If you are participating in the presentations this year, please provide a written report and a copy of your final report presentation by 31 October. If not, please provide a written report by 30 September.

Part 1 - Summary Details

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

CRDC Project Number: **03CSE005**

Project Title: Implications of Bt resistance in *H. armigera*

Project Commencement Date: 1/7/2007 **Project Completion Date:** 30/6/2010

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Part 3 - Final Report **03CSE005**

Background

Bt cotton has been widely adopted by Australian cotton growers. Through its insecticidal activity, the current variety Bollgard II largely prevents damage from the bollworms *H. armigera* and *H. punctigera*. There are two key advantages of growing this variety: - 1. A reduction of the risk associated with growing cotton as the cost of insect control is largely 'built in' to the 'technology component' of the seed; and 2. A marked reduction in the amount of insecticide used on crops. The second factor confers great environmental advantages through a reduction of the contamination of soil and streams by conventional insecticides. Reduced insecticidal applications also greatly diminish on-farm and neighbourhood health risks.

The advantages of Bt cotton will be lost if the target species evolve resistance to the Bt toxins present in transgenic plants. This represents the greatest threat to the long-term viability of such varieties. Given natural rates of mutation, large Helicoverpa spp. population sizes and, as Bt crops are widespread, an intense and sustained selective pressure, the evolution of resistance is perhaps almost inevitable. However the rate of evolution (a change in the frequency of resistance driven by selection), depends on a number of factors including: dominance and genetic basis of the resistance; the availability of host plants that do not produce Bt toxin; gene flow and the existence of fitness costs associated with resistance. Perhaps the most important of those is the degree of dominance. We have shown that Cry2Ab resistance in *H. armigera* is recessive, due to a single gene (Mahon et al. 2007a) and fitness costs are at best not marked and may not be present (Mahon and Olsen 2009, Mahon and Young 2010).

As *H. armigera* is a diploid species, a population will consist of individuals of three genotypes; ones that carry two copies of the resistant allele (homozygous resistant –which here we will designate **rr**); one copy of the resistant allele and one of the wildtype or susceptible allele (heterozygotes **rs**) and, individuals that contain two copies of the susceptible allele (homozygous susceptible **ss**). When used to describe resistance, the term 'dominance' is related to the degree that the heterozygotes are resistant. A dominant form of resistance occurs where both the **rr** and the **rs** genotypes are functionally resistant, whereas a recessive form occurs where only the **rr** geotype is functionally resistant. Resistance is often not completely dominant or completely recessive resulting in **rs** individuals with intermediate levels of resistance.

We found that resistance was recessive in laboratory tests (Mahon et al. 2007a), However, when reporting the frequency of resistance among field insects we showed that the frequency of survivors in positive F_2 tests was often greater than expected (Mahon et al. 2007b). Almost exclusively, positive tests occur when one of the field collected insects was heterozygous. Therefore we expect 1/16 of the F_2 larvae tested to be homozygous resistant and survive the F_2 screen. Instead we often found significantly more than the expected proportion. Perhaps, both field-collected insects were heterozygotes, or, one could be homozygous. However the probability

of choosing two rare heterozygotes is extremely unlikely. The probability of such an event can be calculated as we know have estimated the frequency of the Cry2Ab **r** allele (which we will designate p) as 0.0018; and therefore the frequency of the susceptible allele (q) is 1 – p = 0.9982. Thus the probability of choosing two heterozygous (**rs**) individuals to form a single pair = $(2pq)^2$ = $(0.0035)^2$ = 0.000013 which of course is extremely unlikely. Similarly, another permutation of choosing a homozygote (**rr)** individual as one of the pairs is even more remote with the probability = $(\mathbf{p})^2$ = 0.000006. A far more parsimonious explanation of these observations is that some heterozygotes showed dominance and also survived the F_2 tests, thereby boosting the numbers of survivors above the expected 1/16 proportion. Dominance is common for conventional insecticides and has also been documented for several forms of resistance to the Cry1 class of Bt toxins (reviewed in Ferré and Van Rie 2002).

Conflicting evidence on dominance represents a major problem for our understanding of the likely trajectory of the resistant allele while under selection by Bollgard II. If partially dominant, resistant alleles in the population pose a far greater threat to the sustainability of Bt crops than if they are fully susceptible. Therefore a major component of this study was to assess dominance associated with alleles that confer resistance to Cry2Ab in *H. armigera.* In particular to determine if the genetic background (the collection of genes not directly associated with resistance) of laboratory adapted colonies modified dominance inherent in field collected insects to make it more recessive.

Prior to CSIRO's involvement in Bt resistance monitoring, resistance levels were assessed by screening field-collected insects at a 'discriminating dose' of the commercial Bt formulations of either Dipel or MPV. Traditionally, a discriminating dose is one that kills 99% or 99.9% of susceptible insects. Changes in resistance were to be detected by increases in survival of that discriminating dose. MVP contains the single toxin Cry1Ac while Dipel contains a cocktail of various Cry toxins. Such a resistance monitoring program is appropriate if the resistance encountered proves to be dominant, or nearly so. If a form of resistance that is not dominant is present in the population, this monitoring strategy is extremely inefficient at detecting it while it remains at low frequencies. We can demonstrate this by using the frequency of Cry2Ab as discussed above. If Cry2Ab was completely recessive, only the homozygous resistant genotype **rr** will survive the discriminating dose and they would occur at a frequency of 0.0000032. Thus to have a 50% chance to obtain the first survivor at the discriminating dose one would need to screen nearly a million field collected insects, which is far more than could reasonably be collected in a season. The F_2 technique we introduced (Mahon et al. 2007b) replaced the discriminating dose technique as it is far more sensitive because it detects the more common heterozygotes (0.003) and potentially can detect increasing levels of resistance early enough to take remedial action to retard the rate of increase. The F_2 test fulfilled the need to identify resistance early, however as it required test insects to be reared for two generations before a result could be determined, it is exceedingly labour intensive. Therefore the number of tests that could be performed is limited. Once we established Cry2Ab resistant colony (called SP15), we developed

a simpler, quicker and less labour intensive 'F_1' test. At our instigation and using SP15 under a material transfer agreement to Monsanto as a 'tester colony', this test was initially deployed by S. Addison of Monsanto and soon after CSIRO included it as a major component of the monitoring program. The monitoring program continues to employ F_2 tests, as unlike F_1 tests, they will detect resistance if it is due quite different mechanisims / genes than present in the tester colony. However after an extensive series of tests that examined many separate isolates of Cry2Ab resistance that all appear to be due to mutations at a single gene, (Mahon et al. 2008) we conclude that F_1 tests are a valid means to detect the common form of Cry2Ab resistance.

On deploying the F_1 and F_2 tests side by side, it became clear that the frequency of the resistant gene detected in F_1 tests was far higher (approximately 3 X) than that detected in F_2 tests. Clearly if the higher frequency from the F_1 tests provided the most accurate estimate of what was present, the resistance 'problem' is more substantial than initially thought. Therefore the second major aim of this project was to determine the basis of the differing frequencies obtained when using the two techniques.

While the F_1 tests are simpler, quicker and cheaper than F_2 tests, they still require a significant degree of labour associated with rearing the insects in the laboratory and conducting a bioassay. Given recent advances in technology, DNA-based methods to identify resistance should be within reach to facilitate monitoring for resistance among Lepidoptera. For example, a test has been developed to identify variants of the cadherin gene that confer resistance to Cry1Ac in the pink bollworm (Tabashnik et al. 2000). The same cadherin gene has been shown to be involved in resistance to Cry1Ac in Chinese populations of *H. armigera* (Xu and Wu 2008). Thus it was expected that on isolating an instance of Cry1Ac resistance in Australia the same gene would be a likely candidate as a basis for a DNA test. Fortunately Cry1Ac resistance remains rare in Australia although it should be noted that the Bt monitoring program has recently isolated such resistance in *H. punctigera*. An examination of the cadherin gene in that colony is presently underway.

In contrast to the rare resistance to Cry1Ac, Cry2Ab, resistant strains are readily available for *H. armigera* and *H. punctigera* and prior work (CRC108C) established that for at least *H. armigera,* Cry2Ab resistance was associated with a polymorphism located within linkage group 5. Furthermore, within that linkage group, the gene of interest was located 'nearby' to Bre5, which made it an attractive candidate as a counterpart Bre5 gene in the nematode *C. elegans* was known to be associated with resistance to a Cry toxin. Thus, the third component of this project was to continue work towards identifying the gene that causes resistance to Cry2Ab.

Objectives

1. Establish if the presence of a field genetic background influences dominance of Cry2Ab resistance so that heterozygotes are phenotypically resistant.

The experiments were completed however they gave conflicting results. An unexpected observation was that some F_2 tests provided a negative result when they

were known to be positive. This led to a study on mating competitiveness of heterozygotes. That in turn indicated that insects in the laboratory were mating far less frequently than expected, which readily explains both deviations from the expected frequency of survivors in F_2 tests and as detailed below, the occurrence of false negative tests.

2. Determine why F_1 tests detect Cry2Ab resistance more frequently than F_2 tests.

The complex and demanding tests designed to establish why this effect occurs were completed satisfactorily. However the results were less than informative. These data together with the observations mentioned above regarding mating efficiency in the laboratory led to the study of competitiveness of heterozygotes. Poor mating frequencies was explored as a possible cause of reduced frequencies of positives in F_2 tests using laboratory tests and a sample of F_2 tests performed in the Bt monitoring program. It was clear that false negative F_2 test could and will occur, however this may not be the sole cause of the differences in frequencies observed between the two tests. Importantly, the problem identified cannot affect F_1 tests so they provide true estimates of the resistance status.

3. Continue the development of DNA means to detect resistance.

Progress has been made in the identification of the gene conferring resistance to Cry2Ab. The rate of progress will be greatly enhanced through the *Helicoverpa armigera* genome project which has proceeded to a stage that it is becoming a useful tool.

4. Supplementary objectives added during the course of the project.

During projects of this nature information generated by the project itself or externally generates opportunities and demands that cannot be anticipated. As a consequence the project has made additional contributions. These include:- additional F_1 tests to examine hypothesises to explain an increase in Cry2Ab resistance; the acquisition of a Vip3A clone allowing the production of Vip3A toxin; the selection of Vip3A resistant *H. armigera*; partial characterisation of Vip3A resistant *H. armigera* and *H. punctigera*; and, the production of "superbug" a dual resistant colony that will facilitate F_1 testing for resistance to Cry2Ab and Vip3A in *H. punctigera*. A similar *H. armigera* strain is being prepared.

Methods

Methods used in this study have either been published or are mentioned below when discussing the results.

Results

1. Establish if the presence of a field genetic background influences dominance of Cry2Ab resistance.

Two data sets are available for analysis, one was presented briefly in the final report for CSE109 and the second was developed during this project. The results of both are fully analysed here. The objective of each experiment was to create two parallel colonies that were both homozygous for Cry2Ab resistance but differed in 'genetic background'. In this sense, an individual's genetic background is the

constellation of all genes that it possesses that are not specifically the resistance gene. It would then be possible to test the hypothesis that the possession of a field background converts what is a fully recessive Cry2Ab resistance in a laboratory to a partially dominant one.

The first of the Cry2Ab resistant colonies generated to address this hypothesis possessed the genetic background of a susceptible laboratory colony (GR) through multiple outcrosses to GR and reselection for homozygosity in the F_2 generation. A predecessor of this colony was used when initially determining the dominance of the Cry2Ab resistant colony SP15 (Mahon et al. 2007a) which showed resistance is fully recessive. The second colony also started with SP15 but through repeated crosses to recently colonised field insects followed by reselection to homozygosity in the F_2 generation, the laboratory background was progressively replaced with that typical of field insects. Once the replacement had reached a predetermined level (75% after two outcrosses and reselection, 87.5% after three outcrosses) the colonies were out crossed to its appropriate susceptible colony a final time to produce heterozygotes. These heterozygotes (**rs)** were then again crossed to the appropriate susceptible colony, on this occasion as single pairs rather than mass mating between colonies. The latter part of this process mimics what is performed as F_2 tests on field insects in the Bt monitoring program. The offspring from each single pair were selfed (mated among themselves, brothers x sisters) and their offspring, (F_2) scored for the presence of resistance by exposure to toxin. If the hypothesis was correct, we would expect 1/16 of the F_2 to be resistant when resistance was present in the lab colony background and a higher frequency when a field genome was present. When F_2 screens are scored during the Bt monitoring program, insects that reach third or fourth instar after exposure to Cry2Ab toxin are considered to be resistant. However the growth rate of insects is dependant on temperature and to compensate for any differences during the rearing process and for control mortality, the proportion reaching third instar was adjusted based on the proportion of control (not exposed to toxin) insects that failed to reach that stage using a standard formula (Abbott 1925). In some assays control survival and growth was poor. Data were not included in analyses if control proportions reaching 3rd instar did not exceed 80%. The adjusted proportions of the first and second experiments are presented in Figure 1 and 2 respectively. Statistical analyses of the proportions in the two treatments employed the statistical program GLIM (Crawley, 1993) to generate generalised linear models employing binomial error structures.

Figure 1.

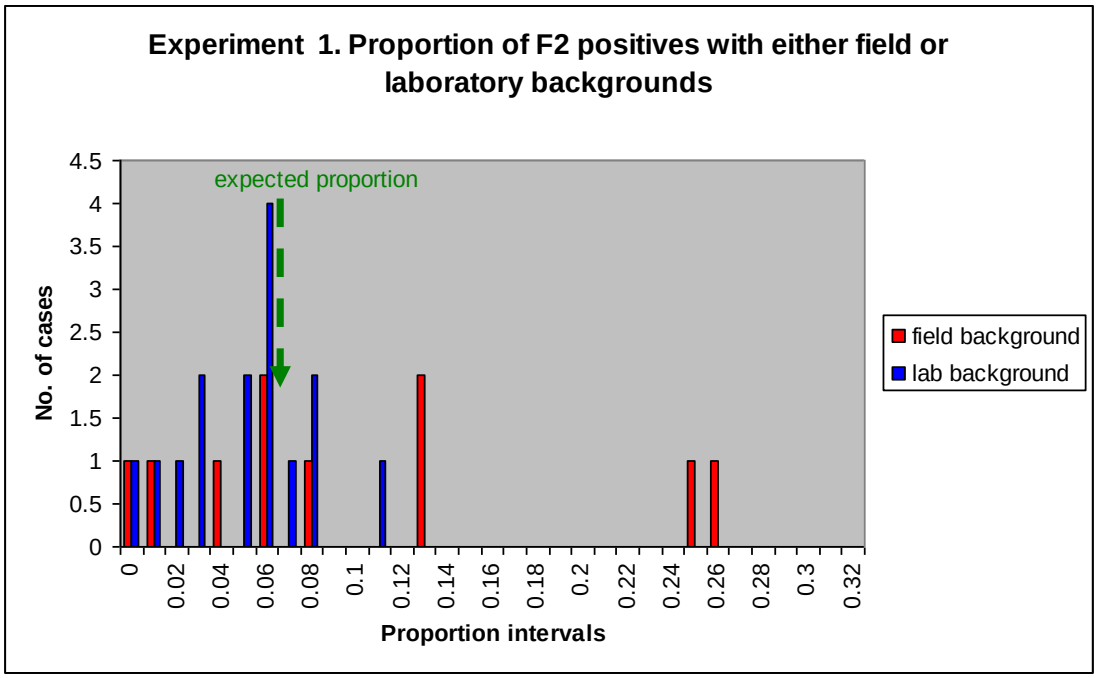
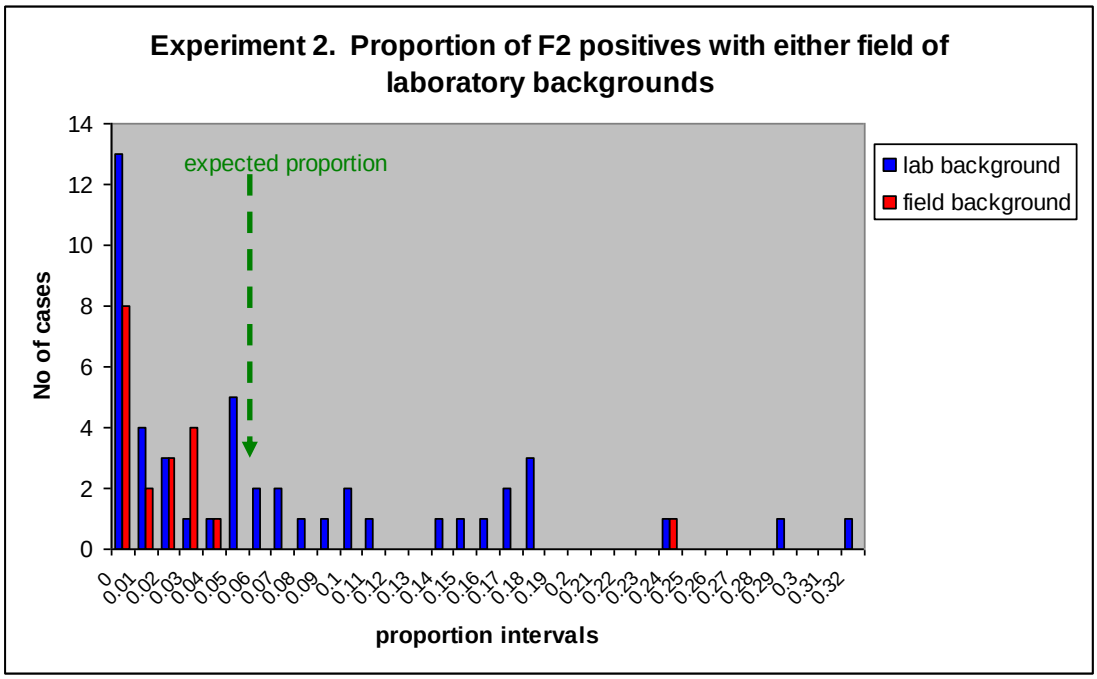


Figure 2.



In the first experiment, individuals with a field background produced a mean proportion survival of 11.97% whereas for those with a laboratory background, the

proportion was 5.01%. The difference is significant ($F_{(1, 23)} = 7.01$, $P = 0.014$) indicating the hypothesis was correct. However on repeating the experiment we found a contrary result. In the second experiment, individuals with a field background produced a mean proportion survival of 2.67% whereas for those with a laboratory background, the proportion was 7.53%. This result is also significant ($F_{(1, 65)} = 7.02$, $P = 0.010$). The basic design of the two experiments was similar, however in the second experiment an additional outcross occurred thus the field insects possessed a greater proportion of field background (87.5%) rather than 75% present in the first experiment. However this is not considered to be the cause of the different result. Helicoverpa colonies isolated from the field often introduce pathogens probably viruses that affect the performance of those strains. Indeed, even the laboratory strains go through periods that may extend over months where they exhibit similar symptoms. In the second experiment, it was clear that the field strain was harbouring such a pathogen and when assayed after 75% of the laboratory background had been replaced (as with the first experiment) control survival was particularly poor. This necessitated an additional outcross to restore vigour before re-testing. Thus we suspect that what should have been an effective protocol to test the hypothesis was unduly influenced by circulating viruses. Given that a correction factor was applied for control survival, that problem should have been overcome, perhaps the presence of a virus reduces the ability of resistant insects to tolerate toxin. As the pathogen was unknown and certainly could not be manipulated, the issue was too complex to take further during this project.

2. Determine why F_1 tests detect Cry2Ab resistance more frequently than F_2 tests.

This component of the project has been completed and a PDF of the recently published paper detailing the work is attached (Mahon et al. 2010). The abstract of this paper is reproduced below and a summary of the paper follows.

> Mahon, R.J. S. Downes, W. James and T. Parker (2010) Why do F_1 screens estimate higher frequencies of Cry2Ab resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae) than do F_2 screens? Journal of Economic Entomology 103: 472- 481.
>
> F_2 and F_1 tests to detect resistance to Cry2Ab in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) were performed during the 2007-2008 summer. F_2 tests indicated a resistance frequency of 0.006, which is similar to the published resistance frequencies for this species during the summers spanning 2002-2006. In contrast F_1 tests indicated a resistance frequency of 0.033. Thus, F_1 tests isolated Cry2Ab resistance alleles almost six-fold more frequently than the F_2 method. A discrepancy might be expected if the F_2 tests detected resistance conferred by more than one locus because F_1 tests identify only the form of resistance present in the tester resistant colony. However, if so, F_2 tests would detect more, not fewer, cases of resistance. In addition, complementation tests on 10 separate isolates indicate that there is only one common form of resistance. We hypothesized that some "resistance alleles" are homozygous lethal if autozygous (as generated in F_2 tests) but not as allozygous homozygotes (as generated in F_1 tests). The hypothesis was extended to accommodate the possibility that alleles at linked loci may be homozygous lethal. Neither of two tests of the hypothesis provided evidence that any alleles that confer resistance are associated with severe fitness costs. Thus we are presently unable to explain the basis of the difference in frequencies between the methods. Because of the simplicity of the F_1 tests, it is difficult to imagine that it overestimates the frequency of resistance and we therefore

accept that this test should provide a more robust method to estimate the frequency of Cry2Ab resistance in *H. armigera*.

The objective was to understand why F_2 tests detected resistance in the same source population at a three fold lower frequency than F_1 tests. We identified two possible explanations. Firstly, perhaps individuals carrying resistance alleles as heterozygotes **rs** were less fit, say unable to mate efficiently, and while scored in F_1 tests where there were no susceptible males to outcompete them, during the F_2 tests such males had to compete directly with susceptible males. If so, the heterozygous male x heterozygous female mating would fail to occur among the first generation of F_2 tests. Such matings were required to produce F_2 offspring that were homozygous **rr** that would survive the Cry2Ab toxin and a positive F_2 would be scored. Secondly, perhaps some forms (alleles)of the resistant gene were homozygous lethal if autozygous, but not as allozygous homozygotes. That statement requires an explanation. In genetic terminology, a homozygous lethal is a form of a gene that when a zygote inherits two copies it proves fatal. An example; if a mutation occurs in the DNA that codes for a vital protein, say an enzyme required for metabolic activity so that when the gene is translated, the enzyme is non-functional. A heterozygote may survive because it possesses one 'good' copy of the DNA and that can code for a functional enzyme. The mutation that confers resistance could be an example. A population may contain a range of versions of the gene (alleles) that possess different mutations (designated as $\mathbf{r}_1$, $\mathbf{r}_2$, $\mathbf{r}_3$ .....$\mathbf{r}_n$). A zygote receiving a combination of different alleles (autozygous) may produce functional enzyme and thus the zygote is viable say $\mathbf{r}_1\,\mathbf{r}_2$ or $\mathbf{r}_3\,\mathbf{r}_5$. However alleles are of the same origin with a common mutation (allozygous) say $\mathbf{r}_1\mathbf{r}_1$ or $\mathbf{r}_5\mathbf{r}_5$ may be fatal. F_1 tests produce autozygous homozygotes while F_2 test produce allozygous homozygotes. This component of the project evaluated the possibility that the presence of allozygous lethals explains the failure of some F_2's to provide positive tests.

In the first part of this component of the project, we tested the resistance status of a series of field-captured males by crossing them to virgin female(s) from a colony homozygous for Cry2Ab resistance (SP15) and then testing their offspring for resistance. This is a standard F_1 test. We know that the allele that confers resistance in SP15 colony (we will call that allele $\mathbf{r}_{sp15}$) is not homozygous lethal, as if it was, a homozygous colony $\mathbf{r}_{sp15}\,\mathbf{r}_{sp15}$ could not be established. Once it was clear that fertile eggs were being produced, the male was recovered and placed with a virgin susceptible female. The latter pairing was dispensed with if the first pairing subsequently failed to yield resistant offspring that implied that the male was not carrying a resistant allele. The second pairing mimicked a F_2 test where one insect of the pair (unusually in this set of experiments we know it is the male) was carrying a resistance allele (we will call this $\mathbf{r}_{field}$) and therefore a positive F_2 test should ensue. If the F_2 tests detected less positives than the F_1 tests (as expected to align with our field frequencies) we can assume that a proportion of positives were missed when scored as F_2's, probably because of fitness costs of some sort. We were able to obtain five pseudo F_2 tests from field-collected males that had earlier proved to be positive

in F_1 tests. Each male also proved positive in the F_2 test. Thus we found no evidence of lethal alleles. Given that we expected three fold more positive F_1 tests than positive F_2 tests this result was unexpected.

The direct test detailed above was only possible on some of the field-derived males and none of the field-derived females involved in F_1 tests. To obtain information from these, a second protocol was developed to utilise insects that proved positive in F_1 tests. For this set of isolates, survivors from positive F_1's must have been autozygous (i.e. $\mathbf{r}_{sp15}\ \mathbf{r}_{field}$) with one resistance allele derived from SP15 and the other from the newly detected **r** allele. The surviving F_1 males were then crossed to GR females and the surviving females to GR males. i.e.

$\mathbf{r}_{sp15}\ \mathbf{r}_{field}$ ♂ x **ss** ♀; $\mathbf{r}_{sp15}\ \mathbf{r}_{field}$ ♀ x **ss** ♂

Pupae produced from these crosses were sexed and allowed to emerge in the absence of the other sex to ensure all were unmated before being set up as single pairs with one of their randomly chosen siblings. The single pairs could be one of four types:

1. $\mathbf{r}_{sp15}\mathbf{s}$ ♂ x $\mathbf{r}_{sp15}\mathbf{s}$ ♀
2. $\mathbf{r}_{sp15}\mathbf{s}$ ♂ x $\mathbf{r}_{field}\mathbf{s}$ ♀
3. $\mathbf{r}_{field}\mathbf{s}$ ♂ x $\mathbf{r}_{sp15}\mathbf{s}$ ♀
4. $\mathbf{r}_{field}\mathbf{s}$ ♂ x $\mathbf{r}_{field}\mathbf{s}$ ♀

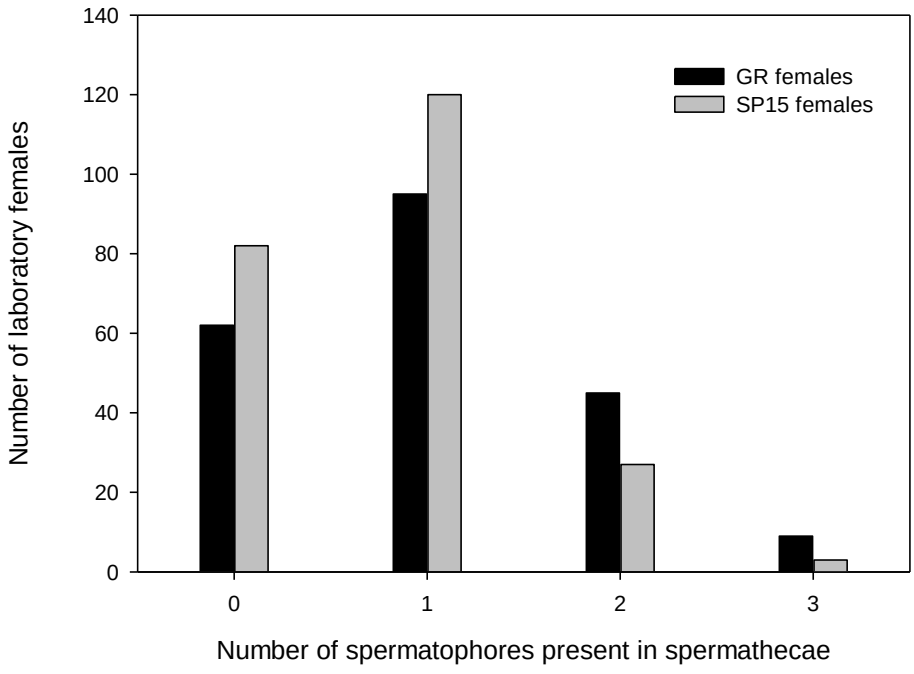
Mating type 4 is the only cross capable of producing the genotype of interest i.e. $\mathbf{r}_{field}\ \mathbf{r}_{field}$ and if that particular field allele is autozygous lethal, should produce no viable resistant insects. Other mating types will yield resistant insects that will be either autozygous (mating type 2 & 3) or the equivalent of SP15 (mating type 1) that we know is not lethal when homozygous. Mating types 1, 2, 3 and 4, should occur at equal frequencies so ¼ of single pairs would be expected to be of type 4. In order to ensure that there was a high probability of encountering a type 4 mating, up to 20 single pairs were evaluated for each separate $\mathbf{r}_{field}$ isolate.

Eight field isolates were examined in this way. For seven of these, no type 4 matings were detected and thus all $\mathbf{r}_{field}$ would be allozygous viable. For one of the tests, 16 single pairs were scored, one of which did not produce resistant insects. However further testing of control insects (ones not exposed to toxin) from that family revealed that this isolate was also homozygous viable.

We concluded that we have no evidence that some alleles are homozygous unviable. In the publication where this work was presented (Mahon et al. 2010) we therefore were left with the alternative that under some conditions matings between heterozygotes are less frequent than expected or some other fitness cost intervenes to limit the production of positive F_2's. However, we have explored fitness costs associated with SP15 (see our previous final report (CSE109)) and our published papers (Mahon and Olsen 2009; Mahon and Young 2010; Lawo et al. 2008) where fitness costs have not been detected, despite extensive study.

Subsequent to publishing the F_1 and F_2 tests comparison work, (Mahon et al. 2010), a new explanation of the differences in detection rates arose. Based on a lead obtained from the work on dominance of Cry2Ab resistance we have developed techniques to enable comparisons of the mating propensity of resistant and susceptible colonies. A honey solution is provided to all caged insects on emergence as a food source. For these tests the honey solution provided to males was 'spiked' with the red dye Rhodamine. Adults feeding on the Rhodamine solution aquire red markings in their soft tissues, especially when viewed by an appropriately configured microscope and UV illumination. Spermatophores passed to females by dyed males when mating are also marked. Therefore, following exposure to a mix of marked and unmarked males, it is possible to dissect female spermathecae and differentiate between marked and unmarked spermatophores to evaluate the mating success. *H. armigera* females can mate on multiple occasions, so a spermatheca may contain more than one spermatophore. Competition cages were established where unmarked females were exposed to an equal mix of marked and unmarked males of different genotypes, and after 48 h, females were killed and their spermathecae examined. Replication of this experiment is underway and when complete, the results will be published separately. However, in the first experiment, both GR and SP15 females were unexpectedly mated infrequently, with many females failing to mate altogether (see Figure 3).

Figure 3.
Frequency of mating by females from two laboratory colonies, GR and SP15.



From Figure 3 it can be seen that a proportion (29% of GR and 35% of SP15) females failed to mate. Based on experience conducting F_1 tests, field females mate less frequently than laboratory adapted females such as GR and SP15. This is not surprising as conditions in the laboratory would be alien to field insects. Therefore, it

is reasonable to expect fewer wild females to mate than found in the above experiment, so it was considered appropriate to explore the impact of limited mating to the outcome of F_2 tests.

The steps of the F_2 test are essentially:-

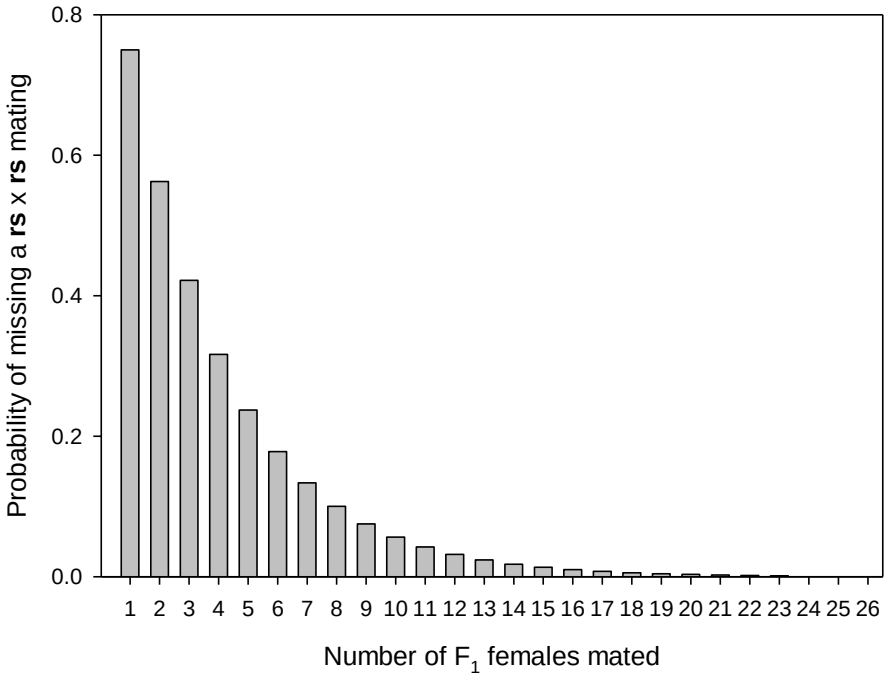
A). mate as a single pair two wild-caught insects;

B). cross their offspring (F_1's - usually a minimum of 20 of each sex) are mated among themselves;

C). screen the neonate offspring (now F_2's) for resistance by exposing them to toxin.

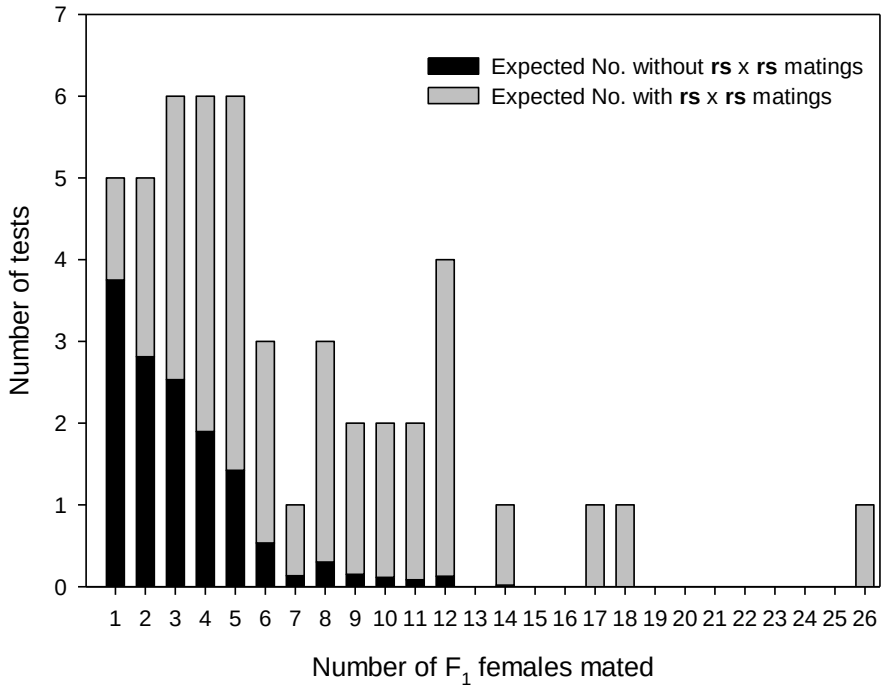
The individuals in step B are only one generation removed from the field so they may mate particularly poorly, and if so influence the probability of detecting a positive in F_2 tests. An example will demonstrate this effect. Consider the situation where either the male or female field insect (step A) was heterozygous for an allele that confers resistance and the extreme example occurs where only one female of the 20 F_1 females mates with one male. As the informative **rs** x **rs** cross (i.e. it will produce a resistant, **rr** insect) occurs in one out of every four matings, (the possible mating pairs are explained above) on 3 out every four occasions fertile eggs are obtained, the F_2 test will be found to produce no resistant insects. Such a test would be assumed to have evaluated two field-collected insects and both would be considered to be homozygous susceptible, whereas the true result should be 3 susceptible and one resistant allele. If two matings occur during step B then the probability of at least one of the matings to be **rs** x **rs** increases and therefore the potential to miss the resistant allele declines (Figure 4). As additional females contribute offspring, the probability of missing the informative cross declines further (Figure 4).

Figure 4.
Probability of missing a mating between two heterozygotes when different numbers of females mate and contribute offspring. It is assumed that one of the field-collected insects was **rs,** the other **ss** which would generate **rs** and **ss** offspring in a ratio of 1:1.



The paucity of matings in the laboratory colonies was established late in the most recent 2009 – 2010 field season. At that stage our CSIRO Canberra laboratory had ceased F_2 screening. However our colleagues Sharon Downes and the Bt monitoring team were still processing F_2's and they kindly scored the number of matings in the prospective female parents (step B) of 59 *H. armigera* F_2 tests. These data provide a profile of the likely mating success in F_2 tests. In 10 of the 59 tests, none of the females mated, and thus no fertile eggs were obtained and obviously no assay could be performed. Of the remaining 49 tests, the number of females fertilised varied from 1 to 26. Twelve of the females were mated twice, which is a minor complication that will be addressed later. If one of the field insects in each pair was indeed a heterozygote, then we can calculate the proportion we expect to detect (Figure 5).

Figure 5.
The distribution of the number of females that mate in step B of the F_2 screens that are:- 1 likely to be missed (lower portion of each bar), 2 likely to be detected (upper portion of each bar), and, 3) the number of fertile females scored for each category is depicted by the cumulative height of each bar.



If, as hypothesised, all 49 tests were from single pairs where one the parents was heterozygote, then the cumulative expectation would be that we would miss 13.56 (28%) of all positive tests. It is evident from Figure 5 that most of these would occur among families where limited numbers of females mated. As mentioned above, in nine of F_2 tests, some females had two spermatophores in their spermathaeca which implies they had mated twice. Among the 1557 *H. armigera* dissected, there were no females that mated more than twice. After recalculating the probabilities to accommodate double matings, the success rate is improved only marginally (expect to miss 12.95 of the positives or 26%). Only a slight improvement was recorded as doubly mated females were largely found in families where significant numbers of step B females mated anyway.

Therefore at least one reason for the difference in efficiency of F_1 and F_2 tests is simply a function of the probability of an informative **rs** x **rs** mating when limited numbers of females mate in step B of the F_2 test. In contrast, the F_1 test is not affected by this problem as to obtain any result from this test, the single female (either a field or laboratory **rr** female) must mate otherwise the test fails. Whether or not this source of error explains all the discrepancy between the two *H. armigera* tests is difficult to say. The error will vary depending on the proportion of females mated in the test. Where a high proportion mate, the result will be accurate and approach the value achieved by the F_1 tests; when few mate the F_2 tests will be less efficient. While

we suspect that some or all of the discrepancy between F_1 and F_2 may be thus explained in *H. armigera* that is probably not the situation in *H. punctigera* as data supplied by Sharon Downes on F_2 tests performed on that species suggest that it mates far more frequently in the laboratory, yet a discrepancy also exists for that species.

3. Continue the development of DNA means to detect resistance.

The final component of the project was to continue attempts to isolate the genes that confer resistance to either Cry1Ac or Cry2Ab in *H armigera*. Published data on Chinese *H. armigera* exists for a form of resistance to Cry1Ac due to the presence of mutations in the cadherin gene coding for a protein found on the surface of cells (Xu and Wu 2008). The mutation prevents the Cry1Ac molecule from binding to the cadherin molecule which is the first step of the pore formation process in the cell membrane that leads to the destruction of the cell and later the death of the insect. Earlier, a similar series of mutations in the cadherin gene of the pink bollworm *Heliothis virescens* had been shown to be associated with Cry1Ac resistance (Gahan et al. 2001). Thus we had an obvious candidate gene to examine if resistance to that toxin was observed during the regular screening of *H. armigera*. To date such resistance has not been observed, however for the last two seasons, the Bt monitoring program has detected several isolates of Cry1Ac resistance in *H. punctigera.* The techniques we expected to use to examine the cadherin gene in *H. armigera* consisted of pairs of primers spanning the cadherin gene to detect deletions/inserts associated with the mobile elements found in both in the Chinese *H. armigera* and *H. virescens* studies. These techniques are now being adjusted to suit the *H. punctigera* cadherin gene and will be applied to survivors of Cry1Ac screens.

The genetic basis of Cry2Ab resistance has also received attention. During an earlier project we attempted to compare binding of the Cry2Ab molecule to brush border membrane vesicles (BBMV) of resistant and susceptible insects. These proved impossible as the Cry2Ab protein was difficult to handle. In our hands, Cry2Ab protein is sticky and either adhered to the walls of containers and cellular debris created during the production of BBMV's or precipitated from solution. An internationally recognised expert in the field, Dr Juan Ferré was also unable to assist as he also encountered the same problems. However in 2009 he advised that he had overcome the technical difficulties. A collaborative agreement was therefore set up between Rod Mahon and Sharon Downes (CSIRO), Juan Ferré (University of Valencia) and Jerone Van Rei (Bayer) to examine Cry2 binding in resistant and susceptible *H. armigera* and *H. punctigera.* The results were recently published (Caccia et al. 2010). Similar to the situation known for Cry1A, Cry2Ab binds to BBMV's of susceptible insects but fails to bind to BBMV's of resistant insects. Interestingly, the failure to bind was found in both resistant *H. armigera* and *H. punctigera* suggesting that the mechanism is similar in both species and although an extrapolation, the genetic basis may also be the same. The implication that a membrane bound protein is involved in Cry2Ab resistance narrowed our focus for the search for the gene of interest. Furthermore, the development of the *H. armigera* genome and the completed silkworm genome are also providing insights and aiding

the search. The current status (September 2010) is that in collaboration with this project, colleagues Dr Tek Tay and Dr Karl Gordon have extended the work that the Bt group and David Heckle performed (CSE108C) and found a mutation in a gene of the Cry2Ab resistant colony SP15 that may be the cause of resistance. Mapping studies confirm that the candidate gene is certainly very closely linked to the gene of interest as there is no observable recombination between it and resistance. However definitive evidence that it is the primary cause of resistance is needed. A decision on that should be available within weeks. If it proves to be the correct gene, the mutation found in SP15 is of a form that should be readily detected in a high-throughput DNA scheme. Such a system would greatly facilitate the scoring of resistance in field populations. The initial practical consequence of such a system would be the simplification of the tasks undertaken by the Bt monitoring program. A longer-term value would be the ability to assay large numbers of insects and therefore undertake fine-scale studies to understand the drivers of the selection for resistance.

4. Supplementary objectives

During the life of this project, additional objectives were addressed. Firstly, during the 2008 Refcom meeting, it was decided that a large data set on the frequency of resistance as detected by F_1 tests was a high priority. The reason behind this was an observed increase in frequency to Cry2Ab resistance in *H. armigera* and the primary cause was suspected to be a decline in the level of Cry1Ac produced by older Bollgard II permitting the survival of homozygous Cry2Ab resistant insects. While *Bt* monitoring was essentially the responsibility of the CSIRO Narrabri laboratory supplemented by contributions by Monsanto's laboratory in Toowoomba, our laboratory also participated. As a result, this project tested 638 and the Bt monitoring project 914 F_1 tests on *H. armigera* during the 2008/09 field season. These data have been analysed and a draft paper is now being prepared (Mahon and Downes. Spatial and temporal distribution of Cry2Ab resistance alleles in Australian *Helicoverpa armigera* and *Helicoverpa punctigera*). The major finding was that heterozygous resistant eggs of both *H. armigera* and *H. punctigera,* especially the latter species were found more commonly on Bollgard II cotton than on alternative crops. This suggests that the level of movement of the occasional insects surviving on Bollgard II to mate with those from non-Bt cotton populations is less than ideal with the offspring of resistant males and females being more frequently found on the Bt crop. In addition we tested the hypothesis that during the latter part of the season higher frequencies of resistance alleles should be detected following a decline in the expression of Cry1Ac. The hypothesis was rejected, as contrary results were found, with higher frequencies being found early in the season when all Helicoverpa are expected to succumb to Cry1Ac. Further work is needed to understand this result but unless a DNA means to detect resistance in far more insects becomes available, the workload to score similar numbers of insects is considered prohibitive.

Another additional objective was undertaken in this project when we developed the capacity to produce Vip3A toxin. During 2009-2010, an effort was made to isolate

a Vip3A resistant colony of *H. armigera*. This followed Monsanto's announcement that Vip3A was to be incorporated into Bollgard III. Using the Vip3A toxin in F_2 tests, Sharon Downes had earlier isolated a Vip3A resistant colony of *H. punctigera*. Thus it became important to establish if similar alleles were present in *H. armigera.* If found, and a colony could be established, we would characterise Vip3A resistance in *H. armigera.* The Bt monitoring team supplied field-collected eggs and employing the F_2 test, two Vip3A resistant isolates (SP85 and SP477) were detected. We have performed complementation tests between the two isolates and established that resistance is due to the same gene. However, before the cotton industry can be confident that this is the only form of resistance present in populations of this species, many more isolates should be subject to complementation tests.

Pooling our 2009-2010 data from this project with those from the Bt monitoring team, it is clear that Vip3A resistance is disturbingly common (0.025 or 2.5%) in field populations of *H. armigera*. Characterisation of the *H. armigera* colonies and the Vip3A resistant *H. punctigera* colony is underway and our early data on dominance (recessive or nearly so) genetic basis (single gene) and cross resistance to Cry toxins (no cross resistance) are being prepared for publication (Mahon Downes and James in prep.). It is now important to establish if Vip3A resistance is associated with fitness costs. Establishing the threat that Vip3A resistance poses to the Australian cotton industry is important. Given its frequency, if there are no evident fitness costs (as is the case with Cry2Ab resistance in *H. armigera*), resistance may evolve quickly after the toxin is introduced. This will be especially critical if the frequency of resistance to Cry2Ab becomes more common than at present in years before the replacement of Bollgard II with the three- gene, Cry1Ac, Cry2Ab and Vip3A expressing Bollgard III.

Many, including the author, anticipate that the Resistance Management Plan (RMP) for the more resistance-robust Bollgard III will be less onerous than the current RMP for Bollgard II. The development of an appropriate RMP for Bollgard III will necessitate a more complete knowledge of the frequency and characteristics of Vip3A resistance particularly concerning fitness costs of the known form of resistance and a comprehensive study to determine if additional forms of resistance occur in field populations.

Since accepting responsibility for the CRDC-funded Bt monitoring program in 2002, our CSIRO Bt resistance laboratory based in Canberra has developed many improvements to the program. Firstly, the commercial formulation of Cry1Ac in the insecticide MPV was replaced with a purer Cry1Ac toxin. The merit of F_2 tests to detect resistance was then established which now provides a far more sensitive method of detecting resistance than the previous reliance on discriminating doses. Then on isolating SP15 using F_2 tests (SP15 was the first Cry2Ab resistant colony of any insect) it became possible to score resistance by F_1 tests which significantly reduced the labour required for F_2 tests. Next, Vip3A toxin was produced in our laboratory enabling F_2 screens for Vip3A resistance. This in turn led to the establishment of a Vip3A resistant *H. punctigera* colony isolated by S. Downes in the Narrabri laboratory in 2008-2009. During the latest season, Vip3A resistant *H.*

armigera colonies were established in our laboratory that now can also be used in F_1 tests. Most recently we have produced an innovation that will further simplify resistance testing. Separate Cry2Ab resistant and Vip3A resistant *H. punctigera* colonies were crossed and their F_2 offspring selected on diet contaminated with both Cry2Ab and Vip3A toxins to produce 'Superbug'. Presently, field-collected *H. punctigera* analysed in F_1 tests are crossed either to homozygous Cry2Ab resistant insects or to homozygous Vip resistant insects to test for resistance. Superbug will simplify that process. Each field insect can be crossed to either a male or female superbug in F_1 tests and all crosses that produce viable offspring can be scored for resistance to both toxins. As field collections and laboratory rearing of collected eggs are both labour intensive processes, the deployment of superbug will reduce the workload of the monitoring program. We have successfully evaluated the efficacy of superbug in the laboratory and expect it will be deployed in the Narrabri F_1 tests during the next (2010-2011) field season. The *H. armigera* equivalent to superbug is now being developed in our laboratory.

Outcomes and conclusions

This project built on earlier work that determined key characteristics of Cry2Ab resistance in *H. armigera*. SP15 was the first Cry2Ab-resistant colony of any insect and much of our work has focused on this strain. Over time, we have established that the characteristics of SP15 are typical of all isolates of Cry2Ab resistance we have examined. In particular, all appear to be due to mutations at the one locus and therefore share the same features. We now know that that gene is responsible for a protein product most likely located on the surface of gut cells that binds to Cry2Ab toxin ingested by larvae (Caccia et al. 2010). Once the toxin is bound, a series of changes occur on the surface of the cell that creates pores in the cell membrane ultimately resulting in the destruction of the cell and subsequently the death of the insect. The toxin does not bind to the mid-gut of resistant insects and therefore they avoid the destructive process.

A major component of this study was to investigate why, when identifying a positive F_2 test; we encounter a greater proportion of homozygous and thus phenotypically resistant insects. If one heterozygous insect was among the two field insects paired to initiate a F_2 test, we would expect $1/16^{th}$ of the F_2 to be homozygous. On occasions we observe many more. The simplest explanation was that the resistance was partially dominant, leading to the survival of heterozygotes. Dominance has been encountered for resistance to Cry1Ac in other Lepidoptera (Ferré and Van Rie, 2002). However in exhaustive tests on SP15 in the laboratory, no evidence of dominance was found (Mahon et al. 2007a). When that study was performed, SP15 had been out-crossed to the susceptible laboratory colony, GR and reselected to homozygosity on multiple occasions. The inevitable consequence of this process was that, with the exception of the gene conferring resistance, and perhaps nearby genes, the entire genome of SP15 was essentially that of GR. GR has been in our laboratory for many years without the addition of field insects, thus it is highly adapted to our laboratory and therefore no-longer typical of field insects. SP15 was therefore also laboratory adapted, however the field insects tested were

not, so it was considered possible that the differing genetic backgrounds may influence the degree of dominance, with the gene conferring resistance expressing dominance when in a field-derived genome. This hypothesis was tested by comparing the number of survivors among contrived F_2 tests where the genetic backgrounds differed, one set with a laboratory background, the other with a recently derived field background. The test proved particularly onerous as the experiments spanned many generations and, partially completed experiments were repeatedly abandoned when field collected insects introduced unknown pathogens. The pathogens caused major mortality and/or poor fitness. Nevertheless two repeats of the experiment were completed but provided conflicting results. In the first, the insects with field genomes did indeed produce more homozygous F_2 than those with the laboratory background. Unfortunately the second experiment produced the opposite result. Thus the experiments were not informative. A puzzling result was that a number of F_2 tests produced no positives despite the experimental design forcing one of the initial pair of the F_2 test to be heterozygous and therefore should have produced a positive F_2 test. To generate a positive test, two heterozygous F_1's must mate to produce functionally resistant homozygotes among the F_2 larvae. This led us to suspect that perhaps the heterozygous males were less competitive when competing for mates than other genotypes. We therefore initiated studies to assess the mating competitiveness of heterozygous and homozygous resistant insects. While this set of experiments is incomplete, it became clear that on occasions few of the F_1s of any genotype mate. If so, distortions in the ratios of matings could readily generate the fluctuations we see in the proportion of survivors. If say only one heterozygous male and heterozygous female mate among the 20 or so males F_1 and 20 females normally used in F_2 tests we would expect ¼ of the F_2 offspring to be functionally resistant. Alternatively, if no matings between heterozygotes occur, but other pairs provide fertile eggs, we would expect no surviving F_2 larvae. That situation was observed and indeed was the trigger for concern about the number of matings. Therefore we conclude that there is no necessity to consider dominance as the reason that on occasions greater than the expected $1/16^{th}$ of F_2 are homozygous and therefore survive the F_2 screen.

The demonstration that all Cry2Ab resistance isolates examined are due to mutations at the same gene (Mahon et al. 2008) justified the deployment of F_1 tests to detect resistance. F_1 tests are simpler, quicker and less labour intensive than F_2 tests deployed to detect new forms of resistance. An anomaly soon became apparent as F_1 tests performed on the same population as F_2 tests yielded different frequencies, with F_1 frequencies being significantly greater. Resolving this anomaly was a major objective for this project. Experiments were designed to determine the basis of the difference. While they were successfully completed, they failed to shed much light (Mahon et al. 2010). However the studies on the mating competitiveness of resistant and susceptible *H. armigera* mentioned briefly above pointed to the possibility that under some circumstances, few laboratory female moths mated. We know that females recently isolated from the field mate more poorly than laboratory adapted females, so this suggested a possible mechanism to explain why F_2 tests might not detect all instances of resistance.

The F_2 process involves three steps. Firstly, a male and a female field-collected insect are mated. Next, their offspring (the F_1) are mated among themselves and, lastly their offspring (now F_2) are screened for resistance. If one of the two field moths was heterozygous for resistance, half the F_1 adults would also be heterozygous. In that case, one out of every four matings between F_1 individuals would be expected to be between two heterozygotes, which is the only cross capable of producing homozygous and therefore functionally resistant offspring to generate a positive result. Thus if matings among F_1 were infrequent, on many occasions false negative F_2 screens would be scored. If matings were common, the probability of the informative cross occurring increases. A sample of the F_1 females from 59 F_2 screens being conducted by the Bt monitoring team in Narrabri was kindly scored for mating to provide a profile of the likely mating success in standard F_2 tests. As anticipated, the frequency of mating among the F_1 females was low, and from those data we could estimate that approximately 26% of F_2 tests would fail to identify a positive test. Further work is required to ensure that this is the only source of error associated with F_2 tests. Fitness costs associated with resistance may also contribute to the discrepancy, although we have yet to detect such costs for Cry2Ab resistance. In contrast, there are no opportunities to generate false negatives during F_1 tests as the two individuals involved in the test (one field insect and one homozygous resistant insect) must mate and provide offspring to yield any result.

While this project has shown that F_2 tests underestimate the true frequency, retention of at least some F_2 tests in the mix of assays performed by the Bt resistance monitoring program is recommended. This is because the F_1 test will only score the forms of resistance we know about and the gene causing the resistance is present and homozygous in the tester colony. Any new forms of resistance caused by mutations in different genes will be undetected by F_1 tests, but most will be captured by F_2 tests. If considered worth the effort, the true probability that a negative test might actually be positive could be calculated by examining the frequency of mating among the F_1 females of each F_2 test.

The deployment of F_2 tests is particularly relevant in the early years of testing for a new form of resistance. Monsanto has indicated that a third toxin, Vip3A will be added to two-toxin Bollgard II to produce Bollgard III which is likely to be deployed by Australian cotton industry in 4 to 5 years. Therefore, the current focus should be Vip3A resistance. We have tested reasonable numbers of separate Cry2Ab isolates obtained through F_2 tests for *H. armigera* and *H. punctigera* and to date only one form of resistance has been detected. Thus there is considerable confidence in employing the F_1 test for that toxin for both species, but as yet, not for Vip3A resistance.

While not among the set objectives of this or earlier projects, for several years the author has been seeking opportunities to examine resistance to Vip3A as it was clearly an important 'backup toxin' if resistance evolved to the Cry1 and Cry2 classes of toxins presently deployed in transgenic cotton. Vip3A is now highly relevant. In 2008, our laboratory developed the ability to produce this toxin and during the 2008-09 field season it was deployed in F_2 tests by the Bt monitoring team. As a result, a *H. punctigera* Vip3A-resistant colony was isolated. During the recent 2009-2010 field

season, the Canberra laboratory undertook to isolate a Vip3A resistant colony of *H. armigera* using F_2 tests. Two such colonies (SP85 and SP477) were established and work immediately began to characterise the resistance present and also in the *H. punctigera* colony earlier established by our Narrabri colleagues.

When our data, and those collected on *H. armigera* by the Bt monitoring team are pooled, we estimate that the frequency of resistance to that toxin at 2.5%. While some small-scale trials by Syngenta of Vip3A-expressing Vipcot™ were conduced during 2003-2004 (Llewellyn, 2008), it is highly unlikely that those plantings provided significant opportunities for selection to elevate the background frequencies of resistance. Thus the detected frequency of 2.5% represents a background frequency untainted by selection from transgenic crops. This value is exceptionally high and some 10 fold higher than the background frequency of Cry2Ab resistance in this species which at the time was considered remarkably high (Mahon et al. 2007b). Further, as discussed above, F_2 tests would underestimate the frequency of resistance; therefore the true frequency is likely to be somewhat higher. The 2.5% frequency for **r** is somewhat ominous as at that frequency, genetic theory indicates that it is unlikely that a marked fitness cost is associated with the resistance. If that prediction proves to be true, given suitable opportunities for selection to occur, Vip3A resistance may evolve quickly. In order to develop an appropriate RMP for Bollgard III, a comprehensive characterisation of Vip3A resistance in both species, including long-term fitness costs studies similar to those performed on Cry2Ab resistant *H. armigera* (Mahon and Young 2010) are merited.

Extension Opportunities

The work reported here continues the flow of inputs made by the Bt groups in both Canberra and Narrabri towards resistance management. For the last 10 years we have provided to the cotton industry relevant information on the frequency and characteristics of resistance to Cry1Ac, Cry2Ab and now Vip3A. These data form the background required to implement an adaptive resistance management program for transgenic cotton. Rod Mahon has presented information on Bt resistance issues at industry meetings on several occasions during the course of this project. Until recently he was an active contributor to the Bt technical panel of TIMS and was a major contributor to the development of a contingency plan for Bt resistance management (Downes et al. 2010). That plan may be implemented if resistance frequencies in either *H. armigera* or *H. punctigera* (particularly Cry2Ab resistance) rise to the extent they become of further concern. Thirteen publications in the international literature by the principal researcher involving Helicoverpa have been produced during the course of this project (listed below). Additional publications, based on data acquired during the project, will appear over the next 12 months.

Publications by the principal researcher during the period this project was active.

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Part 4 – Final Report Executive Summary

This project resolved two long-standing anomalies regarding Cry2Ab resistance in *H. armigera.* The first was the observation that frequently a greater proportion of survivors (assumed to be homozygous resistant insects) were observed in F_2 tests than the expected 1/16. The second was that F_1 tests yield a greater frequency of resistance than F_2 tests. The latter anomaly has created concern as some observers question which test, or indeed do either test, provide reliable data. We have found that both anomalies stem from a common cause, namely that the offspring from single pairs of field-collected insects mate infrequently in the laboratory. As a result some F_2 tests yield a distorted ratio of resistant and susceptible insects. Also when few females mate, the informative mating type (heterozygote x heterozygote) may not occur and a false negative F_2 test is generated. This problem is not shared by F_1 tests as only one round of mating is required (field insect x laboratory homozygous resistant) and if that mating does not occur, viable offspring are not generated and no result can be scored. Thus F_1 tests provide a reliable measure of the frequency of resistance in the population.

While not part of the objectives of the project, studies on Vip3A resistance were also initiated. This proved fortuitous as Monsanto has announced an intention to add the *vip3a* gene to their Bollgard II variety to produce Bollgard III. Our work has permitted the determination of preliminary estimates of the frequency of Vip3A resistance in both species. For *H. armigera* 2.5% of all alleles are of the resistant form and thus resistance is exceptionally high. Preliminary characterisation of Vip3A resistance isolates of both *H. armigera* and *H. punctigera* is underway. Further work to detect and then test through complementation tests additional isolates is required. Other forms of resistance may be present that have quite different characteristics to the ones already examined and therefore pose a different level of threat. Further work is also required to determine if fitness costs are associated with Vip3A resistance before an appropriate RMP can be developed for Bollgard III that causes minimal constraints on growers while protecting the longevity of Bt technology.

When contemplating a RMP for Bollgard III, one key point merits emphasis. The resistance status of Cry2Ab toxin is presently of concern and if the susceptibility of *H. armigera* populations is lost through the evolution of resistance before Bollgard III

is widely deployed, much of the longevity of the new variety will be lost. The existing frequency of Vip3A resistance will ensure that susceptibility to the new toxin will be quickly lost. Then, despite Bollgard III being a three toxin product, it will be rendered the equivalent of a single toxin (Cry1Ac) product, perhaps indistinguishable in efficacy from the early transgenic variety Ingard. Then as functionally a single gene product, a reversion to a more restrictive RMP would be necessary to provide even limited longevity of the susceptibility of *H. armigera* to Cry1Ac. Recently, the Bt monitoring program has identified for the first time several instances of Cry1Ac resistance in *H. punctigera,* so that species would also need to be watched. The possibility that alternative technology companies with a different array of toxins may enter the Australian market is unlikely to offer a practical solution, as all varieties of transgenic cotton already commercialised, or nearing commercialisation, express one or more of the Cry1, Cry2 or Vip classes of toxins derived from *Bacillus thuringiensis*. Cross resistance occurs between different Cry1's for other Lepidoptera (Ferré and Van Rie 2002) and is evident among different Cry2's in *H. armigera* (Mahon et al. 2007a, Caccia et al. 2010). As our isolations of Vip resistance are the first for any species, cross resistance among Vip toxins is yet to be tested, but are also likely. Thus from the authors perspective, in the longer term, if the Cry2Ab resistance situation deteriorates further, the industry should go to whatever lengths necessary to protect the susceptibility of Cry2Ab until Bollgard III becomes available. This might cause pain in the short term, but models of the evolution of resistance incorporating 10 years of data gathered on resistance to the three Bt toxins suggest that the payoff to growers through greatly increased longevity of the Bt technology would eclipse the temporary pain.