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FINAL REPORT - SRDC PROJECT BS95S
SELECTION OF A NON-SUGARCANE GENE FOR CONTROL OF CANEGRUBS

by

P G Allsopp, T K McGhie, K M Shepherd

and G R Smith

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Principal Investigators:

Dr P G Allsopp
Principal Research Officer

BSES
PO Box 651
BUNDABERG Q 4670
Phone (071) 593228

Mr T K McGhie (July 93-Oct 95)
Ms K M Shepherd (Nov 95-June 96)
Research Officers
BSES
PO Box 86
INDOOROOPILLY Q 4068
Phone (07) 33716100

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CONTENTS

1.0 SUMMARY

Four types of non-sugarcane genes were investigated for potential toxicity to canegrubs. They were: toxins from the bacterium *Bacillus thuringiensis* (Bt); plant proteinase inhibitors; plant lectins; and avidin. Each of these was tested in an artificial diet based on wheatgerm and casein and developed within the project.

A Bt toxin with toxicity to canegrubs was not identified. The Bt isolates tested, which had activity against New Zealand grassgrub, bound to the gut lining of larvae of *Antitrogus consanguineus*. This binding proved to be not sufficient for toxicity.

Snowdrop and wheatgerm lectins were shown to reduce canegrub growth rates and increase mortality of canegrubs when fed to grubs. Avidin, a biotin-binding protein, reduced canegrub growth rates but was not toxic to canegrubs.

Gut proteinases from three species of canegrubs were characterised; serine and trypsin proteinases were shown to predominate within an alkaline environment. Proteinase inhibitors with activity against the canegrub gut proteinases were identified; amongst these were those from potato, soybean and wheatgerm. Soybean trypsin inhibitor significantly reduced canegrub growth rates and survival.

A gene construct containing the potato proteinase inhibitor gene was prepared and sugarcane plants transformed with it. Plants have tested positive for the presence of the gene, but none have produced the proteinase inhibitor. A gene construct containing the snowdrop lectin gene was also prepared, and attempts made to transform it into sugarcane plants. No plants containing the gene have yet been regenerated.

2.0 BACKGROUND

Damage caused to sugarcane continues to be a major constraint to increased productivity by limiting yields and the length of the ratoon cycle. Larvae damage the roots and underground parts of the stems of sugarcane, reducing plant growth and in severe cases they kill the plant. Root damage also makes the plant more susceptible to lodging and removal during harvest of the below-ground parts of the stem from which the following year's crop grows. Direct costs, including residual losses, are over $10m each year. This is in a period when canegrubs are under relatively good control. The reliance on a limited number of insecticides and the possible loss of these insecticides, through environmental concerns or increased resistance within canegrubs, is a real threat to the industry. Alternative, economically and environmentally sustainable methods to control canegrubs need to be developed. Increasing host-plant resistance to canegrubs is one alternative strategy that has been identified (Robertson *et al.* 1995).

Genetic transformation of plants to provide protection from insect attack is well established. Introduced insecticidal genes include those coding for toxins from *Bacillus thuringiensis* (Bt), proteinase inhibitors and lectins. The insecticidal Bt gene has been inserted into more than 30 crop species, including sugarcane, cotton, maize, soybean and rice. Plants which contain genes making them resistant to insect attack are currently being

commercialised. Genetic transformation of sugarcane is a reality and provides the opportunity of minimising damage from canegrubs by introducing a gene which codes for an insecticidal protein.

The purpose of project BS95S was to develop methods for selecting a gene for transfer into sugarcane to provide protection from canegrub attack. Three possible sources of genes were targeted for evaluation, and during the project a system to screen gene products was developed.

3.0 OBJECTIVES

- Determine the activity of Bt toxins towards canegrubs
- Determine the toxicity of lectins to canegrubs
- Isolate and characterise the major digestive enzymes of canegrubs
- Identify active insecticidal proteinase inhibitors to the major gut enzymes
- Determine the toxicity of sugarcane cells expressing insecticidal genes to canegrubs
- Recommend candidate genes for further development, based on technical, economic, environmental, regulatory and sugar-quality criteria

Because of the promising results produced and industry concern over large losses from canegrub damage, the last objective was expanded to include the production and biochemical screening of transgenic plants.

4.0 OUTCOMES

- Four types of non-sugarcane genes were investigated for potential toxicity to canegrubs. They were: toxins from the bacterium *Bacillus thuringiensis* (Bt); plant proteinase inhibitors; plant lectins; and avidin.

- An artificial diet based on wheatgerm and casein was developed to enable testing effects of antimetabolic compounds on canegrubs (Allsopp, 1995; Appendix 1).

- A Bt toxin with toxicity to canegrubs was not identified. The Bt isolates tested, which had activity against New Zealand grassgrub, bound to the gut lining of larvae of *Antitrogus consanguineus*. This binding proved to be insufficient for toxicity, because in subsequent artificial diet testing neither of the isolates were toxic to canegrubs or slowed their development (Allsopp, Chilcott and McGhie, 1996; Appendix 2). As new isolates are discovered and tested, one with activity against canegrubs will be identified.

- Snowdrop lectin (*Galanthus nivalis*: gna) and wheatgerm lectin were shown to reduce canegrub growth rates and increase mortality of canegrubs when fed in artificial diet (Allsopp and McGhie, 1996; Appendix 3).

- Avidin, a biotin-binding protein, reduced canegrub growth rates but was not toxic to canegrubs when fed in artificial diet (Allsopp and McGhie, 1996; Appendix 3).
- Gut proteinases from three species of canegrubs were characterised; serine and trypsin proteinases were shown to predominate within an alkaline environment (McGhie, Christeller, Ford and Allsopp, 1995; Appendix 4).

- Proteinase inhibitors with activity against the canegrub gut proteinases were identified. Amongst these were proteinase inhibitors from potato, soybean and wheatgerm (McGhie, Christeller, Ford and Allsopp, 1995; Appendix 4).

- Proteinase inhibitors significantly reduced canegrub growth rates and survival in tests of soybean trypsin inhibitor in an artificial diet (Allsopp, 1995; Appendix 1).

- Proteinase inhibitors from potato and wheatgerm were not purified in sufficient quantities to allow testing in artificial diet studies.

- A gene construct containing the potato proteinase inhibitor gene (pinII) was prepared and used to try to transform into sugarcane plants. Twenty-three plants, 16 from cultivar Q117, three from cultivar Q153 and four from cultivar Q155, have tested positive for the presence of the gene. No plants have been shown to be producing the proteinase inhibitor to date.

- A gene construct containing the snowdrop lectin (gna) gene was prepared, and attempts made to transform sugarcane plants with it. We have not yet been successful in regenerating plants containing the gene.

In conclusion, a number of antimetabolic compounds with activity against canegrubs have been identified, and work commenced to introduce their genes into sugarcane plants. As further antimetabolic compounds are identified and tested, a compound or combination of compounds with even greater activity against canegrubs may be found.

5.0 RESEARCH METHODOLOGY, RESULTS AND DISCUSSION

5.1 Development of a method for testing the effects of antimetabolites on canegrub development and mortality

An artificial diet into which known concentrations of test material can be incorporated is a prerequisite to determine the effects of antimetabolites on canegrubs. The suitability of three diets on the development and survival of canegrubs was tested. One diet based on wheatgerm and casein resulted in the best growth rate and survival of Childers canegrubs (*Antitrogus parvulus*) and negatoria canegrubs (*Lepidiota negatoria*). The same diet without sucrose was less effective, but could be used to test the effects of sugar-binding lectins. Full details of the testing and the composition of the diets are given in the paper in Appendix 1.

The diets were successfully used to test the effects of proteins from Bt (Appendix 2), soybean trypsin inhibitor (a proteinase inhibitor) (Appendix 1), snowdrop and wheat germ lectins (Appendix 3), and avidin (Appendix 3).

5.2 Activity of Bt toxins to canegrubs

The activity of proteins from two New Zealand strains of *Bacillus thuringiensis*, DSIR517 and DSIR1246, was determined against larvae of southern one-year canegrub (*Antitrogus consanguineus*); both strains showed activity against the New Zealand grassgrub, another whitegrub pest. Two DIG-labelled crystal proteins from strain DSIR1246 and three from strain DSIR517 bound to the brush-border membrane vesicles of third instars. However, when fed in artificial diet to second and third instars, the proteins caused no reduction in weight gain or no increased mortality. We concluded that the proteins are dissolved and activated in the gut of *A. consanguineus* and bind to the gut wall, but that the proteins do not insert into the plasma membrane of the gut of *A. consanguineus*. Hence, they are not toxic. Full details of the methodology and results are given in the paper in Appendix 2.

Attempts were made to obtain other strains of Bt with possible activity against canegrubs. The most promising is a strain isolated in Japan (Ohiba *et al.* 1992) with reported activity against a range of ruteline scarabs. However, this strain has been tied up in the patenting process and no material could be released. Late in the project we were made aware of strains with reputed beetle activity from Pakistan (laboratory of Prof. S. Riazuddin). Negotiations are underway to obtain some of this material for testing under project BS163S - 'Canegrub-resistant plants containing antimetabolic compounds'.

5.3 Toxicity of lectins to canegrubs

Snowdrop and wheatgerm lectins were tested in artificial diet against larvae of Childers canegrub and were found to be insecticidal and growth inhibiting. At concentrations as low as 0.5 mg of snowdrop lectin per gram of diet, growth was inhibited after 21 days of feeding and significant mortality was apparent by 28 days. Wheatgerm lectin was active at similar concentrations, although expression of the effects was slower. Full details of the methodology and results are given in the paper in Appendix 3.

5.4 Isolation and characterisation of the major digestive enzymes of canegrubs

The proteinases in the midguts of three canegrubs, noxia canegrub (*Lepidiota noxia*), negatoria canegrub and southern one-year canegrub were investigated to determine the type of proteinase present. pH-activity profiles indicated the presence of serine proteinases and the absence of cysteine proteinases. This was confirmed by the lack of inhibition by specific cysteine proteinase inhibitors. Trypsin, chymotrypsin, elastase and leucine aminopetidase activities were detected by using specific synthetic substrates. These data were used in section 5.5 to identify active insecticidal proteinase inhibitors. Full details of the methodology and results are given in the paper in Appendix 4.

Gut extracts from Childers and negatoria canegrubs have been sent to Dr Angharad Gatehouse, University of Durham, UK, for testing to determine the presence and type of α-amylase activity. No results are yet available.

5.5 Identification of active insecticidal proteinase inhibitors

An *in vitro* screen of 32 proteinase inhibitors produced 9 inhibitors of trypsin, chymotrypsin and elastase which reduced proteolytic activity in three canegrub species by greater than 75%. Although there were slight differences between species, the overall activity was similar in noxia, negatoria and southern one-year canegrubs, indicating that any inhibitory effect should affect a wide range of species. Full details of the methodology and results are given in the paper in Appendix 4.

Soybean trypsin inhibitor, identified above as inhibiting enzyme activity in canegrubs, was tested in an artificial diet against larvae of southern one-year canegrubs. At 10 mg/g of diet the inhibitor reduced the growth rate and survival of second instar larvae. At 1 mg/g of diet the effects were less clear, but there was an apparent response after 21 days on the diet. Full details of the methodology and results are given in the paper in Appendix 1.

Attempts were made to purify a proteinase inhibitor from potatoes, identified above as inhibiting enzyme activity in canegrubs. A range of chromatographic techniques were used to purify the proteinase inhibitor. However sufficient quantities of pure proteinase inhibitor were not obtained to allow testing in artificial diet studies.

5.6 Activity of avidin to canegrubs

Avidin was tested in artificial diet against larvae of southern one-year canegrub and was found to be a growth inhibiting dietary protein. At levels as low as 0.01 mg/g of diet, growth was inhibited by 28 days of feeding. However, avidin caused no significant mortality after 35 days of feeding. Full details of methodology and results are given in the paper in Appendix 3.

5.7 Determination of the toxicity of sugarcane cells expressing insecticidal genes to canegrub

Because of the promising results produced the last objective was expanded to include the production and biochemical screening of transgenic plants. Initially we planned to test the toxicity of sugarcane cells expressing insecticidal genes to canegrubs before moving on to regenerate whole plants. However this approach was unsuccessful. There were problems in producing enough material for artificial diet studies, and it was found that the canegrubs would not feed on media containing callus. Therefore, it was decided to grow potentially resistant plants and test these using the pot-based technique developed in BS132S - 'Plant resistance to canegrubs'.

5.8 Recommendation of candidate genes for further development

Gene constructs coding for two promising canegrub antimetabolites, the potato proteinase inhibitor (pinII) and the snowdrop lectin (gna) were prepared and attempts were made to produce transgenic plants containing these genes. These attempts were successful for the proteinase inhibitor, with 23 plants containing the gene presently growing in the glasshouse. Full details of the methodology are given in Appendix 7. In BS163S - 'Canegrub-resistant plants containing antimetabolic compounds' these plants will be

tested to see whether they are producing sufficient proteinase inhibitor to have an effect on the canegrub. Also in BS163S - 'Canegrub-resistant plants containing antimetabolic compounds', further attempts will be made to produce transgenic sugarcane containing the snowdrop lectin gene.

5.9 Utilisation of transgenic resistance

Breeding crops for resistance offers many advantages over controls such as reliance on synthetic insecticides: (1) it provides season-long protection, (2) insects are often treated at the most sensitive stage, (3) protection is often independent of weather and other environmental conditions, (4) there are no application costs, (5) plant tissues that are difficult to treat with insecticides, such as roots, are protected, (6) only the crop-eating insects are exposed, (7) the material is expressed in plant tissue and does not leach to the environment, (8) the active factor is biodegradable and can be selected so that it is non-toxic to mammals, (9) any residual material is likely to be removed in the milling process and (10) there are possible financial savings. Australian canegrowers are attuned to adopting new higher-yielding cultivars and a canegrub-resistant cultivar with equivalent (or perhaps slightly lower) productivity would have a high adoption rate.

Use of plant genetic manipulation as part of a breeding programme may make significant contributions to the production of insect-resistant crops. Our results suggest that it may be possible to improve resistance in sugarcane to canegrubs by introducing genes producing proteins with antimetabolic effects to insects. Genetic manipulation enables the desired genes to be transferred to the recipient plant without the co-transfer of other undesirable characteristics, thereby speeding up the breeding process and allows the incorporation of genes across incompatibility barriers.

The use of only one resistance mechanism (either natural or engineered) has an inherent danger of leading to the development of resistance in the target insect. This is similar to the use of a single synthetic pesticide for a long period. Several resistance-management strategies for transgenic (and naturally resistant) plants have been evaluated using simulation models. These strategies fall into four basic groups: (1) use of very high doses of one or more toxins, (2) mixtures of plants with high doses of toxin(s) and plants with no toxin expression at all, (3) low doses of toxin interacting with other controls and (4) targeted toxin expression, for example, tissue-specific, time-specific and inducible. Some of these tactics can be used in combination, for example, high doses and tissue-specific expression. However, the types of germplasm and deployment strategies which are best suited for slowing the rate of development of resistance in insects are dependent on mate selection and adult and larval movement characteristics. Hence, they are specific to the target insect. As in other crops, for example cotton, it is impossible to evaluate fully the efficacy of any resistance-management strategy until large areas of transgenic sugarcane are grown. The ideal strategy may vary with the target canegrub for there is a wide variation in species behaviour in the canegrub complex. However, it is possible to evaluate some of the ecological effects or behavioural factors which could influence their effectiveness. The tactic used will be most effective if research is done before naturally resistant or transgenic sugarcanes are deployed. In general, however, we caution against the use of a single resistance mechanism; two or more factors with different mechanisms are desirable and as such proteinase inhibitors, lectins and avidin offer strong potential.

Although host-plant resistance offers many benefits, it does come at a cost. There may be an inherent energy cost to the plant in having to produce an antimetabolite. Phytosynthate, which could contribute to sugar production, may have to be channelled into providing the resistance-tolerance mechanism. Studies in other plants suggest that this cost may be significant, although small. The second cost lies within the breeding process itself; in a discard rate because of the increased number of characters selected for and in the extra direct costs of that selection. This selection is still likely to be necessary even if an agronomically superior cultivar is transformed with an antimetabolite active against canegrubs.

Use of genetically altered lines or lines with enhanced natural resistance may be restricted by community-imposed restraints and by the complex nature of gene transfer in sugarcane. Expression of proteinase inhibitors, snowdrop or wheatgerm lectins or avidin in transgenic sugarcane should cause no problems to consumers. Snowdrop lectin does not appear to be toxic to mammals. Wheatgerm lectin is toxic to mammals and consumption of large amounts of raw egg white, containing avidin, can lead to biotin deficiency in humans. However, Australian commercial sugarcane is not consumed directly. During milling the juice is heated to 103°C and lime is added; this coagulates and removes by clarification nearly all of the protein in the juice. Heating should also reduce or eliminate the effect of proteinase inhibitors, lectins and avidin and lectins should be removed with the bagasse.

To remain sustainable, future management of canegrubs must incorporate a range of control strategies; plant resistance should be part of that arsenal. Under project BS132S - 'Plant resistance to canegrubs', BSES continues to define the nature and extent of natural resistance. We envisage that this could be best incorporated into the breeding programme through the increased use as parents of cultivars with resistance characters (population improvement) rather than by direct selection of resistant clones. The incorporation of resistance traits through genetic engineering offers potential through the use of the transformed cultivar as a parent in the breeding programme or through the improvement of commercial cultivars already selected in the conventional breeding programme. Development of these transgenic lines continues through project BS163S - 'Canegrub-resistant plants containing antimetabolic compounds'.

6.0 PUBLICATIONS

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8.0 ACKNOWLEDGMENTS

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9.0 APPENDICES

Appendix 1 Allsopp PG (1994) An artificial diet suitable for testing antimetabolic products against sugarcane whitegrubs (Coleoptera: Scarabaeidae). *Journal of the Australian Entomological Society* **34**:135-137

Appendix 2 Allsopp PG, Chilcott CN and McGhie TK (1996) 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.

Appendix 3 Allsopp PG and McGhie TK (1996) Snowdrop and wheatgerm lectins and avidin as antimetabolites for the control of sugarcane whitegrubs. *Entomologia Experimentalis et Applicata* **80**:409-414.

Appendix 4 McGhie TK, Christeller JT, Ford R and Allsopp PG (1995) Characterisation of midgut proteinase activities of white grubs: *Lepidiota noxia, Lepidiota negatoria* and *Antitrogus consanguineus* (Scarabaeidae, Melolonthini). *Archives of Insect Biochemistry and Physiology* **28**:351-363.

Appendix 5 Allsopp PG, McGhie TK, Smith GR, Ford R and Cox MC (1995) Progress in the development of cane varieties with resistance to canegrubs. *Proceedings of Australian Society of Sugar Cane Technologists* **17:**97-105.

Appendix 6 Allsopp PG, McGhie TK, Cox MC and Smith GR (1996) Redesigning sugarcane for resistance to Australian canegrubs: a potential IPM component. *Integrated Pest Management Reviews* **1**:79-90.

Appendix 7 Joyce PA, Bernard MJ, Hickman KA, Roberts SA, McGhie TK and Smith GR (1995) Production of transgenic sugarcane with genes for resistance to canegrubs and mosaic virus. Proceedings of the Australian Society for Plant Physiology 35th Conference, No 36.

Appendix 7: Production of transgenic sugarcane containing canegrub antimetabolites

1. Preparation of gene constructs

1.1 emu-pinII-nos construct (coding for potato proteinase inhibitor II)

The pinII gene was cut from the original plasmid, pUCpotII, with restriction enzymes XbaI and KpnI and inserted into the multiple cloning site (MCS) of the pEMU-MCS-N plasmid, flanked by the emu promoter and nopaline synthase (nos) terminator sequence. The plasmid containing the emu promoter had been previously cut with XbaI and KpnI. The resulting construct was then cloned into *E. coli* (strain AC001), for multiplication in order to obtain enough plasmid for microprojectile bombardment.

1.2 ubi-gna-nos construct (coding for snowdrop lectin)

The lectin gene was assembled into a construct containing the Ubiquitin promoter from maize, a stronger promoter than emu. The GNA gene was removed from the p1GNA (GNA gene in MCS of pUC19) plasmid by restriction enzyme digestion with SacI and BamHI. The Sugarcane Mosaic Virus Coat Protein (SCMVCP) gene was cut out of the pUSN (Ubi-SCMVCP-Nos) plasmid, by cutting with the same restriction enzymes, and replaced with the GNA gene to produce the pUGNA construct. The resulting construct was then cloned into *E.coli* (strain DH5α) for multiplication.

2. Plant material

Mature cane tops of various varieties (Q95, Q117, Q124, Q153, Q155) were obtained from BSES Meringa. Leaf discs were prepared and cultured, using standard methods for producing sugarcane embryogenic callus (ie. grown on solid embryogenic callus medium, in the dark at 28°C). After approximately nine weeks of culture, the embryogenic callus was ready for transformation.

Cell suspension cultures were initiated from Q63 callus, and maintained in liquid media, shaking at 28°C in the dark. Protoplasts were prepared from the cell suspension cultures by digesting the cell walls using Vasil I enzymes.

3. Transformation

The transformations were performed using the apparatus and techniques described by Bower and Birch (1992). Briefly, the callus was "shot" with microprojectiles bearing the construct containing the lectin or proteinase inhibitor gene together with the neomycin phosphotransferase (nptII) gene for antibiotic resistance, the latter for use as a selectable marker. The callus material was maintained under antibiotic selection until plantlets with roots developed (in the dark for the first 9 weeks, at 28°C) and plants regenerated under fluorescent lighting with a 12 hr day/night cycle, at 28°C.

4. Testing of transformants

When the plants had grown roots, they were removed from tissue culture, potted up and maintained in the glasshouse.

4.1 Genetic analysis (testing for presence of the gene construct)

Leaf samples were collected fresh, or under liquid nitrogen and extracted immediately or stored at -70°C. DNA was extracted from approximately 200 mg of young leaf tissue using the phenol/chloroform extraction method. Gene specific primers were designed and PCR (Polymerase Chain Reaction) analysis performed. The presence of a DNA band of the correct size indicated the presence of the gene. Southern blot analysis, to confirm PCR data and determine gene copy numbers was not attempted.

4.2 Western analysis (testing for presence of gene products - proteinase inhibitor or lectin)

Transient expression of the pinII gene was detected following electroporation into Q63 protoplasts using the method of Smith *et al.* (1992), confirming that the construct is active in sugarcane plant cells.

To check stable expression, fresh leaf samples were collected from PCR positive (PCR negative plants were included as controls), ground-up and extracted into buffer. The total amount of protein present was quantified against BSA (bovine serum albumin) using the Biorad protein assay system. Following separation by SDS-PAGE on 16.5% Tris-tricine gels, the proteins were transferred electrophoretically onto Polyvinylidene difluoride (PVDF) membranes. The proteins of interest were detected using specific antibodies followed by Enhanced Chemiluminescent staining (ECL) (according to the manufacturer's instructions; Amersham, UK).

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APPENDIX 1

APPENDIX 2

APPENDIX 3

APPENDIX 4

APPENDIX 5

APPENDIX 6

APPENDIX 7

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