FINAL REPORT

For Public Release

Part 1 - Summary Details

CRDC ID: CSE1701

Project Title: Resistance research and monitoring to enhance stewardship of Bt cotton and management of Helicoverpa spp.

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Research Program: 2 Industry

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Part 3 – Final Report

1. Background

The introduction of insecticidal transgenic varieties into the Australian cotton market in the mid-1990's allowed the industry to reduce its pesticide use by more than 90 percent (currently) and is arguably the most important technology the industry uses. However, resistance remains a great threat to the continued availability and efficacy of current and future Bt technologies in Australia. Indeed, CSIRO and Bayer have isolated resistance in the key targets *H. armigera* and *H. punctigera* to all three toxins (Cry1Ac, Cry2Ab, Vip3A) in the current Bollgard 3 varieties. This is set in the context of an emerging global pesticide crisis that could see novel resistant variants of these pests which are selected elsewhere arrive into Australia.

The industry relies on a pre-emptive strategy to slow the development of Bt resistance. This is underpinned by independent monitoring of background resistance frequencies to enable the industry to autonomously respond to emerging issues. Simultaneously, and in collaboration with CSIRO, Bayer runs a parallel Bt resistance monitoring program. In this project, the industry looked to increase its reliance on background frequencies obtained through the program run by Bayer allowing for an increased focus on other high priority stewardship issues related to Bt resistance in *Helicoverpa* species. The project was conducted in the following three parts.

PART 1: Does multiple resistance to Bt toxins in Helicoverpa spp. pose a threat to 3 gene cotton?

(i) Can resistance to multiple toxins be selected?

Currently in *H. armigera* around 5% of individuals in the population are heterozygous (rS) for a Cry2Ab resistance gene or a Vip3A resistance gene which creates a relatively high chance of an insect being resistant to Cry2Ab and Vip3A. Cry1Ac protein level declines as plants age which creates opportunities for selection. We selected dual Cry2Ab/Vip3A resistant colonies (established by crossing Cry2Ab and Vip3A field colonies) over several generations in the laboratory using a low dose of Cry1Ac.

(ii) Is there a disadvantage (fitness cost) associated with being resistant to multiple toxins?

We assessed survival rates of multi-resistant colonies (created from resistant field colonies) on field grown refuge hosts and 3-toxin cotton.

PART 2: Are the frequencies of resistance to 3 gene cotton increasing?

We continued to use the sensitive Cry1A, Cry2A, and Vip3A screens developed previously and prioritised screens against Cry2A and Vip3. This component of the research included support and oversight of monitoring conducted by Bt trait providers. We asked:

(i) Has there been a shift in frequencies of the common recessive resistances?

During 2017/18, we sampled populations of Helicoverpa spp. throughout the industry. We allocated 60% of material to F_1 screens against all toxins, and 40% to F_2 screens (scheduled every 5 yrs and last completed in 2012).

(ii) Do novel dominant forms of Bt resistance exist in field populations of in Helicoverpa spp?

Conducting F_2 screens allowed us to detect any potentially new dominant forms of Bt resistance in field populations as well as isolate novel forms of recessive resistance.

(iii) Is the field performance of Bt technology changing over time?

In 2008 a survey of CCA members determined that 15% of the planted Bt-cotton area briefly carried *Helicoverpa* spp. larvae above threshold levels. Since then the incidence and distribution of larvae has been estimated annually by surveying CCA members. Analysis of this data was part of this project.

(iv) Are molecular tools for Bt resistance validated by monitoring of field populations?

Initially we planned to conduct blind tests of resistance in field collected insects using new molecular tools (developed by CSIRO) vs standard F_1/F_2 screens. However, prior to commencing the work, a sister project was funded (CSE1801) which focusses exclusively on developing a molecular approach to screening that was broader in its scope than the original tool proposed for validation herein. Consequently, to assist with development of the broader molecular tool we examined a number of F_2 and F_1 individuals previously identified as resistant using bioassays during the life of CSIRO's monitoring program. We used two main approaches: (1) screening for previously identified mutations and (2) examining whole genome data for novel mutations. It is anticipated that in 2020/21 the molecular tool developed in CSE1801 will be at a stage that is appropriate for side-by-side validation with current bioassay approaches.

PART 3: What are the characteristics of different variants of Cry2Ab resistance?

We supervised an Indigenous Trainee who used standard laboratory bioassays to investigate differences in the dominance and fitness of 2 different common genetic variants of *H. armigera* Cry2Ab resistance.

2. *Objectives*

No.	Milestone Description	Progress to date
1.1	Assess the effect of low-level selection for Cry1Ac on *H. armigera* Cry2Ab/Vip3A dual resistant lines over time.	Achieved in full.
2.1	In *H. punctigera* assess the survival of multiply resistant heterozygotes and homozygotes on non-Bt cotton relative to susceptibles.	Achieved in full.
2.2	In *H. punctigera* assess the survival of multiple resistant heterozygotes and homozygotes on 3 gene transgenic cotton.	Achieved in full.
3.1	Liaison with CCA to establish collaborators for collection of Helicoverpa spp. material throughout the cotton regions.	Achieved in full.
3.2	Trial the collection of Helicoverpa spp. material using pheromone traps for moths vs eggs at the same selected sites.	Achieved in full.
3.3	Screened Helicoverpa spp. populations using the F_1 method against all Bt toxins deployed in current and imminent products.	Achieved in full.
4.1	Screened Helicoverpa spp. populations using methods to detect any potential novel dominant resistances.	Achieved in full.
5.1	Appropriate questions included in the End of Season CCA Survey, and data analysed for evidence of increasing incidence of survivors over time.	Achieved in full.
6.1	Performed side-by-side screens of resistance with molecular tools vs standard F_1/F_2 screens.	Partially achieved.
7.1	Performed dominance and fitness bioassays on R01 vs R03 variants of *H. armigera* Cry2Ab resistance.	Achieved in full.

3 & 4. Methods and Results

1. Can Cry1Ac resistance be selected for in insects that are already Cry2Ab and Vip3A resistant? YES

To study the potential risk of resistance developing to Bollgard 3 cotton we created a *H. punctigera* colony that is simultaneously resistant to Cry1Ac, Cry2Ab and Vip3A by crossing

colonies resistant to each single toxin and exposing them to all three toxins at once and used this colony to study plant efficacy through the season (see milestone 2 below).

We used F_2 screens to isolate Cry2Ab and Vip3A resistant lines of *H. armigera* that were captured as ongoing colonies. We also identified 3 *H. armigera* families positive for Cry1Ac resistance but none have become an established laboratory line. It therefore has not been possible to create a colony of *H. armigera* with resistance to all three toxins in Bollgard 3 to study the risk of multiple resistance.

To assess whether *H. armigera* could develop resistance to all three toxins in Bollgard 3 (Cry1Ac, Cry2Ab and Vip3A), we therefore exposed our Cry2Ab/Vip3A dual resistant *H. armigera* line (known as DRES and created by crossing HaSP15 with Ha9-477) to low-level selection for Cry1Ac.

The selection was initially set up in Canberra in October 2016 with 2 different cohorts being selected at 15 ng/cm^2 of Cry1Ac (approximately 10 - 20% of the lethal dose for the susceptible line of *H. armigera*, GR). This continued for 4 generations but then died out.

Selection of another 2 cohorts began in July 2017, again at 15 ng/cm^2 of Cry1Ac. After observing an increase in survival after generation 10 in both cohorts, the selective dose was increased to 30 ng/cm^2 of Cry1Ac. At every generation, samples have been collected and stored for future molecular analyses (that are beyond the scope of the current project). LD50 (lethal dose at which 50% of the individuals are killed) was calculated every generation from generation 6.

Figure 1.1 shows that there was an initial increase in LD50 in both cohorts and then some variation around the selective dose of 30 ng/cm^2 of Cry1Ac. However, a dramatic increase in LD50 to over 1000 ng/cm^2 was observed in cohort 2. This dose is about 10 times the LD50 of GR and DRES in the absence of selection. This is > 4 times the discriminating dose for Cry1Ac used in the monitoring program (250 ng/cm^2) and would be considered a positive resistance detection.

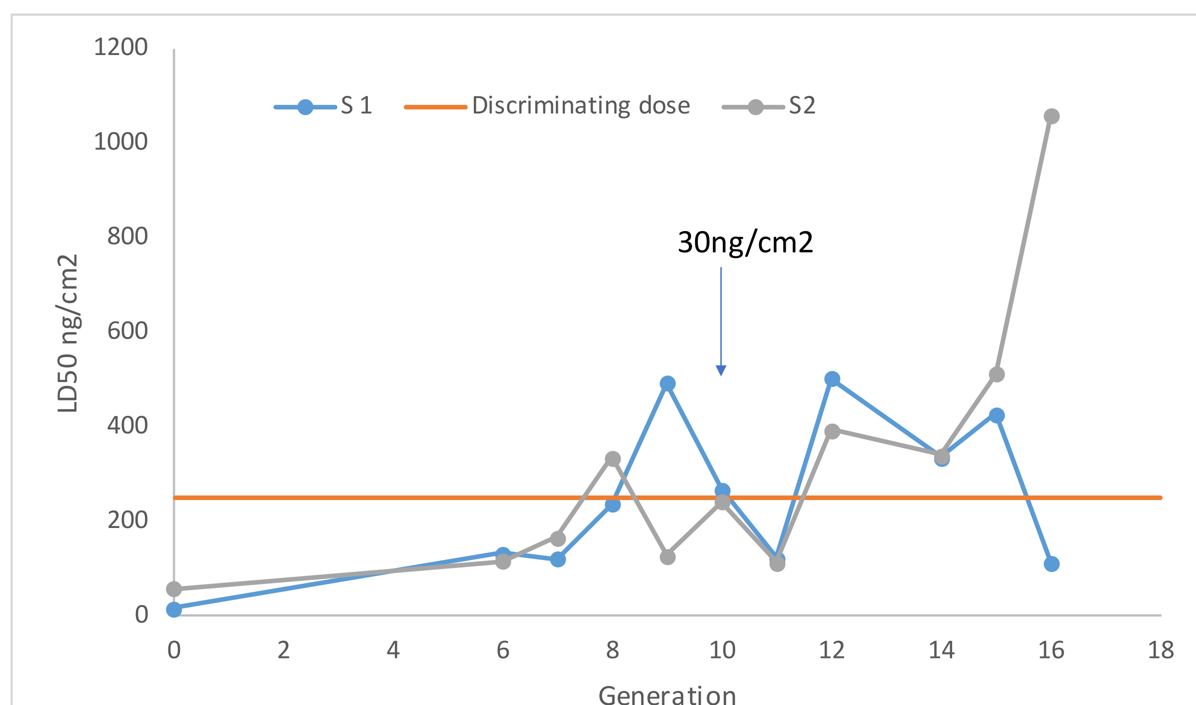

Figure 1.1: LD50 of the two cohorts in Canberra over time. S1 = cohort 1, S2 = cohort 2. A clear increase is observed in S2 but not S1 after 16 generations. The discriminating dose for Cry1Ac in the monitoring program is indicated on the graph (250 ng/cm^2).

H. punctigera resistant to Cry1Ac isolated from the field containing a mutation in the cadherin gene gave a resistance ratio of 113 and similar levels of resistance have been detected in Cry1Ac resistant lines of *H. armigera* from around the world. The mechanism in the line that we selected in this study is unclear and further work would be required to identify the mechanism and characterise the resistance in terms of dominance, etc. Assuming the increase in the LD50 is maintained, **it is clearly possible to select for Cry1Ac resistance in a Cry2Ab and Vip3A background**.

2. Does multiple resistance to Bt toxins in Helicoverpa spp. pose a threat to stacked gene cotton? UNSURE.

In Narrabri we created a *H. punctigera* colony that is simultaneously resistant to Cry1Ac, Cry2Ab and Vip3A (known as TR) by crossing field isolated colonies resistant to each single toxin and exposing them to all three toxins at once. We used established protocols (Mahon and Olsen 2009, JEE 102: 708-716) to rear larvae from these strains on plant material from commercially available cotton varieties grown in fields at Narrabri. All plants were fertilized and irrigated using standard agronomic practices and weeds were spot sprayed with glyphosate, or chipped, as required. No insecticides were applied to any treatments.

Experiments were conducted in 2016/17 and 2018/19. In 2016/17 the cotton was grown at the Australian Cotton Research Institute (ACRI) and larvae were exposed to (a) non-Bt cotton, (b) Bollgard II cotton and (c) Bollgard 3 cotton. In 2018/19 the cotton was grown at Locharba (Bayer's Research Station in Narrabri) and larvae were exposed to (a) non-Bt cotton and (b) Bollgard 3 cotton. In all cases each variety was grown in two adjacent rows that were 20m long and immediately adjacent to the other plant treatment(s). This arrangement was replicated in 4 plots in which the placement of each variety was randomized.

The parental TR strain was challenged as was a susceptible control (LHP) and heterozygotes created by crossing TR with LHP. To examine changes over time, neonates were challenged with plants collected at squaring, flowering and cut-out. During the 2016/17 season we exposed larvae to fresh leaves collected from node 3 of the plant and assessed mortality at day 7. The leaves were kept moist by placing them on top of agar within individual wells in plastic trays. We repeated the experiments in 2017/18 but at day 7 transferred larvae from plastic trays to pots that contained the terminal of the plant (in a small container of water to keep it hydrated) and every 5 days thereafter refreshed material until they died or emerged as moths. The larvae used in experiments had hatched within the preceding 24 hours. In all cases a total of 64 neonates (16 x 4 plots) were set up on plant material. In 2017/18 30 larvae were transferred to pots after day 7 (i.e., 7 or 8 from each of the 4 plots) The survival of larvae was analyzed using ANOVA with plant variety, time throughout the season and larvae genotype as factors.

(1) Neonates challenged for 7 days

Our 2016/17 experiments showed that the impact of insect genotype on survival of larvae after 7 days was dependent on the type of leaf material and consistent throughout the season. When fed non-Bt plant material larvae of all 3 insect genotypes had relatively high (>69%) survival to day 7 (Table 2.1) but at squaring, flowering and cut-out the survival of TR was significantly lower than for the heterozygote and the susceptible strains. No heterozygote or susceptible larvae survived to day 7 on Bollgard II or Bollgard 3 plant material at any time period (Table 2.1). At squaring, flowering and cut-out the TR strain survived on Bollgard II and Bollgard 3 plant material to day 7 at a similar proportion as they did on non-Bt cotton (Table 2.1).

Table 2.1 The proportion survival to 3rd instar of homozygote triple resistant (Cry1Ac/Cry2Ab/Vip3A), heterozygous and susceptible neonates on non-Bt, Bollgard II and Bollgard 3 cotton at squaring, flowering and cut-out. Values presented are means ± SD. Values followed by different letters represent a statistically significant difference at P < 0.05.

	rr (TR)	**rS (TR x GR)**	**SS (GR)**
Squaring			
Non-Bt	0.75 ± 0.11[a]	0.95 ± 0.06[b]	0.94 ± 0.07[b]
Bollgard II	0.69 ± 0.09[a]	0	0
Bollgard 3	0.67 ± 0.07[a]	0	0
Flowering			
Non-Bt	0.79 ± 0.10[a]	0.91 ± 0.08[b]	0.93 ± 0.09[b]
Bollgard II	0.85 ± 0.08[a]	0	0
Bollgard 3	0.77 ± 0.09[a]	0	0
Cut-out			
Non-Bt	0.69 ± 0.10[a]	0.90 ± 0.07[b]	0.91 ± 0.06[b]
Bollgard II	0.82 ± 0.08[a]	0	0
Bollgard 3	0.79 ± 0.09[a]	0	0

(2) Neonates challenged until moth emergence

Our 2017/18 experiments showed that the impact of insect genotype on survival of larvae after 7 days was dependent on the type of leaf material and changed throughout the season. When fed non-Bt plant material larvae of heterozygote and susceptible genotypes had relatively high (>77%) survival to moths (Table 2.2) but at squaring, flowering and cut-out the survival of TR was significantly lower than for the heterozygote and the susceptible strains. No heterozygote or susceptible larvae survived to moths on Bollgard 3 plant material at any time period (Table 2.2). At squaring, the TR strain survived on Bollgard 3 plant material to moths at a similar proportion as they did on non-Bt cotton (Table 2.2). At flowering, around 20% of the TR strain survived on Bollgard 3 which was a significantly lower proportion than for non-Bt cotton at that time period (Table 2.2). No TR larvae survived on Bollgard 3 plant material to moths at cut-out (Table 2.2).

Table 2.2 The proportion survival to moths of homozygote triple resistant (Cry1Ac/Cry2Ab/Vip3A), heterozygous and susceptible neonates on non-Bt and Bollgard 3 cotton at squaring, flowering and cut-out. Values presented are means ± SD. Values followed by different letters represent a statistically significant difference at P < 0.05.

	rr (TR)	**rS (TR x GR)**	**SS (GR)**
Squaring			
Non-Bt	0.26 ± 0.21[a]	0.92 ± 0.11[b]	0.77 ± 0.17[b]
Bollgard 3	0.10 ± 0.07[a]	0	0
Flowering			
Non-Bt	0.37 ± 0.10[a]	0.53 ± 0.08[b]	0.61 ± 0.09[b]
Bollgard 3	0.21 ± 0.06[b]	0	0
Cut-out			
Non-Bt	0.14 ± 0.06[a]	0.40 ± 0.10[b]	0.40 ± 0.09[b]
Bollgard 3	0	0	0

Summary:

Our data suggest that the TR strain carries a fitness cost (observed here as reduced survival) relative to heterozygote and susceptible insects. In 2016/17 when measured over 7 days during the neonate to 3rd instar growth stage this reduced survival of TR was in the order of 10-20% relative to heterozygotes and susceptible insects at all sampled points. In 2017/18 when measured over several weeks during the neonate to moth growth stage this reduced survival of TR was in the order of 50-60% during squaring, 20% during flowering, and 25% during cut-out. Its difficult to draw any firm conclusions from fitness data collected in an unnatural laboratory setting; however, it is possible that any TR insects in natural populations would have a lower survival that susceptible counterparts.

Our data suggest that TR insects may have a relatively high chance of surviving on Bollgard 3 cotton during the first week of their life but that they may not thrive on these plants later in their life. In the field this may present as survival of Helicoverpa larvae beyond the current recommended threshold for Bollgard 3 of 2 larvae/m or 1 larvae >8mm/m (for seedling to cut-out). While it is unlikely that the majority of these larvae will mature, it is possible that some of them may pupate and emerge as moths. They would not be distinguishable from susceptible larvae based on phenotypic methods available to consultants and growers. Following the recommended thresholds would limit opportunities for their selection.

3. Are the frequencies of resistance to 3 gene cotton increasing in *Helicoverpa* spp.? NO

3.1. Liaison with CCA to establish collaborators for collection of Helicoverpa spp. material throughout the cotton regions.

We established successful collaborations with CCA members (Jamie Street, St George; David Parlato, Emerald; Elle Storier, MIA, Hillston) and CSIRO colleagues (Cate Paull, Darling Downs) for collection of material and our immediate project team serviced the Lower Namoi, Upper Namoi, Gwydir, MacIntyre, Mungindi and Macquarie regions. Table 3.1 outlines the number of trips undertaken within each region and the total number of collections submitted to the program (i.e., number of individual fields sampled for eggs).

Table 3.1: A summary of the egg collection effort undertaken in each region.

Region	Collector	Affiliation	No. Trips	No Collections
St George	Jamie Street	CCA	7	52
Emerald	Dave Parlatto	CCA	4	4
MIA	Elle Storier	CCA	10	24
Darling Downs	Cate Paull	CSIRO	4	19
Gwydir	Project Team	CSIRO	3	15
Lower Namoi	Project Team	CSIRO	4	23
Upper Namoi	Project Team	CSIRO	6	23
MacIntyre	Project Team	CSIRO	4	20
Macquarie	Project Team	CSIRO	5	34
Mungindi	Project Team	CSIRO	1	2

These collections translated to 22,489 eggs collected across all sampled valleys for Bt resistance testing during the 2017/18 season. This is within the range of 17,618 to 28,892, and near the average of 23,596, collected for this program over the past decade.

3.2. Trial the collection of *Helicoverpa* spp. material using pheromone traps for moths versus eggs at the same selected sites.

Recently the conventional insecticide monitoring program lead by Dr Lisa Bird (NSW DPI) shifted their collection of material for testing from eggs by hand to moths from traps because comparisons of the two approaches for their program suggested that the latter resulted in more completed bioassays. Within the 2017/18 monitoring season we collected data to assist with determining if this change in collecting protocol also had merit for the Bt monitoring program.

Specifically, we trialled the collection of *Helicoverpa* spp. material using pheromone traps to collect male moths versus hand collecting eggs at the same selected sites within 4 valleys. These comparisons were conducted by our immediate team in the Upper Namoi, Lower Namoi, MacIntyre, and Macquarie regions. At each region there were four sites and each of the regions were visited three times approximately 1 month apart throughout the season

(November, December, January) to collect moths using pheromone traps and eggs using leaf collections in nearby fields.

Within each valley the location of paired sites was determined by the number of eggs that were collected on individual cotton leaves in a field. If two people could collect around 200 leaves containing an egg in 30 mins (which is considered an average yield of eggs based on 15 years of experience) the site was deemed appropriate for setting up a trap. In this case, 2 *H. punctigera* and 2 *H. armigera* traps were set up at random locations in the field that were at least 500 m apart. The traps were set out on the same day of the corresponding egg collections and each trap was emptied the following morning into an individual cloth bag. The egg collections and moth collections were placed into a fridge set to approximately 15 degrees for transport back to the ACRI where they were placed into a cool room (set to 10 degrees). The moths were housed in their original cohorts within 5 L buckets as per our standard rearing procedure.

The following day the material was logged into the Bt monitoring program. For eggs, this involved removal from the cotton leaf and placement onto artificial diet for rearing to moths over the following three to four weeks for eventual single pair mating for F_1 screens. For moths this involved directly setting them up in single pair matings for F_1 screens (i.e., matching males with female partners from our resistant colonies).

To limit the scale of the work to suit available labour, for eggs and moths we limited the numbers set up for F_1 screens to 50 per species and only used Cry2Ab resistant lines (i.e., for *H. armigera*, SP15; *H. punctigera*, HP4.13). If less than 50 moths were captured in a trap or reared from eggs to moths, all of them were set up with partners for F_1 screens. If more than 50 moths were captured in a trap or reared from eggs to moths we selected the fresher and younger looking moths to pair with a resistant female partner. For both situations the field moth was placed into a single pair mating chamber with honey solution and a Cry2Ab resistant partner. Our standard bioassay procedures were used to challenge the resulting offspring from the F_1 screens which were classified as being resistant (rS) or susceptible.

Our analyses of the merit of using egg collection versus trapping to obtain material for the F_1 screens involved the following components:

(1) Average yield for each method

We compared the number of individuals collected by (a) hand over a 30 minute period by two experienced staff and (b) overnight using a set of 2 *H. armigera* and 2 *H. punctigera* pheromone traps. Across all sites a total of 2664 moths were collected by trap and 2553 eggs were collected by hand. However, of the collected eggs only 65% (1675) hatched a healthy neonate. Of the 12 pairwise samples that were collected, in 4 cases at least 20% more moths were obtained than neonates from egg collections, in 5 cases at least 20% more neonates from egg collections were obtained than moths, and in 3 cases the numbers of individuals from both methods were within 20% of each other.

(2) Efficacy of mating for the F_1 screen

For *H. armigera* the proportion of individuals based on total numbers that mated to enable screening was 0.44 for those collected as moths and 0.35 for those collected as eggs. From 4 sites the proportions of mated individuals were at least 10% greater if males were collected as moths versus eggs, and at 8 sites the proportions of mated males were within 10% of each other.

For *H. punctigera* the proportion of individuals based on total numbers that mated to enable screening was 0.52 for those collected as moths and 0.48 for those collected as eggs. From 1 site the proportions of mated individuals were at least 10% greater if males were collected as moths versus eggs, at 3 sites the reverse was true, and at 6 sites the proportions of mated males were within 10% of each other. [At two sites all eggs were *H. armigera*.]

(3) Potential biases in detection of resistance

For *H. armigera* and *H. punctigera* on average from 2009/10 until 2017/18 males and females were equally likely to carry a gene for resistance but in any one year there could be significant biases towards males or females (Tables 3.2.1 and 3.2.2).

Table 3.2.1: A summary for* H. armigera *of the influence of sex on the detection of Cry2Ab resistance using F_1 screens. The data are summed across all cotton regions.

Year	No. Males Tested	No. Males Positive	% Males Positive	No. Females Tested	No. Females Positive	% Females Positive
2017-18	200	12	0.060	138	5	0.0362
2015-16	198	9	0.045	101	3	0.0297
2014-15	229	5	0.022	130	9	0.0692
2013-14	198	6	0.030	136	3	0.0221
2012-13	242	9	0.037	142	6	0.0423
2011-12	260	19	0.073	156	10	0.0641
2010-11	515	29	0.056	390	10	0.0256
2009-10	462	13	0.028	383	22	0.0574
Average	**301**	**13**	**0.042**	**205**	**9**	**0.0443**

Table 3.2.2: A summary for* H. punctigera *of the influence of sex on the detection of Cry2Ab resistance using F_1 screens. The data are summed across all cotton regions.

Year	No. Males Tested	No. Males Positive	% Males Positive	No. Females Tested	No. Females Positive	% Females Positive
2017-18	98	2	0.0204	80	0	0.0000
2015-16	267	4	0.0150	203	4	0.0197
2014-15	118	1	0.0085	101	2	0.0198
2013-14	161	2	0.0124	130	3	0.0231
2012-13	290	4	0.0138	228	1	0.0044
2011-12	197	12	0.0609	162	7	0.0432
2010-11	98	5	0.0510	78	5	0.0641
2009-10	370	12	0.0324	237	3	0.0127
Average	**214**	**6**	**0.0277**	**163**	**4**	**0.0267**

Due to losses associated with rearing eggs to moths and the relatively low proportions of successful matings in F_1 screens to produce families for testing, the sample sizes for comparing potential biases in the composition of resistance individuals collected as eggs versus moths is low (Table 2.2.3). Around 30% more individuals collected as moths versus eggs were tested in the F_1 screen. The only *H. punctigera* that scored positive for Cry2Ab resistance was collected as a moth. Of the 6 *H. armigera* that scored positive for Cry2Ab resistance, half were collected as moths vs eggs. Currently, as part of developing a molecular tool for resistance testing in CSE1801, we are comparing the Cry2Ab resistance frequencies of larger samples of each species collected as moths versus eggs.

Table 3.2.3: A summary for* H. armigera *and* H. punctigera *of the proportion of individuals collected as moths versus eggs that scored positive for being heterozygous for Cry2Ab resistance using F_1 screens. The data are for males and are summed across all cotton regions.

	H. punctigera		***H. armigera***	
	moths	**eggs**	**moths**	**eggs**
Total tests	195	55	208	80
Positive alleles	1	0	3	3
Total alleles	390	110	416	160
Proportion of r (Cry2Ab)	**0.003**	**0.000**	**0.007**	**0.019**

Summary:

Not surprisingly there was considerable variation among sites and across months in the yield of viable material for resistance testing using trapping of moths versus hand collections of eggs. On balance, substantially more trapped moths from the pooled collections were screened than were hand obtained eggs. Logically this reflects losses associated with rearing eggs to moths but may also be due to variation in mating. For instance, for several time points the efficacy of mating in *H. armigera* for the F_1 screen was greater for male moths collected directly from the field versus those reared in the laboratory from field collected eggs. Nonetheless the year to year variation across 8 seasons in resistance frequencies for males versus females potentially complicates using pheromone traps as collecting data only on males may be misleading in some years. Molecular tools can assist with testing this variation more robustly, as well as the potential impact of collection method on resistances frequencies (i.e., in both cases increasing sample sizes to enable meaningful statistical testing). Future consideration of the relative merit of each collection method should also consider differences in resourcing of labour, travel and operating including accessing appropriately trained staff for remote collections.

3.3. Screened Helicoverpa spp. populations against classes of Bt toxins deployed in current and imminent products

Background

Prior to 2002 Bt resistance monitoring involved screening individuals collected directly from field populations (F_0 screens). Since this method only detects dominant resistances, when we took over leadership of the program in 2002 F_2 screens were introduced to estimate Bt resistance frequencies because they can detect heterozygote individuals even when the resistance is recessive (rS) (see Figure 1). They involve testing the grandchildren of pairs of moths raised from eggs collected from field populations, and therefore take about 10 weeks to run. This method tests both alleles from two field collected individuals (4 alleles total), identifies all previously detected and potentially new types of resistance.

In 2007 we introduced into the program the F_1 screen for estimating resistance frequencies which is a shorter version of the F_2 method (see Figure 1). F_1 screens test the offspring of matings between a resistant moth (rr; isolated from F_2 screens and then reared as a colony in the laboratory) and a moth raised from eggs collected from field populations. It takes 5 weeks to conduct and tests both alleles from one field collected individuals (2 alleles total) and can only determine if the field moth has the same resistance as its mating partner. F_1 screens are considered more accurate than F_2 screens since its impossible for assortative mating to influence outcomes.

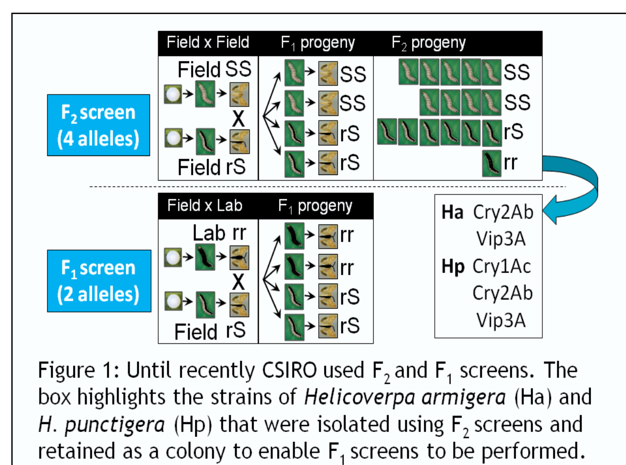

Figure 1: Until recently CSIRO used F_2 and F_1 screens. The box highlights the strains of *Helicoverpa armigera* (Ha) and *H. punctigera* (Hp) that were isolated using F_2 screens and retained as a colony to enable F_1 screens to be performed.

From 2007 to 2012 we used F_2 and F_1 screens in the program. Simultaneously we performed experiments that showed that each of the isolates of Cry2Ab resistance detected using F_2 screens was the same type of resistance that was initially identified. Similarly, work on Vip3A from 2009 to 2012 showed that all newly isolated resistances were the same type that was initially identified. In other words, although frequencies of resistance to some Bt toxins was higher than expected, it seemed that for each species there was only one key type of resistance for each Bt toxin.

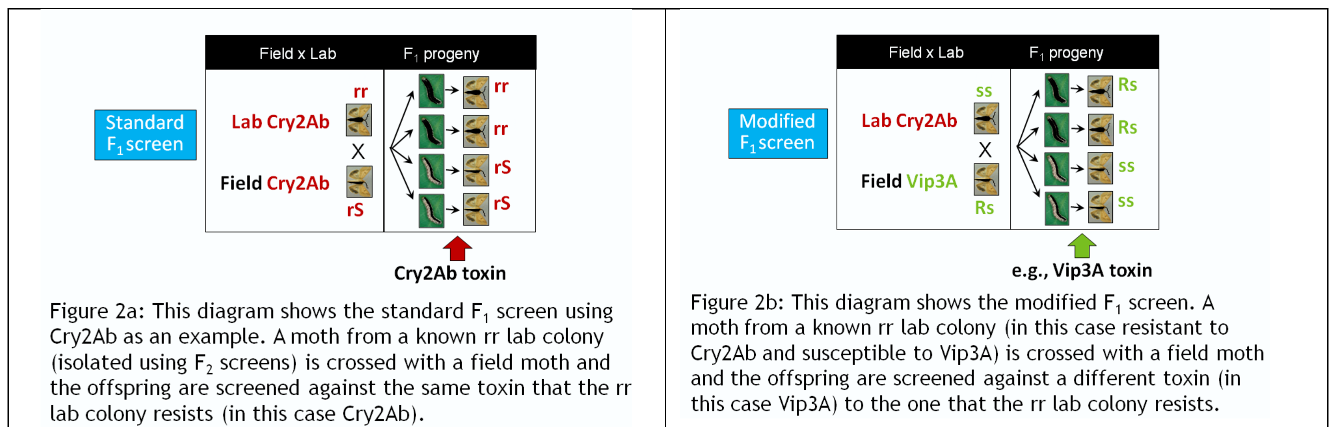

Figure 2a: This diagram shows the standard F_1 screen using Cry2Ab as an example. A moth from a known rr lab colony (isolated using F_2 screens) is crossed with a field moth and the offspring are screened against the same toxin that the rr lab colony resists (in this case Cry2Ab).

Figure 2b: This diagram shows the modified F_1 screen. A moth from a known rr lab colony (in this case resistant to Cry2Ab and susceptible to Vip3A) is crossed with a field moth and the offspring are screened against a different toxin (in this case Vip3A) to the one that the rr lab colony resists.

In 2013 we shifted to performing only F_1 screens to focus on the frequencies of the known common resistances. As well as screening F_1 families against the toxin of interest (e.g., Cry2Ab: Figure 2a), we introduced screens against all relevant Bt toxins (e.g., Cry1Ac and Vip3A: Figure 2b) to detect any new forms of resistance that are dominant. This is effectively an F_0 screen and has been identified as such in Figure 3.

Every 4 or 5 years we scheduled F_2 screens into the program to check for any new resistances that are recessive. Since F_2 screens also detect dominant resistances, during the years that they are preformed the F1 screens only challenge families against the toxin of interest.

In 2017 we begun to augment the monitoring program with molecular diagnostic tools which are currently still being developed (as part of CSE1801). We anticipate the tools will be advanced to a stage where they can be validated by side-by-side screening in 2020/21.

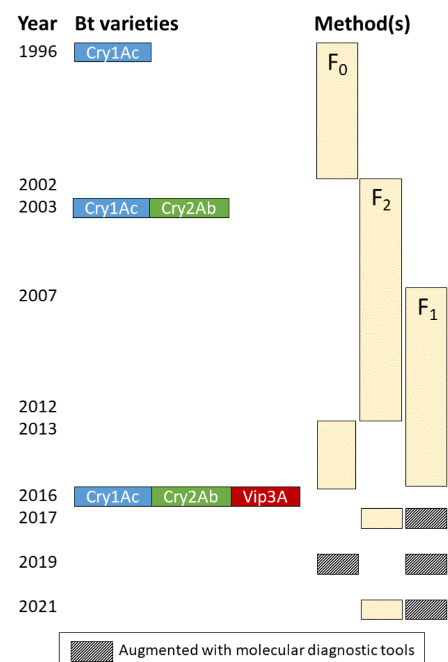

Figure 3: The evolution of screening methods implemented in Australia to monitor populations of key pests for resistance to single, dual and triple toxin Bt cotton varieties.

2017/18 Bt Monitoring Season

During the 2017/18 season F_2 screens were performed for the first time since 2012/13. We also conducted standard F_1 screens that challenged families against the toxin of interest.

F_1 screens

Cry1Ac

Several families of *H. armigera* that carry a gene conferring resistance to Cry1Ac have been isolated using F_2 screens but none have been retained as colonies. Therefore in *H. armigera* it is not possible to perform F_1 screens against Cry1Ac.

In 2017/18 we used the CSIRO strain 9-3784 to screen 222 alleles from *H. punctigera* and isolated 2 cases (0.009) conferring resistance to Cry1Ac (see Table 3.3.1); this frequency is higher than for 2015/16. For *H. punctigera* the cumulative frequency of alleles conferring 9-3784-like resistance to Cry1Ac since 2013/14 is 10/1936 (0.005).

In 2017/18 we used the Bayer strain Gaby to screen 232 alleles from *H. punctigera* and isolated no cases conferring resistance to Cry1Ac; we also did not isolate any resistant families in 2015/16 which was the first time we screened against this strain (n = 232). For *H.*

punctigera the cumulative frequency of alleles conferring Gaby-like resistance to Cry1Ac since 2015/16 is 0/376.

Table 3.3.1: Summary of results from F_1 screens against Cry1Ac using the CSIRO 9-3784 strain of *H. punctigera*. Data are presented as the frequency for that testing season. For each frequency we indicated the number of homozygous (rr) resistant individuals that contributed.

Species	Year	Cry1Ac F_1 screen			
		alleles tested	positive	Freq. of r	No. rr
H. punctigera	2013/14	498	2	0.004	0
	2014/15	394	1	0.003	0
	2015/16	822	6	0.007	1
	2017/18	222	2	0.009	0
	Total	1936	11	0.005	1

Cry2Ab

In 2017/18 we used the CSIRO strain SP15 to screen 676 alleles from *H. armigera* and isolated 18 cases (0.027) conferring resistance to Cry2Ab; this frequency is higher than for 2015/16 (see Table 3.3.2). Of these alleles, two were contributed from one individual that was homozygous (rr) for resistance. For *H. armigera* the cumulative frequency of alleles conferring SP15-like resistance to Cry2Ab since the CSIRO program began (2007/08) is 301/11186, and analyses on this F_1 screen data show that there has not been a significant increase in frequency over time (Goodness-of-fit test, $P<0.05$).

In 2017/18 we used the CSIRO strain Hp4-13 to screen 356 alleles from *H. punctigera* and isolated 2 cases (0.006) conferring resistance to Cry2Ab (see Table 3.3.2); this frequency is lower than for 2015/16. For *H. punctigera* the cumulative frequency of alleles conferring resistance to Hp4-13-like Cry2Ab since the CSIRO program began (2007/08) is 110/5950, and analyses on this F_1 screen data show that there has not been a significant increase in frequency over time (Goodness-of-fit test, $P<0.05$).

Table 3.3.2: Summary of results from F_1 screens of *H. armigera* and *H. punctigera* against Cry2Ab. Data are presented as the frequency for that testing season. For each frequency we indicated the number of homozygous (rr) resistant individuals that contributed.

Species	Year	Cry2Ab F_1 screen			
		alleles tested	positive	Freq. of r	No. rr
H. punctigera	2007/08	194	2	0.010	0
	2008/09	640	30	0.047	1
	2009/10	1138	15	0.013	1
	2010/11	358	10	0.028	0
	2011/12	736	24	0.033	3
	2012/13	518	7	0.014	2
	2013/14	582	5	0.010	0
	2014/15	488	5	0.011	2
	2015/16	940	10	0.011	2
	2017/18	**356**	**2**	**0.006**	**0**
	Total	5950	110	0.018	11
H. armigera	2007/08	278	9	0.032	0
	2008/09	3104	69	0.022	1
	2009/10	1710	37	0.022	0
	2010/11	1810	80	0.044	3
	2011/12	832	33	0.040	1
	2012/13	770	18	0.023	2
	2013/14	684	10	0.015	1
	2014/15	720	15	0.021	1
	2015/16	602	12	0.020	0
	2017/18	**676**	**18**	**0.027**	**1**
	Total	11186	301	0.027	10

Vip3A

Since 2013/14 we used the CSIRO strain 9-477 to perform F_1 screens against Vip3A in *H. armigera.* In 2017/18 we screened 420 alleles from *H. armigera* and isolated 8 cases (0.019) conferring resistance to Vip3A (see Table 3.3.3); this frequency is higher than for 2015/16. Of these alleles, two were contributed from one individual that was homozygous (rr) for resistance. For *H. armigera* the cumulative frequency of alleles conferring 9-477-like resistance to Vip3A since 2013/14 is 30/2230.

Since 2009/10 we used the CSIRO strain 8-48 to perform F_1 screens against Vip3A in *H. punctigera.* In 2011/12, the frequency was 0.095 (7/74); since it was obtained from a relatively small sample it is excluded from the overall summary results and in analyses. In 2017/18 we screened 214 alleles from *H. punctigera* and isolated 0 cases conferring resistance to Vip3A (see Table 3.3.3); this frequency is lower than 2015/16. For *H. punctigera* the cumulative frequency of alleles conferring resistance to Vip3A since the CSIRO program began is 36/3640, and analyses on this F_1 screen data show that there has not been a significant increase in frequency over time (Goodness-of-fit test, $P<0.05$).

In 2017/18 for the first time we used the Bayer strain Vespa to screen 340 alleles from *H. punctigera* and isolated 0 cases conferring resistance to Vip3A.

Table 3.3.3: Summary of results from F_1 screens of *H. armigera* and *H. punctigera* against Vip3A. Data are presented as the final frequency for that testing season. For each frequency we indicated the number of homozygous (rr) resistant individuals that contributed. *Note the very small sample for 2011/12 has been excluded from the total estimates.

Species	Year	Vip3A F_1 screen			
		alleles tested	positive	Freq. of r	No. rr
H. punctigera	2009/10	1144	16	0.014	0
	2010/11	172	3	0.017	0
	2011/12	74	7	0.095	0
	2012/13	284	5	0.018	2
	2013/14	530	6	0.011	2
	2014/15	414	1	0.002	0
	2015/16	882	5	0.011	1
	2017/18	**214**	**0**	**0.000**	**0**
	Total	3640	36	0.010	5
H. armigera	2013/14	642	6	0.009	1
	2014/15	626	10	0.016	1
	2015/16	542	6	0.011	1
	2017/18	**420**	**8**	**0.019**	**2**
	Total	2230	30	0.009	5

F_2 screens

Ambiguity exists around the reliability of F_2 screens for providing absolute estimates of resistance frequencies. This relates to the possibility of assortative mating during the bulk rearing of the F_1 stage of this screen and the relatively recent realisation that resistance to some of the Bt toxins is more diverse than anticipated. For the current program the value of F_2 screens are in isolating new resistances and the data are presented as such below. A table summarising the F_2 screen data from the CSIRO program collected to date is provided in Appendix 1. The specific data for 2017/18 are reported as part of Milestone 4 (below).

Table 3.3.4 lists the isolations of Cry1Ac, Cry2Ab and Vip3A resistant *H. armigera* and *H. punctigera* strains that have been captured as ongoing colonies. In 2017/18 we isolated a new Vip3A resistant *H. armigera* colony (17-294) that is not allelic with the first CSIRO isolated type colony 9-477. We are currently characterising this 17-294 line. In 2017/18 Bayer isolated a new Vip3A resistant *H. punctigera* colony (Pavlov) that is not allelic with their Vip3A resistant *H. punctigera* Vespa colony.

Two previous isolations within *H. punctigera* of Cry1Ac resistance (9-3784 and Gaby) are not allelic. All of the *H. armigera* and *H. punctigera* Cry2Ab resistant isolations are allelic and the type colonies are listed for each organisation in Table 3.3.4.

Table 3.3.4: Isolations of Cry1Ac, Cry2Ab and Vip3A resistant *H. armigera* and *H. punctigera* strains that have been captured as ongoing colonies. Colonies isolated by CSIRO are in black font and those isolated by Bayer are in red font. Within each species and toxin combination those colonies with the same colour background have been demonstrated to be allelic; in some cases those with a different colour background have been demonstrated to not be allelic (refer to text for details). This table does not identify genetic variants within the same colony (see Milestone 7 for an example of such in the *H. armigera* Cry2Ab resistant SP15 colony).

<table>
<tr><th rowspan="2">Species</th><th colspan="3">Toxin</th></tr>
<tr><th>Cry1Ac</th><th>Cry2Ab</th><th>Vip3A</th></tr>
<tr><td rowspan="3">H. armigera</td><td rowspan="3">no isolations cultivated</td><td rowspan="3">4-SP15
8-661</td><td>9-477</td></tr>
<tr><td>17-294</td></tr>
<tr><td>12-Havana</td></tr>
<tr><td rowspan="3">H. punctigera</td><td>9-3784</td><td rowspan="3">4-13
7-Demon</td><td>8-48</td></tr>
<tr><td rowspan="2">11-Gaby</td><td>11-Vespa</td></tr>
<tr><td>17-Pavlov</td></tr>
</table>

Summary

In *H. armigera,* isolations of alleles conferring resistance to Cry1Ac have been detected since 2008 but we have been unable to retain one as a colony (presumably due to fitness costs) for further testing. CSIRO and Bayer have each isolated a unique form of Cry1Ac resistance in *H. punctigera* (9-3784 and Gaby respectively). F_1 data demonstrate that currently in 9-3784-like *H. punctigera* 1% of individuals in the population are heterozygous (rS) for the Cry1Ac resistance gene and there is no indication of changes in resistance frequency since testing started in 2013/14. F_1 screens used in 2015/16 and 2017/18 did not isolate any families with Cry1Ac resistance that was like the *H. punctigera* Gaby strain.

Cry2Ab resistance genes and Vip3A resistance genes were present at detectable levels before Bt cotton expressing these traits was widespread.

Since beginning the F_2 screening program in 2003 we isolated only one form of resistance to Cry2Ab in *H. armigera* (SP15) and *H. punctigera* (Hp4-13) – all of the instances isolated by Bayer have also been of this one form. F_1 data demonstrate that currently in *H. armigera* 5% of individuals in the population are heterozygous (rS) for the Cry2Ab resistance gene and there is no indication of changes in resistance frequency since testing started in 2007/08. F_1 data demonstrate that currently in *H. punctigera* 1% of individuals in the population are heterozygous (rS) for the Cry2Ab resistance gene and there is no indication of changes in resistance frequency since testing started in 2007/08.

Through F_2 screens we isolated 2 forms of resistance to Vip3A in *H. armigera* (9-477, 17-294) – Bayer have isolated a form of Vip3A resistance (Havana) that is different to 9-477 but has not yet been compared against 17-294. Currently in *H. armigera* 4% of individuals in the population are heterozygous (rS) for the 9-477 form of Vip3A resistance gene (based on F_1 data). We have not performed F_1 screens with the 17-294 strain isolated in 2017/18.

Through F_2 screens we isolated 1 form of resistance to Vip3A in *H. punctigera* (8-48) – Bayer have isolated two forms of Vip3A resistance. The Vespa colony is different to 8-48 but the Pavlov colony has not yet been compared to Vespa or 8-48. Currently in *H. punctigera* 0% of individuals in the population are heterozygous (rS) for the 8-48 and Vespa forms of Vip3A resistance gene (based on F_1 data). We have not performed F_1 screens with the Pavlov strain isolated by Bayer in 2017/18.

4. Were novel dominant forms of Bt resistance detected in field populations of *Helicoverpa* spp? NO

The 2017/18 monitoring program used F_2 screens in line with our plans to do so every 4 years; this approach detects all forms of resistance including those that are dominant (for more detail on these methods see the Background to Section 3).

For *H. armigera* we screened:

- 658 alleles against Cry2Ab using F_2 screens and none of the 7 isolated cases of resistance were dominant.
- 640 alleles against Cry1Ac using F_2 screens and the 1 isolated case of resistance was not dominant.
- 658 alleles against Vip3A using F_2 screens and none of the 25 isolated cases of resistance were dominant.

For *H. punctigera* we screened:

- 354 alleles against Cry2Ab using F_2 screens and none of the 6 isolated cases of resistance were dominant.
- 336 alleles against Cry1Ac using F_2 screens and neither of the 2 isolated cases of resistance were dominant.
- 350 alleles against Vip3A using F_2 screens and neither of the 2 isolated cases of resistance were dominant.

5. Is the field performance of Bt technology declining over time? NO

We continued to include questions pertaining to performance of Bt-cotton in the annual end of season survey of Crop Consultant Australia members. These followed from a summer scholarship project supervised by Sharon Downes in 2008 (which collected data starting 2005/06) and aimed to determine the area of Bt-cotton that carried larvae at threshold levels or above. Below we analyse the data collected during the life of this project alongside the larger time series. Note that data from 2018/19 are not yet available from the CCA.

From 2005/06 to 2017/18 the survey included 28 to 63 CCA members and covered 50-75% of the total licensed Bt-cotton area. All valleys were represented. In this report the data are analysed according to climatic region – Tropical (Central Qld), Middle (Gwydir, Mungindi, Macintyre, Namoi, St George, Dirranbandi), Darling Downs, and Cool (Upper Namoi, Macquarie, Southern NSW). Bt-cotton was considered to carry survivors if it was at threshold: at least 2 larvae 3-8 mm/m in at least 2 consecutive checks or 1 larvae > 8mm/m. The Bt-cotton varieties available were Bollgard II from 2005/06 until 2016/17 when Bollgard 3 became the only commercial variety grown in Australia.

The % Bt-cotton with survivors is not increasing: Across years the proportion of the Bollgard II® area that reached threshold ranged from 3 to 21 and did not increase (Fig 1). From 2005 until 2015 the average proportion of the Bollgard II® area that reached threshold was 10%. Since Bollgard 3 was introduced in 2016/17 none of the crop area reached threshold.

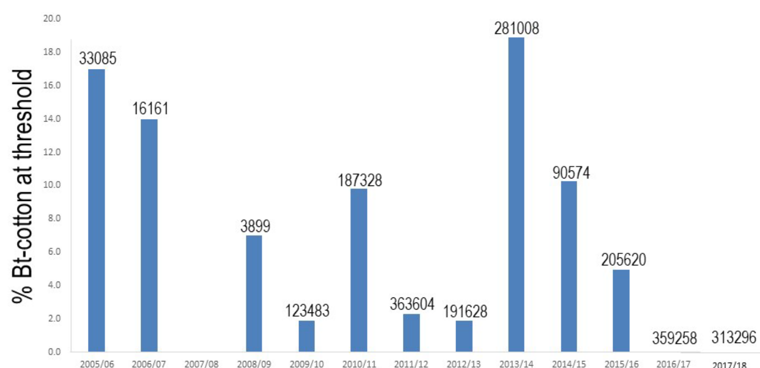

Figure 1: The % Bt-cotton at threshold for each season summed across valleys. The values at the top of each bar are total hectares sampled.

There is no trend among seasons for one region to be more likely to have Bt-cotton with survivors: For instance, in 2009/10 the Downs region had a percentage of Bollgard II® with larvae that was above the average, while in 2013/14 the Tropical region had a percentage of Bollgard II® with larvae that was above the average. The data across all years fit with the dataset for 2005/08 which shows variation among seasons in the proportion of Bollgard II® at threshold for each valley.

Most of the Bt-cotton with survivors was treated: From 2005 to 2015 the percentage of Bollgard II® at threshold that was not treated with a Heliocide varied among seasons from 12 to 85 (Fig 2). From 2012 to 2015 there was a consistent trend for increased treatment of Bollgard II® at threshold. None of the Bollgard 3 grow since 2016 was at threshold.

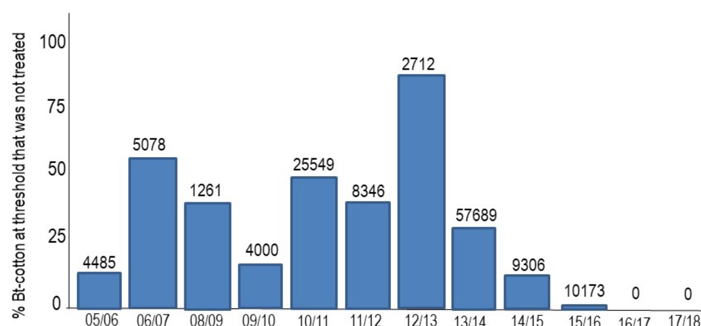

Figure 2: The % Bt-cotton at threshold in each season that was not treated with Heliocide. The values at the top of each bar are total hectares at threshold.

Thresholds were equally as likely to be driven by numbers of medium-large versus small larvae: We introduced a question to distinguish if thresholds were mostly driven by the component concerning medium-large larvae vs. small larvae. This is important because past work demonstrates that a proportion of medium-large large can survive and pupate under Bt-cotton. If surviving larvae are challenged with an insecticide, a greater proportion of medium-large larvae are likely to "escape" this application. When the data were pooled across all regions, the proportional area of Bt-cotton at threshold that was determined by small larvae alone ranged 30 to 86 and there was no pattern among years or regions.

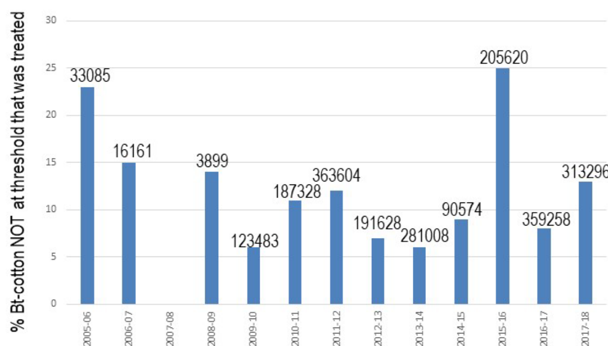

Figure 3: The % Bt-cotton NOT at threshold in each season that was treated with Heliocide. The values at the top of each bar are total hectares inspected.

Bt-cotton is sometimes sprayed for larvae below threshold: From 2005 until 2015 the proportion of the Bollgard II® area that did not reach the Helicoverpa threshold but was sprayed once or more for Helicoverpa larvae ranged from 6 to 25. Since Bollgard 3 was introduced in 2016/17, 8-13% of the area that did not reach the Helicoverpa threshold but was sprayed once or more for Helicoverpa larvae.

Summary: These survey data support frequency estimates which suggest that Bt resistance in Helicoverpa species is not increasing. In some seasons when Helicoverpa larvae reach threshold levels they are not treated which could select for resistance. Conversely, even since the commercialisation of Bollgard 3, around 10% of the crop is sprayed with a heliocide despite Helicoverpa larvae being below the recommended threshold levels for treatment. Our work has shown that Bt resistant larvae of *H. armigera* and *H. punctigera* are killed by the heliocides commonly deployed in cotton systems. Therefore the impact of the prophylactic heliocide treatment on selection for Bt resistance would depend on the relative proportions of resistant versus susceptible larvae in Bt-crops at the time of its application.

6. Are molecular diagnostic tools for Bt resistance developed by CSIRO validated by monitoring of field populations? YET TO BE TESTED

Background

Bt proteins cause toxicity by binding to the gut. Until recently, the Bt resistance in *Helicoverpa sp.* and other Lepidoptera species globally has been largely due to mutations

that cause a loss of function of the protein in the caterpillar gut, reducing or removing the efficacy of the Bt protein. These are mutations in genes involved in processing or binding the Bt protein to the midgut of the insect. More recent discoveries have suggested that some of these mutations involve genes that allow the Bt protein to access the binding target or are involved in the downstream pore formation.

To try to cross validate the molecular methods and the current bioassay methods we examined a number of F_2 and F_1 individuals identified during the life of CSIRO's monitoring program as resistant using bioassay data. We used two main approaches: (1) screening for previously identified mutations and (2) examining whole genome data for novel mutations.

During the course of this project, we commenced another project (co-funded with Bayer and CRDC: CSE1801) that is further developing the molecular testing and developing some of the whole genome sequencing approaches.

Screening for identified mutations

Cry1Ac

There are several known mechanisms of Cry1Ac resistance in Lepidoptera (i.e., loss of function mutations in the cadherin and ABCC2 genes). During the timeline of this project another was discovered in *H. armigera* from China that involves a single point mutation in a single gene that produces **a single amino acid change leading to dominant Cry1Ac resistance**. Loss of function mutations are typically recessive because a mutation in both copies of the DNA inherited from the parents is required. Mutations that change the sequence of the protein can be recessive or dominant and in the China example, a single amino acid change in a Tetraspanin (TSPAN) gene is all that is required for dominant Cry1Ac resistance. No dominant Cry1Ac resistant individuals were identified through the monitoring over the course of this project. However, we have used the published molecular information for this dominant Cry1Ac resistance mutation to test PCR primers so that it can be detected into the future with the molecular tool. These primers used successfully amplified the region of the TSPAN gene involved and can be deployed as a specific test if a dominant line is identified or incorporated into a broader molecular testing regime.

F_2 individuals

To date, F_2 screens by CSIRO identified 3 *H. armigera* families positive for Cry1Ac resistance but none have become an established laboratory line. We therefore focussed our efforts around Cry1Ac in respect to developing the molecular tool for existing Australian *H. punctigera* Cry1Ac mutations.

To date, F_2 screens by CSIRO identified one *H. punctigera* family positive for Cry1Ac resistance that has become an established laboratory line (9-3784). The characterisation of this colony identified a cadherin mutation as the cause of resistance (Walsh et al., 2018). During the life of CSIRO's monitoring program, F_2 screens isolated 5 other *H. punctigera* families that were positive for Cry1Ac resistance. In this project we examined individuals from 4 of those resistant Cry1Ac families at the cadherin loci and did not identify any known or potential resistance alleles. In this context a potential resistance allele is a disruption to the gene somewhere along the length in the coding sequence. These families did not survive to be further characterised and it is possible that the original resistance was the result of fitness or that another resistance allele other than that identified existed in these families.

F_1 individuals

We have material from the numerous Cry1Ac resistant *H. punctigera* families identified throughout the life of CSIRO's monitoring program but did not examine them as part of this project. However, since they were identified via the F_1 screening process with the 9-3784 Cry1Ac resistant line that has already been characterised, they are very likely a mutation in

the same gene. We will undertake to test this assumption during CSE1801 as part of our development of the molecular tool.

Cry2Ab

The Cry2Ab resistance mechanism was identified by CSIRO as a disruption in an ABCA2 gene in *H. armigera* and several different mutations have been identified (Figure 6.1). All mutations so far characterised have been loss of function mutations where the gene is disrupted by a deletion or insertion and the same mechanism has been identified in both *H. armigera* and *H. punctigera*. The loss of the protein encoded by this gene leads to a loss of binding and the Cry2Ab has no effect. This is a recessive resistance mechanism and resistant alleles are readily isolated from the field.

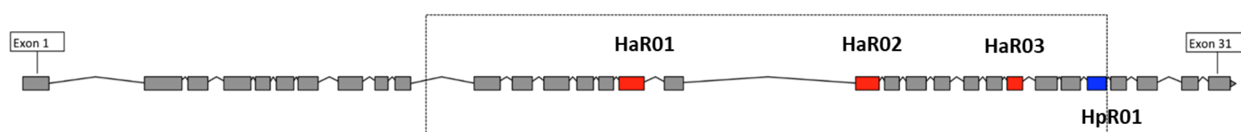

Figure 6.1: ABCA2 gene with the location of the *H. armigera* Cry2Ab resistance mutations in red and the location of the *H. punctigera* mutation in blue.

F_2 individuals

In the 31 F_2 *H. armigera* individuals tested to date from throughout the life of CSIRO's monitoring program, allele R01 represented about 33% of all individuals while the situation seems clearer for *H. punctigera* with almost 75% of individuals displaying allele HpR01 (Figure 6.2).

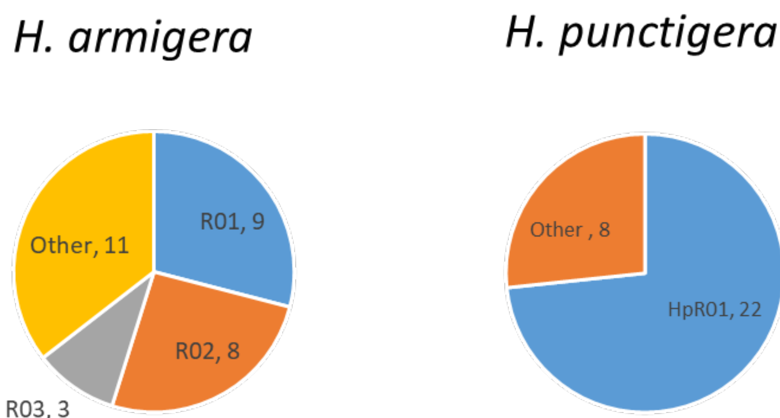

Figure 6.2: ABCA2 mutations identified in historical positive *H. armigera* (n = 31) and *H. punctigera* (n = 30).

F_1 individuals

We examined individuals from approximately 22 F_1 *H. armigera* families scored positive during 2011-12 by PCR and gel electrophoresis. As these individuals were crossed to SP15 to perform the F_1 screen they should be homozygous for HaCry2AbR1 (if the field individual was carrying HaCry2AbR1) or heterozygous suggesting another resistance allele was present. Of the 22 individuals, 11 were homozygous for allele R1 in each of 3 replicates. For the other 11 individuals, at least one replicate appeared to be heterozygous.

We are investigating further to see if the relative size of the resistant allele (100bp smaller) affects the PCR reaction. The next step for the F_1 individuals will be to perform amplicon sequencing to identify the alternative alleles (R2, R3 and others identified in the Cry2Ab resistant F_2 individuals) and to examine more individuals. This is underway with fresh material from the 2017-18 monitoring season.

Vip3A

Work done at CSIRO has identified a gene responsible for Vip3A resistance in *H. armigera* though this is not yet published. In both Ha85 and 477 there is a reduction in expression of the same gene in the midgut of the insect. From PCR and sequencing data we identified SNPs associated with the resistance gene though not the causative mutation.

As part of this project we sequenced Vip3A resistant *H. armigera* individuals from 10 F_2 families from 2014 to 2016 and identified a large number of alleles at the locus thought to cause resistance, all of them different to the resistance alleles in 9-477 and Ha85 (allelic with 9-477). This aligns with the recent discovery of a different mechanism to Ha85 and 9-477 (17-294) suggesting that there is more than one mechanism for Vip3A resistance and that there is not one common mechanism in the field. Furthermore, it appears that the Vip3A resistant *H. punctigera* do not share the same mechanism as Ha85 and 9-477 again suggesting that there is more than one way to be resistant to Vip3A.

Testing for pooled detection

Amplicon sequencing

Amplicon sequencing uses the high sensitivity of PCR combined with high throughput sequencing of the PCR amplicon. This can generate not just the allele frequency but also the diversity around the identified allele. It is possible to pool a large number of individuals as million of sequences can be generated per sample. Furthermore, a large number of different loci from the same individual can be amplified and sequenced.

Amplicon sequencing for allele Cry2Ab allele R1

Known pooled frequencies of the Cry2Ab resistant allele R01 and the susceptible allele in *H. armigera* have been high throughput sequenced with an Illumina Miseq and the alleles quantified to see if quantitative detection of allele frequency is possible in pooled samples. Our analyses suggest that a strong relationship is evident between the pooling frequency and the frequency of resistant and susceptible alleles detected (Table 6.1 and Figure 6.3).

The lowest frequency of resistant alleles to susceptible alleles was 1 resistant allele per 1000 susceptible alleles (as measure by DNA concentration from resistant and susceptible individuals). However, a more reliable detection limit is likely to 1:100. Interestingly the smaller R01 resistant allele (~100bp deletion in the ABCA2 gene) is found at a higher frequency than would be expected in the pools. The smaller size of the resistant allele makes it favourably amplified in a pool, however, log transforming the data allows the construction of a standard curve.

Further refinement of this is ongoing as part of CSE1801 and will be extended to the other resistance alleles for Cry2Ab and the other known alleles for other toxins.

Table 6.1: Allele frequencies detected in pools of susceptible and resistant insects

Pooling (concentration of resistant DNA to susceptible)	GR allele frequency (1 = 100%)	Sp15 allele frequency (1 = 100%)
1:1	0.128	0.872
1:5	0.145	0.855
1:10	0.374	0.626
1:50	0.712	0.288
1:100	0.839	0.161
1:500	0.903	0.097
1:1000	0.918	0.082

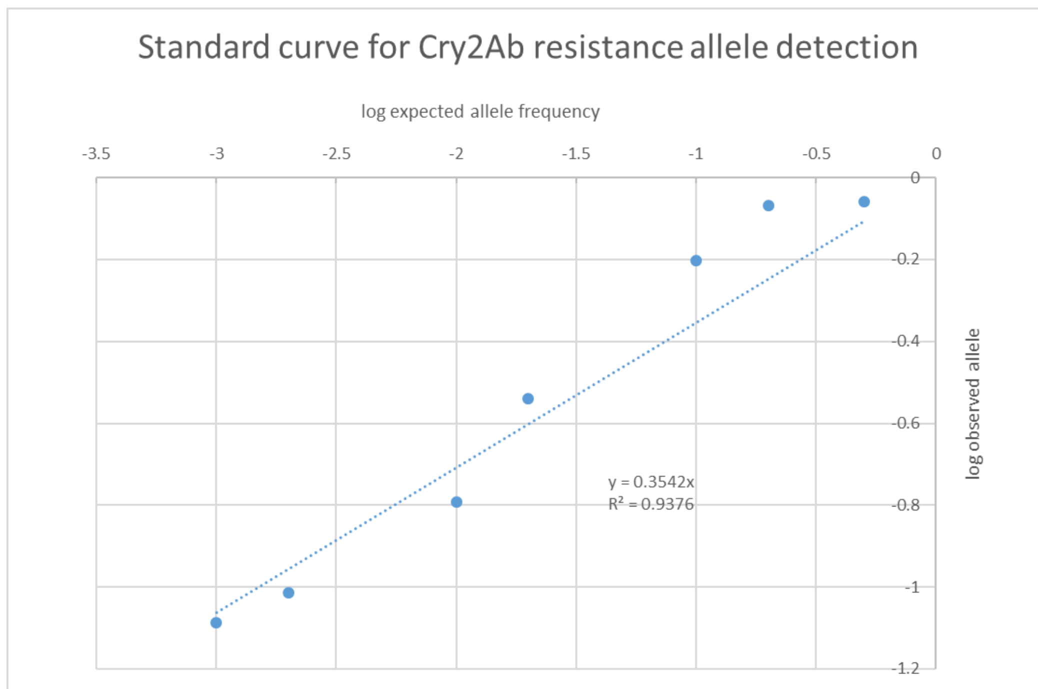

Figure 6.3: A standard curve reflecting pooled DNA frequency of DNA from SP15 (resistance allele R01) and the susceptible line (GR). This suggests detection down to 1 allele in 1000 is possible.

Whole genome sequencing data for new alleles

Whole genome sequencing of individuals is an approach that allows the examination of all known mutations and the discovery of certain types of new mutations. For example, a single resequenced (known as resequencing as we are comparing the data to the existing genome) individual can be examined for all known Bt resistance alleles and all known conventional resistance alleles at the same time including those identified elsewhere and in other species. If a new loss of function mutation is present in a known gene, this can also be identified from the sequencing data. If a completely new mutation, never previously seen in a new gene is present, this must be mapped separately as it would likely be lost in the noise. However, once identified, the whole genome sequencing data becomes a database available for further analysis into the future.

To further understand the genetic changes driving Cry2Ab resistance we sequenced the entire genomes of all the F_2 individuals with unknown mutations as well as several of the more common alleles responsible for the resistance in the lab lines. We are in the process of analysing these data in detail as part of CSE1801 but have immediately identified a number of previously unknown mutations disrupting the ABC transporter (ABCA2) that are involved in the mode of action of Cry2Ab. These are loss of function mutations in the ABCA2 transporter that have not been described or linked to Cry2Ab resistance. None of these sequenced individuals possessed mutations to either Cry1Ac of Vip3A but they did possess and previously identified mutation causing pyrethroid resistance.

Using the output of this project as well as others, we are developing a database of genomic information that can be scanned when any new resistance alleles are described from throughout the world. For example, within a few hours we scanned over 300 samples for the newly described dominant Cry1Ac allele detected in China These 300 resequenced individuals came from around the world. This data includes 80 individuals from Australia: 50 *H. armigera* and 30 *H. punctigera* that were collected since 2006 as part of the CSIRO monitoring program.

Summary

While it was premature to validate the molecular tool alongside standard bioassays in this project, we demonstrated that they can be used to detect known mutations and new mutations in previously identified genes. Furthermore, we demonstrated the utility of a database of sequencing information that can be examined for novel mutations in previously unidentified genes from known resistances overseas. In this project we were not able to completely shadow the monitoring data but we have put in place all of the appropriate protocols and data analysis to examine molecular data into the future. A summary of the options and rough costing for molecular testing can be found in Table 2.

Table 6.2: Options for molecular testing for Bt and conventional resistance into the future.

Method	Output and complexity	Estimated cost per insect
Direct PCR and sequencing	Individual PCR and sequencing of known mutations. Simple but time consuming.	~$10 Only detects known mutations.
Amplicon sequencing	Amplicon sequencing using a pool of primers for multiple targets on pooled of individual insects followed by high throughput sequencing	~$10 for individual moths As low as $0.1 depending on the pooling. Only detects known mutations.
Sequence capture	Uses the sequence of genes involved to capture the sequence of the genes in individuals or pooled insects.	$10-$2 depending on the pooling Detects known and novel mutations in the selected genes
Whole genome sequencing	Allows us to scan through the genome for novel disruptions and would allow us to predict novel genes and mutations.	$100-200 per moth Detects known and novel mutations and provides a database to return to for new mechanism discoveries.

7. Are the characteristics of difference genetic variants of Cry2Ab resistance the same? YES.

Throughout the early development of the molecular tool it became apparent that numerous genetic variants exist of the common *H. armigera* Cry2Ab resistance. During the course of our routine screening we discovered that the SP15 colony housed in Canberra was of genetic variant R03 whereas the version housed in Narrabri was the ‘type’ variant R01. We assumed that the SP15 in both laboratories was R01 and hypothesized that the R03 variant established in Canberra because it somehow contaminated the colony and was fittest.

Herein we tested this hypothesis by characterising the R03 variant of SP15 and comparing it to the known characteristics of the R01 variant. We used the standard surface overlay bioassay methods reported in our previous project reports and numerous scientific articles (e.g., Downes et al. 2010, *J Econ. Entom.* 103:2147-54) starting with a top dose of 4 ug/cm^2 in a 5 step serial dilution (lowest dose, 0.125 ug/cm^2) replicated using 3 cohorts of larvae. Specifically, we examined the dominance and sex-linkage of R03 SP15 *H. armigera* by comparing the dose response of (1) a susceptible laboratory colony of (GR), (2) male R03 SP15 x GR female, (3) female R03 SP15 x GR male, and (4) the R03 SP15 parental strain.

The dose response of larvae were analysed and slopes and LC_{50} estimates were calculated using the logit transformation in GLIM version 3.77 (Payne 1985). Comparisons of LC_{50}’s

were made using models with a common slope. However, where the slopes differed significantly ($p < 0.05$), LC_{50} estimates were calculated with independent slopes.

The bioassay data show no significant variation among the susceptible strain and the reciprocal cross between R03 SP15 and the susceptible strain; that is, the heterozygotes showed a similar dose response to the susceptible strain demonstrating that the resistance is completely recessive (Table 1). This was confirmed by resistance ratios (LC_{50} determined relative to GR) that were close to 1 for both reciprocal crosses (Table 1). Data are not provided for the assay with the R03 SP15 parental strain because the slope was minimal and we could not independently calculate an LC_{50}. The dose responses for R01 SP15 taken from Mahon et al., 2007, J. Econ. Entomol., 100:894–902 show an almost identical pattern (see Table 1 for the resistance ratios).

Table 7.1. Summary of the bioassays data for crosses between the resistant strain R03 SP15 and susceptible strain GR, and GR, exposed to Cry2Ab toxin (micrograms per square centimetre). The resistance ratio for R01 SP15 (taken from Mahon et al., 2007, *J. Econ. Entomol.*, 100:894–902) is provided in brackets for comparison.

Strain/cross	LC_{50}	95%CI	Slope ±SE	Resistance ratio
SS (GR)	0.119 [a]	0.071,0.131	1.205 ± 0.092	-
RS: RR♀ x SS♂	0.112 [a]	0.067,0.125	1.193 ± 0.0751	0.94 (1.26)
RR♂ x SS♀	0.123 [a]	0.077,0.146	1.384 ± 0.0968	1.03 (1.27)

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

We also examined potential cross-resistance by comparing the survival against a discriminating concentration of Vip3A and Cry1Ac (administered separately as distinct assays for each toxin) of (1) a susceptible laboratory colony of (GR), (2) male R03 SP15 x GR female, (3) female R03 SP15 x GR male, and (4) the R03 SP15 parental strain. When data were corrected for control mortality in all cases there was less than 5% survival of larvae against Vip3A and Cry1Ac. This finding is similar to the responses shown by R01 SP15 which is also not cross-resistant to Vip3A or Cry1Ac.

Summary:
There is no indication from our characterisation work that the novel R03 variant of *H. armigera* Cry2Ab resistance poses a different threat to that of the typical R01 variant.

5. Outcomes

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 information from this project will be presented to the Bt Technical Panel of the TIMS committee who will use it to inform:

(1) the risks to Bollgard 3 cotton posed by multiple resistances;

(2) the evolution of resistance and the ecological factors that may drive this process;

(3) the evolution of resistance at a molecular level; and

(4) the current Bt resistance risk across the main cotton growing regions in Australia.

By doing so, it will contribute to a review of the effectiveness of the current Bt resistance management strategy and drive any changes required to maintain its success. It will also be used to evaluate the resistance risk imposed by surviving Helicoverpa larvae on Bt cotton, and to evaluate whether survivors need to be screened as part of any ongoing monitoring.

6. Please describe any:-

a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);

There were no technical advances achieved in this project. Note that the bulk of the development of the molecular tool for resistance testing takes place in CSE1801; please refer to it for details around specific technical advances relating to IP.

b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and

A unique isolation of Vip3A resistant *H. armigera* was isolated. There were no other discoveries in methodology or equipment design.

c) required changes to the Intellectual Property register.

We altered the IP register for this project to capture the detail of the new insect colony isolated during the 2017/18 monitoring season.

7. Conclusion

Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

It is unclear to what degree multiple resistance to Bt toxins in Helicoverpa spp. is a threat to stacked gene cotton. Cry1Ac resistance can be selected for in Helicoverpa spp. insects that are already Cry2Ab and Vip3A resistant. Experiments with a Helicoverpa spp. colony that is simultaneously resistant to Cry1Ac, Cry2Ab and Vip3A suggest that it carries a fitness cost but can nevertheless survive well from the neonate to 3rd instar stage on Bollgard 3 plants. As the larvae mature they are likely to die on Bollgard 3 plants but a small proportion can survive. Following the recommended thresholds should limit opportunities for their selection.

In *H. armigera* several families have scored positive for Cry1Ac resistance but we have been unable to retain them as colonies for various reasons; consequently little is known about this resistance. In *H. punctigera* two different forms of Cry1Ac resistance have been isolated and F_1 data demonstrate that currently <1% of individuals in the population are heterozygous for either of these resistance genes. We isolated one form of resistance to Cry2Ab in *H. armigera* and *H. punctigera* and F_1 data demonstrate that currently 1-5% of individuals in the population are heterozygous for these resistance genes. We isolated 2 forms of resistance to Vip3A in *H. armigera* and currently 4% of individuals in the population are heterozygous for the first isolated form; we have no data on the most recent isolation. In *H. punctigera* currently <1% of individuals in the population are heterozygous for the resistance gene that CSIRO isolated.

These data suggest that currently there is no reason to consider changes to the Resistance Management Plan for Bollgard 3 cotton. However, it will be important to get a more complete understanding of the characteristics of the various Vip3A resistance colonies isolated by CSIRO and Bayer to inform future methods / tools for monitoring resistance (i.e., the number of unique isolations is key for selecting / developing appropriate screening strategies).

While it was premature to validate the molecular tool alongside standard bioassays in this project, we demonstrated that they can be used to detect known mutations and new mutations in previously identified genes. It is essential to validate the molecular tools being developed with standard bioassays in order to translate and incorporate them into any future monitoring programs.

8. Extension Opportunities

This project is part of an ongoing Bt stewardship effort that is integral to decision making around the development of Resistance Management Plan's for Bt cotton. During this project we extended our research findings along with related information on RMP's for Bt cotton in the following industry forums: CRDC Spotlight Magazine, Australian Cottongrower Magazine, TIMS Bt Technical Panel Meeting Presentations, Crop Consultants Australia Seminars, REFCOM presentation, Cotton Production Course, Cotton Conference, and Cotton Pest Management Guide. For the next 12 months we will continue to extend the findings of the work reported herein using the same avenues outlined above.

The research leading on from this project was submitted to CRDC in response to 1920EOI – 0202 'Sustainable management of Helicoverpa species through pre-emptive Bt resistance monitoring'.

9A. List the publications arising from the research project.

Søgaard Jørgensen P, Folke C, Henriksson, Malmros K, Troell, Zorzet A, Living with Resistance project [Peter; Athena Aktipis; Zachary Brown; Yves Carrière; Sharon Downes; Robert R. Dunn; Graham Epstein; Yrjö Gröhn; Govind Tikaramsa Gujar; David Hawthorne; Dusan Jasovsky; Eili Y. Klein; Franziska Klein; Guillaume Lhermie; David Mota-Sanchez; Celso Omoto; Maja Schlüter; H. Morgan Scott; Didier Wernli; Scott P. Carroll], 2019, Coevolutionary governance of antibiotic and pesticide resistance. *Trends In Ecology and Evolution,* Accepted

Carrière Y, Brown ZS, Downes SJ, Gujar G, Epstein G, Omoto C, Storer NP, Mota-Sanchez D, Søgaard Jørgensen P, Carroll SP, 2019, Governing evolution: a socioecological comparison of resistance management for insecticidal transgenic Bt crops among four countries. *Ambio* https://doi.org/10.1007/s13280-019-01167-0

Living with Resistance project [Peter Søgaard Jørgensen; Athena Aktipis; Zachary Brown; Yves Carrière; Sharon Downes; Robert R. Dunn; Graham Epstein; Yrjö Gröhn; Govind Tikaramsa Gujar; David Hawthorne; Dusan Jasovsky; Eili Y. Klein; Franziska Klein; Guillaume Lhermie; David Mota-Sanchez; Celso Omoto; Maja Schlüter; H. Morgan Scott; Didier Wernli; Scott P. Carroll], 2018, Antibiotic and pesticide susceptibility and the Anthropocene operating space. *Nature Sustainability,* 1:632-641 [doi.org/10.1038/S41893-018-0164-3] [Front page feature]

Gomis-Cebolla J, Walsh T, James B, Downes S, Kain W, Wang P, Leonard K, Morgan T, Oppert B, Ferre J, 2018, Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins. *Journal of Invertebrate Pathology*, 155:64-70 [doi: 10.1016/j.jip.2018.05.004]

Luong T, Zalucki M, Perkins L, Downes SJ, 2018, Feeding behaviour and survival of *Bacillus thuringiensis*-resistant and *Bacillus thuringiensis*-susceptible larvae of *Helicoverpa armigera* (Lepidoptera: Noctuidae) exposed to a diet with *Bacillus thuringiensis* toxin. *Austral Entomology*, 57:1-8 [DOI:10.1111/aen.12265]

Walsh T, James B, Chakroun M, Ferre J, Downes S, 2018, Isolating, characterising and identifying a Cry1Ac resistance mutation in field populations of *Helicoverpa punctigera*. *Scientific Reports* 8:2626 (DOI:10.1038/s41598-018-21012-w)

Pearce S, Clarke D, East P, Elfekih S, Gordon K, Jermiin L, McGaughran A, Oakeshott J, Papanikolaou A, Perera OP, V Rane R, Richards S, Tay WT, Walsh T, Anderson A, Anderson C, Asgari S, Board P, Bretschneider A, Campbell PM, Chertemps T, Christeller J, Coppin C, Downes S, Duan G, Farnsworth C, Good R, Han L-B, Han Y, Hatje K, Horne I, Huang Y, Hughes D, Jacquin-Joly E, James W, Jhangiani S, Kollmar M, Kuwar S, Li S, Liu N-Y, Maibeche M, Miller J, Montagne N, Perry T, Qu C, Song S, Sutton G, Vogel H, Walenz B, Xu W, Zhang H-J, Zou Z, Batterham P, Edwards O, Feyereisen R, Gibbs R, Heckel D, McGrathA, Robin C, Scherer S, Worley K, Wu Y, 2017, Genomic innovations, transcriptional plasticity and gene loss underlying the evolution and divergence of two highly polyphagous and invasive Helicoverpa pest species. *BMC Biology* 15:63 [DOI: 10.1186/s12915-017-0402-6]

Whitehouse MAE, Cross D, Mansfield S, Harden S, Johnson A, Harris D, Tan W, Downes SJ, 2017, Do pigeon pea refuges pull their weight as "genetic diluters" to counter Bt resistance in *Helicoverpa* moths? *Crop Protection* 100:96-105

Downes SJ, Kriticos D, Parry H, Paull C, Schellhorn N, Zalucki M, 2017, Perspective on management of *Helicoverpa armigera*: transgenic Bt cotton, IPM and landscapes. *Pest Management Science* 73:485-492

Ives AR, Paull C, Hulthen A, Downes SJ, Andow DA, Haygood R, Zalucki M, Schellhorn NA, 2017, Spatio-temporal variation in landscape composition may speed resistance evolution of pests to Bt crops. *PLoS ONE* 12(1):e0169167 [DOI:10.1371/journal.pone.016916]

Chakroun M, Banyuls N, Walsh T, Downes SJ, James B, Ferré J, 2016, Characterization of the resistance to Vip3Aa in *Helicoverpa armigera* from Australia and the role of midgut processing and receptor binding, *Scientific Reports* 6:24311 [DOI: 10.1038/srep24311]

Downes SJ, Walsh T, Tay WT, 2016, Bt resistance in Australian insect pests. *Current Opinions in Insect Science* 15:78-83

Luong T, Perkins L, Cribb B, Downes SJ, Zalucki M, 2016, Oviposition site selection and survival of susceptible and resistant larvae of *Helicoverpa armigera* (Lepidoptera: Noctuidae) on Bt and non-Bt cotton. *Bulletin of Entomological Research* 2016 Dec:106(6):710-717

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

No online resources were developed as part of this project.

Part 4 – Final Report Executive Summary

The introduction of insecticidal transgenic varieties into the Australian cotton market in the mid-1990's allowed the industry to substantially reduce its pesticide use but resistance continues to threaten its efficacy. Indeed, CSIRO has isolated resistance in the key targets *H. armigera* and *H. punctigera* to all three toxins (Cry1Ac, Cry2Ab, Vip3A) in the current Bollgard 3 varieties. This is set in the context of an emerging global pesticide crisis that could see novel resistant variants of these pests selected elsewhere arrive into Australia.

The industry relies on a pre-emptive strategy to slow the development of Bt resistance. This is underpinned by independent monitoring of background resistance frequencies to enable the industry to autonomously respond to emerging issues, as well as research on other high priority stewardship issues related to Bt resistance in *Helicoverpa* species. The project was conducted in the following three parts.

PART 1: Does multiple resistance to Bt toxins in Helicoverpa spp. pose a threat to 3 gene cotton?

There is a high chance of an insect being resistant to Cry2Ab and Vip3A. Cry1Ac declines as plants age which creates selection opportunities. Our laboratory bioassays demonstrated that it is possible to select for Cry1Ac resistance in a Cry2Ab / Vip3A background.

Experiments with multi-resistant colonies (created from resistant field colonies) challenged with field grown 3-toxin cotton suggest that they carry a fitness cost but can nevertheless survive well from the neonate to 3rd instar stage. As the larvae mature they are likely to die on 3-toxin cotton but a small proportion can survive.

PART 2: Are the frequencies of resistance to 3 gene cotton increasing?

During 2017/18, we used F_1 screens to sample populations of *H. armigera* and *H. punctigera* throughout the industry and did not find evidence of increases over time in the frequencies of resistance to Cry1Ac, Cry2Ab and Vip3A. We also performed F_2 screens and did not isolate any dominant forms of Bt resistance but we did isolate a new recessive Vip3A resistance in *H. armigera*. Our continued survey of CCA members since 2008 supports frequency estimates which suggest that Bt resistance in Helicoverpa species is not increasing.

To assist with development of the molecular tool (as part of CSE1801) we examined F_2 and F_1 individuals previously identified as resistant using bioassays during our monitoring program. We: (1) screened for previously identified mutations and (2) examined whole genome data for novel mutations.

PART 3: What are the characteristics of different variants of Cry2Ab resistance?

There is no indication from our characterisation work that a novel variant of Cry2Ab resistant *H. armigera* poses a different threat to that of the first isolated variant.

Summary: It is unclear to what degree multiple resistance to Bt toxins in Helicoverpa spp. is a threat to stacked gene cotton. Our data suggest that currently there is no reason to consider changes to the Resistance Management Plan for Bollgard 3 cotton. However, it will be important to get a more complete understanding of the characteristics of the various isolated Vip3A resistance colonies to inform future methods / tools for monitoring resistance. It is also essential to validate the molecular tools being developed with standard bioassays to translate and incorporate them into future monitoring programs. Although there currently is no evidence of increasing resistance, it is critical to pre-emptively ascertain any future changes due to, for example, incursions of novel resistances from overseas and/or changing climates driving local selection in Helicoverpa and/or other pests that may carry novel resistance genes to key technologies used in Australia (i.e., Fall Armyworm).

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Appendix 1:

F_2 screen data collected to date during the CSIRO Bt Resistance Monitoring Program.

Table A1: Summary of results from F_2 screens of *H. armigera* and *H. punctigera* against Cry1Ac, Cry2Ab and Vip3A. Data are presented as the frequency for that testing season.

Species	Year	Cry1Ac F_2 screen (alleles)		Cry2Ab F_2 screen (alleles)		Vip3A F_2 screen (alleles)	
		tested	positive	tested	positive	tested	positive
H. punctigera	2002/03	8	0	8	0		
	2003/04	60	0	60	0		
	2004/05	1012	0	1024	1		
	2005/06	468	0	468	0		
	2006/07	712	0	712	2		
	2007/08	1142	0	1142	5		
	2008/09	1088	2	1004	11		
	2009/10	1576	1	1844	10	788	5
	2010/11	164	0	220	2	204	2
	2011/12	544	0	544	14	536	6
	2012/13	572	2	808	8	636	7
	2017/18	**354**	**6**	**336**	**2**	**350**	**2**
	Total	7700	11	8170	55	2514	22
H. armigera	2002/03	136	0	132	1		
	2003/04	280	0	284	2		
	2004/05	364	0	368	0		
	2005/06	900	0	900	4		
	2006/07	524	0	524	5		
	2007/08	772	0	772	4		
	2008/09	804	0	812	8		
	2009/10	484	0	616	6	432	11
	2010/11	624	2	804	17	660	17
	2011/12	572	1	600	8	572	14
	2012/13	476	0	524	5	484	12
	2017/18	**658**	**7**	**640**	**1**	**615**	**25**
	Total	6594	10	6976	61	2763	79