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

Part 1 - Summary Details

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

CRDC Project Number: **CSE008**

Project Title: Evaluating gene silencing technologies to control *H. armigera*

Project Commencement Date: 1July 2007 **Project Completion Date:** 30 June 2010

CRDC Program: 3 Crop Protection

Part 2 – Contact Details

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

(The points below are to be used as a guideline when completing your final report.)

Background

There has been growing interest in recent years in the possibility of using RNA interference (RNAi) to control insect pests such as *Helicoverpa armigera*. RNAi is a very specific method to silence a target gene by introducing double-stranded RNA molecules into the cell in which the gene is expressed (for background reviews, see Gordon and Waterhouse, 2007; Gordon et al., 2009, Obbard et al., 2009). However its application to the control of lepidopteran insects has proven very frustrating. A review currently in press (Terenius et al., 2010) documents and analyses experiments by many researchers around the world to study RNAi in lepidopteran insects (including *H. armigera*), and illustrates the lack of any clear explanation as to why some experiments work and most do not. Among the important factors that appear to affect the effectiveness of RNAi, in addition to being able to deliver sufficient double stranded RNA (dsRNA), are the choice of gene targeted, as well as the tissue in which it is expressed. These factors were also found to be important for RNAi of genes in a beetle, the Western Corn Rootworm (WCRW) (Baum et al., 2007). The relative efficiency with which RNAi can be achieved in beetles allowed this group to study a large number of genes, which were found to vary very significantly in their susceptibility to RNAi.

There have been some very promising results in *Helicoverpa*. Most dramatically, a group at the Chinese Academy of Sciences in Shanghai recently identified a gene for a particular cytochrome P450 that is induced by gossypol (Mao et al., 2007). Knockdown of the gene's expression through transformation of *Arabidopsis* plants to express the dsRNA for the P450 slowed growth of larvae feeding on the plants to which gossypol had been added. This group is extending its work to study the possibility of delivering RNAi via cotton, but no results have yet been published. Work at CSIRO Entomology by a PhD student sponsored by CRDC (Derrick Collinge) showed that delivery of dsRNA or short silencing RNA (siRNA) fragments using either egg injection or feeding to *H. armigera* neonates reduces expression of reporter target genes (Gordon et al 2010), Terenius et al., 2010). There has also been reported successful RNAi application in older larvae by haemocoel injection of dsRNA that targeted a Bt receptor, an aminopeptidase (Sivakumar et al., 2007). These results were supported by studies in *H. armigera* by several international groups (Terenius et al., 2010).

Together, these results suggest that not only is RNAi capable of working in *H. armigera* under some circumstances, but the RNAi triggers can be delivered orally to the insect, a concept that has potential to develop into a plant-based dsRNA delivery system for pest control.

Objectives

The objective of the current research project into control of *H. armigera* using RNAi is to ask whether effective RNAi can be achieved by producing the dsRNA triggers required for RNAi in plant material for feeding directly to larvae. To test this on limited scale, consistent with the pilot nature of this project, we chose a small number of genes from Helicoverpa whose counterparts had been identified as among the most susceptible genes in the WCRW study (Baum et al., 2007).

The 5 genes chosen to test the efficacy of orally delivered dsRNA for RNAi, were selected from CSIRO's EST (expressed sequences tag) library of *Helicoverpa armigera* midgut RNA (Angelucci et al., 2008). These genes are:

* HaC5S00202 (COPI coatomer, beta' subunit).
* HaC5S01162 (COPI coatomer, beta 2 subunit).
* HaC5S00865 (v-ATPase, subunit D).
* HaC5S01607 (ribosomal protein S4).
* HaFLS00593 (gossypol induced cytochrome p450).

Each of these genes are expressed in the larval midgut. The first four are known to be essential genes, and represent the most highly susceptible genes in the WCRW study; the fifth is an enzyme specifically required for gossypol detoxification, i.e. the P450 gene in *H. armigera* required for detoxification of gossypol in cotton (Mao et al 2007).Our work on this project has concentrated on producing five constructs for introduction into *Agrobacterium.* Upon infection by the bacteria of plant cells, expression of the appropriate dsRNA from each construct should interfere with target genes in *H. armigera* larvae that feed on the infected plant tissue.Upon completion of the constructs, they were tested in preliminary infections of *Nicotiana benthamiana*, a relative of tobacco that is particularly amenable to agroinfiltration. Further work explored other hosts as needed, including the potential of performing agroinfiltration experiments in cotton. Rigorous testing of the ability of these dsRNA constructs to trigger RNAi would however probably require extensive testing of transgenic plant material on the scale used by the Chinese groups that first showed this approach to be feasible, but was beyond this pilot project.

Methods

DNA vectors containing suitable dsRNA expression cassettes for introduction into *Agrobacterium* were constructed as described below.A central question in using RNAi for crop protection is whether the expression of dsRNA in plant cells allows effective delivery for gene silencing in insects that feed on the plant material. To test this more rapidly than is possible using transgenic plant material, a technique for achieving transient expression of dsRNA in plant cells has been employed. Working with Peter Waterhouse’s group at CSIRO Plant Industry, (a world leader in RNAi research) we have produced constructs to transiently express dsRNAs in plant material. This approach (called Agroinfiltration) involves treating the leaf with *Agrobacterium* strains carrying expression cassettes for dsRNAs of interest. The somatic plant cells become infected with the *Agrobacterium*, subsequently producing dsRNAs from the expression constructs. This dsRNA can be processed by the plant RNA dicing enzymes to yield siRNAs likely to be effective against feeding larvae.

Results

1. Construction and verification of DNA vectors

We developed 5 Agrobacterium clones with constructs that target the various selected *H. armigera* genes. The clones contain sequences that target COPI coatomer beta subunit; COPI coatomer beta 2 subunit; v-ATPase subunit D; the cytochrome P450 induced by gossypol; and ribosomal protein S4.The various DNA ‘kits’ and primers for the genes were acquired and the sequences re-cloned into a vector suitable for dsRNA synthesis upon *Agrobacterium*-mediated delivery to plant tissue. The following steps were carried out:

Designed primers to PCR each of the genes. PCR products for each gene were produced with attB1 and attB2 ends to enable Gateway recombination. PCR products were run on gels and their correct size confirmed.

1. Using the Gateway Technology cloning system, PCR products were cloned into pDONR 221. Restriction enzyme (XhoI and XbaI) digestion confirmed that the correct orientation of the genes in the donor vector was achieved.
2. Each gene was then fully sequenced to confirm its identity and that the clones were full-length.
3. A second recombination reaction was used to insert the two copies (sense and anti-sense) of the target gene into the pHELLSGATE vector. Restriction enzyme (XhoI and XbaI) digestion confirmed the correct orientation of the genes in the destination vector. Transcription across these paired, inverted copies of the insert gives a hairpin RNA.
4. The pHELLSGATE+target gene construct was transferred into *Agrobacterium* (GV3101) by the triparental mating. Restriction digests confirmed insertion of pHELLSGATE vector containing the target gene into *Agrobacterium*.

2. Evaluate the efficacy and variability of Agrobacterium infection in cotton and tobacco.

In order to use agroinfiltration to deliver *Agrobacterium* for dsRNA production, we first asked whether the introduction of a gene expressing a visible reporter, the green fluorescent protein (GFP) was possible with the agroinfiltration process. The first plant selected was *N. benthamiana*, due to its being particularly amenable to agroinfiltration. We have successfully expressed green fluorescent protein (GFP) in leaves of growing *N. benthamiana* plants, achieving widespread infection and expression visible under UV lamps. However, we were less successful at infecting cotton as the relatively robust cotton leaf resists our method of infection. Essentially the technique is to force a solution of *Agrobacterium* containing the DNA construct under pressure through stomata allowing it to disperse between the upper and lower surfaces of the leaf. Leaf cells coming in contact with the construct become infected and begin to express the desired GFP protein. The cotton leaf resisted the hydrostatic pressure applied. Considerable effort was expended to overcome this by varying conditions in order to fully dilate stomata that might allow easier access to the mesophyll cells within the leaf, without marked success.

3. E**valuate insect responses to agroinfiltration of dsRNA-expressing Agrobacterium.**
(a) *Nicotiana benthamiana*,

Initial experiments to ask whether RNAi could be achieved upon agroinfiltration were conducted using the tobacco relative *N. benthamiana*. These assays involved using the constructs listed above for agroinfiltration. Bioassays for the effect of gossypol, and for the ability of the cyp450-dsRNA to render insects susceptible to gossypol, involved painting a solution of pure Gossypol (purchased from Sigma) on to the leaves, followed by feeding to neonate larvae. Gossypol was applied at a concentration of 100 ug/ml, which significantly retards *H. armigera* larval growth in comparison to controls (Mao et al., 2007). Control experiments were carried out omitting either agroinfiltration, or using only water, or non-insect reporter genes such as GFP (green fluorescent protein). These control bioassays on *N. benthamiana* showed that overall larval growth on the plants was slower and much more variable than on the standard diet, with the Larval Development Index (LDI) reduced from 3.5-4 on diet (over the time-course used) to a broader range of 2.3-3.8 on plants. There was marked inter-plant variability in larval survival, possibly due to variation in plant defence

chemicals. This variability was also evident in larvae raised on samples treated with gossypol and/or subject to agroinfiltration, making the experiment very difficult to interpret. The effects on larvae, if any, were masked by the inherent variability in the experiment.

We also performed experiments using late second instar larvae rather than neonates on the *N. benthamiana* leaves, based on the experiments described by Mao et al. (2007) who also used older larvae in their studies on tobacco and Arabidopsis. Control survival was improved, however some variability remained within and between experiments. The results from one experiment, in Table 1, showed no significant evidence that application of dsRNA-expressing *Agrobacterium* strains rendered the larvae susceptible to gossypol; indeed these treated larvae grew better than those subject only to gossypol in this experiment, illustrating the variability inherent in this approach. Although agroinfiltration of strains expressing dsRNA designed to knockdown the other target genes apparently resulted in slower growth for some targets (eg v-ATPase, subunit D and COPI coatomer, beta 2 subunit) this effect was neither pronounced nor apparent in all experiments. Overall, this tobacco-related plant was concluded to be not an ideal host for larvae, and unsuitable for this experimental approach.

Table 1.
Results on *N. benthamiana* using late 2nd instar, 16 larvae per assay

Treatment	**Scored at day 5**	
	%survival	LDI
Control - artificial diet	100	3.69
Tobacco leaves	100	3.19
Gossypol	94	3.2
gossypol + cyp450-dsRNA	100	4
v-ATPase, subunit D dsRNA	88	3.07
COPI coatomer, beta 2 subunit dsRNA	94	3
COPI coatomer, beta' subunit dsRNA	88	3.79
ribosomal protein S4 dsRNA	100	3.19

(b) Cowpea

In view of the variable plant effects seen with *N. benthamiana*, an alternative host, cowpea (*Vigna unguiculata*), has been tested. Agroinfiltration has proven possible with this species, although the injection process is not as easy as in tobacco. However, it has the marked advantage that bioassays are consistent and there appears to be no variable effect due to plant agroinfiltration on *H. armigera* larvae in the controls (Table 2).

Table 2.
Testing larval growth on cowpea

Diet type	**Dead**	**Alive**	**%Alive**	**1**	**2**	**3**	**4**	**5**	**LDI**
Plant 1	0	4	100			3			2.25
Plant 2	2	14	87.5			14			3.00
Plant 3	1	11	91.667		2	9			2.82
Plant 4	1	14	93.333			14			3.00
Plant 5	2	13	86.667		1	12			2.92

We next tested the effect of gossypol on larval survival and growth. Again, the results were somewhat variable, but overall there was little effect on larvae, with the LDI not

differing significantly from the control; this illustrated the ability of *H. armigera* larvae to cope with the main allelochemical found in cotton. Addition of the dsRNA targeting the detoxification gene by agroinfiltration of the appropriate strain, gave quite variable results, with elevated mortality evident in one experiment but generally no significant effect on the LDI compared to controls.

We then assessed the efficacy of three other unique constructs targeting selected genes in *H. armigera*. Some intriguing results were obtained, with the dsRNA targetting the two COPI coatomer subunits noticeably retarding the LDI over the first 7 days of one bioassay; the effect declined after this point and all larvae successfully proceeded to pupate. None of the constructs evoked a marked or extended suppression of larval growth in these assays.

Table 3.

Results on cowpea using neonates, 16 larvae per assay.

Treatment	**Scored at day 7**		**Scored at day 12**		***Scored at day 22**
	%survival	LDI	%survival	LDI	% pupation
Control	94	3.6	94	3.73	94
gossypol	100	2.4	nd	nd	nd
gossypol + cyp450-dsRNA	92	2.67	92	3.63	nd
v-ATPase, subunit D dsRNA	100	2.94	nd	nd	nd
COPI coatomer, beta 2 subunit dsRNA	94	2.73	94	3.2	88
COPI coatomer, beta' subunit dsRNA	88	2.79	88	3.29	88

(* transferred to artificial diet @ day 14)

(c) Cotton

Repeated attempts to achieve agroinfiltration for cotton failed to provide any evidence of success: in experiments using control reporter genes, no GFP fluorescence was observed. Leaves at any stage were found to be too rigid to allow the bacterial solution to enter stomata. Further experimentation, involving changes to the growth and humidity conditions under which the plants were grown, failed to overcome this problem within the time available, although more extensive practice might achieve successful results. Under these conditions, it was judged pointless to attempt direct agroinfiltration of the dsRNA-expressing bacterial strains.

Brief summary and notable achievements

We have successfully developed constructs designed to generate dsRNA targeting specific genes of *H. armigera* and further demonstrated we can express a reporter gene by agroinfiltration in the tobacco relative *N. benthamiana* and in cowpea. We have performed several experiments investigating differing growth conditions in an attempt to produce cotton that will respond to the injection procedure. We have focused our attention on cotton in order to attempt to replicate work by a Chinese group that targeted gossypol deactivation using RNAi. However to date, experiments with cowpea remain the most informative performed, and while some experiments were suggestive on an impact, overall the results did not indicate a positive effect of the constructs.

Outcomes

No technical advances of commercial significance were foreseen. This pilot study solely concerned experiments conducted to test biosassays of larvae on plants treated with agroinfiltration to express dsRNA designed to silence larval genes. Control bioassays on *N. benthamiana* showed that larval growth on different plants is highly variable, making it impossible to determine any responses to agroinfiltration. This variability observed means that we have found it necessary to identify a better experimental system. Results of control experiments using cowpea were encouraging allowing experiments to be performed to test the effects of agroinfiltration of dsRNA-expressing strains. However no conclusive evidence for an RNAi effect was obtained. Further work is still needed to overcome technical difficulties to performing agroinfiltration in cotton. This methodology for RNAi testing applied remains novel, but has not been verified by the limited work possible under this grant. It is likely that we may need to use transgenic plants to verify plant-mediated RNAi in larvae.

No changes are required to the IP register.

Conclusion

Recent advances in molecular biology have opened possible new control technologies. CSIRO's large genomics effort is delivering not only the complete genome of *H. armigera*, but has also sequenced many thousands of genes expressed in the midgut of larvae. Gene silencing by RNAi will allow us to exploit the genomics results for pest control. RNAi involves introduction of double-stranded RNA (dsRNA) into a target species, resulting in highly specific gene silencing. By reducing the expression of any essential gene, RNAi could be used as an effective method of protecting crop plants from insect damage or of controlling the propagation of the pest insects themselves.

Most workers attempting to achieve reproducible and effective RNAi-knockdown of genes in Lepidoptera such as *Helicoverpa armigera* have found this to be very challenging, for reasons that are not clear (eg Terenius et al., 2010). However the report by Mao et al. (2007) suggests that the plant-based expression of dsRNA is an effective way of achieving knockdown of biologically important genes. In this pilot project, we asked whether any evidence of RNAi-caused knockdown could be observed upon transient expression of dsRNA in plant tissue. This approach proved to be challenging for a number of reasons, and no consistent evidence of RNAi was observed in any bioassay.

The use of alternative hosts was also explored, and we have also tested constructs using cowpea as a host. Injections are more successful than with cotton, and several bioassays have been performed using *H. armigera* neonates fed cowpea leaves infected with experimental constructs. Unfortunately, none of the constructs or experiments induced increased mortality or modified larval growth relative to controls (buffer only injected leaves). Further cowpea experiments showed no significant difference in mortality between the injected clones and the controls.

These experiments indicated that it would be necessary to undertake a full-scale project replicating the complete experiment reported by Mao et al. (2007) by first making transgenic plants (Arabidopsis or tobacco). Although an initial attempt to transform the clones into Arabidopsis, by using the floral dip method, was made, it was not possible to successfully develop this technique within the time frame of this project. Demonstration of

the efficacy of RNAi will eventually also require expression in cotton that has been genetically modified to express suitable constructs.

Extension Opportunities

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

At this stage no further work is planned.. However CSIRO may pursue further work in this area if funding becomes available. Meanwhile we expect large private and commercial research groups overseas to extend findings published by the CAS group in 2007, and test whether RNAi can be delivered by genetically-modified cotton to silence the cytochrome P450 enzyme responsible for detoxifying gossypol.

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

None at this stage.

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

Further information on the Helicoverpa genome project is available at: http://insectacentral.org/helicoverpa/

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Part 4 – Final Report Executive Summary

H. armigera is one of the major pests faced by the Australian cotton industry. Widespread uptake of transgenic Bt-cotton has reduced the costs and environmental and health impacts associated with the use of chemical insecticides to control this pest. However, genes for Bt-resistance have been detected in *H. armigera*, and the danger of resistance to the limited array of Bt toxins available to control Lepidoptera, means that novel biological methods of pest control continue to be of interest.

Recent advances in molecular biology have opened possible new control technologies. CSIRO's large genomics effort is delivering not only the complete genome of *H. armigera*, but has also sequenced many thousands of genes expressed in the midgut of larvae. Successful gene silencing by RNAi would allow us to exploit the genomics results for pest control. RNAi involves introduction of double-stranded RNA (dsRNA) into a target species, resulting in highly specific gene silencing. By reducing the expression of any essential gene, RNAi could be used as an effective method of protecting crop plants from insect damage or of controlling the propagation of the pest insects themselves.

Using the limited resources available to this project, we asked whether any evidence of RNAi-caused knockdown could be observed upon transient expression of dsRNA in plant tissue. This approach proved to be challenging for a number of reasons, and no consistent and significant evidence of RNAi was observed in any bioassay. A small number of *H. armigera* genes, all expressed in the larval midgut, were targeted. The first four are known to be essential genes, and the fifth is an enzyme specifically required for gossypol detoxification. Initial experiments were conducted to test biosassays of larvae on *Nicotiana benthamiana*. These control bioassays on different plants showed that larval growth on control and mock-treated plants was quite variable; even when older larvae were tested, no significant effects were observed. The variability observed demanded that we have found it necessary to find a better experimental system. Results of control experiments using cowpea were encouraging, but no evidence for RNAi was obtained upon transient expression of the dsRNA constructs. This work suggests that a full-scale project to evaluate genes for their effectiveness as RNAi targets will be required, making use of transgenic plants, such as Arabidopsis or tobacco in the first instance.