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Part 2 - Contact Details

Admin Contact: Mr (Title) | Mark (First Name) | Hardwick (Last Name)

Organisation: CSIRO Entomology
(name of organisation that will be administering the funding)

Postal Address: GPO Box 1700
PO Box
Canberra (Town) | ACT (State) | 2601 (Postcode)

02 6246 4008 (Phone) | 02 6246 4095 (Fax) | Mark.Hardwick@ento.csiro.au (Email)

Principal Researcher: Dr (Title) | Raymond (First Name) | Akhurst (Last Name)

Organisation: CSIRO Entomology

Postal Address: GPO Box 1700
PO Box
Canberra (Town) | ACT (State) | 2601 (Postcode)

02 6246 4123 (Phone) | 02 6246 4173 (Fax) | raya@ento.csiro.au (Email)

Project Supervisor: Dr (Title) | Raymond (First Name) | Akhurst (Last Name)

Organisation: CSIRO Entomology

Postal Address: GPO Box 1700
PO Box
Canberra (Town) | ACT (State) | 2601 (Postcode)

02 6246 4123 (Phone) | 02 6246 4173 (Fax) | raya@ento.csiro.au (Email)

Plain English Summary

Sucking pests of cotton (aphids and mirids) that are expected to become more significant as the use of synthetic chemical sprays is replaced by the deployment of transgenic cotton. The objective of this project was to characterise genes encoding proteins toxic for sucking pests of cotton. Eight lectins and extracts prepared from over 100 strains of bacteria from three different genera were tested by bioassay for their toxicity to three major sucking pests: the green mirid, cotton aphid and silverleaf whitefly. Extracts of several bacterial strains were found to be toxic for the sucking pests; no strain was most toxic for all three pests. One protein with toxicity for cotton aphid was purified.

Background to the project

The commercial introduction of transgenic cotton expressing the cry1Ac gene of *Bacillus thuringiensis* has been accompanied by a significant reduction in the use of insecticidal sprays (Fitt and Wilson, 2000). An expected side effect of this reduction is the increased occurrence of sap-sucking insects, such as mirids and aphids, that were previously controlled by sprays directed at heliothis. The identification of strains of bacteria in the CSIRO Entomology culture collection that produce insecticidal proteins suggested a solution to the expected increase in problems associated with sap-sucking pests.

The green mirid, *Creontiades dilutus*, is currently recognised as the major sap-sucking pest of cotton and in the northern growing areas is considered the second most important pest of cotton. The insects' feeding on cotton at the seedling and pre-squaring stages results in prolongation of the maturation period and subsequently reduced yield and greater exposure to damage by other pests and weather. Insecticidal sprays that can be used for treating green mirid outbreaks are expensive and add to the general environmental concern about the use of insecticides by the cotton industry.

Two species of aphid are recognised as pests of cotton in Australia, the cotton aphid (*Aphis gossypii*) and the green peach aphid (*Myzus persicae*). The principal damage attributed to aphids is due to the production of honeydew that contaminates the lint and causes difficulties in the spinning process. However, aphids may also impact on yield by feeding and by virus transmission. The latter has not been confirmed as a problem at present but may become so if aphid numbers increase due to the reduction in spraying. The cotton aphid can be controlled with sprays of soft synthetic chemical insecticides but the green peach aphid, currently a lesser pest, is much more refractory to insecticides and could become the major aphid pest with the reduction in the use of sprays for heliothis control.

The silverleaf whitefly, *Bemisia tabaci* biotype B, is recognised as a potential, rather than a current, pest. It has been found in the Qld and NSW cotton growing regions. It is considered to pose the greatest risk to those areas where there is a continuous supply of suitable crops likely to allow *Bemisia* to build up into large numbers. Regions such as the Emerald and Ord River regions and parts of the Darling Downs where combinations of intensive horticulture that have extended periods of cotton cultivation and wet season soybean are particularly at risk. Northern NSW is considered less threatened but is likely to experience problems in years where good rains over winter allow a continuous supply of suitable weed species. The potential for damage will be considerably increased should geminiviruses, which are vectored by *Bemisia,* invade cotton.

The options for non-chemical control measures are limited by the feeding behaviour of the sucking pests. They are vulnerable to predation and parasitism but are generally not exposed to pathogens which, except for fungi, must be ingested. However, the development of transgenic technology for plants allows the transformation of the plant to express the toxic agents produced by insect pathogens. There have been two reports of *Bacillus thuringiensis* (Bt) insecticidal crystal proteins (ICPs) toxic for aphids but little investigation of other sources of peptide toxins.

Objectives

This project sought to contribute to the development of cotton plants resistant to mirids and other sap-sucking pests by identifying, isolating and characterising genes encoding proteins toxic for these pests. Although extracts from more than 100 strains of insecticidal bacteria from three genera and 11 lectins were tested against three pest species, no suitably toxic proteins were isolated. However, toxic proteins were detected in several strains and further analysis of these strains can be expected to produce genes that can be employed to make transgenic cotton resistant to sucking pests.

Methodology

Insect Collection/Culture

A colony of the cotton aphid, *Aphis gossypii*, was maintained on cotton plants at 22°C and ambient humidity with a 14:10 day:night light cycle.

The green mirid, *Creontiades dilatus*, could not been maintained in culture and was collected in a sweep net from lucerne fields near Canberra and Cowra between December and March/April. The mirids were transferred from the sweep net to a cage containing pots of glasshouse-grown lucerne for transport to the lab. Mirids were left on the lucerne for at least one day to exclude any injured during collection and were used within two weeks of collection (usually within one week).

The silverleaf whitefly, *Bemisia tabaci* Biotype B, was maintained as a laboratory culture on hibiscus plants at 25°C and 20-24% RH.

Preparation of Toxins

It was essential that test samples be sterile as the growth of any bacterium in the bioassay media invariably resulted in control deaths.

Xenorhabdus and *Photorhabdus*

Each strain was grown in 10 ml Luria-Bertani broth for 24 h at 28°C. The bacteria were lysed by the addition of 1 mg ml^{-1} lysozyme (RT, 30 min). The culture was centrifuged at 4000 rpm in an Eppendorf centrifuge for 10 min and the supernatant sterilised by filtration through a 0.45µ filter. As the toxins are very sensitive to freeze/thaw, the filtrate was used in the bioassay immediately.

E. coli clones expressing the *toxb4* and *toxV16* genes of *Xenorhabdus nematophila* and *Photorhabdus luminescens*, respectively, were grown overnight at 37°C in Luria-Bertani broth containing 150µg ml^{-1}. The bacteria were lysed by the addition of 1mg ml^{-1} lysozyme (RT, 30 min). These preparations had to be used without any further sterilisation (i.e. filtration) or storage.

The dosages used were chosen arbitrarily because we have no means of measuring the level of toxin production by *Xenorhabdus*, *Photorhabdus* or *E. coli*.

B. thuringiensis

B. thuringiensis strains were grown to sporulation in 100 ml Luria-Bertani broth (Sambrook *et al.*, 1989) in a 500ml baffled flask shaken at 200rpm at 28°C. Cultures were examined daily after 3d and harvested when >80% of cells had sporulated, formed crystals and lysed. Although most strains reached this stage in 3-4d, some were not ready until 6d. The spores and crystals were harvested by centrifuging the culture medium in 50 ml aliquots at 6000g at 4°C for 10 min and discarding the supernatant. The pellets were washed twice by suspension in 10 ml water and re-centrifugation.

The spores had to be removed before bioassay because they germinated in the testing medium and the subsequent growth was lethal for the insects. The spores were removed by centrifugation after dissolving the crystals overnight at room temperature. The final spore/crystal pellet was resuspended in 20mM sodium carbonate at pH10 to dissolve the crystals. This suspension was centrifuged at 6000g at 4°C for 10 min and the supernatant was removed and centrifuged once more. The final supernatant was filtered through a 0.2µ filter and the protein concentration estimated by measuring OD_{280} (OD_{280} of 1.1 = 1 mg ml^{-1} Cry protein). The concentration of protoxin was adjusted to 2 mg ml^{-1} and the protoxin solution was stored in 200µl aliquots at -80°C until required. As required, an aliquot was thawed on ice, the requisite amount was taken for bioassay, and the remainder discarded to avoid freeze/thaw cycles.

The dosages used in this screening program were about 20-fold greater than the dosage of Cryt1Ac required to kill *H. armigera*. The reason for the high dosage is that the crystals of native *B. thuringiensis* strains often contain multiple Cry proteins (as many as nine have been recorded) and these are not present in equimolar amounts. For example, the Cry1D component of *B. thuringiensis* serovar *aizawai* strain HD133 is only 2% of the crystal protein (Masson *et al.*, 1998). The screening dosage was set on the assumption that we may need to identify a protein that comprised only 5% of the protoxin solution. Ideally we would have used a dosage that was five times higher but were unable to keep the protoxin in solution at the concentration required.

B. thuringiensis toxins were also tested as activated toxins. The spore/crystal suspensions were prepared and washed as described above. The crystals were dissolved and protoxin activated by resuspending the spore/crystal pellet in 50 mM NH_4HCO_3, 0.5 mM EDTA, 1 mM E64, 10 mg ml^{-1} trypsin at 37°C. Trypsin activity was stopped by the addition of PMSF (5µl ml^{-1}). The incubation time required for each strain was determined by sampling at 0.25, 0.5, 1, 2 and 4h and running an SDS-PAGE gel to determine the extent of activation/degradation. The trypsin-activated toxin was aliquoted and stored at -80°C until required. As above, repeated freezing and thawing was avoided.

Six novel proteins cloned and expressed in *B. thuringiensis* as part of a GRDC project were tested by bioassay against cotton aphid.

Lectins

Eight lectins were provided by Dr T.J. Higgins, CSIRO Plant Industry; each was tested at a concentration of 0.09 mg ml^{-1}. This was approximately half the dosage used for testing *B. thuringiensis* protoxin solutions.

Bioassays

Bioassays with cotton aphids were conducted in 19mm diameter perspex arenas with 120µl Auclair's (1965) diet contained in a parafilm sandwich across the top of the arena. The toxin sample was added to the diet in a 1:9 ratio. Two days before the assay, adult aphids were transferred to Auclair's diet. Any nymphs produced the following day were discarded and only those produced on the second day were used for bioassay. In general, two replicates of five nymphs per arena were used for each toxin sample, with the nymphs in the two replicates being derived from different parents. The arenas were incubated under the same conditions used for culturing cotton aphid and the numbers of surviving aphids recorded daily for five days. Any insects dying within 24h were considered as having been damaged by handling and these data were not included.

Mirid bioassays were conducted in 55mm diameter petri dishes, with a modified Debolt (1982) diet. The toxin sample was added to the diet in a 1:9 ratio and 150µl pipetted onto the petri dish lid. The diet was then covered by a 7.5cm square piece of parafilm that was stretched to cover the full area of the lid. The mirids could not be standardised for age because they were field-collected insects. The mirids were removed from the transport cages by aspiration, anaesthetised with carbon dioxide, transferred onto the parafilm, and covered with the petri dish base; only one mirid was placed in each arena because they are cannibalistic. The arenas were incubated at 22°C. The mirids were exposed to the toxin/diet for three days during which mortality, deposition of frass droplets and the diet condition were checked daily for three days. After three days the mirids were transferred to a petri dish containing a fresh green bean. Any individuals that died in the first 24h or deposited less than five frass droplets were excluded from the data. The final data were recorded at 5d.

Glass tubing (70 mm length, 25 mm diameter) was used to make testing arenas for whitefly bioassays. The base was plugged with black foam plastic and the top with parafilm "sandwich" containing 200µl Auclair's (1965) diet containing 10% toxin. Newly emerged (<24h) adults were aspirated in groups of 30 into a 25 mm vial. The vial was then butted against the opened base of the testing arena. When all of the whiteflies migrated up into the arena, the base was re-plugged and the arena incubated at 25°C and 20-24% RH. The bioassay was scored at 1, 5 and 7d; any insects dead at 1d were excluded from the data.

Cloning and Expression of cry *Genes from a Mirid-toxic Strain,*

The *B. thuringiensis* strain most toxic for the green mirid, JLF17.18.6, was chosen for further characterisation.

The strain was analysed by PCR to test for the known *cry* genes (Table 1). The 21 *cry* gene primer sets enable detection of 48 different *cry* genes; primer pair # 28, which detects the *SpoOA* gene, was included with every analysis as a control for DNA quality. The 988kbp and the 1025bp amplicons from primer pairs #2 and #27, respectively, were directly sequenced using Big Dye Terminator Cycle sequencing and analysed by BLASTN (ANGIS) to confirm that they contained *cry1* and *cry9* sequences, respectively. These amplicons were cloned into the pGEM-T Easy Vector (Promega) using the manufacturer's protocol.

Table 1. Characteristics of PCR primers used in analysis of JLF17.18.6

Primer Pair #	Primer sequence (5' to 3')	Genes recognised	Product size (bp)	Primer Tm (°C)	Reference
1	CCGGTGCTGGATTTGTGTTA AATCCCGTATTGTACCAGCG	Cry1Aa,b,c, d, e	492	60 60	Carozzi *et al.* 1991
2	CCGAGAAAGTCAAACATGCG TACATGCCCTTTCACGTTCC	*Cry1Aa,c d, 1Ca,b, 1Db, 1Ea,b, 1Fa,b, 1G, 1H* *cry1Ab*	988 910	60 60	Carozzi *et al.* 1991
3	CAAGCCGCAAATCTTGTGGA ATGGCTTGTTTCGCTACATC	*Cry4A, Cry4B*	797	60 58	Carozzi *et al.* 1991
6	AGGTGCCAACTAACCATGTT GATCCTATGCTTGGTCTAGT	*Cry3A, (Cry3B)*	1060	58 58	Carozzi *et al.* 1991
7	GATGCCTTATCAGATGAAGT ATATCTGAACGAATAGAACC	*Cry5Aa,b, Cry12A*	745 (5A), 748 (12A)	56 54	CSIRO Entomology
8	ATGATTATTGATAGTAAAAC ATTATTATACCAATCCGAAT	*Cry6A*	1425	48 50	CSIRO Entomology
9	ATGATTTTAGGGAATGGAAA AGGAGTATAAGCATTTAGTA	*Cry6B*	1185	52 52	CSIRO Entomology
12a	TATAATCAATTCCGTAGAGA CATAATTTGGTCGTTCTGTT	*Cry1Ba,b,c* *Cry1Ia,b*	636-639 636	52 54	CSIRO Entomology
15	TACAATTGTTTAAGTAATCC TTATACGTATCCACACAATG	*Cry1Da,b*	601	50 54	CSIRO Entomology
16	CAGATACCCTTGCTCGTGTAA ATAGGCCCGTGCTCCACCAGG	*Cry2Aa,b*	1072	62 84	Asano *et al.*, 1993
17	ACTGTTACAAGGCTAGCGAC AATCCTTTACAAACACCGTT	*Cry2Ac*	281	60 54	CSIRO Entomology
18	GGATCCTTGTGTTGAGATA ATGAAACTAAAGAATCCAGA	*Cry1Ia,b*	1145	54 52	Gleave *et al.*, 1993
22	ATATGAAATATTCAATGCTC ATAAATTCAAGTGCCAAGTA	*Cry10A*	613	50 52	CSIRO Entomology
23	CCAGCATTAATAGCAGTAGC TGTACACATTTGAGTAAAAA	*Cry11A, 11B*	385	58 50	CSIRO Entomology
24	ACTAGTAATACAGGTAGTCC TGAGACGAATAGTCATCTGG	*Cry13A*	922	56 58	CSIRO Entomology
25	TCTAATGTTAATGCGTTGGT ACTCTTTGTGTATATTCATT	*Cry14A*	742	54 50	CSIRO Entomology
27	AGATGTTATTGGAAGCGGTA GAACTAAGAAATGC(G/A)TATTGCC	*Cry9Aa, 9Ba, 9Da* *9Ca,*	1025 1022	56 ~60	CSIRO Entomology

Table 1 (ctd). Characteristics of PCR primers used in analysis of JLF17.18.6

Primer Pair No.	Primer sequence (5' to 3')	Genes recognised	Product size (bp)	Primer Tm (°C)	Reference
28	AGGGAATAAATGAACGATAAAC TAACCGAATAAGGACGAAATAG	*Bt spoOA*	857	58 60	CSIRO Entomology
29	AACAATCGAAGTGAACATGA TTTGGTACCTCCTGTACCCAC	*Cry3Ca*	940	54 64	CSIRO Entomology
30	AATTTATGACAGAAGTAGAA TATCCATTGTTCATAAGTGG	*Cry7Aa,b*	460	50 54	CSIRO Entomology
31	GAAACGTATCCAACGTATCT ATACGTAAGGAATGGACTGT	*Cry8A, 8B, 8C*	639	56 56	CSIRO Entomology
32	GAACAACTCCTACCAATCGAC GCGTTCACGATGCTTCTCTCG	*Cry15Aa*	880	62 66	CSIRO Entomology

Genomic DNA was prepared for library construction. Strain JLF17.18.6 was grown to an OD_{600} of 0.6, centrifuged, and washed three times in 100 mM NaCl, 10 mM Tris-HCl, 20 mM EDTA, pH 8.0. The pellet was resuspended in 1.5 ml of 150 mM NaCl, 100 mM EDTA, pH 8.0 and 3 mg lysozyme was added. After incubation at 37°C for 2 h, 1.5 ml of 100 mM NaCl, 100mM Tris-HCl, pH8.0, 2% SDS was added and the solution incubated at 60°C for 30 min. The solution was extracted with an equal volume of phenol/chloroform and DNA precipitated by the addition of 2.5 volumes of 100% ethanol. The precipitate was spooled on a glass hook, washed with 70% ethanol and air dried. The precipitate was then dissolved in 100 mM NaCl, 100 mM Tris-HCl, 10 mM EDTA, pH 8. RNA was removed by addition of 100μg ml^{-1} RNAse A and incubation at 37°C for 6 h. Proteinase K was added (to 100 μg ml^{-1}) and the solution incubated at 37°C for 2.5 h. Proteins were removed by phenol/chloroform extraction and the DNA precipitated in ethanol/sodium acetate. The sample was centrifuged (4K, 15 min., SS34 rotor) and the pellet washed in 70% ethanol. The pellet was air dried and then dissolved in TE pH 8.0 buffer (Sambrook *et al.*, 1989) overnight at 4°C.

A genomic DNA library was prepared in a plasmid vector. Genomic DNA was digested with *MboI* and the digest run on low melting point agarose gel. The gel region containing fragments of 8-14kb was excised and the DNA recovered by agarase digestion of the gel followed by ethanol precipitation. The DNA fragments were ligated to pBluescript vector that had been digested with *BamHI* and phosphatased. Electroporation was used to transform the recombinant plasmid into *E. coli* strain DH10β. The electroporation reaction was titred by plating on LB/IPTG/X-gal agar plates. Restriction analysis showed that the mean insert size in recombinant cells was 8kb (4-10kb). Over 5000 colonies were plated onto LB agar and transferred to nylon membranes according to the manufacturer's instructions.

The 988kbp and the 1025bp amplicons from primer pairs #2 and #27, respectively, were labelled with ^{32}P by nick translation and used to screen the membranes. Positive clones were picked off, re-plated and re-screened twice to ensure that single clones were obtained.

Plasmid DNA was isolated from the positive clones by alkaline lysis. The plasmids were partially sequenced to obtain sequence data for both termini of the inserts and to identify which clones contained a complete *cry* gene. Restriction analysis was used to identify restriction enzymes that could be used in subcloning without cutting the insert. The insert of clone 72 was excised with *SacI* and *PstI* and ligated into the shuttle vector pHT315 pHT315 cut with the same enzymes. The insert of clone 17 was excised with *SalI* and *SacI* and ligated into the pHT315 cut with the same enzymes. The recombinant pHT315 plasmids were electroporated into the non-methylating *E. coli* strain SCS110, re-isolated from SCS110 and then electroporated into CryB, a *cry*$^-$ strain of *B. thuringiensis.* The *B. thuringiensis* transformants were grown to sporulation and examined by phase contrast microscopy for the presence of crystals.

Results

Bioassays

Xenorhabdus and *Photorhabdus*

The data for bioassays of extracts of *Xenorhabdus* and *Photorhabdus* cultures conducted with cotton aphid, the green mirid and silverleaf whitefly are summarised in Table 2. Silverleaf whiteflies were the most susceptible species, with eight of the 20 extracts tested killing 100% and 12 killing more than 85%. Only one of 12 extracts tested killed >85% green mirids and two of 20 killed >85% cotton aphids. There was considerable variation in the toxicity of *Xenorhabdus nematophila* strains, with one (S.carp.It) killing all green mirids and others killing none. A similar variation, though over a narrower range, was noted in the cotton aphid bioassays.

Table 2. Toxicity to sucking insects of extracts of *Xenorhabdus* and *Photorhabdus* cultures

Species	Strain	% mortality[1] at 5d		
		Cotton aphid	Green mirid	Silverleaf whitefly
X. nematophila	All/1	71	0	89
	A24	62	40	100
	AN6	100	0	0
	BK/1	48	18	100
	CB/1	31	80	0
	F1/1	100	69	98
	Mex/1	75	13	86
	Pi/1	30	0	29
	Scarp.It	45	100	38
	XnNach	68		100
	XnOhio/1	73	50	100
X. beddingii	Q58/1	23		5
X. bovienii	T363	16	0	23
Xenorhabdus sp.	CB/2A/W	54		100
P. luminescens	Hm/2	11		100
	D1/1	10	0	89
	HI/1	31		100
	K81/2	7		
	V16/1	36		50
P. temperata	C1/1	13		16
Photorhabdus sp.	Q614/2	38		100

1 Adjusted for control mortality

When *toxb4* and *toxV16,* toxin genes cloned from *X. nematophila* and *Photorhabdus luminescens*, respectively, were expressed in *E. coli*, no significant toxicity for cotton aphid or green mirid three insect species was detected (Table 3). There was appreciable mortality among the whiteflies.

Table 3. Toxicity to sucking insects of extracts of recombinant *E. coli* expressing toxin genes from *X. nematophila* and *P. luminescens*

Clone	% mortality[1] at 5d		
	Cotton aphid	Green mirid	Silverleaf whitefly
toxb4 (*X. nematophila*)	5	0	22
toxV16 (*P. luminescens*)	0	1	50

[1] Adjusted for control mortality

B. thuringiensis

Solubilised protoxins from 87 *B. thuringiensis* strains were bioassayed against cotton aphid, 81 against the green mirid and 13 against silverleaf whitefly (Table 4). Most strains had no toxicity and none had the high levels of toxicity observed for *Xenorhabdus* and *Photorhabdus*. The most toxic strains were DR2.12.1, JL17.18.6 and DR2.4.5, which killed 50% cotton aphid, 29% green mirid and 36% silverleaf whitefly, respectively.

Table 4. Toxicity of solubilised protoxin of *B. thuringiensis* to three species of sucking pests of cotton

Strain	% mortality[1] at 5d		
	Cotton aphid	Green mirid	Silverleaf whitefly
8L365	0	6	
8L367	0	4	
AS2.1.1	0	10	
CAA436	0		
CAA436	0		
DR1.1.2	13	11	15
DR1.1.2	0		
DR2.1.1	8	0	4
DR2.10.37	0	1	
DR2.11.1	0	0	0
DR2.12.1	50		
DR2.12.2	0		
DR2.13.3	7	7	12
DR2.14.5	6	0	0
DR2.15.5	0	0	3
DR2.2.2	5	0	7

[1] Adjusted for control mortality

Table 4 (ctd). Toxicity of solubilised protoxin of *B. thuringiensis* to three species of sucking pests of cotton

Strain	% mortality at 5d		
	Cotton aphid	Green mirid	Silverleaf whitefly
DR2.4.5	5	1	36
DR2.7.28	0	13	
DR2.8.30	11	0	0
DR2.9.32	0	1	0
GB1.3.2	7	0	
GB1.7.1	13	0	
JLF10.10.33	5	0	
JLF10.11.38	0	0	
JLF11.1.1	0	0	
JLF13.10.73	0	0	
JLF13.10.74	8	0	
JLF13.2.11	3	0	
JLF13.4.25	0	0	
JLF13.5.37	0	0	
JLF14.3.29	0	0	
JLF15.4.66	0	0	
JLF17.12.4	11	0	
JLF17.17.1	0	0	
JLF17.17.7	7	10	
JLF17.18.6	0	29	
JLF17.22.7	13	0	
JLF17.5.1	6	0	
JLF17.5.10	0	0	
JLF17.5.5	9	10	
JLF19.10.6	13	0	
JLF2.4.1	0	0	
JLF2.5.6	0	0	
JLF2.7.6	3	0	
JLF21.1.3	3	0	
JLF21.3.22	0	0	
JLF21.8.70	0	0	
JLF3.10.2	0	0	
JLF3.12.56	13	0	
JLF4.1.10	0	0	
JLF4.13.62	0	0	
JLF4.5.2	17	0	
JLF4.6.27	11	0	
JLF7.12.63	0	0	
JLF7.3.8	0	0	
JLF7.4.17	20	0	
JLF8.11.47	10	0	

Table 4 (ctd). Toxicity of solubilised protoxin of *B. thuringiensis* to three species of sucking pests of cotton

Strain	% mortality at 5d		
	Cotton aphid	Green mirid	Silverleaf whitefly
JLF9.12.67	0	0	
JW3.2.2	0	12	
JW3.2.5	2	19	
JW3.2.6	8	10	
JW4.5.12	8	0	
JW4.6.21	0	0	
KJ1.7.2	0	0	
MCOO 1.11.1	0	0	
MCOO 1.8.66	0	0	
PS.NG 1.10.1	3	10	
RA.AS 1.1.1	6	0	
RA.AS 1.16.2	22	18	
RA.AS1.17.4	0	0	
RA.AS 1.24.5	11		
RA.AS 1.25.4	17	0	
RA1.4.1	0	0	
RA1.5.1	8	0	
SR 2.2.1	0	0	
TW1.10.7	0	10	
TW1.8.11	7	0	2
TW1.8.2	0	0	
TW1.8.3	10	10	
VB1.2.9	0	11	
WA 2.3.1	26	0	
WA 2.6.10	0	0	9
WA 2.7.1	7	11	
WA 3.4.9	0	20	
WA 3.6.1	0	6	17
WA3.7.3	5	13	
WM 1.6.3	16	0	

The protoxins of *B. thuringiensis* must be proteolytically activated to produce an active toxin. Some insect species that are insensitive to *B. thuringiensis* protoxins because they lack suitable proteases for activating the protoxin are killed when fed with trypsin-activated toxin. As transgenic cotton has been found to activate the Cry1Ac protoxin, we tested some trypsin-activated toxins against cotton aphid and green mirid. Activation did not increase the toxicity of any of them (Tables 5, 6).

Table 5. Effect of activated *B. thuringiensis* toxin on cotton aphid

Strain	% mortality[1] at 5d	
	Protoxin	Activated toxin
JLF17.22.7	13	16
JLF7.4.17	20	26
JLF8.11.47	10	24
RA.AS1.16.2	22	17
TW1.8.3	10	3
WM1.6.3	16	6

[1] Adjusted for control mortality

Table 6. Effect of activated *B. thuringiensis* toxin on the green mirid

Strain	% mortality[1] at 5d	
	Protoxin	Activated toxin
DR1.1.2	11	0
DR2.13.3	7	0
DR2.7.28	13	9
JLF17.18.6	29	22
JLF 17.5.5	10	11
RA.AS 1.16.2	18	0
VB1.2.9	11	13
WA3.4.9	20	0
WA3.7.3	13	1
WA2.7.1	11	10

[1] Adjusted for control mortality

Six Cry proteins from genes that had been cloned from various *B. thuringiensis* strains in other projects were bioassayed against cotton aphid. One protein, from the *pd45* gene of strain JLF10.11.38, showed toxicity and a dose response (Table 7).

Table 7. Effect of single *B. thuringiensis* protoxins on cotton aphid

Source Strain	Gene	Dosage (mg ml^{-1})	% mortality[1] at 5d
JW3.2.5	*iz105*	0.14	0
	iz98	0.18	4
	pAM2	0.16	3
JLF7.4.17	*pBt 5.1a*	0.20	8
	pBt 5.2	0.20	0
JLF10.11.38	*pd45*	0.07	21
	pd45	0.29	91

[1] Adjusted for control mortality

Lectins

Eight lectins were bioassayed against cotton aphid and green mirid (Table 8). None killed more than 30% of the insects tested.

Table 8. Toxicity of lectins to sucking insects

Lectin	% mortality[1] at 5d	
	Cotton aphid	Green mirid
ANGO	26	30
Carbonic	5	0
GSL Lectin	9	0
GSR Lectin	19	0
Taro-T1	11	0
WA-T1	0	22
WB-Lectin	5	0
Wheat Lectin WGA	22	0

[1] Adjusted for control mortality

Cloning and Expression of cry *Genes from a Mirid-toxic Strain*

PCR analysis of strain JLF17.18.6 with 21 primer pairs for *cry* genes yielded amplicons from seven pairs of *cry* primers and for the control (*SpoOA*) primers. Only the amplicons from primers for *cry1*, for *cry9* and for *SpoOA* were of the size predicted (Table 9). There is a very high probability that the amplicons that differ from the expected size are false positives. They were accorded low priority.

Table 9. PCR products obtained from *B. thuringiensis* strain JLF17.18.6

Primer pair #	Genes recognised	Product(s) (bp)	
		Expected	Actual
1	*Cry1A*	492	360, 492
2	*Cry1A, 1C, 1Db, 1E, 1F, 1H*	988	988
22	*Cry10A*	613	980, 1390, 1510
27	*Cry9*	1025	1025
29	*Cry3A*	940	860
30	*Cry7A*	460	800, 860
28	*SpoOA*	857	857

Sequencing of the amplicons from primer pairs #2 and #27 indicated that JLF17.18.6 carries *cry1* and *cry9* genes. Sequencing of multiple clones showed that 988bp "amplicon" from primer pair #2 was actually two amplicons with 79% identity and that both were most similar to *cry1* genes but were less than 90% identical to any gene in the databases. These data suggested that JLF17.18.6 has two *cry1* genes that are novel. The amplicon from primer pair #27 had 96% identity to the corresponding sequence of *cry9A*; this suggested the presence of a *cry9* gene, possibly in the *cry9A* family.

When the genomic library was screened with these three amplicons, 13 positive clones were identified. Five were positive for the *cry9* amplicon, seven for one of the *cry1* amplicons and one for the other *cry1* amplicon; the last was also weakly positive for the first *cry1* amplicon probe. Sequencing of the termini of the inserts from these 13 clones showed that three contained complete *cry* genes; these three represented the three amplicons.

Restriction mapping and further sequencing showed that two clones contained a partial *cry* gene as well as a complete one. Clone 17 contained a complete gene that was most similar to *cry1D*, but with too little identity to be a *cry1D* gene, and a partial *cry2* gene. Clone 65 contained a complete *cry9* gene with less than 80% identity to *cry9C* and a partial *cry1* gene. However, two of the clones may lack essential 5′ sequence. No promoter could be identified for clone 72 (*cry1*) or the *cry9* gene of clone 65 which also lacked the ORF1 sequence that is commonly found associated with *cry9C*. When the three inserts were subcloned into the shuttle vector pHT315 and transformed into an acrystalliferous strain of *B. thuringiensis*, only clone 17 produced crystals. As these crystals were not obtained until June 2000, no mirids were available for bioassay before the project terminated.

Discussion

The most promising leads for toxins for the sucking pests of cotton came from the bacteria *Xenorhabdus* and *Photorhabdus*, of which the most effective species was *Xenorhabdus nematophila*. Extracts of cultures of some *X. nematophila* strains killed 100% of all three species. However, no one strain killed 100% of each species, indicating that several insecticidal agents may be involved.

Two families of insecticidal proteins produced by *Xenorhabdus* and *Photorhabdus* have been identified. One, with a molecular weight of 1,000,000Da, is a complex of native complexes encoded at four gene loci (Bowen and Ensign, 1998; Bowen *et al.*, 1998). The other family is a single unit protein with a molecular weight of 40,000Da (East *et al.*, 1998). Proteins of this latter class produced from genes cloned from *X. nematophila* strain A24 and *Photorhabdus luminescens* strain V16 and expressed in *E. coli* were not toxic for cotton aphid or the green mirid although they were toxic for silverleaf whitefly. It is not clear what was the basis for the toxicity exhibited by the *X. nematophila* and *P. luminescens* culture extracts (Table 2). The toxicity may have been due to the large toxin complex or the 40kDa proteins expressed in *E. coli* may differ significantly from those produced in their native strains. Further research being conducted by Dr P. East, CSIRO Entomology, is likely to resolve this matter.

Although none of the protoxins killed 100% of any of the species, two *B. thuringiensis* strains were identified as potentially useful. Strain JLF17.18.6 killed 29% of the green mirids and strain DR1.12.1 killed 50% of the cotton aphids. Strain JLF17.18.6 was characterised in an effort to identify the Cry protein with toxicity to green mirid. The five *cry* genes isolated from this strain belong to two classes (*cry1, cry9*) that are usually toxic for Lepidoptera. Only one gene from JLF17.18.6 was expressed within this project and it cannot be assessed until the next summer when the green mirids become available once more.

It is possible that the toxicity for green mirid obtained with the protoxins of JLF17.18.6 is due to a novel *cry* gene that could not be identified with PCR. A technique that we developed in a GRDC-funded project may provide the means for us to identify, clone and express this gene.

None of the lectins tested was found to be sufficiently toxic to either the green mirid or cotton aphid to warrant further investigation. Although the toxicity of some lectins was near that of the solubilised protoxin mixtures, the comparison is between a single lectin and multiple protoxins. The toxicity achieved with a single lectin was not sufficiently great that one might anticipate effective field control.

The protoxin produced from the *pd45* gene may be adequately toxic for cotton aphid. The dosage required to kill 90% of the aphids was about 1.5 times greater than the dosage of Cry1Ac required to kill 90% of first instar *H. armigera*. The level of Cry1Ac in INGARD™ cotton is not sufficient throughout the whole season to kill all *H. armigera* (Fitt and Wilson, 2000). However, it is not necessary to achieve the same kill rates for aphids as for *Helicoverpa* and a lower efficacy might be acceptable for aphid control. Further assays are required to confirm the toxicity of the *pd45* product.

If the *pd45* product proves to have inadequate toxicity to achieve the level of aphid control that is required by growers, it might still have some value. Since there are so few proteins with toxicity for sucking pests, the *pd45* protein might be used as a lead molecule for a toxin discovery program or for protein engineering.

As well as identifying one protein toxic for the sucking pests of cotton, this project also provided some valuable leads. The bacteria *Xenorhabdus* and *Photorhabdus* and two strains of *B. thuringiensis* were identified as sources of useful toxins. Further research is required to identify the specific proteins toxic for the green mirid and cotton aphid which are expected to become increasingly important pests as the use of synthetic chemical sprays declines with expanded deployment of transgenic cotton.

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Assessment of the likely impact of the results and conclusions of the research project for the cotton industry. Where possible include a statement of the costs and potential benefits to the Australian Cotton industry and future research needs

The increasing deployment of transgenic cotton for control of Lepidopteran pests is being accompanied by reductions in the use of synthetic chemical insecticides. A consequence of these reductions is expected to be increased problems with minor pests such as the green mirid and cotton aphid that are not killed by the current transgenic cotton constructs. This project has shown that there are proteins that can be utilised for controlling these secondary pests and has identified bacterial strains from which the cognate genes can be cloned. More research will be required to clone the relevant genes, make constructs suitable for use in cotton and test them in commercial varieties.

Description of the project technology (e.g. commercially significant developments, patents applied for or granted, licenses, etc)

The identification of strains of *Xenorhabdus*, *Photorhabdus* and *B. thuringiensis* that produce proteins toxic for sucking pests is the most significant project technology. They can form the basis for ongoing research to identify the specific proteins responsible for toxicity.

A technical summary of any other information developed as a part of the research project including discoveries in methodology, equipment design, etc.

Recommendations on the activities or other steps the may be taken to further develop, disseminate, or to Exploit the Project Technology

Investigation of the toxicity of the *pd45* protein for cotton aphid and green mirid to determine the LC_{50} dosages is warranted. Further investigation of the *Xenorhabdus* and *Photorhabdus* toxins is clearly important and will be conducted.

A list of publications arising from the research project

Nothing has been published to date.

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