CRDC

FINAL REPORT

Resistance of *Helicoverpa armigera* to Cry2A

CSE 96C

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Part 1 - Summary Details

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

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28/9/04

1. Background

In project CSE72C we selected a strain (BX) of H. armigera that is resistant to Cry1Ac, the active component of INGARD cotton. By characterising this strain, we showed that *H. armigera* is capable of developing a level of resistance that allows it to complete its development from egg to fertile adult on young, glasshouse grown INGARD cotton plants. We also showed that the resistance is due to a loss of the higher affinity Cry1Ac binding site, which is the only resistance mechanism identified in field populations of a Cry1A-resistant species (diamondback moth). This suggests that the BX strain is representative of the resistance that could occur in field populations of *H. armigera.* However, experience with other species shows that Bt resistance can also arise through other mechanisms, indicating that examination of other resistant *H. armigera* strains (e.g. our VRX strain) is warranted.

We have shown that the BX strain is cross-resistant to the Cry1Aa and Cry1Ab toxins. However, it is not resistant to the Cry2Aa toxin and is not expected to be resistant to Cry2Ab.

It is evident from experience with the resistance of *H. armigera* to chemical insecticides and its demonstrated ability to develop resistance to Cry1Ac that single-gene cotton is not a sustainable control strategy. The second generation of transgenic cotton (BOLLGARD II) contains both the *cry1Ac* and *cry2Ab* genes. This combination was expected to significantly reduce the ease with which *H. armigera* can develop resistance to these plants. However, a mutagenesis approach adopted by Dr J. Daly in project CSE73C has shown that *H. armigera* is at least capable of low level cross-resistance to Cry2Aa, as well as to Cry1Ac (Line EMS21). This result shows that the cotton industry needs to prepare itself for resistance to Cry2Ab as it has with Cry1Ac.

As Line EMS21 was derived by mutagenesis, it may not reflect resistance that will occur in field populations. It will therefore be desirable to generate Cry2A resistance as we did for Cry1A resistance, by selecting field-collected insects with Cry2Ab.

2. Objectives

The aim was to determine the magnitude and nature of resistance to Cry2Ab by *H. armigera* so that an appropriate resistance strategy can be implemented to sustain the use of cry1Ac/cry2Ab transgenic cotton. In the first instance, we set out to establish a Cry2Ab-resistant strain by laboratory selection. Based on our experience in selecting for Cry1A-resistance, we expected that the selection process would be lengthy and that we may only succeed towards the end of the project, at which time we would undertake a preliminary characterisation.

The project was successful in generating not only one but three Cry2Ab-resistant strains. Preliminary investigation of one strain showed it does not confer cross-resistance to Cry1A toxins.

3. Methods

All Cry2Ab selections and bioassays were made with a spore/crystal mixture from a recombinant strain of *B. thuringiensis* provided by Dr Luke Masson, National Research Council of Canada, which produces the Cry2Ab protoxin alone. The choice of the spore/crystal mixture was made for two reasons. First, separation of the spore and crystal required the use of sucrose gradient centrifugation that is very labour-intensive and therefore expensive at best and was not very efficient for this strain. Secondly, we previously demonstrated the successful use of a spore/crystal mixture in selecting for Cry1A resistance. In preparing Cry2Ab for this project, we made sufficient quantity to support projects CSE102C and CSE104C in 2002/03.

Dose/response bioassays were conducted using the diet incorporation technique. Although this technique has the disadvantage of requiring substantially more toxin than the surface contamination method, it produces more reproducible data. The amount of toxin required was reduced by scoring the bioassay on growth rate, assessed by scoring the number of larvae that developed to third instar by 7 d, rather than on mortality. The LDI used will shortly be published in Bird and Akhurst (2004, J. Econ. Entomol.). Each bioassay was replicated at least three times and the data combined for probit analysis using the POLO-PC software (LeOra Software) to estimate IC_{50}, the concentration required to halve the growth rate.

Resistance ratios were determined by dose/response bioassays of the selected strain and the ANGR strain as the susceptible baseline control. ANGR was chosen as the susceptible strain because its susceptibility to Bt toxins is more similar than the GR strain to the susceptibility of field strains. Resistance ratio was calculated as the ratio of IC_{50} values. The use of LC_{50} is method of calculation is standard in the vast majority of studies of resistance to Bt toxins throughout the world because the inherent variation in dose/response data for Bt toxins is lowest at this point, making it a more reliable indicator of resistance than LC_{90}. The use of the inhibitory indicator (IC_{50} or EC_{50}) rather than the mortality indicator (LC_{50}) has been accepted as valid in the scientific literature (Liao *et al*, 2002, J. Invertebr. Pathol. 80: 55-63; Wu *et al.*, 2002, J. Econ. Entomol. 95: 826-831). It should be noted that the use of IC_{50} produces an estimate of the resistance ratio that is about 10 times greater than the estimate obtained with LC_{50}.

Selection for Cry2Ab Resistance

Selection commenced in 2001 when survivors of high dosages in bioassays of Kununurra and Bundaberg strains were pooled and added to survivors from screening bioassays of populations from Emerald (neonates on 10μg Cry2Ab ml^{-1} diet) and Bundaberg (3^{rd} instar larvae on 50μg Cry2Ab ml^{-1} diet) F_1 strains. Larvae from these screens and bioassays that survived to the 3^{rd} instar at day 7 were bulk reared and the progeny, designated the TAB '01 strain, were screened at doses from 2 – 20μg ml^{-1} diet of Cry2Ab spore/crystal mix. The choice of selection based on growth rate rather than survival was made because our experience in selecting for Cry1A resistance in *H. armigera* showed that the sublethal effects of Bt toxins are such that selection on the basis of survival could not be sustained.

TAB '01 was bioassayed four times at irregular intervals for six generations. The resistance ratio, determined by comparison of the LC_{50} values for TAB '01 and ANGR, ranged between 2- and 7-fold.

The TAB '01 strain suffered from inbreeding depression because of the intensity of selection pressure. Survivors from bioassays of Cry2Ab conducted with the EMS21 and GRS (an outcross of the susceptible GR strain with a field strain from sunflower in Griffith) strains were subsequently added to TAB '01. Despite efforts to improve the vigour of this strain by the addition of genetic diversity from these strains, the TAB '01 strain was terminated late in 2002.

Selection of TAB '02 commenced at the start of the 2002/3 season. Field collected *H. armigera* eggs from the Namoi, Gwydir and Griffith areas in New South Wales and F_1 strains from Katherine (NT), Emerald and Goondiwindi (QLD), Moree, Wee Waa, Griffith, and Hillston (NSW) were screened by rearing larvae on diet containing Cry2Ab at a concentration of $\geq 0.74 \mu g\ ml^{-1}$ (spore/crystal mix). The screening dose was reduced significantly compared with the previous season to improve detection of heterozygotes for incompletely dominant resistance mechanisms.

A total of 386 larvae developed to the third instar by day 7 (249 from F_1 strains, 137 from field collected) from these screens. These insects were pooled as the TAB '02 strain and reared to adults on artificial diet without Cry2Ab. Neonates of subsequent generations were selected at $0.74 \mu g$ Cry2Ab ml^{-1} for 7 days, after which they were transferred to toxin-free diet to complete their development.

TAB '02 was reared for three generations, with only two generations being subjected to selection because of difficulty in maintaining the strain under sustained selection. In the third generation, the TAB '02 strain was outcrossed to the ANGR strain because of concerns about inbreeding depression; the outcrossed strain was designated TABOC. Selection was relaxed for the following generation and recommenced in generation 5; selection pressure was increased to $6 \mu g$ Cry2Ab ml^{-1} and was maintained each generation thereafter.

Test for Cross-resistance in TABOC

TABOC was tested for cross-resistance to the four Cry proteins to which *H. armigera* is known to most susceptible. Diet incorporation bioassays were conducted with spore/crystal mixtures of Cry1Ab, Cry1Ac and Cry2Ab and solubilised protoxin of Cry2Aa and was scored at 7 d using the number of larvae reaching third instar as the indicator of growth rate. Cry2Aa was used as a solubilised protoxin because it was produced by Dr W. Moar (Auburn University, USA), in *E. coli* rather than *B. thuringiensis* and so did not form crystals.

Selection for dual resistance to Cry1Ac and Cry2Ab

The IS strain was the result of a highly Cry1Ac-resistant strain that had been outcrossed to three separate susceptible field strains (originating from Emerald, Bundaberg and Boggabri), which were combined and reselected on diet containing $42 \mu g\ ml^{-1}$ Cry1Ac (spore/crystal mixture). The VR strain, initially established from Gippsland, Vic., by Dr Neil Forrester, and maintained under selection in our laboratory for approximately ten

years is also highly resistant to Cry1Ac. These Cry1A-resistant strains of *H. armigera* were selected on diet containing both Cry1Ac and Cry2Ab toxin for three generations; the dual selected strains were designated IS2Ab and VR2Ab.

4. Results

Selection for Cry2Ab Resistance

When the TABOC strain was bioassayed in generation 6 by diet incorporation, the resistance ratio was 33-fold (Table 1). Following a further four generations of continuous selection at 6μg ml^{-1}, the resistance ratio exceeded 100-fold.

Table 1. Development of resistance to Cry2Ab in the TABOC strain as the result of laboratory selection.

Generation No. [a]	Selection dose (μg/ml diet)	IC_{50}(μg/ml diet)	95% confidence interval	Slope	Resistance Ratio
1	0.74	NT[b]			-
2	no selection	NT			-
3	0.74	NT			-
4	no selection	NT			-
5	0.74	NT			-
6	6.00	10.0	7.8 - 12.7	2.0	33
7	6.00	24.5	14.9 - 34.5	2.8	82
8	6.00	NT			-
9	6.00	74.8	45.7 - 97.6	2.8	249
10	6.00	32.1	25.0 - 40.2	2.0	107
11	6.00	NT			

[a] Generations 1-3 were the TAB '02 strain. The third generation was outcrossed to ANGR creating the TABOC strain

[b] NT - not tested

Test for Cross resistance in TABOC

Bioassays conducted on the TABOC strain in generation 10 indicate that there is resistance to Cry2Aa as well as very low level cross-resistance to the Cry1 class of toxins (Table 2).

Table 2. Cross-resistance of the Cry2Ab-resistant TABOC strain. The relative susceptibilities of Cry2Ab susceptible (ANGR) and resistant (TABOC) strains of *H. armigera* to Bt insecticidal proteins were determined. Cry1Ac, Cry1Ab and Cry2Ab were tested as a spore/crystal mix; Cry2Aa was tested as a solubilized protoxin.

Toxin	Toxin			TABOC			Resistance ratio
	IC_{50} (μg/ml)	95% CI	Slope	IC_{50} (μg/ml)	95% CI	Slope	
Cry1Ab	0.2	0.2 - 0.3	2.6	2.6	1.4 - 4.3	0.8	13
Cry1Ac	0.5	0.4 - 0.7	2.6	5.6	4.2 - 7.2	1.3	11
Cry2Aa	0.1	0.06 - 0.11	1.3	14.1	9.4 - 18.6	2.1	141
Cry2Ab	0.3	0.2 - 0.4	2.2	32.1	25.0 - 40.2	2.0	107

Selection for dual resistance to Cry1Ac and Cry2Ab

The IS2Ab and VR2Ab strains were found to have retained their extremely high levels of resistance to Cry1Ac while acquiring moderate levels of cross resistance to Cry2Ab (Table 3). It is evident from this data that significant resistance to both Cry1A and Cry2A toxins can co-exist within individual insects and that gene stacking alone will not prevent *H. armigera* from feeding on Cry1A/Cry2A transgenic cotton.

Table 3. Cross-resistance in two Cry1A-resistant strains. Relative susceptibilities to Cry1Ac and Cry2Ab of Cry1A-resistant strains selected for three generations against both Cry1Ac and Cry2Ab and a Cry1A/Cry2A susceptible (ANGR) strain of *H. armigera* were determined. Cry1Ac and Cry2Ab were tested as a spore/crystal mixes.

Parameter	Cry1Ac			Cry2Ab		
	ANGR	IS2Ab	VR2Ab	ANGR	IS2Ab	VR2Ab
IC_{50} (μg/ml diet)	0.5	>5120	>5120	0.3	17.7	16.6
95% CI	0.4 - 0.7	nd	nd	0.2 -0.4	12.8 -24.6	12.3 - 22.0
Slope	2.6	nd	nd	1.8	2.1	2.2
Resistance ratio	-	>4655	>4655	-	59	55

5. Conclusions

The project was successful beyond expectations in that it produced not one but three strains of *H. armigera* with resistance to Cry2A. The development of Cry2A resistance in three independent strains after only three generations of selection, coupled with the discovery of Cry2A resistance alleles in field collected insects (in project CSE104C), demonstrates that the optimism that Bollgard II cotton would provide a solution to the resistance issue may have been misplaced.

The lack of cross-resistance to Cry1A toxins in the TABOC strain shows that the "gene from hell" that would confer resistance to any Cry toxin has not been detected. Although TABOC has slight cross-resistance to Cry1Ac (RR = 11), this is within the normal variation in susceptibility of *H. armigera* field strain (Wu *et al.*, 1999, J. Econ. Entomol. 92: 273-278) and is not considered a specific threat.

However, the selection for Cry2Ab resistance in the previously Cry1A-resistant strains (IS2Ab and VR2Ab) shows that resistance for both toxins in Bollgard II can co-exist within one insect. It appears that the additive effects of the fitness costs associated with either resistance does not present an insuperable burden for the insect, at least as it feeds on artifical diet. These strains need to be tested on cotton to determine if the double burden can be supported in the field. It might be noted that a highly Cry1A-resistant *Heliothis virescens* strain (RR >10,000) cannot survive on conventional cotton.

It appears that the resistance detected in the TABOC strain may differ from that of the SP15 strain obtained by the use of the F_2 screen (CSE104C). It is very unlikely that a strain that can be selected for resistance in three generations from a starting culture of a few hundred insects is based on a highly recessive gene, as appears to be the case for SP15. In addition, the resistance ratio for the TABOC strain is much less than that of the SP15 strain, which suggests that the strains have different resistance mechanisms or that the TABOC strain is far from homozygous for the resistance allele detected in SP15. However, if TABOC had the same allele as SP15, it should have quickly moved to homozygosity under the selection regime imposed. It therefore appears that *H. armigera* has at least two options for resistance to Cry2A. The implications of this need to be examined through a thorough characterisation of all of the Cry2A-resistant strains.

6. How research has addressed the Corporation's three Outputs - Economic, Environmental and Social

This project has produced evidence of the ease with which *H. armigera* is able to develop resistance to the Cry2Ab toxin and that this pest is capable of supporting resistance to both toxins produced by Bollgard II. Consequently, it provides a strong argument for the need to maintain an effective resistance management strategy if the economic and environmental benefits of Bt cotton are to be sustained.

7. Summary

The development of Cry2A resistant *H. armigera* is the first reported example of specific selection for Cry2Ab resistance, although Dr Rod Mahon (CSE104C) has detected Cry2Ab resistance in *H. armigera* through an F_2 screen and Jurat-Fuentes *et al.* (2003, Appl. Environ. Microbiol. 69: 5898-5906) reported Cry2Aa resistance in two *H. virescens* strains selected against Cry1Ac. The mechanism in the TABOC strain is apparently significantly different from that of the SP15 strain of *H. armigera* as described above and from the *H. virescens* strains in that it does not provide cross-resistance to Cry1A.

There is no intellectual property that can be secured by patent but the TABOC strain may have significant value in developing a diagnostic for Cry2 resistance that may be useful beyond *H. armigera*. It is proposed that this strain, as also the SP15 strain, be retained in-house while their IP value is more fully assessed.

8. Future activities

The Cry2A resistant strains developed in this project are being more fully characterised in a new CRDC funded project. Determination of fitness cost and mode of action will be

used, in conjunction with F_1 and F_2 screens of field populations to assess the nature and level of threat to the Australian cotton industry posed by Cry2 resistance. It is hoped that funding can be secured to enable a postgraduate student to utilise these strains, and a Cry1A resistant strain, in a proteomic analysis that will provide the basis for development of rapid diagnostics for resistance.

The results of this project and the early part of the new project will be published in the international scientific literature.

9. Publications arising from the research

Akhurst, R., Olsen, K., Bird, L. and Mahon, R. (2004). Resistance to Cry2Ab in *Helicoverpa armigera*. Abstr.37th Ann Conf. Soc. Invertebr. Pathol., Helsinki. p.78. (attached)

10. Online resources

None developed.

11. Assessment of the likely impact of the results and conclusions of the research project for the cotton industry.

The discovery of the facility with which *H. armigera* can develop substantial resistance to Cry2A indicates that TIMS may need to re-evaluate the resistance management strategy. Data gathered over the next six months will be critical for informing the industry on the sustainability of the current refuge strategy. It is evident that, if Bollgard II is compromised before the next generation of transgenic cotton (e.g. VipCot) becomes commercially available, the industry can expect to bear significant economic and environmental costs.

Final Report Executive Summary

It is evident from experience with the resistance of *H. armigera* to chemical insecticides and its demonstrated ability to develop resistance to the Cry1Ac toxin produced in INGARD™ that single-gene cotton is not a sustainable control strategy. The second generation of transgenic cotton (BOLLGARD II) prodcues both the Cry1Ac and Cry2Ab toxins. This combination was expected to significantly reduce the ease with which *H. armigera* can develop resistance to these plants. However, a mutagenesis approach adopted by Dr J. Daly has shown that *H. armigera* is at least capable of low level cross-resistance to a Cry2A toxin (Cry2a), as well as to Cry1Ac. This result shows that the cotton industry needs to prepare itself for resistance to Cry2Ab as it has with Cry1Ac.

The aim of this project was to determine the magnitude and nature of resistance to Cry2Ab by *H. armigera* so that an appropriate resistance strategy can be implemented to sustain the use of cry1Ac/cry2Ab transgenic cotton. In the first instance, we set out to establish a Cry2Ab-resistant strain by laboratory selection. Based on our experience in selecting for Cry1A-resistance, we expected that the selection process would be lengthy and that we may only succeed towards the end of the project, at which time we would undertake a preliminary characterisation.

The project was successful beyond expectations in that it produced not one but three strains of *H. armigera* with resistance to Cry2A. The development of Cry2A resistance in three independent strains after only three generations of selection, coupled with the discovery of Cry2A resistance alleles in field collected insects (in project CSE104C), demonstrates that the optimism that BOLLGARD II™ cotton would provide a solution to the resistance issue may have been misplaced.

Preliminary characterisation of the first of the laboratory-selected Cry2A-resistant strain (TABOC) showed that is not associated with resistance to Cry1Ac and therefore would not be expected to survive on early season BOLLGARD II™. It appears that the resistance detected in the TABOC strain differs from that in the strain obtained by the use of the F_2 screen (SP15). It therefore appears that *H. armigera* has at least two options for resistance to Cry2A.

It is too early at this time to identify the likely consequences of Cry2A resistance in *H. armigera*. In a successor project to this and CSE104, we will establish whether we have detected one or more types of Cry2A resistance in *H. armigera*. We shall also determine the dominance of the resistance alleles in the TABOC and SP15 strains and the fitness cost associated with that resistance. This will enable us to develop a refuge strategy to minimise the risk of resistance that would undermine the sustainability of BOLLGARD II™ cotton. Until there is a better understanding of the nature and extent of the threat posed by Cry2A resistance, the cotton industry would be well advised to adopt a cautious attitude to its management of BOLLGARD II™.

ATTACHMENT

Conference presentation:

Akhurst, R., Olsen, K., Bird, L. and Mahon, R. (2004). Abstr. 37th Ann. Conf. Soc. Invertebr. Pathol., Helsinki. p.78.

Resistance to Cry2Ab in *Helicoverpa armigera*

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Transgenic cotton expressing the Cry1Ac and Cry2Ab toxins of *Bacillus thuringiensis* (BOLLGARD II™) being introduced into Australia is expected to reduce the risk of the evolution of resistance by *H. armigera* to these and similar toxins. This cosmopolitan pest has demonstrated its capacity for developing resistance to synthetic chemical insecticides and to the Cry1A toxins, such as the Cry1Ac produced in the first generation of transgenic cotton. Investigation has rapidly confirmed the capacity for *H. armigera* to develop resistance to Cry2Ab. Five lines of *H. armigera*, including a field derived line obtained from an F_2 screen, with various levels of resistance to Cry2Ab have been established in our laboratory using several approaches. Some lines also have some resistance to Cry1Ac. Characterisation of resistance in these lines will be discussed.