# BSES Limited

**FINAL REPORT SRDC PROJECT BSS237**

**IDENTIFICATION OF CANEGRUB-RESISTANT TRANSGENIC SUGARCANE LINES FOR COMMERCIAL EVALUATION**

**by**

**RJ Geijskes, KA Nutt and PG Allsopp**

**SD04013**

**Contact:**
Dr Jason Geijskes
Research Officer
BSES Limited
PO Box 86
Indooroopilly Q 4068
Telephone: 07 3331 3375
Facsimile: 07 3871 0383
Email: jgeijskes@bses.org.au

*Funding for this activity was provided in part by the sugar industry and the Commonwealth Government through SRDC, and is gratefully acknowledged. BSES is not a partner, joint venturer, employee or agent of SRDC and has no authority to legally bind SRDC, in any publication of substantive details or results of this project.*

**BSES Limited Publication**
**SRDC Final Report SD04013** **June 2004**

**Copyright © 2004 by BSES Limited**

*All rights reserved.* No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, recording, or otherwise, without the prior permission of BSES Limited.

**Warning:** Our tests, inspections and recommendations should not be relied on without further, independent inquiries. They may not be accurate, complete or applicable for your particular needs for many reasons, including (for example) BSES Limited being unaware of other matters relevant to individual crops, the analysis of unrepresentative samples or the influence of environmental, managerial or other factors on production.

**Disclaimer:** Except as required by law and only to the extent so required, none of BSES Limited, its directors, officers or agents makes any representation or warranty, express or implied, as to, or shall in any way be liable (including liability in negligence) directly or indirectly for any loss, damages, costs, expenses or reliance arising out of or in connection with, the accuracy, currency, completeness or balance of (or otherwise), or any errors in or omissions from, any test results, recommendations statements or other information provided to you.

**CONTENTS**

**Page No**


**SUMMARY** .......... i

**1.0 BACKGROUND** .......... 1

**2.0 OBJECTIVES** .......... 2

**3.0 NEGOTIATIONS ON IP AND RESEARCH AGREEMENTS TO ALLOW COMMERCIAL RELEASE OF TRANSGENIC PLANTS** .......... 3

**3.1 Previously obtained genes** .......... 3

**3.1.1 *Nicotiana alata* proteinase inhibitor** .......... 4

**3.1.2 Potato proteinase inhibitor II** .......... 4

**3.1.3 *Galanthus nivalis* lectin** .......... 4

**3.2 Novel genes** .......... 4

**3.2.1 Avidin5**

**3.2.2 Fusolin** .......... 5

**3.2.3 Antifeeding effect/Amber disease** .......... 5

**3.2.4 Blood-bank lectin** .......... 5

**3.2.5 Microprotein toxins** .......... 6

**3.3 Promoter and terminator sequences** .......... 6

**3.4 Selection genes** .......... 6

**3.5 Transformation technologies** .......... 6

**4.0 INSECTICIDAL PERFORMANCE OF CURRENT TRANSGENICS EXPRESSING SNOWDROP LECTIN AND PROTEASE INHIBITORS** .......... 6

**4.1 Transformation and tissue-culture method** .......... 6

**4.2 Confirmation of gene insertion** .......... 7

**4.3 Confirmation of gene expression** .......... 8

**4.4 Screening** .......... 9

**5.0 TEST OF ADDITIONAL PRODUCTS FOR ANTIMETABOLIC EFFECTS ON CANEGRUBS AND INCORPORATION OF ACTIVE ONES INTO THE PROGRAM** .......... 17

**5.1 IMB toxins** .......... 17

5.2 *Serratia entomophilia* strains .................................................... 19

6.0 PRODUCTION OF ADDITIONAL TRANSGENIC LINES .......................... 24

6.1 Using snowdrop lectin and protease inhibitors .................................... 24

6.2 Using other gene constructs.................................................................... 24

6.2.1 Transformation of Q117 and Q205$^{\phi}$ with *Avidin* ...................... 24

6.2.2 Transformation of Q117 and Q205$^{\phi}$ with *Fusolin* .................... 24

6.2.3 Transformation of Q117 and Q205$^{\phi}$ with Antifeeding effect/Amber disease gene............................................................ 25

6.2.4 Transformation of Q117 and Q205$^{\phi}$ with *Da10-12*.................. 25

7.0 OUTPUTS.......................................................................................... 25

8.0 EXPECTED OUTCOMES, FUTURE NEEDS AND RECOMMENDATIONS.......................................................................... 25

9.0 PUBLICATIONS ARISING FROM THE PROJECT .................................... 26

10.0 ACKNOWLEDGMENTS ........................................................................ 26

11.0 REFERENCES.................................................................................... 26

## SUMMARY

This project aimed to progress canegrub-resistant transgenic sugarcane to recommendations for field testing by negotiating IP and research agreements to allow commercial release of transgenic plants, determining the insecticidal performance of current transgenics expressing snowdrop lectin and protease inhibitors, testing additional products for antimetabolic effects on canegrubs and incorporating active ones into the program, and producing additional transgenic lines using snowdrop lectin and protease inhibitors and based on other cultivars.

The rationale behind this approach was that the incorporation of transgenic sugarcane lines containing antimetabolite genes would aid in the development of robust pest management systems. Initial experiments conducted as part of BSS163 using transgenic sugarcane lines containing antimetabolite genes showed some of these lines had significant effect on canegrub weight gain. The further testing of these transgenic lines and of new lines containing other antimetabolites could identify commercially viable lines for release to the industry. The project also tested additional potential toxins.

Antimetabolite genes and other technologies used to make transgenic sugarcane plants are often subject to intellectual property claims that complicate the path to commercialisation. Therefore, in order to commercialise a product, freedom to operate must be negotiated with the owners of relevant IP rights.

Genes used in transgenic lines produced during BSS163 were all subject to third-party IP restrictions. Attempts to negotiate freedom to operate with these genes during the project were unsuccessful.

Suitable Material Transfer Agreements were negotiated to obtain the *Fusolin* and *Avidin* genes.

Pot trials (five trials over three years at two locations and with two species of canegrubs) for testing transgenic lines for antibiosis towards canegrub larvae were unable to clearly screen transgenic lines. No transgenic line tested had a statistically significant effect on the weight gain of canegrubs.

New potential toxins from two sources were tested, but none were effective against greyback canegrubs.

The production of transgenic lines containing the *PinII*, *NaPI* or *GNA* genes based on other cultivars was attempted using the new transformation technology developed as part of BSS242. Cultivars Q117 and Q165$^{\Phi}$ were initiated into tissue culture. Subsequent transformation experiments with this technique yielded no transgenic lines.

Plant-transformation experiments with the *Avidin*, *Fusolin* and *Da10-12* genes have commenced and will be completed under CRC-SIIB project 1bii 'Environmentally sustainable canegrub resistance'.

## 1.0 BACKGROUND

Canegrubs, the larvae of endemic melolonthine beetles, are the most important insect pests affecting the sugar industry in Australia. The 19 species of canegrub are placed in four genera, *Dermolepida*, *Lepidiota*, *Antitrogus* and *Rhopaea*. Collectively, canegrubs cost the industry about $12 million a year in damage and control costs, although uncontrolled populations could cause such severe damage as to leave parts of the industry unviable (Robertson *et al.* 1995). Management of canegrubs is highly dependent on two controlled-release formulations of chloropyrifos, suSCon® Blue and suSCon® Plus, and on the liquid insecticide Confidor®. Plant resistance to canegrubs has not been a breeding or selection criterion. However, the failure of suSCon Blue in some highly alkaline soils (Robertson *et al.* 1998), combined with the difficulty of reapplying insecticide into ratoon crops to provide control over high insect populations, has focused research into investigating options to increase plant resistance. Research to investigate the level of natural resistance in sugarcane and some related germplasm is being pursued for potential application in conventional breeding programs (Allsopp *et al.* 1995, 1996b, 1997; Allsopp & Cox 2002).

Another option is to engineer insect-resistance genes from other plant species into sugarcane. The first phase of this approach is to use biochemical or feeding assays to identify candidate compounds that increase the level of resistance to canegrubs, and then to transfer the genes encoding these compounds into sugarcane. Potential compounds, such as protease inhibitors, toxins from *Bacillus thuringiensis*, lectins, avidin and alpha-amylase inhibitor, were tested in BS95S and BSS163, and suitable candidates were identified (McGhie *et al*. 1995; Allsopp *et al*. 1996a, 1997, Allsopp & McGhie 1996) during these screens. Genes for the potato protease inhibitor II (Murray & Christeller 1994), the *Nicotiana alata* protease inhibitor (Atkinson *et al.* 1993) and the lectin gene of the snowdrop plant, *Galanthus nivialis* (van Damme *et al.* 1987), were obtained, constructed into expression plasmids and transformed into sugarcane (Allsopp *et al.* 1995). Canegrubs feeding on lines with two of these antimetabolites had significantly reduced growth during pot trials (Nutt *et al.* 1999).

To provide a useful outcome/output to the industry, further tests of the insecticidal action of these transgenic lines to determine the most effective line against a range of canegrub species was required. This project aimed firstly to test the biological efficacy of the transformed lines against greyback and southern 1-year canegrubs. This would be extended to other species when enough material of transgenic lines becomes available.

The level of expression of the transgenic product in the plant achieved in BSS163 was reasonably low and there is potential for improvement. We aimed to incorporate into the genetic constructs new promoter and/or regulator sequences when these become available, eg BBTV promoter and termination sequences. We also aimed to produce transgenic plants based on cultivars other than Q117; these may have higher levels of expression than so far achieved.

Antimetabolites from a variety of other sources are continually being developed and some may be more useful than those currently being used. If the genes for two or more compounds could be introduced into plants, then this may provide a combination against which the insect would find it difficult to develop resistance. We aimed to source useful

antimetabolites and incorporate these into the transformation program. Where feasible and subject to agreement with IP holders, we aimed to produce plants containing various combinations of transgenes, so that different types of resistance are pyramided to minimize any evolution or selection of resistant insects.

The production and commercial use of canegrub-resistant transgenic sugarcane has considerable IP implications. The project would negotiate the commercial use of this technology and approval from Office of the Gene Technology Regulator (OGTR), which replaced GMAC during the project, for field trials and release.

## 2.0 OBJECTIVES

The project aimed to progress canegrub-resistant transgenic sugarcane to recommendations for field testing through the following objectives:

- Negotiate IP and research agreements to allow commercial release of transgenic plants.
- Determine insecticidal performance of current transgenics expressing snowdrop lectin and protease inhibitors.
- Test additional products for antimetabolic effects on canegrubs and incorporate active ones into the program.
- Produce additional transgenic lines using snowdrop lectin and protease inhibitors and based on other cultivars.

The rationale behind this approach was that the incorporation of transgenic sugarcane lines containing antimetabolite genes would aid in the development of robust pest management systems. Initial experiments conducted as part of BSS163 using transgenic sugarcane lines containing antimetabolite genes showed some of these lines had significant effect on canegrub weight gain. The further testing of these transgenic lines and of new lines containing other antimetabolites could identify commercially viable lines for release to the industry. The project also tested additional potential toxins.

***Objective 1 - Negotiate IP and research agreements to allow commercial release of transgenic plants.***

Antimetabolite genes and other technologies used to make transgenic sugarcane plants are often subject to intellectual property claims that complicate the path to commercialisation. Therefore, in order to commercialise a product, freedom to operate must be negotiated with the owners of relevant IP rights.

Genes used in transgenic lines produced during BSS163 were all subject to third-party IP restrictions. Attempts to negotiate freedom to operate with these genes during the project were unsuccessful.

Suitable Material Transfer Agreements have been negotiated to obtain the *Fusolin* and *Avidin* genes.

***Objective 2 - Determine insecticidal performance of current transgenics expressing snowdrop lectin and protease inhibitors.***

Pot trials (five trials over three years at two locations and with two species of canegrubs) for testing transgenic lines for antibiosis towards canegrub larvae were unable to clearly screen transgenic lines. No transgenic line tested had a statistically significant effect on the weight gain of canegrubs.

***Objective 3 - Test additional products for antimetabolic effects on canegrubs and incorporate active ones into the program.***

New potential toxins from two sources were tested, but none were effective against greyback canegrubs.

***Objective 4 - Produce additional transgenic lines using snowdrop lectin and protease inhibitors and based on other cultivars.***

The production of transgenic lines containing the *PinII*, *NaPI* or *GNA* genes based on other cultivars was attempted using the new transformation technology developed as part of BSS242. Cultivars Q117 and Q165[(b] were initiated into tissue culture. Subsequent transformation experiments with this technique yielded no transgenic lines.

Plant-transformation experiments with the *Avidin*, *Fusolin* and *Da10-12* genes have been conducted and analysis of the resulting transgenic lines has been commenced and will be completed under CRC-SIIB project 1bii ‘Environmentally sustainable canegrub resistance’.

## 3.0 NEGOTIATIONS ON IP AND RESEARCH AGREEMENTS TO ALLOW COMMERCIAL RELEASE OF TRANSGENIC PLANTS

Antimetabolite genes and other technologies used to make transgenic sugarcane plants are often subject to intellectual property claims that complicate the path to commercialisation. Therefore, in order to commercialise a product, freedom to operate must be negotiated with the owners of relevant IP rights.

### 3.1 Previously obtained genes

Genes used in transgenic lines produced during BSS163 were all subject to third-party IP restrictions. Attempts to negotiate freedom to operate with these genes during the project were unsuccessful.

### 3.1.1 *Nicotiana alata* proteinase inhibitor

The *NaPI* gene encoding the *Nicotiana alata* proteinase inhibitor (Atkinson *et al.* 1993) was originally obtained under material transfer agreement (MTA) from the University of Melbourne's (Victoria) commercialisation body Hexima. However, the original MTA made no prevision for future commercialisation of any product containing this gene. Transgenic sugarcane plants were made using this gene during BSS163.

Several discussions have been held with Leading Dog Consulting (acting for Hexima) on the commercialisation of transgenic lines without significant progress. It has become apparent that sugarcane is not an important crop to Hexima and that their principal interests are elsewhere.

### 3.1.2 Potato proteinase inhibitor II

The proteinase inhibitor isolated from potato *Solanum tuberosum* (Murray and Christeller 1994) was shown to cause a reduction in canegrub weight during feeding trials in BS95S. The *PinII* gene encoding the potato proteinase inhibitor II was obtained under material transfer agreement from Hort+Research, New Zealand.

Advice following patent searches and consideration of previous research agreements show no clear ownership by Hort+Research New Zealand of rights to the *PinII* gene. Hence, no agreement with Hort+Research on the use of the gene is necessary. There are, however, some claims from The Netherlands on rights to the gene that are being explored further. This is a complex area and may well need some significant patent attorney input to resolve. However, the possibility of resolving these issues in the short term and with little financial outlay is limited.

### 3.1.3 *Galanthus nivalis* lectin

The lectin isolated from the snowdrop plant *Galanthus nivalis* (van Damme *et al.* 1987) was shown to cause a reduction in canegrub weight during feeding trials in BS95S. Syngenta is the fourth owner of GNA since we first obtained research access to this gene. Syngenta appears to have no interest in sugarcane, and this is reflected by our inability to pursue this issue with them. We have previously attempted to use contacts within Syngenta to expedite progress on this issue, currently without any success and our attempts to obtain a response from Syngenta has also met with no response. Discussions with other sugarcane groups also working with GNA indicate that ours is not a unique situation.

## 3.2 Novel genes

Despite our best efforts to obtain a new antimetabolite that we can transfer into sugarcane progress has only been achieved in the last year of the project. The *fusolin* gene from CSIRO and the *avidin* gene from Hort+Research have been obtained recently and a few

others remain as options. However, unless we can sign an MTA that is advantageous to the industry, we will not proceed to test that second group of genes.

#### 3.2.1 Avidin

The *Avidin* gene isolated from the chicken *Gallus gallus* has been shown to produce a highly effective product against canegrubs (Allsopp and McGhie 1996), but avidin is also toxic to plant cells. Hort+Research holds a patent that describes the expression of plant noxious genes such as avidin by targeting these compounds to plant vacuoles.

An MTA was requested in December 2000 to use this technology for the expression of avidin in sugarcane. Negotiations were ongoing with Hort+Research to negotiate an MTA that was acceptable to the future commercialisation of any products arising from the research. The MTA, which includes the terms for potential commercial use, was signed in November 2003, and the *Avidin* constructs have now been received. These genes have been prepared in plant expression vectors and plant-transformation experiments have commenced. There is, however, insufficient time remaining in this project to generate and test these transgenic lines.

#### 3.2.2 Fusolin

*Fusolin* genes, derived from entomopox viruses, which have potential activity against canegrubs, have been identified at CSIRO Entomology. We plan to test this type of gene in sugarcane and have been attempting to negotiate a satisfactory MTA with CSIRO Entomology. An MTA was signed in March 2004 to obtain the *Fusolin* genes and these have now been received. These genes have been prepared in plant expression vectors and plant-transformation experiments have commenced. There is, however, insufficient time remaining in this project to generate and test these transgenic lines.

#### 3.2.3 Antifeeding effect/Amber disease

An antifeeding effect gene has been isolated from the bacterium *Serratia entomophilia* (Nunez-Valsez and Mahanty 1996, Giddens et al 2000), a pathogen of New Zealand grass grub. Bacterial strains and genes have now been obtained without an MTA from Canterbury University, New Zealand. The *Serratia* strains are currently undergoing feeding trials to determine if they have an effect on canegrub growth and development. Any further work with this source would fall outside of the time frame of this project.

#### 3.2.4 Blood-bank lectin

The Australian Red Cross Blood Service had identified an interesting plant-derived lectin with similar properties to GNA. After a series of meetings and exchange of documents we could not negotiate an agreement that was beneficial to the Australian sugar industry. Red Cross wanted to retain all rights to any discovery or commercialisation of these materials, and expected BSES to provide its canegrub expertise for free and test the efficacy of this compound at the project's expense. This unrealistic proposal was rejected.

#### 3.2.5 Microprotein toxins

Twelve microproteins derived from unspecified venoms were obtained from the Institute for Molecular Bioscience, University of Queensland. None of the toxins was effective against canegrubs using the micro-injection technique and no further negotiation was attempted.

### 3.3 Promoter and terminator sequences

The promoter and terminator sequences used in both the selection and target genes during this work are the same. The Maize ubiquitin promoter has been patented, although this patent was not lodged in Australia and is, therefore, available to use for the Australian industry. The nopaline synthase terminator from *Agrobacterium tumefaciens* is to the best of our knowledge free of patent claims and is, therefore, also available to use.

### 3.4 Selection genes

The gene used for the selection of transgenic lines, neomycin phosphotransferase (*nptII*), which detoxifies kanamycin, genetecin and paromomycin in transformed cells is to the best of our knowledge free of patent claims and is, therefore, also available to use.

### 3.5 Transformation technologies

Transgenic sugarcane lines were produced by microprojectile bombardment using a ‘home-made’ particle inflow gun. The transformation of plant tissues using microprojectile bombardment is subject to IP.

## 4.0 INSECTICIDAL PERFORMANCE OF CURRENT TRANSGENICS EXPRESSING SNOWDROP LECTIN AND PROTEASE INHIBITORS

### 4.1 Transformation and tissue-culture method

All transformed plants were produced by microprojectile bombardment using a ‘home-made’ particle inflow gun. Selection of transformed callus was done using the kanamycin resistance gene.

We have some concerns about the effect of somaclonal variation on the pot trials conducted to date, especially the contribution of somaclonal variation, induced by the callus tissue culture-transformation system, to the level of resistance apparent in the different pot trials. This concern is best evidenced by our inability to obtain a reasonable correlation between the biochemical data on the level of transgene performance and the resistance of the plants in the trial. Some detailed analysis of protein expression and stability in root sections over time is required to confirm the potential of this approach. The targeting of proteins to intercellular organelles also needs to be investigated as a means of improving the level of protein accumulation and protein stability in root tissues.

### 4.2 Confirmation of gene insertion

Gene insertion was confirmed by PCR followed by Southern analysis on most lines prior to inclusion in pot trials. A sample the PCR screening results is shown in Figure 1. A sample of Southern blot results obtained is shown in Figure 2.

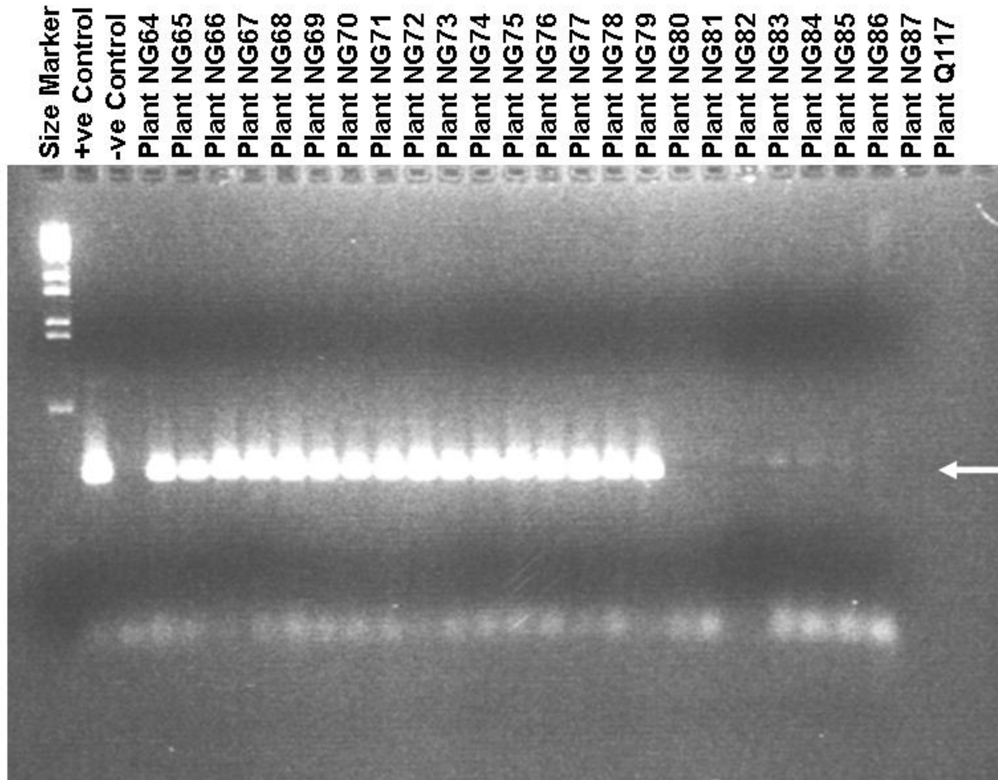


**Figure 1 Sample picture of PCR screening results used for confirmation of transformation with *GNA* gene**

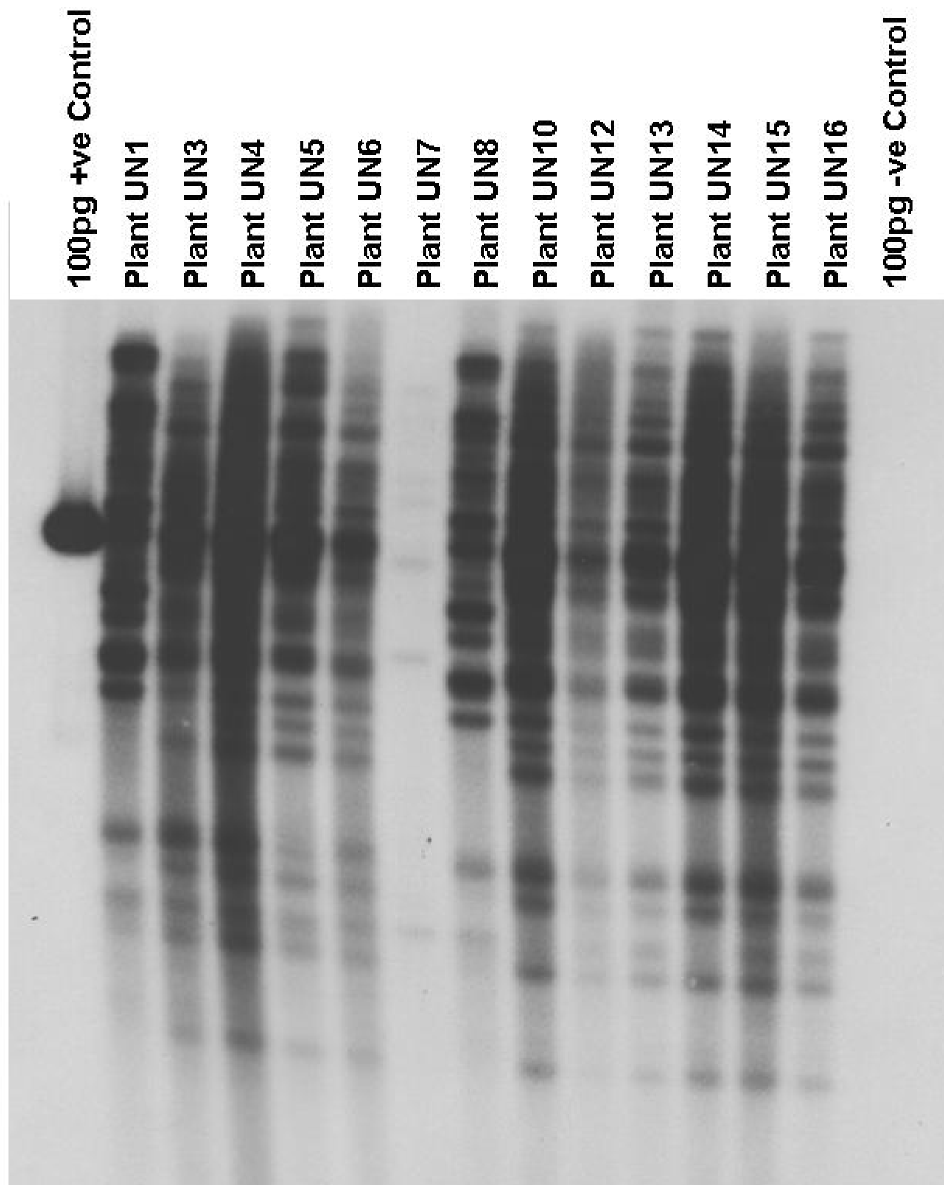


**Figure 2 Sample picture of Southern blot results used for confirmation of transformation**

### 4.3 Confirmation of gene expression

Gene expression was confirmed by western analysis although not all plants had been tested prior to inclusion in pot trials. A sample the western blot results obtained is shown in Figure 3.

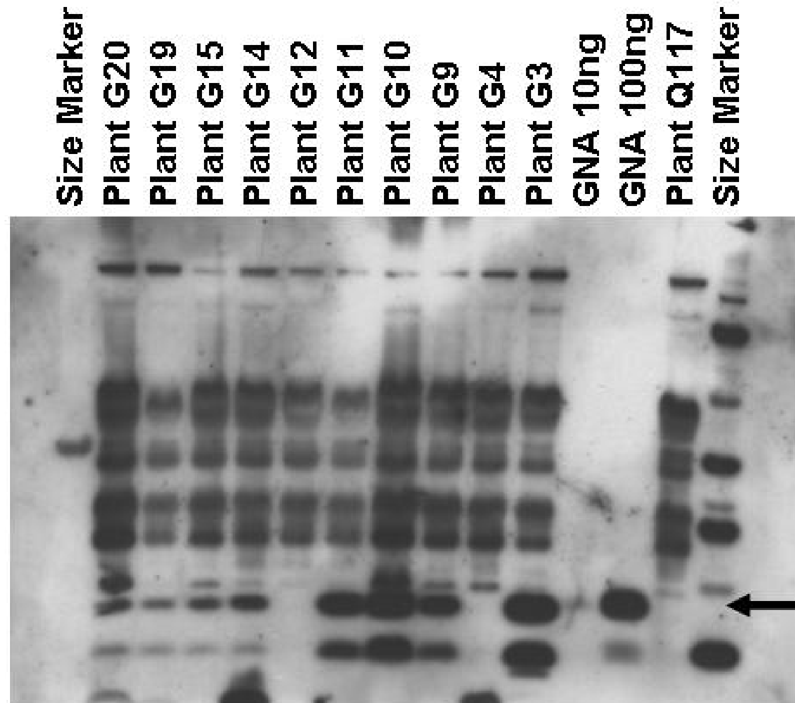


**Figure 3 Sample picture of western blot results used for confirmation of gene expression**

### 4.4 Screening

We screened a total of 152 transgenic lines in five pot trials to determine their effects on growth rate and mortality of southern 1-year canegrub or greyback canegrub.

Pot trials for canegrub feeding were conducted in a similar way to those described in Smith *et al.* (2000). Briefly, transgenic and control sugarcane lines are germinated from one eye setts in 20 L drums of sandy soil. Trials 1-4 were conducted with three replicates of each line in a randomised complete-block design; Trial 5 had six replicates in an effort to improve trial precision and in line with the recommendation of the review panel. The plants were grown for a minimum of 3 months before infestation with young third-instar canegrubs. Three grubs were placed into each pot and allowed to feed for 6-8 weeks. Trials were harvested by recovering the grubs from each pot and recording the weights and mortality for each line. Mean weight gain of surviving grubs in each pot was analysed by analysis of variance; the analyses were first run with number of grubs surviving and initial mean weight of grubs in each pot and a rating of the amount of roots remaining (scale 1-3) as covariates. Means were separated by Tukey's test.

Trial 1 (Woodford 2000) tested 12 lines engineered with genes to express both the *Nicotiana alata* proteinase inhibitor and snowdrop lectin (NG plants in Table 1) using the *Ubi* promoter against southern 1-year canegrub. There was a strong trend to significant differences in grub weight gain among the lines tested ($F = 2.22$ , $df = 13,19$, $P = 0.056$),

with root rating a significant covariate (P = 0.0008, coefficient 0.45). However, no transformed line had weight gains significantly different from the O6 and Q117 controls.

**Table 1** **Trial 1 (Woodford 2000), mean weight gains of *Antitrogus consanguineus* larvae feeding on the roots of transgenic sugarcane lines derived from Q117 and engineered with genes to express both the *Nicotiana alata* proteinase inhibitor and snowdrop lectin (NG plants) using the *Ubi* promoter**

| Line | Weight gain (g)* |
|---|---|
| NG9 | 1.47 |
| NG18 | 1.48 |
| NG19 | 1.02 |
| NG20 | 1.00 |
| NG26 | 1.06 |
| NG31 | 1.09 |
| NG32 | 0.43 |
| NG45 | 0.96 |
| NG52 | 1.13 |
| NG62 | 1.10 |
| NG66 | 0.86 |
| NG72 | 0.42 |
| | |
| O6 | 0.40 |
| Q117 | 1.13 |

Trial 2 (Bundaberg 2001) tested 25 lines engineered with a gene to express the *Nicotiana alata* proteinase inhibitor (N plants in Table 2) and 17 lines engineered with both the *Nicotiana alata* proteinase inhibitor and snowdrop lectin (NG plants in Table 2). There appeared to be significant differences in grub weight gain among the lines tested (F= 1.49, df = 44,126, P = 0.045), with initial mean weight a significant covariate (P < 0.0001, coefficient -0.77). However, the Tukey test detected no significant difference between any line – the former apparent differences being due to chance.

**Table 2** **Trial 2 (Bundaberg 2001), mean weight gains of *Antitrogus consanguineus* larvae feeding on the roots of transgenic sugarcane lines derived from Q117 and engineered with a gene to express the *Nicotiana alata* proteinase inhibitor (N plants), both the *Nicotiana alata* proteinase inhibitor and snowdrop lectin (NG plants), or neomycin phosphotransferase (kanamycin resistant control, NPT plants) using the *Ubi* promoter**

| Line | Weight gain (mg)* |
|---|---|
| N96 | 590.49 |
| N97 | 402.02 |
| N98 | 715.38 |
| N103 | 612.80 |
| N104 | 587.94 |
| N105 | 620.05 |
| N108 | 577.92 |
| N114 | 614.27 |
| N121 | 710.56 |
| N122 | 577.07 |
| N131 | 503.70 |
| N132 | 613.46 |
| N133 | 487.81 |
| N136 | 570.84 |
| N139 | 641.35 |
| N140 | 439.80 |
| N147 | 670.38 |
| N149 | 460.07 |
| N151 | 573.36 |
| N156 | 624.12 |
| N196 | 577.93 |
| N199 | 425.50 |
| N201 | 503.70 |
| N212 | 645.12 |
| N216 | 467.77 |
| NG17 | 530.08 |
| NG21 | 659.27 |
| NG30 | 596.36 |
| NG34 | 623.64 |
| NG37 | 563.80 |
| NG47 | 613.44 |
| NG54 | 595.36 |
| NG56 | 618.23 |
| NG57 | 495.77 |
| NG62 | 711.56 |
| NG63 | 647.17 |
| NG68 | 564.50 |
| NG70 | 612.07 |
| NG76 | 527.70 |
| NG83 | 553.15 |

| | |
|---|---|
| NG84 | 682.41 |
| NG85 | 704.57 |
| | |
| NPT8 | 618.60 |
| O6 | 747.54 |
| Q117 | 731.09 |

Trial 3 (Woodford GH7 2001) tested 17 lines engineered with a gene to express the snowdrop lectin (G plants in Table 3) and 34 lines with a gene to express the potato proteinase inhibitor II (UP plants in Table 3). There appeared to be significant differences in grub weight gain among the lines tested ($F= 1.69$, $df = 53,98$, $P = 0.012$), with initial mean weight ($P < 0.0001$, coefficient -0.75) and root rating ($P = 0.0046$, coefficient 0.13) significant covariates. However, the Tukey test detected no significant difference between any line and the Q117 control, although lines UP119 and UP223 registered very low weight gains.

**Table 3 Trial 3 (Woodford GH7 2001), mean weight gains of *Antitrogus consanguineus* larvae feeding on the roots of transgenic sugarcane lines derived from Q117 and engineered with a gene to express the snowdrop lectin (G plants), the potato proteinase inhibitor II (UP plants), or neomycin phosphotransferase (kanamycin resistant control, NPT plants) using the *Ubi* promoter**

| Line | Weight gain (g)* |
|---|---|
| G5 | 0.900 ABC |
| G6 | 1.372 ABC |
| G49 | 1.623 ABC |
| G59 | 1.374 ABC |
| G76 | 1.366 ABC |
| G77 | 1.499 ABC |
| G78 | 1.445 ABC |
| G81 | 2.130 A |
| G82 | 1.834 ABC |
| G90 | 1.670 ABC |
| G92 | 1.633 ABC |
| G94 | 1.052 ABC |
| G95 | 1.807 ABC |
| G109 | 1.366 ABC |
| G116 | 1.435 ABC |
| G127 | 1.819 ABC |
| G140 | 1.881 AB |
| | |
| UP24 | 1.593 ABC |
| UP27 | 1.475 ABC |
| UP29 | 1.450 ABC |
| UP91 | 1.400 ABC |
| UP96 | 1.453 ABC |

| | |
|---|---|
| UP100 | 1.569 ABC |
| UP119 | 0.626 BC |
| UP124 | 1.838 ABC |
| UP129 | 1.651 ABC |
| UP140 | 1.460 ABC |
| UP174 | 1.550 ABC |
| UP223 | 0.496 C |
| UP224 | 1.678 ABC |
| UP225 | 1.044 ABC |
| UP229 | 1.644 ABC |
| UP231 | 1.662 ABC |
| UP232 | 1.553 ABC |
| UP233 | 1.821 ABC |
| UP248 | 1.428 ABC |
| UP249 | 1.232 ABC |
| UP255 | 1.480 ABC |
| UP263 | 1.555 ABC |
| UP264 | 1.415 ABC |
| UP277 | 1.713 ABC |
| UP286 | 1.212 ABC |
| UP288 | 1.910 AB |
| UP349 | 1.369 ABC |
| UP354 | 1.662 ABC |
| UP357 | 1.935 ABC |
| UP368 | 1.346 ABC |
| UP381 | 1.282 ABC |
| UP389 | 1.108 ABC |
| UP395 | 1.647 ABC |
| UP408 | 1.328 ABC |
| | |
| NPT8 | 1.476 ABC |
| O6 | 1.143 ABC |
| Q117 | 1.168 ABC |

*Means followed by the same letter are not significant different at the 5% level.

Trial 4 (Woodford GH8 2001) tested 56 lines engineered fromQ124 with genes to express the both the snowdrop lectin and the potato proteinase inhibitor (PG plants in Table 4) using the *Ubi* promoter against greyback canegrub. In the analysis of grub weight gain, both initial weight ($P < 0.0001$, coefficient -1.16) and root rating ($P = <0.0001$, coefficient 0.28) contributed significantly as covariates. There was no significant difference among lines ($F = 1.00$, $df = 57,108$, $P = 0.49$) (Table 4).

**able 4** **Trial 4 (Woodford GH8 2001), mean weight gains of *Dermolepida albohirtum* larvae feeding on the roots of transgenic sugarcane lines derived from Q124 and engineered with a gene to express both the snowdrop lectin and the potato proteinase inhibitor II (PG plants) using the *Ubi* promoter**

| **Line** | **Weight gain (g)*** |
|---|---|
| PG1 | 1.0647 |
| PG3 | 0.9703 |
| PG4 | 1.1526 |
| PG5 | 1.9231 |
| PG6 | 1.6813 |
| PG7 | 2.0696 |
| PG9 | 1.7098 |
| PG12 | 1.8567 |
| PG15 | 1.9069 |
| PG18 | 2.2051 |
| PG19 | 1.3251 |
| PG20 | 1.7415 |
| PG21 | 1.4818 |
| PG30 | 1.6909 |
| PG31 | 1.5851 |
| PG32 | 1.9327 |
| PG33 | 1.9091 |
| PG34 | 1.6455 |
| PG36 | 1.7783 |
| PG38 | 1.4861 |
| PG39 | 1.4970 |
| PG41 | 0.9100 |
| PG43 | 1.3895 |
| PG46 | 1.5498 |
| PG47 | 1.1070 |
| PG49 | 1.3544 |
| PG52 | 1.5861 |
| PG53 | 1.9090 |
| PG54 | 1.3534 |
| PG56 | 1.6083 |
| PG57 | 1.5034 |
| PG61 | 1.3576 |
| PG63 | 1.0560 |
| PG64 | 1.5166 |
| PG67 | 1.7271 |
| PG68 | 1.6617 |
| PG69 | 1.3851 |
| PG71 | 1.8531 |
| PG72 | 1.7481 |
| PG73 | 2.2436 |
| PG74 | 1.3039 |
| PG75 | 0.8971 |

| | |
|---|---|
| PG83 | 1.3072 |
| PG84 | 1.9672 |
| PG87 | 1.6906 |
| PG88 | 2.0248 |
| PG89 | 1.4531 |
| PG90 | 1.4558 |
| PG92 | 1.2984 |
| PG95 | 1.2192 |
| PG96 | 1.3427 |
| PG97 | 1.8696 |
| PG99 | 1.9746 |
| PG100 | 1.5485 |
| PG101 | 1.4700 |
| PG102 | 1.0779 |
| | |
| Q124 | 1.5550 |
| O3 | 1.5823 |

Trial 5 (Woodford and Bundaberg 2003) tested 16 lines engineered from Q117 with genes to express the snowdrop lectin (G plants in Table 5), 10 lines to express the *Nicotiana alata* proteinase inhibitor (N plants in Table 5), and 1 line to express both the *Nicotiana alata* proteinase inhibitor and snowdrop lectin (NG plants in Table 5) using the *Ubi* promoter against southern 1-year canegrub. In the analysis of grub weight gain, only initial weight contributed significantly as a covariate (P = 0.0055, coefficient -0.76). There was no significant site* line interaction (F = 1.07, df = 29,114, P = 0.39), and no significant site difference (F = 0.61, df = 1,2, P = 0.52). There were apparent significant differences among lines (F = 1.94, df = 29,114, P = 0.0074) (Table 5), but means were not separated by Tukey's test although lines such as G94, G95, N129 and N220 gave weight increases <70% of weight gains on untransformed Q117. Lines that had been previously tested retained their rankings of weight gain, giving confidence in the trial design.

**Table 5 Trial 5 (Woodford and Bundaberg 2003), mean weight gains of *Antitrogus consanguineus* larvae feeding on the roots of transgenic sugarcane lines derived from Q117 and engineered with a gene to express the snowdrop lectin (G plants), the *Nicotiana alata* proteinase inhibitor (N plants), *Nicotiana alata* proteinase inhibitor and snowdrop lectin (NG plants), and neomycin phosphotransferase (kanamycin resistant control, NPT plants) using the *Ubi* promoter**

| Line | Weight gain (mg)* |
|---|---|
| G3 | 626.3 |
| G5 | 597.0 |
| G9 | 594.2 |
| G10 | 665.8 |
| G21 | 686.7 |
| G26 | 603.1 |
| G27 | 792.1 |
| G33 | 724.6 |
| G37 | 701.4 |
| G49 | 595.6 |
| G76 | 682.1 |
| G94 | 454.9 |
| G95 | 496.6 |
| G97 | 525.0 |
| G101 | 678.7 |
| G145 | 603.7 |
| | |
| N118 | 660.7 |
| N129 | 466.7 |
| N131 | 776.9 |
| N132 | 667.4 |
| N147 | 728.2 |
| N151 | 633.9 |
| N152 | 536.4 |
| N153 | 520.8 |
| N201 | 519.2 |
| N220 | 470.0 |
| NG77 | 639.9 |
| | |
| NPT 2 | 550.9 |
| O6 | 667.6 |
| Q117 | 715.5 |

Our pot trials (five trials over three years at two locations and with two species of canegrubs) for testing transgenic lines for antibiosis towards canegrub larvae were unable to clearly screen transgenic lines. The results from the first four trials indicated a lack of reproducibility in the trial results that compromised the ability to identify useful transgenic lines. At the project review, this problem was highlighted and the review panel made recommendations that were implemented in trial 5. This trial was conducted using

two locations containing the same transgenic and control lines. These trial results were an improvement on trials 1-4, due to the increased number of replicates. Additional controls suggested by the panel were not included, as both plant material and space were limited. The statistical confidence was much greater in this final trial and some of the variation within the trial data had been removed. However, no transgenic lines tested had a statistically significant effect on the weight gain of canegrubs.

The significant negative effect of initial grub weight as a covariate in Trials 2-5 was not unexpected – smaller grubs were expected to put on proportionately more weight than larger larvae that were closer to final size. We also blocked larvae on their initial weight to reduce the impact of this parameter on the statistical analyses.

In Trials 1, 3 and 4, root rating at the end of the experiment was a significant positive covariate. This indicates that available food may have been limiting in smaller plants or plants heavily damaged. We suggest that future trials be done with much larger plants to reduce the impact of this parameter.

We also suggest further studies into the expression of genes within the root system to determine whether expression levels and location of expression of transgenes in the root system can be modified to increase canegrub ingestion of antimetabolites.

## 5.0 TEST OF ADDITIONAL PRODUCTS FOR ANTIMETABOLIC EFFECTS ON CANEGRUBS AND INCORPORATION OF ACTIVE ONES INTO THE PROGRAM

Alternative antimetabolites were identified from three sources, but a satisfactory use-agreement could not be signed with one. Twelve potential nerve toxins were obtained from the Institute of Molecular Bioscience, University of Queensland, were tested using a novel injection technique.

An alternative pathogen was also obtained for testing. *Serratia entomophilia* is a pathogen of New Zealand grass grubs (*Coleoptera Zealandica*) which is used as a biocontrol agent for control of these pests. Feeding trials of *Serratia entomophilia* to greyback canegrubs (*Dermolepida alboretium*) were conducted to determine whether these *Serratia* strains were also pathogenic to canegrub species.

### 5.1 IMB toxins

Twelve toxins were supplied by the Institute of Molecular Biosciences, University of Queensland, in freeze-dried 100 μg aliquots contained in 1.5 mL eppendorf tubes. Each toxin pellet was resuspended in 20 μL of 0.9% sodium chloride. The organophosphate insecticide tebupirimfos (93% active ingredient) was used as a positive control because of its availability and known effectiveness against canegrubs. Physiological saline (0.9% sodium chloride) was used as a negative control.

***Methods***

In experiment 1, third-instar greyback canegrubs were each injected with 0.5 μL of either one of the resuspended toxins, tebupirimfos or saline. Up to 15 canegrubs were treated with each formulation and were observed for signs of morbidity and/ or mortality at 1, 3 and 24 h and 1, 2 and 3 weeks after injection. Injections were carried out with hand-pulled glass capillaries (outside diameter of 1 mm, internal diameter of 0.5 mm, length of 10 cm). Canegrubs were held using 'stocks' fashioned from plastic razor-blade holders and injected at the suture line between the labrum and the clypeus. Manoeuvring of pipette and injection of samples was achieved using a micromanipulator borrowed from Dr Paul Ebert at the University of Queensland.

In experiment 2, injection volumes were increased to 2 μL and each treatment generally reduced to three replications; the small quantities of toxins only allowed one grub to be treated with some toxins. The increased volume raised the toxin level to ~10 μg, well above the level which could have been realistically injected in the first trial. Once again, 0.9% sodium chloride was used as the negative control.

***Results***

In experiment 1, injection of tebupirimfos gave 100% mortality within 36-48 hours (Table 6). Corrected mortality rates for all toxins indicate that none were effective against greyback canegrub (Table 6).

High mortality rates in the negative controls performed on the 22/3 and 26/3 were of concern. Dark lesions at or near the injection site were noticed on several grubs during routine observation. Negative controls were performed at the end of the day, tiredness and rougher than normal handling possibly inducing trauma causing higher mortality. Unfortunately, by the time deaths were observed/ recorded, body discolouration had reached such a point as to hide any sign of injection site lesions. Such lesions can be indicative of disease.

**Table 6 Percentage canegrub mortality 1 and 3 weeks post injection in experiment 1**

| | | | | 1wk | 3wk | 1wk | 3wk |
|---|---|---|---|---|---|---|---|
| **Date** | **Treatment** | **vol. uL** | **Reps** | **% mortality** | | **% mortality corrected** | |
| **13/03/2001** | control +ve(100%) | 0.5 | 10 | 100.0 | 100.0 | | |
| **20/03/2001** | control-ve | 0.5 | 15 | 13.3 | 13.3 | | |
| | T1 | 0.5 | 15 | 6.7 | 13.3 | 0.0 | 0.0 |
| **21/03/2001** | control-ve | 0.5 | 15 | 13.3 | 13.3 | | |
| | T2 | 0.5 | 12 | 6.7 | 6.7 | 0.0 | 0.0 |
| **22/03/2001** | control-ve | 0.5 | 15 | 53.3 | 53.3 | | |
| | T3 | 0.5 | 15 | 6.7 | 6.7 | 0.0 | 0.0 |
| | T4 | 0.5 | 15 | 6.7 | 6.7 | 0.0 | 0.0 |
| **26/03/2001** | control-ve | 0.5 | 15 | 46.7 | 46.7 | | |
| | T5 | 0.5 | 15 | 6.7 | 20.0 | 0.0 | 0.0 |
| | T6 | 0.5 | 15 | 40.0 | 40.0 | 0.0 | 0.0 |
| | T7 | 0.5 | 15 | 6.7 | 6.7 | 0.0 | 0.0 |
| | T8 | 0.5 | 15 | 13.3 | 13.3 | 0.0 | 0.0 |
| **27/03/2001** | control-ve | 0.5 | 15 | 20.0 | 26.7 | | |
| | T9 | 0.5 | 15 | 6.7 | 6.7 | 0.0 | 0.0 |
| | T10 | 0.5 | 15 | 20.0 | 26.7 | 0.0 | 0.0 |
| | T11 | 0.5 | 15 | 13.3 | 13.3 | 0.0 | 0.0 |
| | T12 | 0.5 | 15 | 6.7 | 13.3 | 0.0 | 0.0 |
| | | | | | | | |

In experiment 2, using higher volumes, there was no mortality from any of the toxins (Table 7).

**Table 7 Percentage canegrub mortality 1 and 3 weeks post injection in experiment 2**

| | | | | 1wk | 3wk | 1wk | 3wk |
|---|---|---|---|---|---|---|---|
| Date | Treatment | vol. uL | Reps | mortality | | % mortality correct. | |
| 2/04/2001 | control-ve | 2.0 | 3 | 30.0 | 30.0 | | |
| | NaCl/ NaBenz. | 2.0 | 3 | 0.0 | 0.0 | 0.0 | 0.0 |
| | T3 | 2.0 | 2 | 0.0 | 0.0 | 0.0 | 0.0 |
| | T5 | 2.0 | 1 | 0.0 | 0.0 | 0.0 | 0.0 |
| | T7 | 2.0 | 1 | 0.0 | 0.0 | 0.0 | 0.0 |
| | T8 | 2.0 | 1 | 0.0 | 0.0 | 0.0 | 0.0 |
| | T9 | 2.0 | 3 | 0.0 | 0.0 | 0.0 | 0.0 |
| | T10 | 2.0 | 2 | 0.0 | 0.0 | 0.0 | 0.0 |
| | T12 | 2.0 | 2 | 0.0 | 0.0 | 0.0 | 0.0 |
| | NaCl + colouring | 2.0 | 3 | 0.0 | 0.0 | 0.0 | 0.0 |

***Conclusions***

None of the potential toxins tested using the injection technique had any statistically significant affect on the canegrubs tested. In future, injection procedures could include increased observation to determine if there is any correlation between mortality and injection site lesion. Negative controls may need to be carried out at the beginning and end of injecting sessions, to take account of handling variables. Given awareness of these factors, further testing of toxins via injection has potential.

### 5.2 *Serratia entomophilia* strains

Feeding trials to determine if *Serratia* strains had any effect on greyback canegrub weight gains were conducted in duplicate.

***Materials and Methods***

We obtained approximately 100 third-instar greyback canegrubs from a Burdekin canefield and held them at 25ºC for 48 hours to check for mortality and disease. Healthy grubs were weighed and sorted into five weight groups before randomly assigning one from each weight group to each of the five treatments. Three replicates of five grubs were prepared for each treatment and each replicate was randomly assorted into a block for analysis. Treatments were:

- no food
- Carrot only
- *E. coli* DH5a control
- *Serratia* +ve strain BC4B
- *Serratia* +ve strain A1MO2
- *Serratia* –ve strain UC7
- *E. coli* DH5a Cos 31

Bacterial cultures were grown overnight in LB media and concentrated to 1.5 x $10^9$ cells per 10 µL. We placed 10 µL of culture to a 1.6 g piece of carrot and allowed a grub to feed on it. At 2, 4 and 8 weeks post inoculation, the grubs were observed for activity and appearance. Grubs were weighed at 2, 4 and 8 weeks post inoculation in trial 1. Weights were recorded at 2, 4 and 6 weeks post inoculation in trial 2. Grubs were transferred to new containers with fresh sand and fresh diet after each observation.

We analysed weight gains over time, using survival and the initial weight as covariants. Only in the no-food treatment was there any significant mortality and this treatment was omitted from the analyses.

***Results and Discussion***

In feeding trial 1, there were no significant differences in grub weight gain among the lines tested ($F = 0.62$, df = 5,10, $P = 0.69$) over the 8-week trial (Table 8). There was, not unexpectedly, a significant increase in weight over time ($F = 19.95$, df = 2,24, $P < 00001$), but no time*line interaction ($F = 0.58$, df = 10,24, $P = 0.81$).

**Table 8 Feeding trial 1, Weight gains of *Antitrogus consanguineus* larvae feeding on carrot treated with potential canegrub pathogens after 2, 4 and 8 weeks**

**Serratia Feeding Trial - 1 2004**

| | | | **Grub weights at Week 2** | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Line** | **Rep** | **Av Init. Wt.** | **Grub 1** | **Grub 2** | **Grub 3** | **Grub 4** | **Grub 5** | **Survival** |
| **No Food** | 1 | 3.960 | 4.398 | 4.709 | 5.011 | 2.810 | 1.921 | 5 |
| | 2 | 3.999 | 4.579 | 3.672 | 4.385 | 2.740 | 4.101 | 5 |
| | 3 | 4.500 | 4.561 | 6.092 | 2.655 | 3.492 | 3.840 | 5 |
| **Carrot only** | 1 | 4.460 | 3.116 | 3.384 | 5.798 | 5.776 | 4.970 | 5 |
| | 2 | 3.996 | 1.576 | 3.869 | 5.131 | 4.606 | 4.318 | 5 |
| | 3 | 4.059 | 3.058 | 3.711 | 3.936 | 4.557 | 4.884 | 5 |
| **E. coli -ve** | 1 | 3.627 | 4.301 | 5.101 | 2.812 | 2.591 | 4.865 | 5 |
| | 2 | 4.753 | 3.700 | 4.885 | 5.251 | 5.125 | 5.702 | 5 |
| | 3 | 4.164 | 4.079 | 3.957 | 4.950 | 3.287 | 4.927 | 5 |
| **Ser -BC4B** | 1 | 4.143 | 3.829 | 4.485 | 5.495 | 4.644 | 1.992 | 5 |
| | 2 | 3.965 | 4.329 | 4.035 | 3.912 | 3.201 | 3.816 | 5 |
| | 3 | 4.398 | 5.052 | 3.593 | 5.692 | 4.856 | 3.582 | 5 |
| **Ser-A1MO2** | 1 | 4.119 | 5.091 | 4.337 | 5.193 | 4.899 | 1.963 | 5 |
| | 2 | 4.430 | 4.898 | 4.884 | 3.253 | 6.301 | 3.187 | 5 |
| | 3 | 3.780 | 4.922 | 4.563 | 4.044 | 3.272 | 3.658 | 5 |
| **Ser-UC7** | 1 | 3.549 | 4.100 | 4.197 | 3.279 | 2.387 | 5.338 | 5 |
| | 2 | 4.288 | 4.979 | 4.175 | 4.633 | 4.655 | 3.383 | 5 |
| | 3 | 4.746 | 3.428 | 4.486 | 4.235 | 5.351 | 5.097 | 5 |
| **Cos31 E.coli** | 1 | 4.256 | 4.908 | 4.297 | 4.876 | 3.908 | 3.621 | 5 |
| | 2 | 3.227 | 3.561 | 3.372 | 4.733 | 2.540 | 2.618 | 5 |
| | 3 | 5.001 | 5.038 | 4.957 | 4.476 | 4.804 | 4.522 | 5 |

| | | | **Grub weights at Week 4** | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Line** | **Rep** | **Av Init. Wt.** | **Grub 1** | **Grub 2** | **Grub 3** | **Grub 4** | **Grub 5** | **Survival** |
| **No Food** | 1 | 3.960 | 4.491 | 4.704 | 4.916 | 2.944 | 2.512 | 5 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 2 | 3.999 | 4.453 | 3.694 | 4.395 | | 4.065 | 4 |
| | 3 | 4.500 | 4.388 | 5.205 | 2.512 | 3.447 | 3.752 | 5 |
| **Carrot only** | 1 | 4.460 | 3.966 | 3.681 | 5.560 | 6.313 | 5.160 | 5 |
| | 2 | 3.996 | 2.181 | 4.237 | 5.479 | 4.913 | 4.787 | 5 |
| | 3 | 4.059 | 3.781 | 4.071 | 4.407 | 5.120 | 5.114 | 5 |
| **E.coli -ve** | 1 | 3.627 | 4.329 | 5.522 | 3.354 | 3.081 | 5.109 | 5 |
| | 2 | 4.753 | 4.234 | 5.262 | 5.094 | 5.565 | 5.984 | 5 |
| | 3 | 4.164 | 4.486 | 4.449 | 5.108 | 3.526 | 5.003 | 5 |
| **Ser -BC4B** | 1 | 4.143 | 4.325 | 4.763 | 5.606 | 4.903 | 2.582 | 5 |
| | 2 | 3.965 | 4.324 | 4.397 | 4.258 | 3.686 | 4.012 | 5 |
| | 3 | 4.398 | 5.288 | 3.819 | 5.811 | 5.265 | 4.057 | 5 |
| **Ser-A1MO2** | 1 | 4.119 | 5.674 | 4.676 | 5.535 | 5.319 | 2.615 | 5 |
| | 2 | 4.430 | 4.985 | 4.994 | 3.868 | 6.229 | 3.449 | 5 |
| | 3 | 3.780 | 4.938 | 4.886 | 4.088 | 6.830 | 4.050 | 5 |
| **Ser-UC7** | 1 | 3.549 | 4.199 | 1.622 | 3.835 | 3.052 | 5.559 | 5 |
| | 2 | 4.288 | 5.168 | 4.537 | 5.022 | 5.098 | 3.722 | 5 |
| | 3 | 4.746 | 3.807 | 5.081 | 4.907 | 5.341 | 4.998 | 5 |
| **Cos31 E.coli** | 1 | 4.256 | 5.371 | 4.574 | 4.958 | 4.024 | 4.187 | 5 |
| | 2 | 3.227 | 3.693 | 3.889 | 4.836 | 3.142 | 3.133 | 5 |
| | 3 | 5.001 | 5.484 | 5.031 | 4.686 | 5.073 | 4.559 | 5 |

**Grub weights at Week 8**

| Line | Rep | Av Init. Wt. | Grub 1 | Grub 2 | Grub 3 | Grub 4 | Grub 5 | Survival |
|---|---|---|---|---|---|---|---|---|
| **No Food** | 1 | 3.960 | 4.437 | 4.041 | 3.753 | | | 3 |
| | 2 | 3.999 | 4.204 | | 2.854 | | | 2 |
| | 3 | 4.500 | 4.260 | 4.963 | | | 3.543 | 3 |
| **Carrot only** | 1 | 4.460 | 4.185 | 3.964 | 4.531 | 5.356 | 4.022 | 5 |
| | 2 | 3.996 | | 3.670 | 4.974 | 4.528 | 4.161 | 4 |
| | 3 | 4.059 | 3.713 | 4.174 | 4.592 | 4.518 | 4.099 | 5 |
| **E.coli -ve** | 1 | 3.627 | 4.046 | 4.857 | 3.452 | 3.220 | 4.643 | 5 |
| | 2 | 4.753 | 4.098 | 4.679 | 4.380 | 4.521 | 5.066 | 5 |
| | 3 | 4.164 | 4.293 | | 5.568 | 3.257 | 4.169 | 4 |
| **Ser -BC4B** | 1 | 4.143 | 4.249 | 4.006 | 4.560 | 4.463 | 2.455 | 5 |
| | 2 | 3.965 | 3.738 | 4.365 | 3.673 | 3.697 | 3.731 | 5 |
| | 3 | 4.398 | 4.794 | 3.734 | 5.744 | 4.800 | 4.415 | 5 |
| **Ser-A1MO2** | 1 | 4.119 | 4.214 | 3.759 | 4.937 | 5.015 | 2.777 | 5 |
| | 2 | 4.430 | 4.474 | 4.488 | 3.898 | 6.141 | 3.180 | 5 |
| | 3 | 3.780 | 4.906 | 4.512 | 3.954 | 3.859 | 4.559 | 5 |
| **Ser-UC7** | 1 | 3.549 | 3.865 | 4.567 | 3.961 | 3.555 | 4.675 | 5 |
| | 2 | 4.288 | 4.325 | 4.246 | 4.642 | 4.507 | 3.724 | 5 |
| | 3 | 4.746 | 3.532 | 4.670 | 4.231 | 4.894 | 4.803 | 5 |
| **Cos31 E.coli** | 1 | 4.256 | 4.814 | 4.252 | 4.757 | 3.803 | 3.759 | 5 |
| | 2 | 3.227 | 3.986 | 3.713 | 4.277 | 3.139 | 3.288 | 5 |
| | 3 | 5.001 | 4.797 | 4.495 | 4.569 | 4.565 | 4.390 | 5 |

In feeding trial 2, there were no significant differences in grub weight gain among the lines tested (F = 0.40, df = 5,10, P = 0.849) over the 6-week trial (Table 9). There was, not unexpectedly, a significant increase in weight over time (F = 657.7, df = 2,24, P < 00001), but no time*line interaction (F = 0.55, df = 10,24, P = 0.84).

**Table 9 Feeding trial 2, Weight gains of *Antitrogus consanguineus* larvae feeding on carrot treated with potential canegrub pathogens after 2,4 and 6 weeks**

**Serratia Feeding Trial - 2 2004**

| | | | Grub weights at Week 2 | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Line** | **Rep** | **Av Init. Wt.** | **Grub 1** | **Grub 2** | **Grub 3** | **Grub 4** | **Grub 5** | **Survival** |
| **No Food** | 1 | 2.8432 | 2.263 | | 3.094 | 2.395 | 2.045 | 4 |
| | 2 | 2.9622 | 3.086 | 1.879 | | 2.851 | 2.85 | 4 |
| | 3 | 3.0182 | 2.126 | 2.537 | 3.435 | 3.151 | 2.539 | 5 |
| **Carrot only** | 1 | 2.807 | 3.285 | 2.559 | 2.725 | 2.924 | 3.551 | 5 |
| | 2 | 2.8088 | 3.111 | 3.39 | 3.016 | 3.422 | 2.219 | 5 |
| | 3 | 3.1296 | 3.572 | 2.949 | 4.009 | 3.041 | 2.738 | 5 |
| **E.coli -ve** | 1 | 2.9592 | 3.641 | 3.284 | 2.955 | 2.38 | 3.592 | 5 |
| | 2 | 2.7958 | 3.377 | 3.302 | 2.761 | 3.298 | 2.691 | 5 |
| | 3 | 2.606 | 2.975 | 3.931 | 2.797 | 2.39 | 2.829 | 5 |
| **Ser -BC4B** | 1 | 2.6586 | 3.252 | 2.881 | 3.187 | 3.158 | 2.912 | 5 |
| | 2 | 2.9708 | 2.292 | 3.089 | 3.544 | 2.912 | 3.335 | 5 |
| | 3 | 2.9768 | 3.796 | 2.238 | 2.489 | 3.47 | 3.94 | 5 |
| **Ser-A1MO2** | 1 | 2.8172 | 3.462 | 3.592 | 3.112 | 2.777 | 2.759 | 5 |
| | 2 | 2.7064 | 2.934 | 4.046 | 2.34 | 2.25 | 2.408 | 5 |
| | 3 | 3.1082 | 2.761 | 3.713 | 3.245 | 2.853 | 2.743 | 5 |
| **Ser-UC7** | 1 | 2.675 | 2.72 | 3.469 | 3.169 | 2.529 | 2.424 | 5 |
| | 2 | 2.8318 | 3.188 | 3.243 | 2.341 | 2.656 | 4.132 | 5 |
| | 3 | 3.1712 | 3.025 | 4.242 | 2.808 | 2.759 | 3.515 | 5 |
| **Cos31 E.coli** | 1 | 3.0402 | 3.041 | 3.752 | 3.155 | 2.997 | 3.379 | 5 |
| | 2 | 3.0482 | 2.811 | 2.642 | 4.315 | 2.594 | 3.277 | 5 |
| | 3 | 2.5168 | 2.374 | 2.798 | 2.229 | 3.423 | 2.917 | 5 |

| | | | Grub weights at Week 4 | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Line** | **Rep** | **Av Init. Wt.** | **Grub 1** | **Grub 2** | **Grub 3** | **Grub 4** | **Grub 5** | **Survival** |
| **No Food** | 1 | 2.8432 | | | 3.109 | | | 1 |
| | 2 | 2.9622 | 2.768 | | | | | 1 |
| | 3 | 3.0182 | | | | | | 0 |
| **Carrot only** | 1 | 2.807 | 3.626 | 2.91 | 3.207 | 3.467 | 4.011 | 5 |
| | 2 | 2.8088 | 3.293 | 3.852 | 3.544 | 3.601 | 2.758 | 5 |
| | 3 | 3.1296 | 3.953 | 3.256 | 4.504 | 3.642 | 3.398 | 5 |
| **E.coli -ve** | 1 | 2.9592 | 3.75 | 3.542 | 3.153 | 2.87 | 3.988 | 5 |
| | 2 | 2.7958 | 3.874 | 3.769 | 3.191 | 3.959 | 3.314 | 5 |
| | 3 | 2.606 | 3.467 | 4.136 | 3.529 | 2.984 | 3.304 | 5 |
| **Ser -BC4B** | 1 | 2.6586 | 3.683 | 3.217 | 3.502 | 3.596 | 3.42 | 5 |
| | 2 | 2.9708 | 2.901 | 3.543 | 3.817 | 3.616 | 3.66 | 5 |
| | 3 | 2.9768 | 4.148 | | 2.968 | 3.758 | 4.247 | 4 |
| **Ser-A1MO2** | 1 | 2.8172 | 3.857 | 3.772 | 3.518 | 3.351 | 3.288 | 5 |
| | 2 | 2.7064 | 3.29 | 4.232 | 2.785 | 3.002 | 3.054 | 5 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 | 3.1082 | 2.652 | 4.017 | 3.66 | 4.158 | 3.122 | 5 |
| **Ser-UC7** | 1 | 2.675 | 3.074 | 3.737 | 3.582 | 3.178 | 2.775 | 5 |
| | 2 | 2.8318 | 3.899 | 3.779 | 2.853 | 2.882 | 4.464 | 5 |
| | 3 | 3.1712 | 3.491 | 4.727 | 3.27 | 3.381 | 3.942 | 5 |
| **Cos31 E.coli** | 1 | 3.0402 | 3.532 | 4.045 | 3.56 | 3.338 | 3.623 | 5 |
| | 2 | 3.0482 | 3.265 | 3.152 | 4.134 | 3.319 | 3.818 | 5 |
| | 3 | 2.5168 | 2.846 | 3.395 | 2.863 | 3.842 | 3.282 | 5 |

| | | | **Grub weights at Week 6** | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Line** | **Rep** | **Av Init. Wt.** | **Grub 1** | **Grub 2** | **Grub 3** | **Grub 4** | **Grub 5** | **Survival** |
| **No Food** | 1 | 2.8432 | | | | | | 0 |
| | 2 | 2.9622 | | | | | | 0 |
| | 3 | 3.0182 | | | | | | 0 |
| **Carrot only** | 1 | 2.807 | 2.823 | 3.484 | | 4.476 | 4.429 | 4 |
| | 2 | 2.8088 | 3.796 | 4.511 | 4.284 | 4.07 | 3.721 | 5 |
| | 3 | 3.1296 | 4.332 | 3.733 | 4.659 | 4.682 | 4.129 | 5 |
| **E.coli -ve** | 1 | 2.9592 | 4.659 | | 3.634 | 3.641 | 4.372 | 4 |
| | 2 | 2.7958 | 4.318 | 4.203 | 3.929 | 4.905 | 4.379 | 5 |
| | 3 | 2.606 | 4.026 | 4.63 | 4.16 | 3.206 | 3.714 | 5 |
| **Ser -BC4B** | 1 | 2.6586 | 4.802 | 3.764 | 4.292 | 4.436 | 4.229 | 5 |
| | 2 | 2.9708 | 3.792 | 4.277 | 4.395 | 4.073 | 4.264 | 5 |
| | 3 | 2.9768 | 4.488 | | 3.49 | 4.094 | 4.623 | 4 |
| **Ser-A1MO2** | 1 | 2.8172 | 4.459 | 4.298 | 4.389 | 4.287 | 3.92 | 5 |
| | 2 | 2.7064 | 4.098 | 4.795 | 3.486 | 3.776 | 4.186 | 5 |
| | 3 | 3.1082 | 2.866 | 4.502 | 4.575 | 4.571 | 3.796 | 5 |
| **Ser-UC7** | 1 | 2.675 | 3.144 | 4.386 | 4.108 | 3.828 | 3.548 | 5 |
| | 2 | 2.8318 | 4.7 | 4.47 | 3.596 | 3.299 | 5.244 | 5 |
| | 3 | 3.1712 | 4.285 | 5.184 | 3.973 | 4.23 | 4.622 | 5 |
| **Cos31 E.coli** | 1 | 3.0402 | 4.611 | 4.707 | 4.458 | 4.252 | 4.179 | 5 |
| | 2 | 3.0482 | 3.872 | 3.767 | 4.587 | 4.095 | 4.253 | 5 |
| | 3 | 2.5168 | 3.761 | 3.934 | 3.517 | 4.568 | 4.021 | 5 |

***Conclusions***

In two replicated trials, *Serratia entomophilia* strains, which cause amber disease in New Zealand grass grubs, did not have any statistically significant effect on the weight gain or mortality of *Antitrogus consanguineus* larvae.

The *Serratia* and *E. coli* containing the cosmid 31 did not give any obvious signs of either disease or anti-feeding affect.

These *Serratia* strains may still be effective against other species of canegrubs and feeding trials to investigate this are planned for next year’s grub season and will be completed within CRC-SIIB project 1bii.

# 6.0 PRODUCTION OF ADDITIONAL TRANSGENIC LINES

## 6.1 Using snowdrop lectin and protease inhibitors

The production of transgenic lines containing the *PinII*, *NaPI* or *GNA* genes based on other cultivars was attempted using the new transformation technology developed as part of BSS242. Cultivars Q117 and Q165$^{\Phi}$ were initiated into tissue culture. Subsequent transformation experiments with this technique yielded no transgenic lines.

## 6.2 Using other gene constructs

Additional genes for canegrub resistance were transformed into sugarcane cultivars Q117 and Q205$^{\Phi}$. We had planned to use a range of new genes for transformation work during this project, but negotiations to obtain access to promising third-party genes were difficult and access was obtained only towards the end of the project. Transformation experiments are currently underway with the *Avidin* and *Fusolin* genes and some plants were also produced using the proteinase-inhibitor gene *Da10-12* isolated during BSS2207.

### 6.2.1 Transformation of Q117 and Q205$^{\Phi}$ with *Avidin*

Avidin isolated from the chicken *Gallus gallus* was shown by Allsopp and McGhie (1996) to be highly effective against canegrubs. However, high levels of avidin are also toxic to plant cells.

Hort+Research holds a patent that describes the expression of plant noxious genes, such as avidin, by targeting these compounds to plant vacuoles. An MTA was requested in December 2000 to use this technology for the expression of avidin in sugarcane. The Several rounds of negotiations were necessary with Hort+Research to negotiate an MTA that was acceptable to the future commercialisation of any products arising from the research. An MTA, which includes the terms for potential commercial use, was signed in November 2003 and the *Avidin* constructs have now been received. These genes have been prepared in plant expression vectors and plant-transformation experiments have commenced. There is, however, insufficient time remaining in this project to regenerate and test these transgenic lines. This will be done by BSES within CRC-SIIB project 1bii.

### 6.2.2 Transformation of Q117 and Q205$^{\Phi}$ with *Fusolin*

The *Fusolin* gene derived from an entomopox virus that has possible activity against canegrubs has been identified at CSIRO Entomology. We hoped to test this gene in sugarcane and attempted to negotiate a satisfactory MTA with CSIRO Entomology. A MTA was signed in March 2004 and the *Fusolin* genes have been received. These genes have been prepared in plant expression vectors and plant-transformation experiments have commenced. There is, however, insufficient time remaining in this project to regenerate and test these transgenic lines. This will be done by BSES within CRC-SIIB project 1bii.

### 6.2.3 Transformation of Q117 and Q205$^{\Phi}$ with Antifeeding effect/Amber disease gene

An antifeeding affect gene has been isolated from *Serratia entomophilia* (Nunez-Valsez and Mahanty 1996, Giddens et al 2000), a parasite of New Zealand grass grub (another scarab). Bacterial strains and genes for amber disease have been obtained without an MTA from Canterbury University, New Zealand. Production of expression constructs and subsequent transformation of these genes into sugarcane has not commenced as we would like to have the results of feeding trials before investing more time with this potential resistance gene.

### 6.2.4 Transformation of Q117 and Q205$^{\Phi}$ with *Da10-12*

A potential proteinase-inhibitor gene from greyback canegrub was identified during project BSS2207. This gene was transferred into Q117 under the control of the *Ubi* promoter. Screening of plants using an antibody produced to the bacterially expressed Da10-12 protein has failed to detect any expression of the Da10-12 proteinase inhibitor in any transgenic lines.

## 7.0 OUTPUTS

- The rial design for pot-based screening of transgenic lines has been improved and will be used in future assays.
- A method for screening potential insect toxins by microinjection has been developed and can be used in future assays.
- No transgenic lines proved to have a significant effect on canegrub weight gain.
- No transgenic lines can be recommended for commercial release.
- No new toxins or biocontrol agents were identified

## 8.0 EXPECTED OUTCOMES, FUTURE NEEDS AND RECOMMENDATIONS

Milestones 9 could not be achieved in the near future as the agreement on the experimental use of the *fusolin* or *avidin* gene has only recently been obtained. Transformation experiments have commenced with these genes, but any transformed plants would be available for planting for pot-trial towards the end of 2005. These would then be suitable for testing early in 2006, coinciding with the occurrence of third-instar canegrubs. We feel that this is an unreasonable delay to the current project and these activities have been incorporated into the CRC-SIIB project 1bii ‘Environmentally sustainable canegrub resistance’.

Our impression from negotiations with Novartis (and other groups) is that they see the commercialisation of transgenes in sugarcane as a low-priority issue because they can not easily understand how they can control the use of the gene and obtain a return on investment. In other crops, a license charge is imposed on the grower at the point of seed sale. Novartis’ concerns focus on how this can be imposed in a vegetatively propagated

crop, when a license charge could be imposed, and who (the industry in general or individual growers) would pay the license charge. These are issue in common to the use of any transgenic technology within the Australian sugar industry, and resolution is beyond the scope and resources of a single project.

We believe that the industry needs to develop an agreed position on this issue. We suggest that SRDC needs to take an active role in developing such an agreed position.

In the meantime, field testing of our transgenic lines would provide additional data that will help convince companies such as Novartis that commercialisation is worth progressing. This will be done through the CRC-SIIB.

Future development of this approach to control root feeding pests would also benefit from further analysis of protein expression and stability in root sections over time. The targeting of proteins to intercellular organelles also needs to be investigated as a means of improving the level of protein accumulation and protein stability in root tissues.

## 9.0 PUBLICATIONS ARISING FROM THE PROJECT

Allsopp PG, Nutt KA, Geijskes RJ and Smith GR. 2000. Transgenic sugarcane with increased resistance to canegrubs. In: *Sugarcane Pest Management in the New Millennium* (eds Allsopp PG and Suasa-ard W), pp. 63-67. International Society of Sugar Cane Technologists, Brisbane.

Nutt KA, Allsopp PG, Geijskes RJ, McKeon MG and Smith GR. 2001. Canegrub resistant sugarcane. *Proceedings of the International Society of Sugar Cane Technologists* 24(2): 582-583.

## 10.0 ACKNOWLEDGMENTS

We thank all the staff who have been associated with this work and made valuable contributions to progress, especially Michael McKeon, George Bade and Norm McGill. We also thank our Australian and overseas colleagues and collaborators for access to various compounds and genes to trial against canegrubs. The *PinII* and *Avidin* genes were obtained under research agreement from HortResearch of New Zealand, *Fusolin* genes from CSIRO-Plant Industry, *Serratia* strains and anti-feeding genes from Canterbury University of New Zealand, *NaPI* from The University of Melbourne and *GNA* from Syngenta.

## 11.0 REFERENCES

Allsopp PG, Chilcott CN & McGhie TK 1996a. Activity of proteins from two New Zealand strains of *Bacillus thuringiensis* against larvae of *Antitrogus*

*consanguineus* (Blackburn) (Coleoptera: Scarabaeidae). *Australian Journal of Entomology* 35:107-112.

Allsopp PG & Cox MC. 2002. Sugarcane clones vary in their resistance to sugarcane whitegrubs. *Australian Journal of Agricultural Research* 53: 1111-1136.

Allsopp PG & McGhie TK. 1996. Snowdrop and wheatgerm lectins and avidin as antimetabolites for the control of sugarcane whitegrubs. *Entomologia Experimentalis et Applicata* 80:409-414.

Allsopp PG, McGhie TK, Cox MC & Smith GR. 1996b. Redesigning sugarcane for resistance to Australian canegrubs: a potential IPM component. *Integrated Pest Management Reviews* 1: 79-90.

Allsopp PG, McGhie TK, Smith GR, Ford R & Cox MC. 1995. Progress in the development of cane varieties with resistance to canegrubs. *Proceedings of the Australian Society of Sugar Cane Technologists* 17: 97-105.

Allsopp PG, Shepherd KM, Smith GR, Cox MC & Robertson SK. 1997. Use of host-plant resistance as a component in IPM for canegrubs. *In* Allsopp PG, Rogers DJ and Robertson LN (Eds) *Soil invertebrates in 1997*. pp. 102-105. Bureau of Sugar Experiment Stations, Brisbane.

Atkinson AH, Heath RL, Simpson RJ, Clarke AE & Anderson MA. 1993. Proteinase inhibitors in *Nicotiana alata* stigmas are derived from a precursor protein which is processed into five homologous inhibitors. *Plant Cell* 5: 203-213.

Giddens SR, Tormo A & Mahanty HK. 2000. Expression of the antifeeding gene anfA1 in Serratia entomophilia requires RpoS. *Applied and Environmental Microbiology* 66: 1711-1714.

McGhie TK, Christeller JT, Ford R & Allsopp PG. 1995. Characterization of midgut proteinase activities of white grubs: *Lepidiota noxia*, *Lepidiota negatoria*, and *Antitrogus consanguienus* (Scarabaeidae, Melolonthini). *Archives of Insect Biochemistry and Physiology* 28: 351-363.

Murray C & Christeller JT. 1994.Genomic nucleotide sequence of a proteinase inhibitor II gene. *Plant Physiology* 106: 1681.

Nunez-Valsez ME & Mahanty HK. 1996 The amb2 locus from *Serratia entomophilia* confers anti-feeding effect on larvae of *Costelytra zealandica* (Coleoptera: Scarabaeidae). *Gene* 172: 75-79.

Nutt KA, Allsopp PG, McGhie TK, Shepherd KM, Joyce PA, Taylor GO, McQualter RB & Smith GR. 1999. Transgenic sugarcane with increased resistance to canegrubs. *Proceedings of the Australian Society of Sugar Cane Technologists* 21: 171-176.

Robertson LN, Allsopp PG, Chandler KJ & Mullins RT. 1995. Integrated management of canegrubs in Australia: current situation and future research directions. *Australian Journal of Agricultural Research* 46: 1-16.

Robertson LN, Chandler KJ, Stickley BDA, Cocco RF & Ahmetagic M. 1998. Enhanced microbial degredation implicated in rapid loss of chlorpyrifos from the controlled-release formulation suSCon® Blue in soil. *Crop Protection* 17: 29-33.

Smith GR, Nutt KA & Allsopp PG. 2000. Final Report - SRDC project BSS163 Canegrub resistant plants containing antimetabolic compounds. BSES Publication SD00002.

Van Damme EJM, Allen AK & Peumans WJ. 1987. Isolation and characterization of a lectin with exclusive specificity towards mannose from snowdrop (*Galanthus nivialis*) bulbs. *FEBS Letters* 215: 140-144.