were then removed and allowed to pupate in 30 ml containers containing ~10 ml of rearing diet. The first experiment used presquare cotton and in the later experiments both early squaring and fruiting plants (after boll-set) were employed.

Space in the growth rooms was limited and thus restricted the number of larvae that could be challenged. As a very small proportion completed development on Bollgard II, few insects from this treatment survived to enable comparisons of survival to adult, fecundity etc.

In order to boost numbers and improve the accuracy of our estimates of survival on Bollgard II, we undertook four additional experiments using field-collected Bollgard II, with conventional cotton as controls (Sicot 71BR, Sicot 71BR BII). For these experiments, fruiting structures and/or fresh leaves were collected weekly from ACRI experimental plots, dispatched by air to our Canberra laboratory and provided to larvae in individual containers. Plant material was kept alive by immersing the stalks in a pot of water within the container and plant material was refreshed once or twice weekly, as needed, until larvae pupated. The experiment was performed with plants at three different growth stages (presquare, early squaring and after boll-set). The experiment with fruiting Bollgard II was repeated to collect additional data.

Conventional cotton. These experiments were conducted to compare the performance of the resistant genotypes on conventional vs Bollgard II cotton, as well as investigating potential fitness costs on cotton as a refuge crop. Insects of each genotype were confined individually to plants of conventional cotton (Siokra V-16 and Sicot 189) in the plant room experiment as described above. One experiment was performed when cotton had developed to the presquare stage, the other when at an early squaring stage. Individuals were reared through to adult emergence. In the first (prequare) experiment, adults were also assessed for fertility, fecundity and longevity.

Since it became apparent after the first fitness experiments on conventional cotton, that any costs that could be attributed to the resistant gene would be small, we conducted further growth and mortality experiments using excised cotton leaves so we could test larger numbers of insects to yield robust data for analysis. Approx. 100 neonate larvae of each genotype were tested individually in each of two experiments. Two varieties of conventional cotton plants (Siokra V-16, Sicot 71), grown in the glasshouse, were used in each experiment. One experiment used presquare cotton and the other early squaring cotton. Plant material was kept alive by immersing the stalks in a pot of water inside the container and material was refreshed once or twice weekly, as needed, until larvae pupated. Individuals were reared until adult emergence.

Pigeon pea. Larvae were reared individually on field-grown excised pods of the popular refuge crop, pigeon pea, in two experiments. Larvae and pods were contained in 9 cm diameter petri-dishes with a 5 ml mixture of 2% agar and 0.1% sorbic acid for moisture and microbial control. Pods were replaced and larvae assessed, twice weekly. Approximately 100 larvae of each genotype were reared to adulthood in each experiment.

Heterozygote dominance in a field background (on-going)

This experiment involved the progressive replacement in the resistant strain, SP15, of its laboratory genome (where the allele has proven to be recessive) with that from field collected *H. armigera* (where the allele may be only partially recessive). The proportion of laboratory genome replacement by field genomes increased with each outcross to field insects. Individual outcrossed heterozygous individuals (RS) were mated to a single susceptible (SS) field insect. Their offspring were selfed (mated among themselves) and neonates (F_2s) screened at a dose of Cry2Ab that ensured that only RR individuals should survive. This

process mimics our F_2 screen we use to isolate resistance alleles. Controls lines were generated by outcrossing SP15 to the laboratory strain GR and heterozygous individuals mated to another GR insect to mimic the above procedure. If dominance was present, a larger proportion than the expected 1/16 of the screened F_2's would indicate that heterozygous individuals were also surviving. The hypothesis that the genetic background influences the degree of dominance would be supported if an excess of survivors occurred among isofemale lines from the field-introgressed cohort, but not from the laboratory-introgressed group.

Molecular / biochemical techniques

Brush border membrane vesicles (BBMVS)
There are no published reports of binding studies of Cry2Ab in Lepidoptera. We employed techniques successfully employed to study binding of Cry1Ac toxin to BBMVS from larvae midguts followed by competition binding assays (Liao et al 2005). Midguts of 4th instar were dissected and cleaned in MET buffer (300mM Mannitol, 5mM EGTA, 17mM Tris-HCl, pH 7.5). Brush border membrane vesicles (BBMV) were prepared by the MgCl methods as described by Wolfersberger et al. (1987) and stored at −80°C .

Cry2Ab toxin
Cry2Ab toxin used was produced 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 concentration of toxin was estimated following electrophoretic separation of proteins 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).

Proteases

The gut protease profiles of susceptible and resistant strains were compared by zymogram analysis to detect changes in proteases associated with resistance. Midguts from 4th instar larvae were dissected and retained at 4°C. Samples from the different strains were centrifuged at 14,000*g* for 15 minutes at 4°C and the supernatant removed. The protein concentration was quantified by Bradford protein analysis.

Proteins in gut extracts were separated in a 10% SDS electrophoresis gel run at 4°C. The gel was then incubated in 50mM Tris-HCl buffer (pH7.5) containing 2% casein for 2 h at 4°C, followed by a 20 min incubation at room temperature. Protein was visualised by staining with the general protein dye, coomassie blue. Where proteinase activity was present on the gel, casein was degraded. Such areas were visible as clear bands against a blue background.

Degradation of Cry2Ab by gut extracts
Protease degradation of the Cry2Ab was tested by determining the stability of activated Cry2Ab toxin in gut juice from susceptible and resistant strains. Cry2Ab toxin was added to gut extracts (produced as above) and incubated at room temperature for 10 minutes. Proteins were then separated by electrophoresis on a 10% SDS gel and stained with coomassie blue.

Proteomics

BBMVs prepared as above were cleaned with trichloroactic acid, freeze dried and proteins dissolved in extraction buffer (7M urea, 2M thiourea, 30mM DTT, 2% CHAPS, 0.5% Triton-X). The samples were placed on an acrylamide gel strip and iso-focused overnight at 500v to separate proteins on their pH. The iso-focused proteins were then further separated by 10% SDS electrophoresis gel at 10C. Gels were stained with Coomassie blue to reveal areas 'spots' of protein.

Results

Investigation, selection and preparation of Cry2Ab resistant strains for experiments.

- *Prepare by selection with toxin, Cry2Ab-resistant lines SP15, SP202 and TABOC to be homozygous for resistant alleles (if possible). Determine LC_{50} and degree of dominance of the alleles that confer resistance in the two lines with bioassays of the appropriate crosses and backcrosses to a susceptible strain.*

SP15, SP202 and a third strain, SP566, all of which were derived from the field by the F_2 screening method, were outcrossed to a susceptible strain to increase vigour, and then re-selected to produce separate homozygous resistant strains. SP15 had already been fully characterised, details of which are included in the annual report for an associated project (CSE 108C). In summary, the level of resistance is high (> 6,800 fold), resistance is inherited as a single major gene and is completely recessive. The form of resistance characterised by SP15 is surprisingly common in *H. armigera* populations, so confirmation that it is recessive is important. Had it exhibited a degree of dominance, there would have been far greater concern that the resistant alleles would quickly increase in frequency through selection. Resistance is not sex-linked and, importantly, there is no cross resistance to Cry1Ac.

TABOC, which was derived through laboratory selection of a colony established from *H. armigera* collected in 2002/03 was characterised in this project. It exhibits some dominance with the F_1s from a cross between the susceptible GR and TABOC exhibiting a resistance ratio of 5 relative to GR (Table 1A) and consequently, proved difficult to select to homozygosity. A homozygous (or close to homozygous) strain was achieved through screening the offspring of isofemale lines (2005 data in Table 1). Relative to SP15, the level of resistance in the near-homozygous generations was comparatively low (≈ 70 fold). TABOC was also found to exhibit resistance to Cry1Ac (106 fold) which proved to be allelic with the Cry1Ac resistance present in the BX strain (note the elevated resistance ratio (RR) of the cross between BX and TABOC in Table 1B). The RR to Cry1Ac was readily increased following selection with Cry1Ac to 501 fold (Table 1B).

The resistance present in TABOC proved to be induced by a different gene or genes than the gene that confers resistance in SP15 as the cross between the two strains yielded a low resistance ratio. If allelic, we would expect a RR of somewhere between 27 and 6000 (Table 1A).

Table 1. Alleleisim tests and resistance of TABOC to Cry2Ab (A) and Cry1Ac (B)

A. Cry2Ab

Strain/cross	LC_{50} µg/cm^2 Cry2Ab toxin	95%CI	RR
2004: allelism test SP15			
GR (susceptible)	0.16	0.81, 0.3	-
TABOC	4.25	3.51, 5.15	27
TABOC x GR	0.75	0.57, 1.03	5
TABOC x SP15	0.55	0.39, 0.85	3
SP15	na	na	>6000
SP15 x GR	0.07	0.05, 0.09	0.4
2005: reselected TABOC			
ANGR (susceptible)	0.07	0.06, 0.09	-
TABOC	4.93	4.02, 6.03	70

B. Cry1Ac

Strain/cross	LC_{50} µg/cm^2 Cry1Ac toxin	95%CI	RR
2005: allelism test BX			
ANGR (susceptible)	0.12	0.09, 0.16	-
TABOC selected Cry1Ac	61.51	46.83, 81.05	501
TABOC selected Cry2Ab	12.98	9.86, 17.04	106
BX	100.83	76.22, 134.3	821
TABOC Cry1Ac x BX	40.914	31.87, 52.61	333
TABOC Cry1Ac x ANGR	0.84	0.65, 1.09	7
BX x ANGR	0.67	0.51, 0.86	5

- *Determine through complementation tests if the Cry2Ab strains established from F_2's (ie SP15 and SP202) are allelic, and when available, perform similar tests with TABOC.*

Since this objective was framed, additional Cry2Ab resistance strains were established and therefore it was possible to examine the genetic relationship of five separate isolates. Complementation tests were carried out between SP15, SP202, SP566, Ha405 and Ha738 all isolated from F_2 screens; the latter two by Sharon Downes group in Narrabri as part of the Bt monitoring program. SP15 proved to be allelic with all four isofemale lines. This component of the project has been prepared for publication and submitted to Journal of Economic Entomology. Rather than duplicate an extensive description here, a PDF of the draft paper is attached (Mahon, Olsen and Downes (in prep)).

This finding is important as it means that the one form of resistance at a single locus prevails in *H. armigera* populations rather than multiple forms of resistance determined by different loci. If the instances of resistance were due to mutations at different loci, each mutation would

have been quite rare. From a resistance management perspective, the later situation would have been preferable. Nevertheless, there is a positive aspect to this finding. At least we know (and increasingly understand) this form of resistance and therefore it is possible to make reasonable assumptions about how resistance to Cry2Ab will evolve in *H. armigera* populations.

Interestingly, a laboratory selected Cry2Ab resistant strain (IS2Ab) derived from the Cry1Ac resistant strain, BX, was also shown to share the same form of resistance as that found in SP15. This strain was outcrossed with field-collected material in 2003 to improve vigour, and we assume that the gene conferring resistance to Cry2Ab was either introduced into the BX colony at this time or it was always present in the colony and only became apparent following selection with Cry2Ab toxin. This is of importance as it reinforces other data that indicate that the Cry2Ab resistance predated the introduction of Bollgard II.

The Cry2Ab resistance in the laboratory selected strain (TABOC) showed no complementation when crossed to SP15 and is thus due a different gene or constellation of genes (see above and Table 1B). That it differs from SP15 was not surprising, since TABOC exhibits a lower level of resistance than SP15 and some dominance.

- *Establish near-isogenic lines of the resistant strains, (SP15, and depending on the outcome of complementation tests, SP202 and TABOC) and a susceptible strain through several generations of outcross to the susceptible strain and re-selection of the resistance, in preparation for fitness experiments.*

All field derived strains produced by F_2 screens available for complementation tests when this milestone was formulated subsequently proved to be allelic to SP15 and thus did not merit independent study. Further, the laboratory selected strain, TABOC, proved to exhibit only low-level resistance to Cry2Ab (≈ 70 fold) (see above and Table 1A). This level was achieved by selection by progeny testing individual iso-female lines and pooling 'high-expressors' and thus is probably the maximum level attainable for that colony. The low level of Cry2Ab resistance of TABOC, and more particularly the presence of the BX form of Cry1Ac resistance (see above and Table 1B) made it an unsuitable candidate for the study of fitness costs associated with Cry2Ab resistance as this form of Cry1Ac resistance has already been shown to be associated with fitness costs (Bird and Akhurst 2007). Clearly it would have been impossible to differentiate fitness costs due to resistance to Cry1Ac from those of Cry2Ab.

Our experiments on fitness costs concentrated on the Cry2Ab resistant strain SP15 as a representative of the form of Cry2Ab resistance present in Australian populations of *H. armigera.* Four, and later, six outcrosses of SP15 to a susceptible strain (GR) were completed during the course of these experiments. After each outcross, the F_2 generation was re-selected. Through this process, SP15 was made both homozygous for resistance and to share a similar genetic background to the susceptible strain. Theoretically the two should have 93.75% and 98.44% of their genomes in common after the fourth and sixth outcross respectively.

TABOC was similarly outcrossed and re-selected three times in preparation for these experiments but was not used for the reasons outlined above.

- *Establish an F_3 generation of an outcross between the homozygous resistant strains and a susceptible strain. Using progeny testing and discriminating doses to differentiate genotypes, ascertain the frequency of genotypes among F_3 prior to commencing a pupal diapause experiment.*

Preliminary diapause experiments were conducted, and a method devised that would allow the differentiation of each genotype, homozygous resistant (RR), heterozygous (RS) and homozygous susceptible (SS). The frequency of genotypes among the F_3 was ascertained in the control populations. This preliminary milestone was completed during the first year and more substantial experiments performed subsequently are addressed below.

Performance of larvae on Bollgard II cotton. Relative fitness of the three genotypes, homozygous resistant (SP15), homozygous susceptible (GR) and heterozygotes (F_1 progeny of SP15 x GR).

- *Undertake an experiment to investigate the effect of diapause on the homozygous susceptible, homozygous resistant and heterozygotes for SP15 and TABOC, by inducing diapause in an outcrossed line of the resistant strain, which contains all three genotypes at known frequencies.*

Diapause was induced in separate groups of the three genotypes as well as in two mixed populations. Controls (a sub-set of the individuals set up for the diapause experiments) were established for each group. Controls for the separate lines of genotypes were reared at a standard temperature (25°C) and assessed for differences in vigour. Controls of the populations with mixed genotypes were reared at 25℃ and progeny tested to determine the frequency of specific genotypes. Due to difficulties associated with a change of rearing practices (diet changed from chickpea to soya) too few pupae of any genotype survived diapause for the experiment to be informative.

The F_3 technique employed in this experiment required progeny testing each individual and that proved to be logistically difficult. The complexity was introduced to minimise improved performance from hybrid vigour in the heterozygote generated when GR and SP15 were crossed. However to a very large extent, once SP15 and GR were multiply outcrossed, hybrid vigour should have been minimal. Consequently in subsequent experiments, a simple process that did not necessitate progeny testing was adopted. This involved comparison of the performance in diapause of the SP15, GR colonies, and heterozygotes were generated by crossing the two colonies. Two further experiments were conducted using this method and after a prolonged period in diapause (6 months) pupae were allowed to emerge.

Of the pupae that entered diapause, similar proportions of each genotype successfully emerged after diapause was 'broken' (Figure 1). There was no indication that RR or RS genotypes were less fit under diapause conditions. The mean weight of SP15 pupae entering diapause was marginally greater than that of the susceptible strain and the heterozygotes (Figure 2) and was similar to that of the non-diapause control pupae which were reared at 25ºC (data not shown). Following return to non-diapause conditions, adults of all genotypes emerged in a similar pattern (Figure 3). Females emerged first (with some females breaking diapause while still under diapause conditions) with males trailing a few days later (Figure 3). These experiments provided no evidence of fitness cost associated with either the heterozygous or homozygous resistant genotypes.

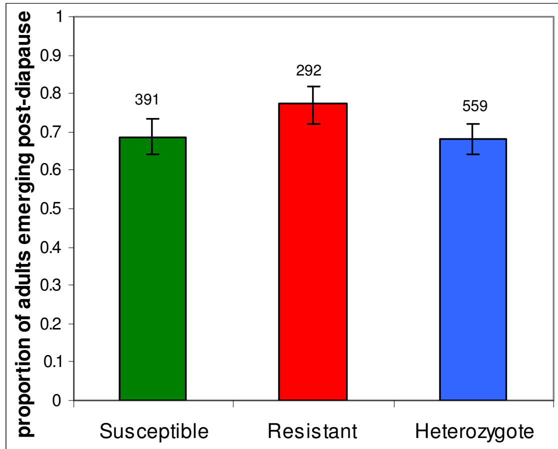

Figure 1. Proportion (± 95% CI) of adults of the three genotypes (SS, SR and RR), emerging from pupae after six months in diapause. Numbers above bars represent the combined number of pupae used in two experiments.

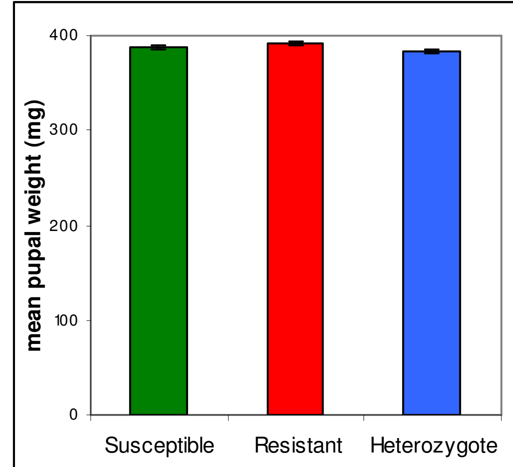

Figure 2. Weight (mg ± SE) of pupae of the three genotypes (SS, SR and RR) in which diapause was induced in two experiments

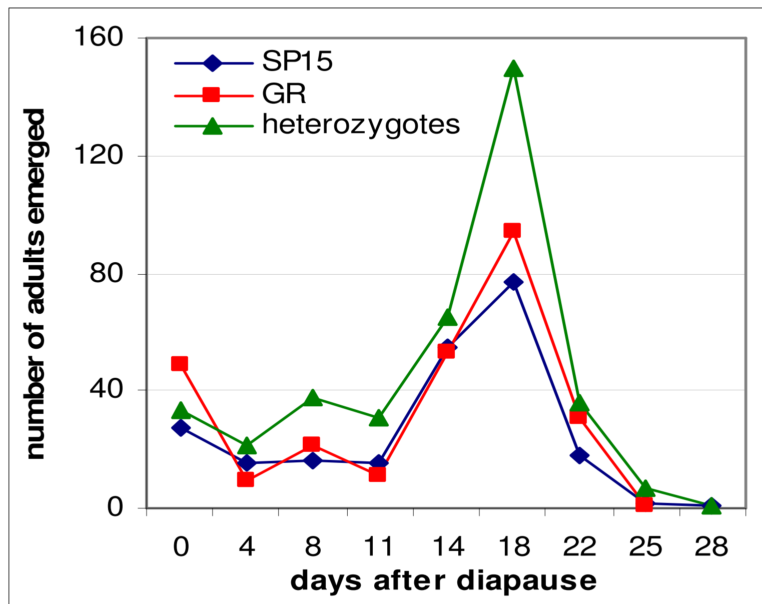

Figure 3. Pattern of emergence over time, for adults of the three genotypes (SS, SR and RR) emerging after six months in diapause.

In supplementary experiments we investigated the incidence of summer diapause (prolonged pupal stage following larval exposure to temperatures above 33°C) in nine genotypes of laboratory and fields strains of *H. armigera* to establish if insects resistant to Bt toxins Cry1Ac and Cry2Ab suffered additional mortality. Only a small proportion of insects entered diapause under the conditions employed, and even fewer survived to become adults. However, it was of interest that the only individuals that survived diapause were Cry1Ac resistant (BX x ANGR) and Cry2Ab resistant (SP15 x GR) heterozygotes. The diapause method used was clearly challenging to this species.

- *Measure the survival and rate of development of homozygous and heterozygous larvae of the resistant strains relative to a near-isogenic susceptible line grown on conventional cotton. Rear larvae through to adults, collecting additional data such as pupal weights, numbers of adults emerging and their fertility and fecundity. Repeat experiment.*
- *Measure the survival and rate of development of homozygous and heterozygous larvae of the resistant strains relative to a near-isogenic susceptible line grown on Bollgard II as a food source. Rear larvae through to adults, collecting additional data such as pupal weights, numbers of adults emerging and their fertility and fecundity. Repeat this experiment.*

Experiments addressing these two objectives will be considered together as comparisons between them are informative. No homozygous susceptible or heterozygotes survived to pupation on presquare or early squaring Bollgard II, whether grown in the growth room or field, and survival of homozygous resistant individuals was very low and similar on both sources of Bollgard II (Table 2). There was an increased level of survival of all genotypes on the fruiting Bollgard II, especially those from the plant room, but numbers were low. When supplied field grown Bollgard II, larger sample sizes were possible however survival rates were low, even for SP15. From a resistance management perspective, the poor survival of SP15 homozygotes, even on older Bollgard II® where we would expect Cry1Ac titres to be reduced was encouraging. This mortality clearly reflects the strain's sensitivity to the residual Cry1Ac (presumably at low concentration) present in the maturing plants.

Table 2. Survival of larvae of three genotypes (RR, RS and SS) reared to adults on presquare, early squaring and fruiting Bollgard II® cotton grown in plant growth rooms and in the field.

Origin of Bollgard II cotton:	***plant room***		***Field grown***	
Genotype	*% Survival to adult (95% CI)*	*Total tested*	*% Survival to adult (95% CI)*	*Total tested*
Presquare plants (1 experiment each)				
Susceptible	0	52	0	218
Heterozygote	0	91	0	220
Homozygous resistant	2.08 (0.39, 10.89)	48	0	221
Early squaring plants (2 experiments – plant room, 1 experiment – field)				
Susceptible	0	46	0	179
Heterozygote	0	76	-	-
Homozygous resistant	3.39 (0.93, 11.54)	59	2.54 (1.17, 5.43)	236
Fruiting plants (squares, flowers, bolls) (2 experiments each)				
Susceptible	12.5 (2.24, 47.09)	8	1.64 (0.75, 3.53)	366
Heterozygote	5.26 (0.93, 24.63)	19	1.72 (0.79, 3.7)	348
Homozygous resistant	31.25 (14.16, 55.6)	16	8.47 (6, 11.83)	354

Neither homozygous resistant larvae nor heterozygotes exhibited detectable fitness costs that impacted on their growth and development on conventional cotton, relative to the performance of susceptible larvae (Figures 4 and 5, Table 3) although in some cases the results for the susceptible and the resistant strains differed significantly. This difference, especially in earlier experiments, was been attributed to the relative vigour of the strains at the time of the experiment as there was no consistent trend towards one strain. Additional effort was put into monitoring the vigour of all strains used in later experiments. Larvae which survived through to adults grew more slowly on Bollgard II than conventional cotton, irrespective of genotype (Figure 4).

Table 3. Survival of larvae of three genotypes (RR, RS and SS) reared to adults on presquare, early squaring and fruiting conventional cotton grown in plant growth rooms and in the field.

Origin of conventional cotton:	*Grown in plant room*		*Field grown*	
Genotype	*% Survival to adult (95% CI)*	*Total tested*	*% Survival to adult (95% CI)*	*Total tested*
Presquare plants	(1 experiment – growth room)			
Susceptible	60 (46.18, 72.39)	50	-	-
Heterozygote	63.4 (53.64, 72.12)	101	-	-
Homozygous resistant	74.5 (61.13, 84.45)	51	-	-
Early squaring plants	(1 experiment each)			
Susceptible	84.44 (71.21, 92.25)	45	81.03 (69.14,89.06)	58
Heterozygote	94 (83.78, 97.94)	50	-	-
Homozygous resistant	50 (35.53, 64.47)	42	78 (64.76, 87.25)	50
Fruiting plants (squares, flowers, bolls)	(2 experiments each)			
Susceptible	-	-	50.45 (41.22, 59.67)	109
Heterozygote	-	-	54.05 (44.8, 63.03)	111
Homozygous resistant	60 (38.66, 78.12)	20	48.65 (39.55, 57.84)	111

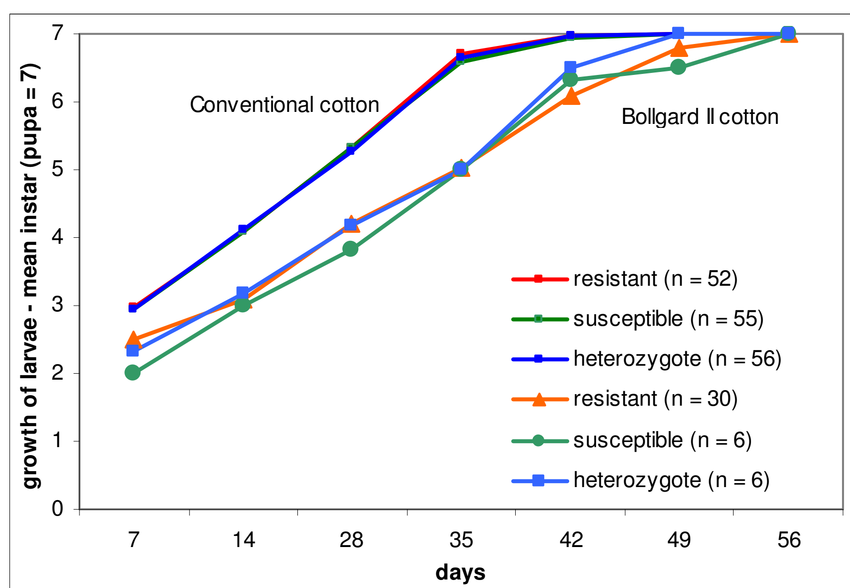

Figure 4. Growth of larvae (mean instar) of the three genotypes (homozygous susceptible, heterozygote and homozygous resistant) recorded weekly until pupation, on excised field-grown leaves, terminal and bolls of fruiting conventional and Bollgard II cotton.

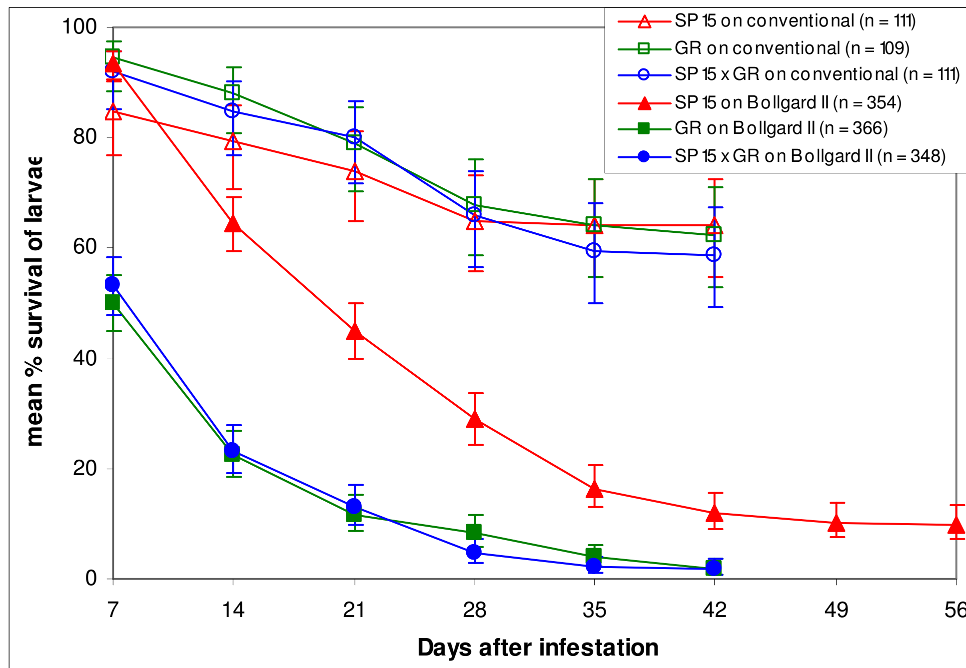

Figure 5. Survival (+ 95%CI) of larvae of the three genotypes (homozygous resistant SP15, homozygous susceptible GR and heterozygotes, F_1 progeny of SP15 x GR) recorded weekly until pupation, on excised field-grown leaves, terminal and bolls of fruiting conventional and Bollgard II cotton.

Data on pupae weights and developmental time (neonate to adult) is summarised in Table 4. Fecundity / fertility and longevity data, collected from single pair matings of emerged adults, are presented in Table 4. Pupae were significantly smaller for all genotypes when fed on Bollgard II as opposed to conventional cotton, and females laid fewer eggs. However, when reared on conventional cotton, no fitness costs were evident for the RR or RS genotypes for any of the fitness parameters examined (pupae weight, developmental period, longevity, fertility and fecundity). This finding was important, as from a resistance management perspective, the lack of fitness costs associated with resistant genotypes in the absence of toxin is disappointing. In other species, such fitness costs have proven to be important in retarding the evolution of resistance to Bt toxins.

Table 4. Pupal weights and development time (neonate – adult) of three genotypes (RR, RS and SS) reared to adults on presquare, fruiting conventional and fruiting Bollgard II cotton grown in plant growth rooms or in the field.

Genotype	*Number of pupae*	*Mean pupal weight (mg ± SE)*	*Time: neonate – adult emergence (days ± SE)*
Presquare conventional plants (growth room – 1 experiment)			
Susceptible	35	235 ± 40	33.2 ± 0.6
Heterozygote	70	242 ± 29	31.2 ± 0.5
Resistant	40	260 ± 41	30 ± 0.6
Early squaring conventional plants (growth room – 1 experiment)			
Susceptible	41	366 ± 9.6	34.3 ± 0.4
Heterozygote	54	372 ± 7.7	34.4 ± 0.4
Resistant	22	368 ±12	36.5 ± 0.8
Early squaring conventional plants (field – 1 experiment)			
			(Days to pupation ±SE)
Susceptible	49	306 ± 8	(23.4 ± 0.64)
Resistant	42	303 ± 7.4	(27.1 ± 0.68)
Early squaring Bollgard II plants (field – 1 experiment)			
Resistant	7	233.8 ± 22.1	54.4 ± 2.3
Fruiting conventional plants (field – 2 experiments)			
Susceptible	65	305 ± 5.4	39.8 ± 0.6
Heterozygote	61	317 ± 6.7	39.2 ± 0.6
Resistant	65	292 ± 7.9	39 ± 0.7
Fruiting Bollgard II plants (field – 2 experiments)			
Susceptible	6	204 ± 22.3	49.5 ± 1.2
Heterozygote	6	205 ± 11.8	48.5 ± 2.5
Resistant	37	242 ± 9.5	49.5 ± 2

Table 5. Fertility, fecundity and longevity of three genotypes (RR, RS and SS) reared to adults on presquare, fruiting conventional and fruiting Bollgard II cotton grown in plant growth rooms.

Genotype	*% fertile adults*		*Fertile eggs/female*	*Longevity of adults*	*Number of*
	male	*female*	*± SE*	*(days ± SE)*	*adults*
Presquare conventional plants (growth room – 1 experiment)					
Susceptible	28.6	56.3	618 ± 206	9.4 ± 0.6	33
Heterozygote	65.6	56.3	754 ± 227	10.5 ± 0.4	67
Resistant	61.9	62.5	824 ± 261	10.9 ± 0.4	38
Fruiting conventional plants (field – 2 experiments)					
Susceptible	40	62.2	678 ± 97.7	11.2 ± 5	57
Heterozygote	70.4	87.1	752 ± 89.8	15.1 ± 5.5	58
Resistant	76.7	95.8	724 ± 77.8	17 ± 5.3	54
Fruiting Bollgard II plants (field – 2 experiments)					
Susceptible	n/a	n/a	468 (4♀)	11.5 ± 2.5	6
Heterozygote	n/a	n/a	412 (2♀)	13 ± 4.3	6
Resistant	46.7	86.7	401 ± 104 (13♀)	12 ± 5.5	32

n/a – estimate not available as too few males survived

- *Measure the survival and rate of development of homozygous and heterozygous larvae of the resistant strains relative to a near-isogenic susceptible line grown on selected refuge crops as the food source. Rear larvae through to adults, collecting additional data such as pupal weights, numbers of adults emerging and their fertility and fecundity. Repeat this experiment.*

We identified conventional cotton and pigeon pea as the most relevant crops to test as refuge crops. Conventional cotton was considered in the above experiments. In addition to those experiments, we investigated the survival and development of ~100 individuals of each genotype (susceptible, heterozygote and homozygous resistant) reared on excised leaves of glasshouse grown conventional cotton. Data on fertility and fecundity was collected in the first plant experiment, on presquare conventional cotton grown a plant room (data presented in Table 4). Since there was no indication of a fitness cost in this area, no further data fertility and fecundity was collected in these experiments. In addition, differences between the two cotton varieties siocra and sicot were negligible thus the data form each have been pooled.

In this experiment mortality was low for all genotypes (Figure 6) however the susceptible genotype exhibited slightly

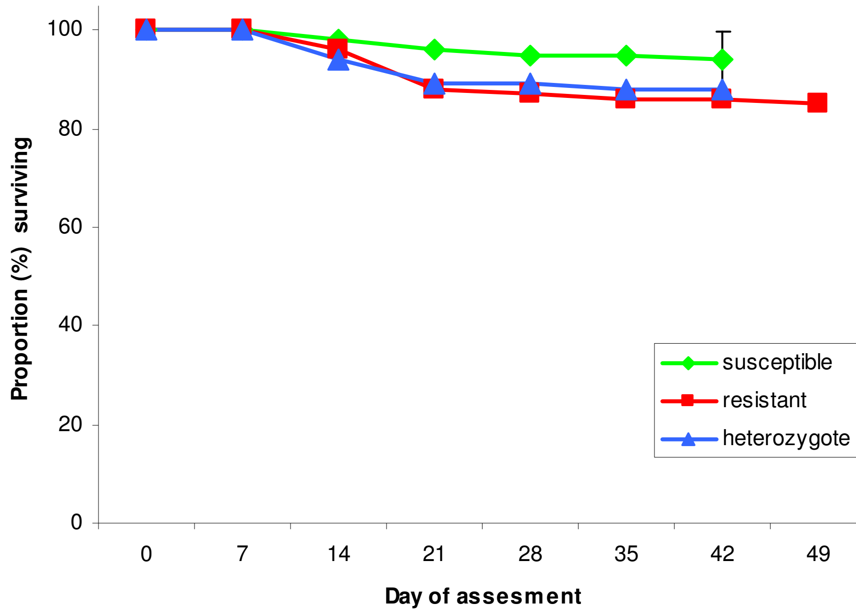

Figure 6. Survival of SS, RS and RR individuals from neonate to adult moth when fed glasshouse-grown cotton. To prevent clutter, only a single representative 95% CI is drawn for the susceptible genotype proportion (94 out of 100) on day 42.

Growth rates of the three genotypes were also similar (Figure 7) and again there was no indication of fitness costs expressed inby the resistant phenotypes.

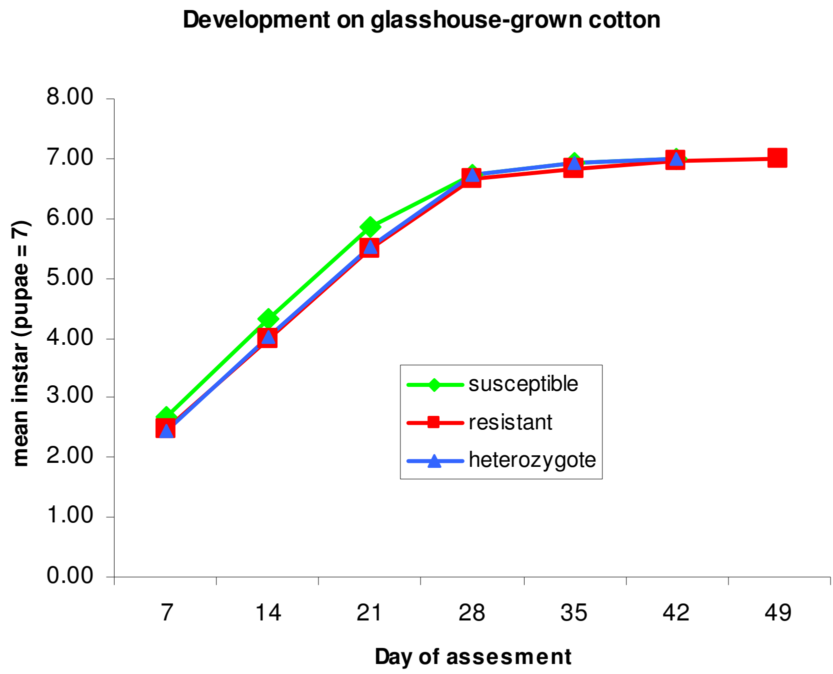

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Figure 7 Development of SS, RS and RR individuals from neonate to adult moth when fed glasshouse grown cotton.

Pigeon pea

The survival and development of ~200 individuals of each genotype were evaluated when reared on excised field-grown pigeon pea pods, in two experiments. Comparisons between experiments are not valid as it was inevitable that conditions and food quality would subtly different. Mortality of all genotypes was low and differences among genotypes within an experiment were not significant (note the overlapping 95% CI's in Figure 8)

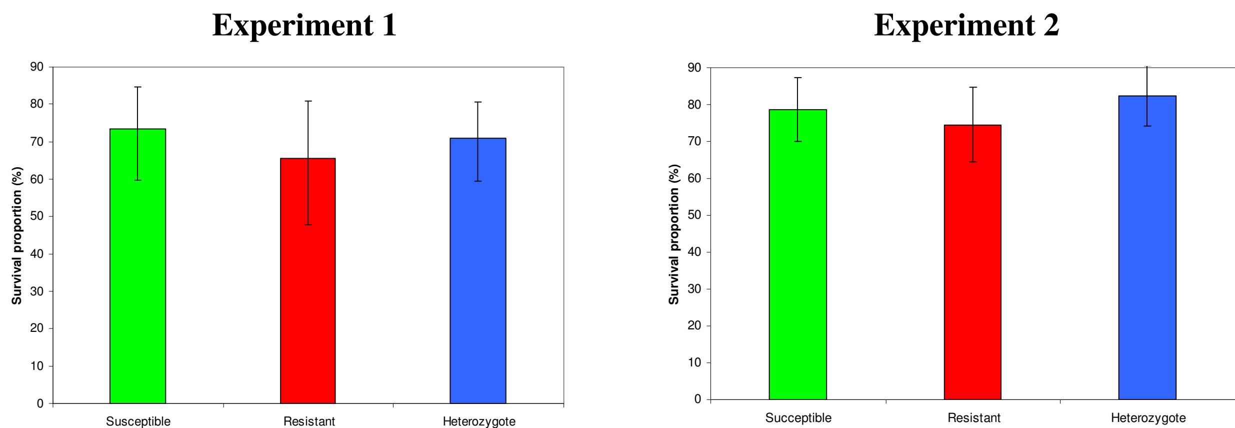


Figure 8. Proportion of SS, RS and RR individuals surviving in two experiments from neonate to adult moth when fed field-grown pigeon pea. Error bars are 95% CI's.

Within experiments, pupal weights of all genotypes were similar (Table 6) and differences were not significant.

Table 6. Weight of pupae of RR RS and SS genotypes when larvae were reared on field-grown pigeon pea.

Experiment 1

Genotype	Pupae Weight mg ± se
SS	329.99[a] ± 6.96
RR	348.58[a] ± 9.52
RS	332.25[a] ± 5.80

Experiment 2

Genotype	Pupae Weight mg ± se
SS	339.38[a] ± 1.42
RR	326.24[a] ± 1.58
RS	345.80[a] ± 1.87

[a] Means with common superscript are not significantly different (P > 0.05).

The duration of larval life and pupal life was also examined (Table 7). In the first experiment the heterozygote took significantly longer to pupate than either SS or RR group. In contrast, in the second experiment, the RR genotype pupated slightly (but significantly) earlier. The duration of the pupal stage of the genotypes did not differ in the first experiment however in the second, the RR genotype was subtly longer. It is relevant that while the RR individuals pupated earlier their pupal life was extended so effectively peak emergence was similar for all genotypes.

Table 7. Duration of larval stages (neonate to pupae) and pupal stage (pupation to adult emergence of SS, RR and RS genotypes when reared on pigeon pea

Experiment 1

Genotype	Time to pupation Days ± se	Pupae to adult Days ± se
SS	19.52[a] ± 0.14	10.53[a] ± 0.06
RR	19.31[a] ± 0.37	9.90[a] ± 0.34
RS	20.60[b] ± 0.38	10.33[a] ± 0.28

Experiment 2

Genotype	Time to pupation Days ± se	Pupae to adult Days ± se
SS	20.48[a] ± 0.41	9.71[a] ± 0.41
RR	18.70[b] ± 0.31	10.64[b] ± 0.31
RS	19.63[a] ± 0.45	9.25[a] ± 0.45

[a, b] Means with common superscript are not significantly different (P > 0.05).

Larval instars were scored on days 4,7,11,18,21,25 and 28. Assigning the first instar a value of one, the second two etc and pupae a value of 7, the 'average instar' for each genotype on each scoring occasion is presented in Figure 9. While in the first experiment there may have been a tendency for the resistant insects to develop slower, in the second experiment the reverse situation occurred but for both experiments any differences in growth rate were minor.

Experiment 1

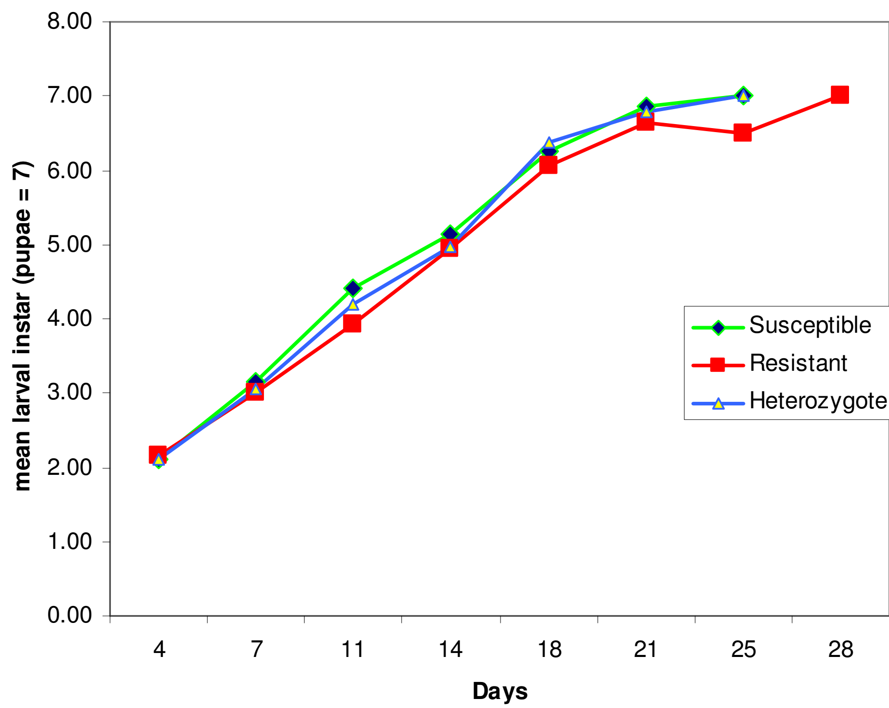
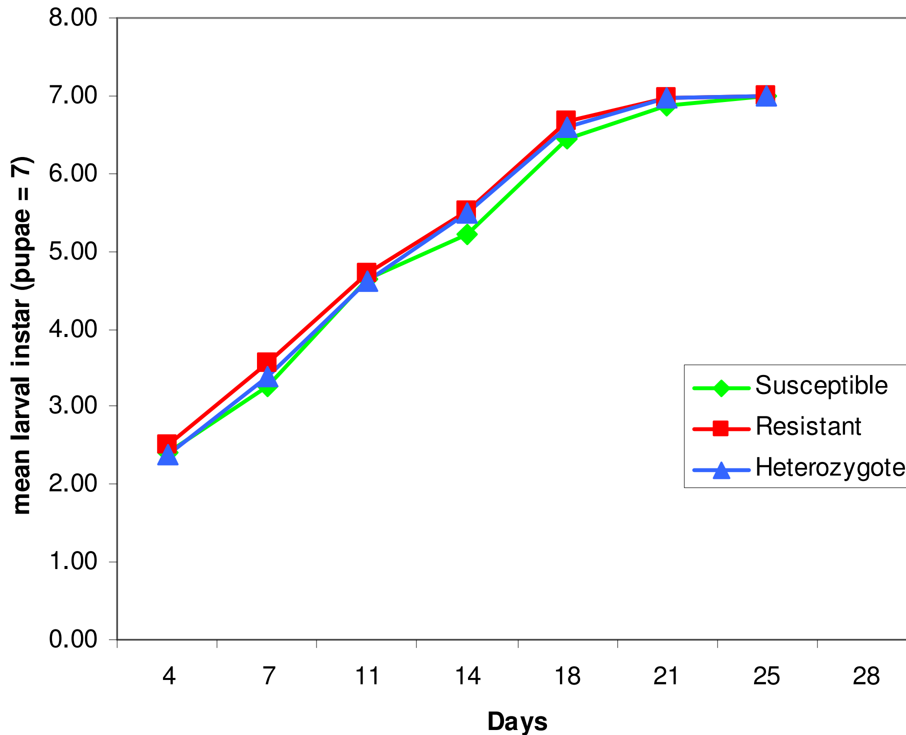


Fig 9 Growth rates of RR SS and RS genotypes on Pigeon pea.

Experiment 2



- *Assess possible fitness costs associated with Cry2Ab resistance in the field derived strain (SP15) when exposed to temperature stress. The survival and development of individuals of each genotype (susceptible, heterozygote and homozygote) will be assessed at each of three temperature regimes (low, medium, high). Large numbers of insects will be assayed in order to provide the power to detect slight differences in fitness and allow robust statistical analysis of the data. The experiment will be repeated once.*

An assessment of the fitness costs associated with Cry2Ab resistance in the field derived strain (SP15) when exposed to temperature stress was investigated in two experiments using high, medium and low temperature regimes. Both experiments yielded very similar results and for brevity only the second experiment will be considered here. Insects were reared on standard diet.

Mean pupal weights (± SE) for each genotype and temperature treatment are presented in Table 8 . Clear differences between the treatments are evident and pupae were larger for all genotypes at the medium temperature. Within each treatment, the heterozygotes were largest but the difference between them and RR were not significant. Pupal size of SS genotypes was smallest for each treatment; significantly smaller than RS on all occasions and smaller than RR for the hot treatment. There are no indications of fitness costs exhibited by RR or RS for this parameter.

Table 8 : Stress expt 2 Comparisons of pupal weights of cohorts of SS, RR and RS exposed to cool medium or hot temperartures. Different superscript letters indicate that means for genotype for that treatment are significantly different. N ≈ 100 for each genotype at each temperature.

	Genotype Pupae wt (mg) ±SE		
Treatment	**SS**	**RR**	**RS**
cool	**367.9[a] ± 2.78**	**371.8[ab] ± 2.79**	**375.9[b] ± 2.97**
medium	**393.7[a] ±3.06**	**395.9[ab] ±2.87**	**401.5[b] ±2.31**
hot	**317.1[a] ±2.82**	**338.4[b] ±3.41**	**340.7[b] ±2.55**

[a, b] Means with common superscript are not significantly different (P > 0.05).

The time taken for larvae to reach pupation for each genotype and temperature treatment are presented in Table 9. A similar picture to that described above for the pupal weight emerges. Clear differences between the treatments were expected and are evident with the fastest development occurring at the higher temperatures. Within each treatment, SS genotypes exhibited the slowest development rate. Interestingly, at the medium temperature regime the time to pupation was significantly different among all genotypes. Again, there are no indications of fitness costs for either RR or RS individuals on comparison of this component of their lifecycles.

Table 9: Duration of larval development for each genotype under three temperature regimes.

	Genotype Time to pupation (days)±SE		
Treatment	**SS**	**RR**	**RS**
cool	**29.4[a] ± 0.31**	**27.46[b] ± 0.38**	**28.14[b] ± 0.31**
medium	**15.89[a] ±0.18**	**14.51[b] ±0.13**	**15.52[c] ±0.16**
hot	**10.77[a] ±0.09**	**10.61[ab] ±0.09**	**10.46[b] ±0.06**

The growth rate of each genotype and temperature regime are presented in Figure 10
Part of the information presented in this figure is captured in the analyses on time to pupation (Table 9) however from inspection of Figure 10 it is clear that for all treatments, the development rates for early larval stages are remarkably similar for the genotypes at each treatment.

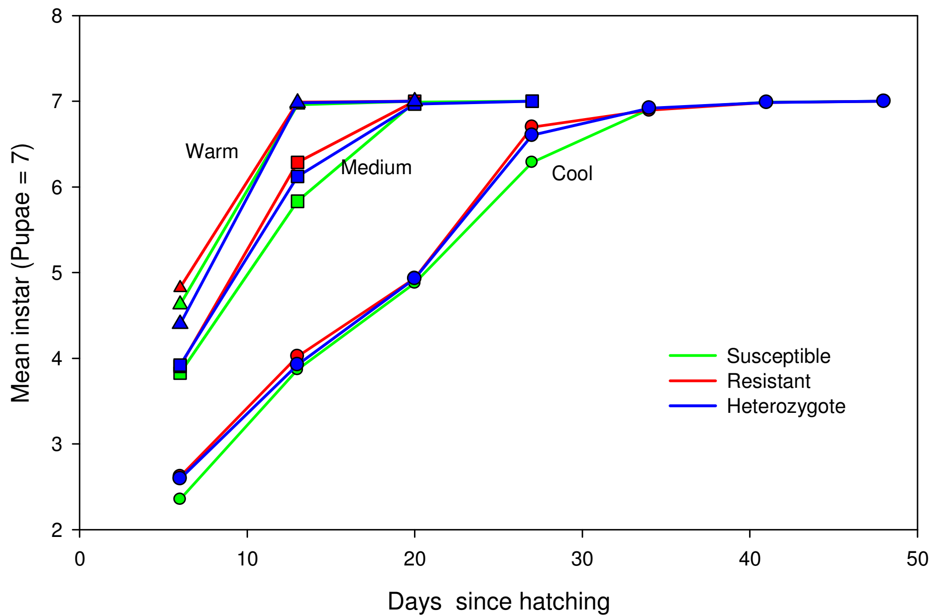


Figure 10 Growth rates of RR SS and RS genotypes when reared under three temperature regiemes, cool medium and warm.

- *To assess the extent of dominance of a field-derived allele that confers resistance to Cry2Ab toxin when in a field genetic background. This involves the progressive replacement of laboratory genome (where the allele has proven to be recessive) with that from field collected H. armigera where the allele may be only partially recessive.*

The first experiment to investigate this hypothesis has been completed. Isofemale lines of the resistant strain, SP15 that had 75% of their genetic background replaced with field material were assayed on a screening concentration of toxin as an F_2 generation. Similarly, the control isofemale lines (lines outcrossed to the susceptible strain GR rather than field material) were also screened. The results (Figure 11) are not conclusive but significantly more variation ($F_{(20,17)} = 5.71$; $P<0.05$) was seen in the field lines (N = 21) with between 0 % and 23% of F_2 larvae surviving the screen as 3rd or 4th instar larvae. The expected proportion is 6.25% for each line. Interestingly, the two field lines showing the highest survival (22 and 23%) were the only lines which also exhibited increased survival as F_1 larvae where we expected (50:50 heterozygotes: susceptible ratio), on a screening concentration of toxin. In the laboratory-based iso-female lines (n = 18) the results were more uniform and the range was 0% to 10.8%, which is within the expected range (Figure X). This expected distribution was also observed in the mass-mated lines with field and laboratory-based backgrounds (range: 0 – 11%, N = 15). One concern that arose from these experiments was the frequency of lines where the resistant allele was not detected. This implies that the F_2 screening system would also 'miss' some instances of resistance. This question will receive further attention in future experimentation.

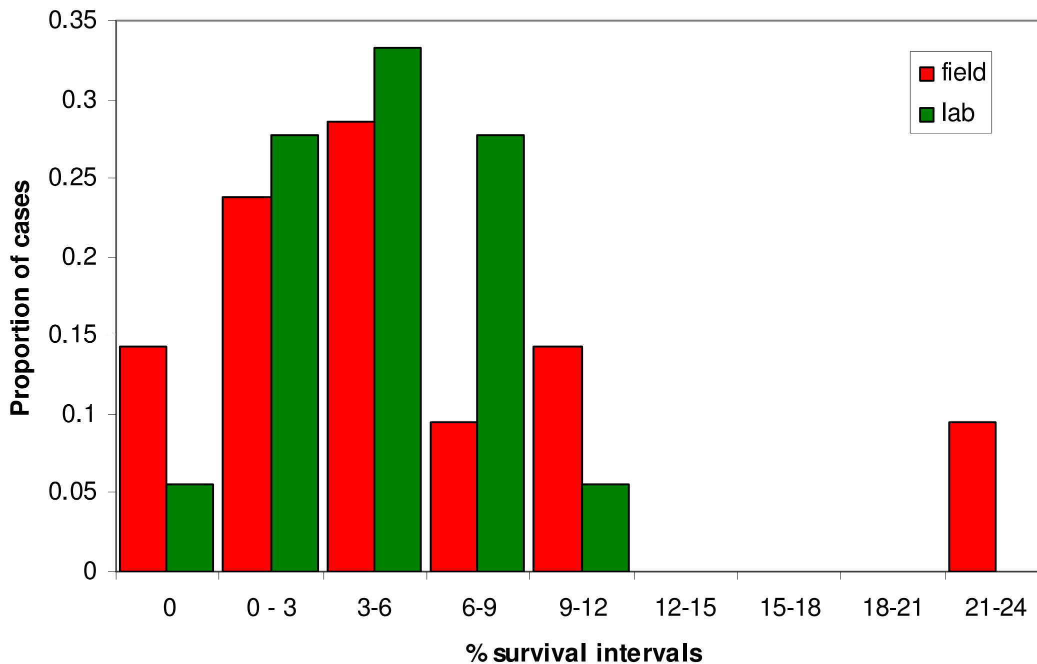


Figure 11: Distribution of survivors in isofemale lines with and without genetic backgrounds derived from the field or laboratory colonies.

Molecular and biochemical investigation of Cry2Ab resistance.

- *Determine if Cry2Ab binds specifically or non-specifically to* H. armigera *midgut tissues.*

Attempts to ascertain if Cry2Ab binds specifically or non-specifically to *H. armigera* midgut tissues were thwarted by the very high level of non-specific binding by Cry2Ab. Because there is substantial non-specific binding to the membrane used in the ligand blot (probably to materials from the polyacrylamide gel), we could not determine if specific binding occurs. We found no evidence for a difference in Cry2Ab binding to BBMV proteins of SP15 and GR when we tested binding in ligand blots. Attempts to identify binding proteins on two dimensional gels by ligand blot have been unsuccessful.

We sought to enlist the help of a Spanish colleague, Dr Juan Ferré who is internationally recognised for his Cry toxin binding work. However he was unable to assist as he too has encountered the same problem. Because attempts to block the non-specific binding to the membrane were unsuccessful, this line of research was discontinued.

- *Compare the gut protease profiles of TABOC, SP15 and the susceptible strain and their effect on the Cry2Ab toxin.*

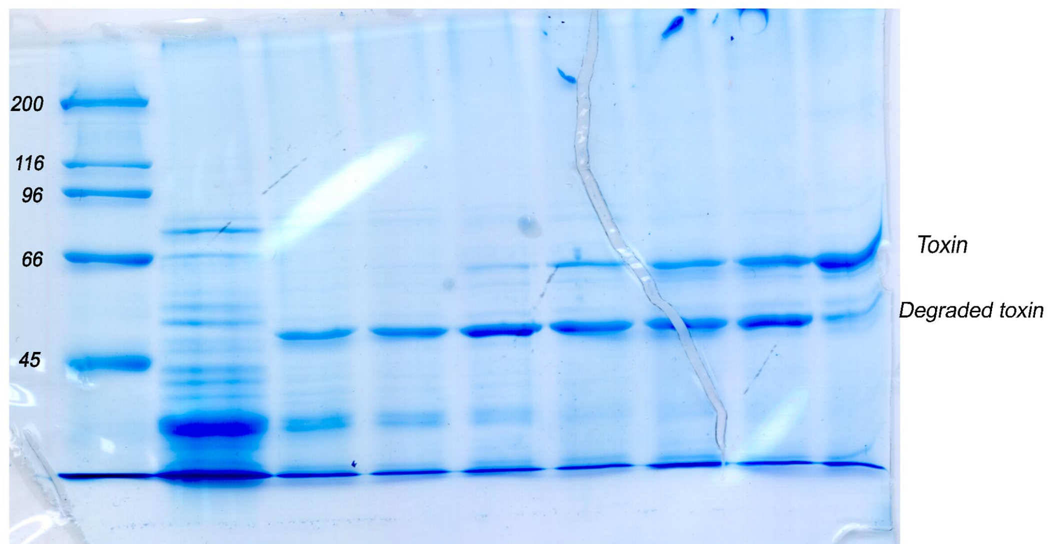
The digestion of Cry2Ab protein by the Cry2Ab-resistant TABOC strain and the susceptible ANGR strain were compared. An extra (35kDa) band was detected in the TABOC strain. However, this proved to be unrelated to resistance as, although it was absent from ANGR, it

was seen in two other Cry2Ab-susceptible strains. Western blotting showed that all of the Cry2Ab was precipitated in the gut juice of both TABOC and ANGR, in contrast to Cry1Ac, which was present in both supernatant and gut juice precipitate of ANGR. There was a small, quantitative difference between TABOC and ANGR in some bands on the western blot. However, it seems unlikely that this difference could account for the resistance.

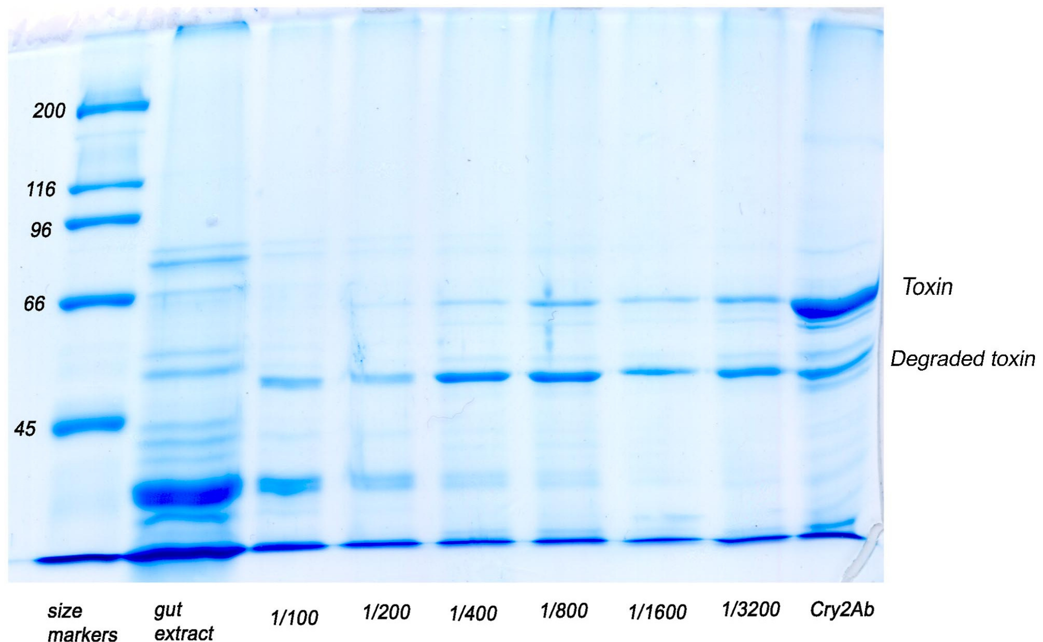
We also compared the rate of digestion of Cry2Ab protein in GR and SP15 and comparative gels are presented in Figure 12. Digestion appeared to be subtly more effective in SP15 than GR. Note the amount of degraded toxin at all concentrations of the SP15 gut juice relative to that from GR in the figure.

Figure 12: Digestion of Cry2Ab toxin by gut juices from GR and SP15 larvae.

GR



SP15



- *Identify proteins expressed at different levels in Cry2Ab-resistant and -susceptible strains by the use of proteomics.*

We have undertaken a proteomic analysis of SP15 and GR after repeated backcrosses so that the both strains share most variability. A variety of differences between resistant and susceptible strains were detected.

Proteomic comparisons of SP15 (Cry2A-resistant) and the near-isogenic susceptible GR strain have revealed six differences (Figure 13). Although most take the form of differential expression, one appears to be a shift in mass or a complete loss of expression in SP15. It is not immediately evident that any of the differentiating proteins are the primary cause of resistance. We need to examine further resistant and susceptible lines to establish a correlation with the presence of such differences and resistance. If the correlation 'holds up' we hope to determine through the use of MALDI-TOF equipment (Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry) the types of proteins that differ in the two strains, and thus home in on more likely candidate proteins that might be associated with the resistance.

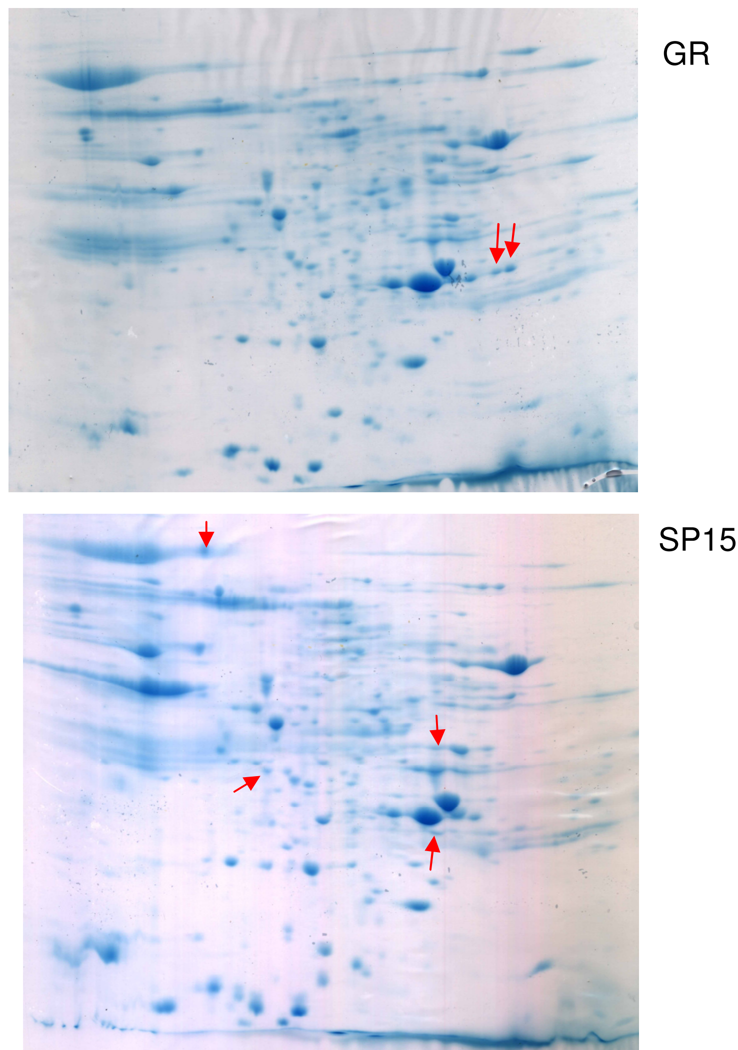


Figure 13. Two dimensional separation of proteins present in BBMV from GR and SP15. Arrows indicate differences in the two gels.

- *Determine the extent to which the Cry2Ab toxin is sequestered in TABOC, SP15 and the susceptible strain.*

Other researchers have reported that insects with altered gut chemistry can avoid intoxication by Bt toxins. We have found that gut extracts of *Helicoverpa* can precipitate Cry2Ab protoxin and it is possible that precipitation may change the mode of action/efficacy of the toxin. We explored that possibility by comparing Cry2Ab precipitate from gut extracts of our resistant (SP15) and susceptible (GR) strain and found that the gut extract of SP15 has an increased capacity to degrade the Cry2Ab toxin compared to the susceptible strain (above). This confounds efforts to compare levels of sequestration. We have also measured the levels of proteinases but have found no marked differences between SP15 and GR.

- *Investigate a possible association of the Bre-5 gene with Cry2Ab resistance in H. armigera.*

This objective replaced the enhanced repair mechanism investigation because of a redirection of effort to pursue the Bre-5 gene after a possible association with Cry2Ab resistance in *H. armigera* was established in a previous project, CSE 108C (see 2006 final report for that project).

It has been shown that a mutant form of the Bre-5 (Bt-Resistance) gene in *C. elegans* leads to resistance to a Bt toxin. The Bre-5 gene produces an enzyme that attaches a carbohydrate to the gut surface proteins and lipids and this carbohydrate is necessary for Bt toxin binding. In an earlier CRDC-funded project, we established that both the Cry2Ab resistance and an equivalent gene to BRE-5 maps to the linkage group (5), suggesting that Bre-5 may also be responsible for Cry2Ab resistance in *H. armigera*. If true we would have been able to develop a powerful tool to detect Cry2Ab resistance. To explore this relationship we sequenced a 420 base pair region of the Bre-5 gene (Figure 14) that linkage analysis found differs between a susceptible (GR) and resistant (SP15) colonies. Individuals from our three Cry2Ab resistant strains (SP15, SP202 and SP566), from our lab susceptible strains and from field-collected *H. armigera* were examined. While we found some DNA sequence differences (that result in 3-4 amino acid changes) between the susceptible and Cry2Ab resistance strain, the association between Bre-5 and resistance was not confirmed. More recent data indicating recombination between Bre-5 and resistance indicates that Bre-5 while close to the gene conferring resistance may not be the actual gene. Our colleague David Heckel continues to pursue the gene responsible.

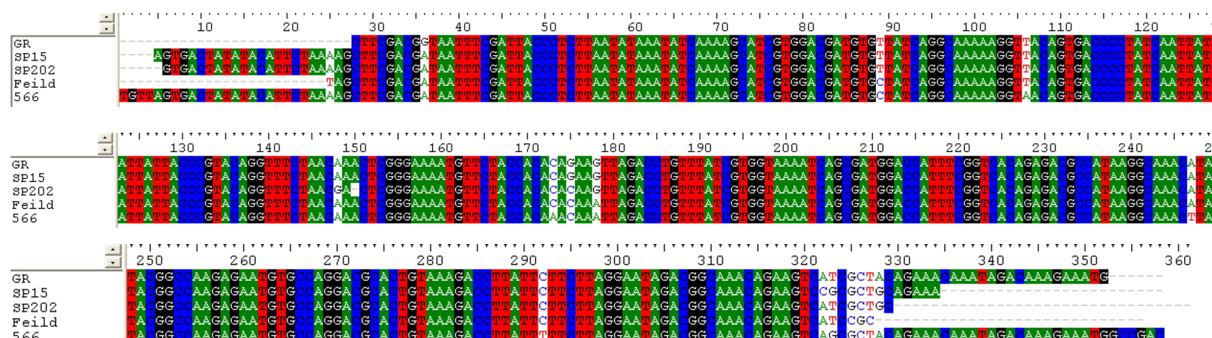


Figure 14. Sequences of a 420 base pair region of the Bre-5 gene in five individual *H. armigera* GR, SP15, a field insect, SP202 and 566 (two Cry2Ab isolates from the field). Note the variability (in the uncoloured regions of the alignments) but the variability is not confined to resistant insects.

- ***Prepare the results of the experiments for publication.***

Several papers, that in part include the work performed in this project, have been accepted for publication in international scientific journals, presented at conferences or published in the Australian Cotton Grower. They are listed in a later section of the report. An additional manuscript (attached) is presently under review that presents data on the allelisim tests of the isolates of Cry2Ab resistance and several other manuscripts are at various stages of preparation.

Outcomes and Conclusion

Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

The pyramided Bt toxins in Bollgard II are expected to ensure this variety of cotton is resilient to the development of resistance by *Helicoverpa* spp. Throughout the period Ingard was deployed in Australia, the focus of the resistant management strategy was to ensure that Cry1Ac resistant individuals remained rare until a two-toxin variety became available. While the resistance management plan, (limits on growing Ingard; the use of refuges; pupae busting; restrictions on planting times etc), appears to have achieved that objective, the frequency of Cry2Ab resistance in *H. armigera* populations is far more common than anticipated. At the time this project was initiated, we had isolated resistance on three separate occasions. Now CSIRO and Monsanto have isolated a total of 17 instances of Cry2Ab resistance.

This project focused on determining key characteristics of the resistance and the fitness of homozygous and heterozygous resistant *H. armigera* relative to the fitness of susceptible insects. SP15 was the first Cry2Ab-resistant colony established and much of our work has focused on this strain. During this project, and in part a previous project, the resistance present in SP15 was been characterised. We now know many of the important features of this form of resistance that will affect the rate of evolution of resistance. These are:-

- SP15 proved to be highly resistant to Cry2Ab. Larvae would readily cope with the level of that toxin present in Bollgard II.
- SP15 was fully susceptible to Cry1Ac. If at the time *H. armigera* eggs are laid on Bollgard II the expression of the *cry1Ac* gene was optimal, larvae would be very susceptible to this toxin in Bollgard II.
- SP15 was recessive in the laboratory. In contrast, when it and subsequent resistant colonies were first isolated, they behaved as if the resistance was partially dominant. The laboratory studies of SP15 and other resistant strains were conducted after they had been repeatedly outcrossed to a laboratory susceptible colony. It is possible that the discrepancy is a consequence of the different genetic backgrounds i.e. once outcrossed, the constellation of genes from the laboratory colony that replaced the field genetic

background modified the dominance. We have begun to investigate this hypothesis. Dominance is critical, because even if heterozygotes have a slight advantage over susceptible insects, the rate of evolution of resistance is greatly increased.

- Resistance in SP15 is due to a single gene. Furthermore, all of the isolations of Cry2Ab resistance thus far tested (N=5) carry variants at the one locus (gene) that confer resistance. If, as we speculate, the as yet untested isolates are also allelic, the frequency of resistance is more common than the alternative option where there were many genes conferring resistance.

SP15 has no detectible dominant or recessive fitness costs. We exposed individuals of the three genotypes to a range of differing environments, including different diets, conventional cotton, Bollgard II, and pigeon pea, differing temperature regimes and diapause conditions. On occasions, minor differences in fitness parameters were detected however there were no consistent differences that could be assigned to fitness costs. On some occasions, the resistant genotypes proved to be more fit than the susceptible insects and on other occasions the reverse was observed. This variability was assigned to subtle differences in the vigour of the colonies that is a regular feature of Lepidopteran cultures.

Normally, but not invariably, resistant forms of genes are deleterious in the absence of the toxin /insecticide that they are resistant to. It is unfortunate that the SP15 form of resistance exhibits no fitness costs. Had such costs been present it would have tended to oppose increases in frequency of the allele that may occur in the presence of the toxin (ie Bollgard II). Without costs, when opportunities occur for resistant insects to increase in frequency relative to the susceptible genotypes (say on late-season Bollgard II) the frequency will remain stable at that new level until the next opportunity arises. There will be no decline in the frequency when larvae are not exposed to the toxin either in mandated or unstructured refuges.

The expected outcome of this project was enhanced knowledge of Cry2Ab resistance. Based on these findings, if it was necessary, recommendations would have been made to modify the resistance management strategy for Bollgard II. The above summary of what we have learned leads the authors to believe that while we remain concerned, we do not recommend modification of the RMS at this stage. However our findings do focus attention on the critical role played by the resistance monitoring program in detecting any increase in frequency of Cry2Ab resistance. If rapid changes in frequency are detected the RMS should be flexible enough to respond.

The research on mechanisms of resistance hoped to provide the basis for the development of diagnostic tools and in part has given indications of where to focus our attention. This work is continuing.

1. Please describe any:-
   a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);
   b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and
   c) required changes to the Intellectual Property register.

Acknowledgments

The assistance from our colleagues Sharon Downes and Trudy Staines (ACRI) have provided tremendous support for this project through discussions and by providing field material for analysis in Canberra. The technical team has great experience and skills and it is fitting that they are acknowledged as authors. Additional expert assistance was given by Lisa Bird, Corrine Philips, Mick Neave and Cathy Smith.

Extension Opportunities

2. Detail a plan for the activities or other steps that may be taken:
   (a) to further develop or to exploit the project technology.
   (b) for the future presentation and dissemination of the project outcomes.
   (c) for future research.

Dr Mahon has presented information on Bt resistance issues at industry meetings on many occasions during the course of this project and expects to continue to do so. The Bt group also has contributed a number of articles to the Australian Cottongrower on aspects of our research. More formal publications in international literature have been produced (listed below) and additional papers, based on the data acquired during the project, will appear over the next 12 months.

8. A. List the publications arising from the research project and/or a publication plan.
   (NB: Where possible, please provide a copy of any publication/s)

<u>Peer-reviewed journals/books</u>

Mahon, R. J., K. M. Olsen, K. A. Garsia, and S. R. Young, 2007. Resistance to *Bacillus thuriengensis* toxin Cry2Ab in a strain of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Australia. *J Econ. Entomol.* 100: 894 – 902.

Downes, S.J., R. Mahon, and K. Olsen, 2007. Adaptive resistance management in Australia for Bt-cotton: current status and future challenges. *Journal of Invertebrate Pathology* 95: 208 - 213.

Mahon R., K. Olsen, S. J. Downes, and S. Addison, 2007. Frequency of alleles conferring resistance to the Bt toxins Cry1Ac and Cry2Ab in Australian populations of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *J. Econ Entomol* (in press).

Bird, L.J. and Akhurst, R.J., 2005. The fitness of Cry1A-resistant and -susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on transgenic cotton expressing sub-optimal levels of Cry1Ac. *J. Econ. Entomol.* 98(4):1311-1319.

Liao C., Brooks, L., Trowell, S.C. and Akhurst, R.J., 2005. Binding of Cry δ-endotoxins to brush border membrane vesicles of *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae). *Insect Sci.* 12, 231-240.

Ma, G., Roberts, H., Sarjan, M., Featherstone, N., Lahnstein, J., Akhurst, R. and Schmidt, O., 2005. Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactivated by a coagulation reaction in the gut lumen of resistant *Helicoverpa armigera* larvae? *Insect Biochemistry and Molecular Biology* 35: 729-739.

Olsen, K.M., J. C. Daly, H. E. Holt and E. J. Finnegan, 2005. Season-long Variation in Expression of the Cry1Ac Gene and Efficacy of Bt Toxin in Transgenic Cotton against Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae). *J. Econ. Entomol*. 98: 1007-1017.

Olsen, K.M., J. C. Daly, E. J. Finnegan and R. J. Mahon, 2005. Changes in Cry1Ac Bt Transgenic Cotton in Response to Two Environmental Factors: Temperature and Insect Damage. *J. Econ. Entomol*. 98: 1382-1390.

Akhurst, R. and Bird, L., 2005. Managing resistance to *Bacillus thuringiensis* toxins. In: Ngo, D.B., Akhurst, R.J. and Dean, D.H. (eds). *Biotechnology of Bacillus thuringiensis, Vol. 5.* Science and Technics Publishing House, Hanoi. 247-258

Liao C., Trowell, S.C. and Akhurst, R.J Akhurst., 2005. Purification and characterisation of Cry1Ac toxin binding proteins from the brush border membrane of *Helicoverpa armigera* midgut. *Curr. Microbiol.* 51, 367-371

Conference papers:

Mahon, R.J., Olsen, K., Young, S. and Downes S.J. (2006). Resistance to Cry1Ac and Cry2Ab toxins in Australian populations of the cotton bollworm *Helicoverpa armigera* Beltwide Cotton Conference San Antonio, January 2006.

Bird, L. and Akhurst, R. (2005). Variability of fitness costs associated with Cry1A resistance in *Helicoverpa armigera* on cotton and alternative refuge crops. XXXVII Ann. Meeting, Soc. Invertebr. Pathol., Anchorage.

Downes S.J, Mahon R, (2005) What is the current situation for Bt resistance in field populations of *Helicoverpa armigera* in Australia? *Australian Entomological Society Meeting*, Canberra, Australia.

Downes S.J, Mahon R, Olsen K, Young S. (2005). Resistance to Cry2Ab toxin in the cotton bollworm *Helicoverpa armigera* in Australia. *Pacific Rim Conference on the impacts of* Bacillus thuringiensis *on the environment,* Victoria, Canada.

Bird, L. and Akhurst, R. (2005). Host plant effects on fitness costs associated with Cry1A resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae). 6th Pacific Rim Conference on the Biotechnology and Environmental Impact of *Bacillus thuringiensis*, Victoria BC, Canada.

Bird, L.J. and Akhurst, R.J. (2004). The relative fitness of Bt resistant and susceptible *Helicoverpa armigera* - implications for the refuge strategy. XXII Int. Congr. Entomol., Brisbane.

Australian Cottongrower articles:

Rossiter L, Downes S J, Mahon RJ, 2006. Helicoverpa: species mix, parasitism and resistance monitoring. *The Australian Cotton Grower* Dec-Jan:66-69.

Kay, A. Allen, S, Kauter, G Mahon, R. and Bange, M. 2006. Strategies for success at Beltwide. *The Australian Cotton Grower* Feb-Mar:28-32.

Posters:

Mahon R., K. Olsen, S. Young and J. Armstrong (2007). Cry2Ab resistance and fitness costs in *Helicoverpa armigera* in Australia. *World Cotton Research Conference*, Texas, September, 2007.

Olsen, K. M., R. J. Mahon and S. J. Downes (2005). Resistance to Cry2Ab toxin in the cotton bollworm *Helicoverpa armigera* in Australia: Implications for the cotton industry. *Australian Entomological Society Meeting*, Canberra, Australia, December 2005.

Young S. R., K. M. Olsen and R. J. Mahon. (2005) presented at the Combined Invertebrates Conference in December 2005 "Induction of Summer Diapause in Cry1Ac and Cry2Ab Bt Resistant and Susceptible Colonies of *Helicoverpa armigera* in Australia" *Australian Entomological Society Meeting*, Canberra, Australia December 2005.

Ma, G., Roberts, H.L.S., Featherstone, N., Sarjan, M., Roush, R. T., Akhurst, R. and Schmidt, O. (2004). Tolerance to the *Bacillus thuringiensis* endotoxin Cry1Ac in a laboratory *Helicoverpa armigera* strain is based on immune induction, which is transmitted by a maternal effect. XXII Int. Congr. Entomol., Brisbane

Mahon, R.J., K. M. Olsen and K. A. Garsia (2004) Resistance to Cry2Ab in Australian populations of *H. armigera* XXII Int. Congr. Entomol., Brisbane

Mahon, R.J., K. M. Olsen and K. A. Garsia (2004) Resistance to Cry2Ab in Australian populations of *H. armigera* Cotton Conference Gold Coast August

B. Have you developed any online resources and what is the website address?

None.

Part 4 – Final Report Executive Summary

Transgenic cotton has proved to be valued by Australian cotton growers and over 80% of all cotton grown is Bollgard II. The benefits, largely accrued through a reduction in the use of insecticides, have resulted in some cost savings but markedly improved environmental outcomes. A return to a state where 10 sprays per season are required for *Helicoverpa* control would be unwelcome. Yet the industry is presently reliant on only one transgenic insecticidal variety of cotton - Bollgard II. Resistance to the toxins Cry1Ac and Cry2Ab present in this variety is the greatest challenge to transgenic cotton's long term sustainability.

Work by CSIRO Entomology has shown that resistance to Cry1Ac remains rare as we have not yet encountered Cry1Ac resistance in the Bt monitoring program. However, we know that an uncommon but potent form of resistance exists in *H. armigera* populations in China, and it is prudent to assume it is present within Australian populations. Of more immediate concern is the presence of Cry2Ab resistance in Australian populations of this species. We have shown that a variant form (an allele) of a single gene confers resistance to this toxin in *H. armigera*. That allele is present at a frequency of four in every thousand copies of the gene so it is certainly available to respond to selection.

Since isolating this form of resistance in 2002, CSIRO Entomology's 'Bt group' in Canberra and Narrabri has been studying aspects of the resistance to evaluate the threat it poses to the Australian cotton industry. This project has contributed much to our understanding of the Bt resistance. In addition we possessed a laboratory strain called TABOC, that was selected to be resistant to Cry2Ab, and we also examined its characteristics and compared them to the field-derived form of resistance.

We examined five of the seventeen separate isolations of Cry2Ab resistance in *H. armigera* to determine their genetic relationship. For all isolations tested to date, resistance was found to result from alleles at the one locus. As the characteristics such as growth and survival rates of larvae were similar for the remaining isolates, we speculate that that they too may be the same form of resistance. In contrast, the resistance present in the laboratory-selected strain TABOC proved to be the result of variants at differing genes or perhaps a constellation of genes.

We examined the performance of a representative (SP15) of the field-derived form of Cry2Ab resistance when fed Bollgard II. Because SP15 is susceptible to Cry1Ac, it performed poorly on younger cotton, but significantly better than susceptible insects on older cotton; presumably when the Cry1Ac titre had declined. Nevertheless, survival rates of resistant insects was low <10%. Importantly, in laboratory tests and when challenged on Bollgard II, larvae carrying one copy of the 'resistant gene' (heterozygous) proved to be susceptible to Cry2Ab. This is important as had heterozygotes proved to show an advantage, the threat posed by this form of resistance would have been markedly enhanced.

In many instances where insects develop resistance to an insecticide or to a Bt toxin, individuals carrying the resistance are less fit than susceptible ones. Thus in the absence of the selective agent (in our case host plants other than Cry2Ab-expressing toxin Bollgard II) resistant

insects perform poorly. Parameters such as survival and growth rates, fertility and fecundity can be affected in individuals carrying 'resistant genes'. Such 'fitness costs' of resistance tend to retard the evolution of resistance as on non-challenging environments (in our situation, *H. armigera* growing on refuge crops, weeds or alternative hosts) the frequency of resistance declines. We challenged SP15 under a range of conditions aimed to expose the presence of fitness costs – growth on pigeon pea, conventional cotton during diapause and when exposed to different temperature regimes – however, no costs were detected.

Finally, a component of this project concerned the mechanism of Cry2Ab resistance. An unexpected problem was encountered when this was addressed. The purified Cry2Ab toxin proved to be 'sticky' and adhered to cellular material and components of the substrates used to examine its binding. Nevertheless, progress was made in comparing the array of proteins expressed in gut cells that may lead us to identify the causes of resistance.