BSES Limited

BSES
Sugarcane *for the future*

FINAL REPORT – SRDC PROJECT STU032

CHARACTERISATION OF PROTEINASE INHIBITORS FROM CANEGRUBS

FOR POSSIBLE APPLICATION TO GENETICALLY ENGINEER PEST-

DERIVED RESISTANCE INTO SUGARCANE

by

KA NUTT

SD05007

Contact:
Kerry Nutt
Research Officer
BSES Limited
PO Box 86
Indooroopilly Q 4068
Telephone: 07 3331 3375
Facsimile: 07 3871 0383
Email: knutt@bses.org.au

Australian Government
**Sugar Research and
Development Corporation**

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 SD05007   **May 2005**

CONTENTS

SUMMARY

The primary objective of this research was to identify, isolate and clone a proteinase inhibitor from the haemolymph of a canegrub species with activity against the major midgut proteases of the source insect. The potential for using this canegrub-derived proteinase inhibitor as a novel source of insect resistance in transgenic sugarcane was also to be determined.

The research undertaken in this project has demonstrated that canegrub haemolymph protease inhibitors have activity against commercially purified enzymes and serine proteases found in crude midgut extracts. A cDNA encoding a potential canegrub protease inhibitor (DA10-12) was cloned, but it did not have activity against the major canegrub midgut proteases. However, this protein has potential for modification into a serine protease inhibitor suitable for use as a novel insect-resistance transgene. The possibility of using haemolymph derived inhibitors as novel antimetabolites in a canegrub management strategy based on transgenic plants was also explored. This research suggests that proteins with properties similar to those of DA10-12 will require the presence of a signal peptide and/or codon optimisation for successful expression in sugarcane.

The most significant outcome of this research was the cDNA cloning and partial characterisation of an *Ascaris* family protease inhibitor from greyback canegrub, *Dermolepida albohirtum*. The research outlined in this report is the first investigation of protease inhibitors in the haemolymph of scarab larvae, and is the first report of an *Ascaris* family inhibitor that does not inhibit a serine protease.

The project has formed the basis of a PhD thesis submitted to the Queensland University of Technology.

1.0 BACKGROUND

Numerous approaches, using both biological and chemical agents, have been investigated in an attempt to control canegrubs. Natural resistance to canegrubs has not been a key criterion in the sugarcane breeding program, as synthetic insecticides have offered low cost and effective control since the late 1940s. Current management of canegrubs is highly dependent on synthetic insecticide formulations. However, with evidence of insecticide failure in some areas and increasing environmental pressure, reliance on insecticides as a form of control is becoming precarious and growers are now encouraged to use whole-farm management strategies.

Development of transgenic plants with increased resistance to canegrubs is one option that is being explored, and the ability to engineer canegrub resistance into sugarcane has now been established. However, the industry currently relies on sourcing the transgenes from external agencies and is, therefore, faced with potential unfavourable commercialisation terms. Additionally, as with the use of a single chemical insecticide, reliance on only one resistance mechanism has the inherent danger of leading to the development of resistance in the target insect. The use of more than one resistance mechanism offers the greatest potential for long lasting control. Proteinase inhibitors present in canegrub haemolymph offer an opportunity to develop new resistance genes for inclusion in the canegrub management strategy.

The primary objective of this research was to identify, isolate and clone a proteinase inhibitor from the haemolymph of a canegrub species with activity against the major midgut proteases of the source insect. The potential for using this canegrub-derived proteinase inhibitor as a novel source of insect resistance in transgenic sugarcane was also to be determined.

The rationale for this project was based on the earlier observations by Thomas *et al.* (1994, 1995a,b) who suggested that proteinase inhibitors isolated from pest insects could be used as an effective transgene for the control of the same pest, as well as other insect species.

2.0 OBJECTIVES

The specific objectives of the project were to:
- Demonstrate that canegrub haemolymph contains protein proteinase inhibitors that regulate the proteolytic activity of canegrub midgut enzymes by characterising the proteinase inhibitor profiles of the haemolymph of four species of canegrub, across four genera.
- Identify, clone and sequence a gene(s) encoding a canegrub proteinase inhibitor.
- Express the proteinase inhibitor protein from the cloned sequence, in sufficient quantity for an artificial diet feeding trial against canegrubs.
- Introduce the gene into sugarcane cells to determine whether it can be expressed *in planta*.

All of the objectives were achieved.

Objective 1: Characterise the proteinase inhibitor (PI) profiles of the haemolymph of four species of canegrub, across four genera

A survey of the haemolymph extracts from four species of canegrubs (*Dermolepida albohirtum*, *Antitrogus consanguineus*, *Lepidiota noxia*, *Rhopaea magnicornis*) showed proteinase inhibitor activity, and the use of specific substrates and commercial enzymes confirmed the presence of trypsin, chymotrypsin and elastase inhibitors in the haemolymph. Parallel studies, utilising the same substrates, identified inhibitory activity of haemolymph against enzymes in midgut extracts of these canegrubs. Interestingly, the inhibitory activity of *D. albohirtum* haemolymph against *D. albohirtum* midgut trypsin-like enzymes was stable after boiling for 5 minutes. However, activity could be destroyed by including 1% β-mercaptoethanol in the solution, suggesting that the inhibitor(s) contains disulphide bonds. Assays of enzymes present in the midgut extracts showed that the enzyme types utilised by the four species were similar, but the four species have different numbers of enzymes as determined by electrophoretic mobilities. Despite these differences, the haemolymph proteinase inhibitors affected midgut enzymes from all species similarly. These findings complement reports on proteinase inhibitors present in other insect species and confirm the haemolymph of canegrubs to be a source of proteinase inhibitors with potential for use as novel transgenes.

Objective 2: Identify, clone and sequence gene(s) encoding a canegrub proteinase inhibitor

Greyback canegrub (*D. albohirtum*) was chosen as the source species in the initial search for a putative proteinase inhibitor. The reasons for this were threefold: it is the most damaging species, it is a large insect and larvae are easier to collect than those of other species. The target enzymes for the initial inhibitor search were trypsin-like enzymes, being the major enzyme type present in the midguts of all four species of canegrub. Midgut enzymes for the initial search were also sourced from *D. albohirtum*.

Inhibitory activities for these enzymes were detected in fractions of haemolymph from *D. albohirtum* after gel filtration chromatography. The fractions with the highest activity contained a protein (designated DA10-12) with a relative molecular mass (Mr) of ~6 kDa, as determined by electrophoresis under reducing conditions on polyacrylamide gels. The N-terminal sequence of DA10-12 was determined by Edman degradation and facilitated the cloning of the DA10-12 cDNA using 5′/3′ RACE. The cDNA of DA10-12 was isolated from larval fat bodies and has an open reading frame which codes for a 79-residue polypeptide with a hydrophobic NH_2-terminal sequence (19 residues) that appears to be a signal peptide. The deduced amino-acid sequence shows that DA10-12 is a basic protein, which includes a high percentage of proline residues and 10 cysteine residues, and has a mature molecular weight of 6.29 kDa.

A search of the databases demonstrated that the pattern of cysteine residues in DA10-12 is similar to the pattern found in proteinase inhibitors from the *Ascaris* inhibitor family. Alignment of the DA10-12 amino acid sequence with sequence from four members of the

Ascaris family revealed that the canegrub protein has less than 50% homology with these inhibitors. However, besides the 10 cysteine residues and the location of the reactive site, there is little homology within the *Ascaris* family. Interestingly, the alignment suggests that glutamine occupies the P_1 position of the DA10-12 reactive site, which is rare in serine protease inhibitors.

Objective 3: Express the proteinase inhibitor protein from the cloned sequence, in sufficient quantity for an artificial diet feeding trial against canegrubs.

To facilitate a more detailed biochemical analysis of DA10-12, the cDNA was inserted into a bacterial expression vector and the protein expressed in *Escherichia coli*. Soluble DA10-12 was produced in *E. coli* with the aid of a glutathione-S-transferase N-terminal fusion, which also assisted in the purification of recombinant protein by affinity chromatography. Assay of the recombinant protein against the assumed target, trypsin-like enzymes from *D. albohirtum* midgut, revealed the protein to be biologically inactive. This was subsequently shown to be a property of DA10-12 itself, rather than a consequence of production in *E. coli*.

DA10-12 was purified to homogeneity from *D. albohirtum* haemolymph using gel filtration chromatography, followed by ion exchange chromatography. The native protein was also unable to inhibit midgut trypsin- and chymotrypsin-like enzymes, as well as the commercially available enzyme equivalents. It is believed that either the target enzyme for this inhibitor is not a serine protease, or the proline residue at the putative P_1' position of the reactive site may be hindering its activity.

Objective 4: Introduce the sequence into sugarcane cells to determine whether it can be expressed in planta

The length of time required to produce transgenic sugarcane plants meant that the plant transformation research had to begin before biochemical characterisation of DA10-12 was complete. The mature DA10-12 cDNA was inserted into a reliable plant-expression vector, under the control of the maize *Ubiquitin* promoter, and introduced into sugarcane cells using a well established biolistic transformation system. Although the presence of the DA10-12 transgene could be detected by PCR analysis of DNA from leaves of regenerated plants, no protein could be detected in the leaves by western analysis. Results of RT-PCR analysis of a few plants suggest that the absence of transprotein is the result of inefficient translation or degradation of the protein in the cytosol.

3.0 RESEARCH METHODOLOGY, RESULTS AND DISCUSSION

The following summarises material presented in my PhD thesis. A copy of the thesis will be sent to SRDC on completion of the degree.

3.1 Proteinase inhibitors in the haemolymph of canegrubs

3.1.1 Methods

Haemolymph and midgut extracts were prepared from *L. noxia*, *A. consanguineus*, *R. magnicornis* and *D. albohirtum*. Enzymatic activity was measured for midgut enzymes of the four species, using synthetic substrates for trypsin, chymotrypsin and elastase. Inhibition of proteolytic activity of commercially available and canegrub midgut enzymes by canegrub haemolymph was also determined. The assay results were plotted as absorbance versus volume of haemolymph in order to extrapolate the volume of haemolymph required to reduce enzyme activity by 50%. Caseinolytic midgut proteases from the four species were examined using zymography (electrophoresis of proteases in gels containing casein). Similarly, proteins resistant to proteases from the four species were examined using reverse zymography (electrophoresis of proteins in gels containing casein, which is subsequently treated with protease solution).

3.1.2 Results

3.1.2.1 Enzymatic activity assay

Trypsin-like, chymotrypsin-like and elastase-like activities were detected in the midgut extracts of all four species, but all species had different proteolytic rates on each of the substrates (Table 1). Midgut extracts of all species had highest activity when using the chymotrypsin substrate SAAPFpNA and the elastase substrate SAAPLpNA, lower activity with the trypsin substrate L-BApNA, and lowest activity with the elastase substrate SAAApNA. Trypsin-like and chymotrypsin-like activities were highest in the *R. magnicornis* extract, while elastase-like activity was highest in extracts from *D. albohirtum*. Midgut extracts from *D. albohirtum* had the lowest trypsin-like activity, *L. noxia* the lowest activity on SAAApNA, and *A. consanguineus* midgut the lowest chymotrypsin-like and elastase-like (SAAPLpNA) activity.

Table 1 **Mean (±s.e.) activity of trypsin-like, chymotrypsin-like and elastase-like enzymes in larval midgut extracts (nmol/min/µL gut)**

Species	Mean gut vol (µL)	Substrate			
		L-BAPNA	SAAPFpNA	SAAApNA	SAAPLpNA
D. albohirtum	247 ± 0.045 (n=16)	5.37 ± 0.06 [B]	40.83 ± 0.07 [AB]	1.23 ± 0.02 [A]	25.02 ± 0.04 [A]
R. magnicornis	77 ± 0.014 (n=3)	12.66 ± 0.18 [A]	74.17 ± 0.08 [A]	1.17 ± 0.01 [AB]	19.98 ± 0.02 [AB]
A. consanguineus	89 ± 0.040 (n=3)	9.20 ± 0.05 [AB]	37.14 ± 0.78 [B]	0.70 ± 0.04 [AB]	11.21 ± 0.16 [B]
L. noxia	75 ± 0.010 (n=3)	11.10 ± 0.04 [AB]	47.09 ± 0.96 [AB]	0.48 ± 0.01 [B]	18.14 ± 0.03 [AB]
		K-W statistic=10.38 P=0.016	K-W statistic=10.42 P= 0.015	K-W statistic=10.38 P= 0.016	K-W statistic=10.38 P= 0.016

A number of pooled extracts were used to calculate mean gut volume. Within each substrate column, means followed by the same letter are not significantly different at a 5% level of probability.

3.1.2.2 Inhibition studies

Titration of midgut enzymes with haemolymph indicated that up to 80% of proteolytic activity is inhibited by haemolymph protease inhibitors (Fig. 1) and that midgut enzymes from any one species are inhibited by haemolymph from all species (Fig. 2). However, the abilities of haemolymph to inhibit midgut enzyme activity differed significantly among species. In general, chymotrypsin-like midgut enzymes and enzymes using the elastase substrate SAAPLpNA were inhibited by a lower volume of haemolymph from *A. consanguineus* than from any other species. Comparison of haemolymph efficacy across the midgut enzymes using SAAApNA is not possible, due to the differences in the concentration of enzyme used for each species. Generally, for each midgut the highest volume required to reduce activity was from *A. consanguineus* and the lowest volume was from *D. albohirtum*. Purified trypsin, chymotrypsin and elastase were also inhibited to various degrees by all samples of haemolymph.
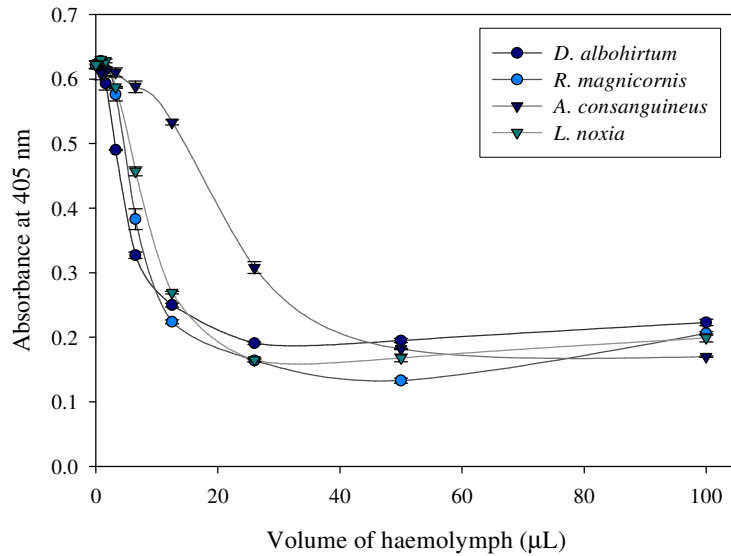
Figure 1   **Inhibition of *D. albohirtum* trypsin-like midgut enzymes titrated with increasing concentrations of haemolymph.   This plot is typical of results for inhibition of midgut enzymes from the four canegrub species examined. Error bars indicate standard error of the mean.**
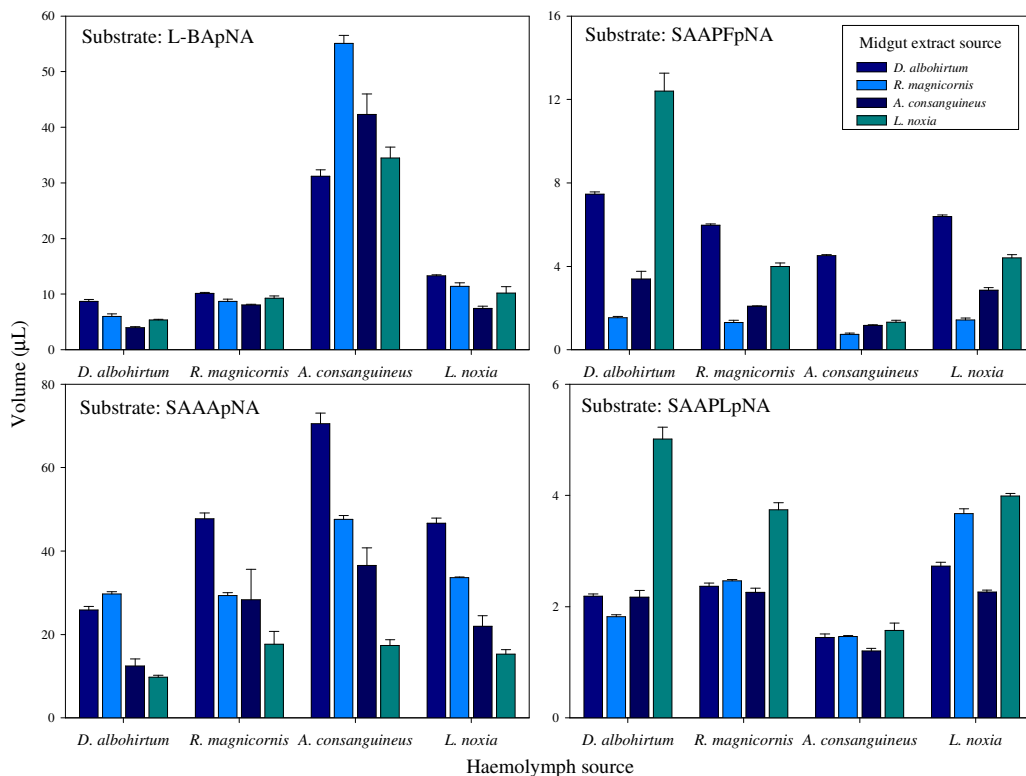
Figure 2   **Volume of haemolymph required to inhibit 50% of enzyme activity from canegrub midgut extracts.   Values (mean ± s.e.) were extrapolated from inhibition assay data plotted as haemolymph volume versus absorbance, as illustrated in Fig. 1. Note the different scales in these four graphs. Error bars indicate standard error of the mean.**

3.1.2.3   Zymogram gel electrophoresis

The electrophoretic patterns of the caseinolytic enzymes are unique for each of the four species of canegrub (Fig. 3). Both *D. albohirtum* and *R. magnicornis* midgut extracts contained three bands, while *A. consanguineus* contained five bands, and *L. noxia* four bands. None of the species have bands with identical electrophoretic mobilities.
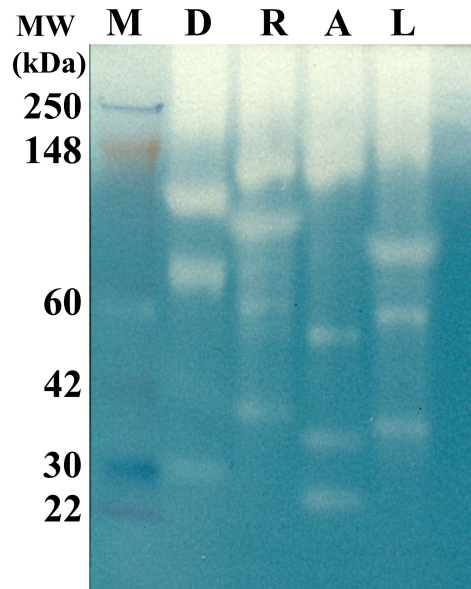
Figure 3   **Zymograph profile of caseinolytic enzymes from canegrub midgut extracts. The clear bands represent activity of caseinolytic enzymes from midgut extracts of *D. albohirtum* (D), *R. magnicornis* (R), *A. consanguineus* (A), and *L. noxia* (L) separated by polyacrylamide gel electrophoresis. NOVEX MultiMark protein molecular mass (kDa) marker (M).**

3.1.2.4   Reverse zymography

Several proteins resistant to enzymatic degradation were seen in the haemolymph of each canegrub species tested (Fig. 4). Overall, more proteins appear resistant to chymotrypsin than to canegrub midgut enzymes, and fewer proteins again are resistant to trypsin. In all canegrub species, enzyme resistant proteins that were stable to boiling were those with a relative molecular weight less than 50 kDa. These proteins were also the ones that varied the most between enzyme solutions.
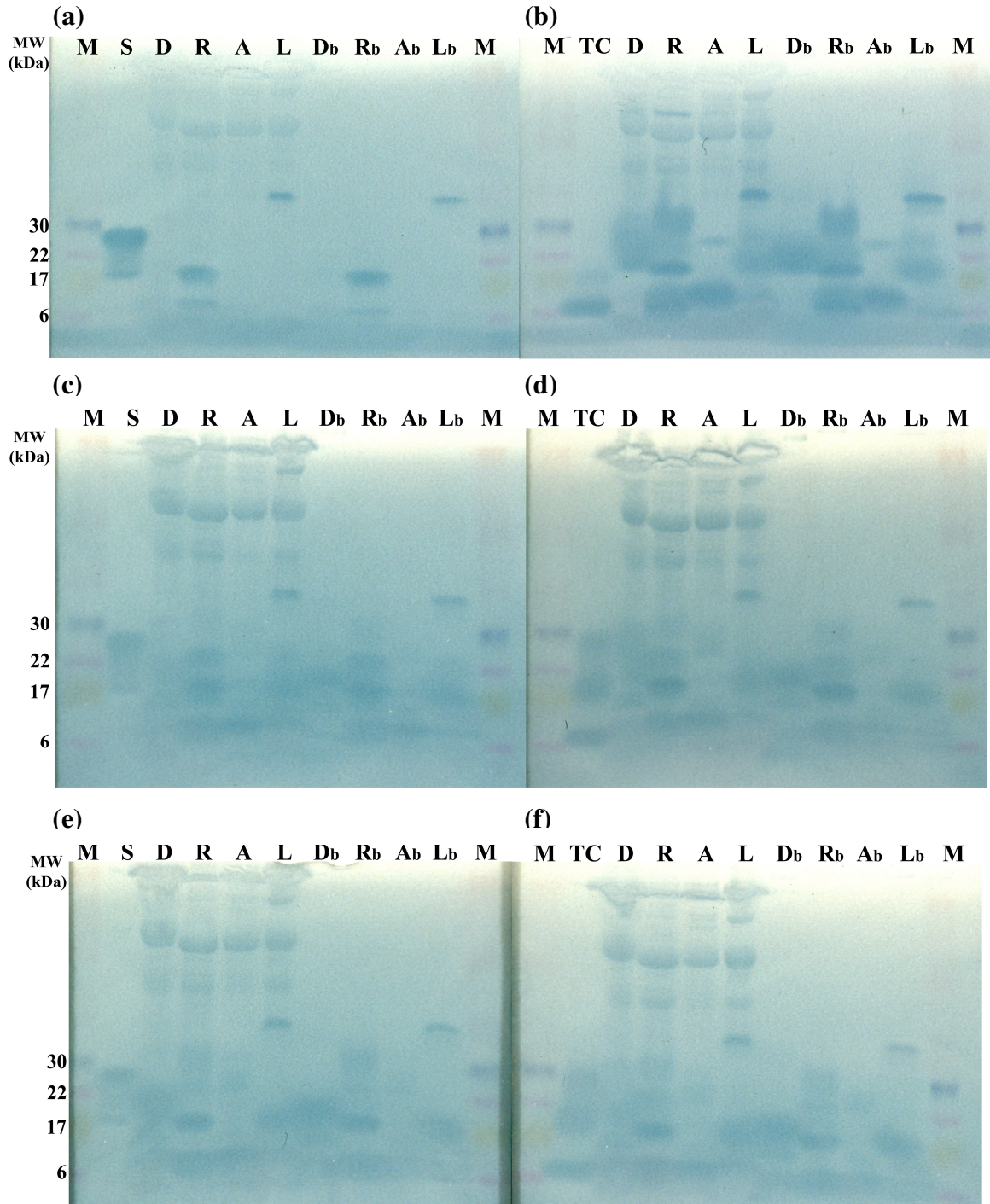
Figure 4   **Profiles of enzyme resistant proteins from canegrub haemolymph. Haemolymph proteins from *D. albohirtum* (D), *R. magnicornis* (R), *A. consanguineus* (A), and *L. noxia* (L) were separated by polyacrylamide gel electrophoresis in gels containing blue casein. Enzyme resistant proteins were visualised as blue bands, after incubating the gel in trypsin (a), chymotrypsin (b), or midgut extracts from *D. albohirtum* (c), *R. magnicornis* (d), *A. consanguineus* (e), and *L. noxia* (f). Heat stable proteins can be seen in the boiled haemolymph samples of the four canegrub species (Db, Rb, Ab, Lb). NOVEX MultiMark protein molecular mass (kDa) marker (M).**

3.1.3 Conclusions

The midgut enzyme assay results presented above support the findings of McGhie *et al.* (1995) and provide data on two previously unstudied species, *D. albohirtum* and *R. magnicornis*. My results also demonstrate that canegrub haemolymph protease inhibitors have activity against commercially purified enzymes and serine proteinases found in crude midgut extracts.

3.2 Identification and cDNA cloning of a putative proteinase inhibitor

3.2.1 Methods

Haemolymph and midgut extracts were prepared from *D. albohirtum*, but the haemolymph was boiled for 5 min to remove 95% of the protein content. Proteins in the boiled haemolymph were fractionated using gel filtration chromatography and all fractions collected were tested for inhibitor activity. Proteins identified as potential inhibitors were N-terminally sequenced, enabling the design of primers for cDNA amplification by 5′/3′ RACE (rapid amplification of cDNA ends). mRNA isolated from larval fat bodies was used to synthesise cDNA, from which the cDNA sequence of the potential proteinase inhibitor was amplified and cloned.

3.2.2 Results

3.2.2.1 Gel filtration chromatography

Separation of proteins by gel filtration chromatography resolved the fractions containing proteinase inhibitor activity into three groups. The best inhibition of enzyme activity appeared to coincide with the elution of the Mr ~6 kDa protein (DA10-12) and a protein of Mr ~4.5 kDa, in the second group of fractions and a protein of about 4 kDa in the third group of fractions. It was not possible to derive N-terminal sequence from the ~4.5 and ~4 kDa proteins, due to their low concentrations, but a sequence of 18 amino-acid residues was obtained for DA10-12.

3.2.2.2 Cloning and sequencing

The final cDNA sequence for DA10-12 was 400 bp in length, including a 16 base poly(A) tail (corresponding to the $(T)_{16}$ of the oligo-dT anchor primer used in 3' RACE). The sequence contains an open reading frame of 240 bp in reading frame three, which translates into an amino-acid sequence of 79 residues (Fig. 5).

```
    1  AAAACAAATTCTTAGAGAGTATTTGTTGTTGCTGCTTCATCGTTTACAAA
   51  CATAAAATGAAATTTATTTACTCTGCGTTGCTGCTCTTCATTTTATTTGT
           M   K   F   I   Y   S   A   L   L   L   F   I   L   F   V    15
  101  TTCAGCATTGGCAGCTGTACCTAAATGCGGTGAAAATGAAGAATACAAAA
           S   A   L   A   A   V   P   K   C   G   E   N   E   E   Y   K   T    32
  151  CTTGTGGTCCAGCATGCCACCCAACCTGCTCCAAACCAGACGTATCAACC
           C   G   P   A   C   H   P   T   C   S   K   P   D   V   S   T    48
  201  ATAGCGTGTCCACAGCCTTGTATTTCTGGATGTTTCTGCAAGGGTGGGTT
           I   A   C   P   Q   P   C   I   S   G   C   F   C   K   G   G   F    65
  251  CTTGACTAACAATAAAGGCAAATGTGTACCAAAGAATAAATGTTGAACTT
           L   T   N   N   K   G   K   C   V   P   K   N   K   C   *    79
  301  TTTGAAATGTTATGTACATTTTTTTAAGTTTGTTATGCTCTATAATTTTT
  351  ATAAAGTGA**AATAAA**TTTTAAAAGATGTGAAAGTAAAAAAAAAAAAAAAA
```

Figure 5 **cDNA sequence of DA10-12 cloned using 5′/3′ RACE. The deduced amino acid sequence is shown beneath the cDNA sequence, with the predicted signal peptide shown in italics. The amino-acid residues underlined with the solid line are those that match the N-terminal sequencing results of DA10-12, while the residues underlined with the dotted line are those that match the sequence obtained from DA-3. A putative polyadenylation signal is shown in bold.**

The deduced amino-acid sequence showed similarity to the *Ascaris* family of protease inhibitors, characterised by the presence of five disulphide bonds in a single small protein domain of 61-62 residues (Babin *et al.* 1984). A conserved location for the reactive site has been established for three of the *Ascaris* inhibitors. If the reactive site of DA10-12 is located in the same position, the P_1 position residue is expected be a glutamine, a residue rarely found in this position among serine proteinase inhibitors (Laskowski *et al.* 1987; Irving *et al.* 2000; Hejgaard 2001).

3.2.3   Conclusions

The cDNA sequence for DA10-12, a putative proteinase inhibitor, was cloned from the fat bodies of *D. albohirtum* larvae. Based on the cysteine residue alignment and partial sequence homology, DA10-12 may be considered to be a new member of the *Ascaris* inhibitor family.

3.3 Expression and purification of DA10-12

3.3.1 Methods

3.3.1.1 Production and purification of recombinant DA10-12

A sequence encoding a Tobacco Etch Virus (TEV) protease cleavage site was added to the 5′ end of the DA10-12 cDNA, using standard PCR techniques. This fragment was cloned into two Gateway™ (Invitrogen) destination vectors to produce plasmids for expression of DA10-12 as either a GST fusion (pGST-DA10-12) or a 6xHis fusion (pHis-DA10-12) protein. The two DA10-12 expression vectors were transformed into *E. coli* strain, BL21-AI™ and the bacteria were tested for DA10-12 production.

Purification of recombinant DA10-12 was performed using only the soluble fraction of cell lysate from *E. coli* BL21-AI expressing GST-DA10-12. GST-DA10-12 was purified from the cell lysate by passing through a GSTrap affinity chromatography column (Pharmacia Biotech). Removal of the GST-tag from the purified fusion protein was achieved using TEV protease, which cleaved the recognition sequence included between the tag and DA10-12. The cleavage reaction was boiled to precipitate any residual fusion protein, the cleaved GST tag and the TEV protease, while recombinant DA10-12 remained in solution. Assessment of native folding of the DA10-12 portion of the GST fusion was achieved by assaying the protein against *D. albohirtum* trypsin-like midgut enzymes.

3.3.1.2 Purification of native DA10-12

Purification of DA10-12 from boiled *D. albohirtum* haemolymph utilised gel filtration chromatography followed by ion-exchange chromatography through a HiTrap™ SP HP column (Amersham Biosciences) attached to a Shimadzu Class-VP HPLC System. The inhibitory activities of any peaks collected from the ion exchange column were measured against *D. albohirtum* midgut trypsin-like and chymotrypsin-like enzymes, as well as trypsin and chymotrypsin.

3.3.2 Results

##### 3.3.2.1 Production and purification of recombinant DA10-12

A high level of expression was achieved when DA10-12 was produced as a fusion protein in *E. coli* BL21-AI, but it was largely (GST-fusion) or wholly (6xHis-fusion) produced in the insoluble form. Inhibition assays showed that the soluble GST-DA10-12 and recombinant DA10-12 had no detectable activity against *D. albohirtum* trypsin-like midgut enzymes.

3.3.2.2 Purification of native DA10-12

DA10-12 purified by ion exchange chromatography, failed to inhibit the activity of trypsin-like and chymotrypsin-like midgut enzymes from *D. albohirtum*, as well as the activity of chymotrypsin and trypsin (Fig. 6). Interestingly, fractions collected from the

flow through fraction of the column reduced the activity of trypsin-like and chymotrypsin-like *D. albohirtum* midgut enzymes and chymotrypsin, whilst the activity of trypsin remained unaffected.
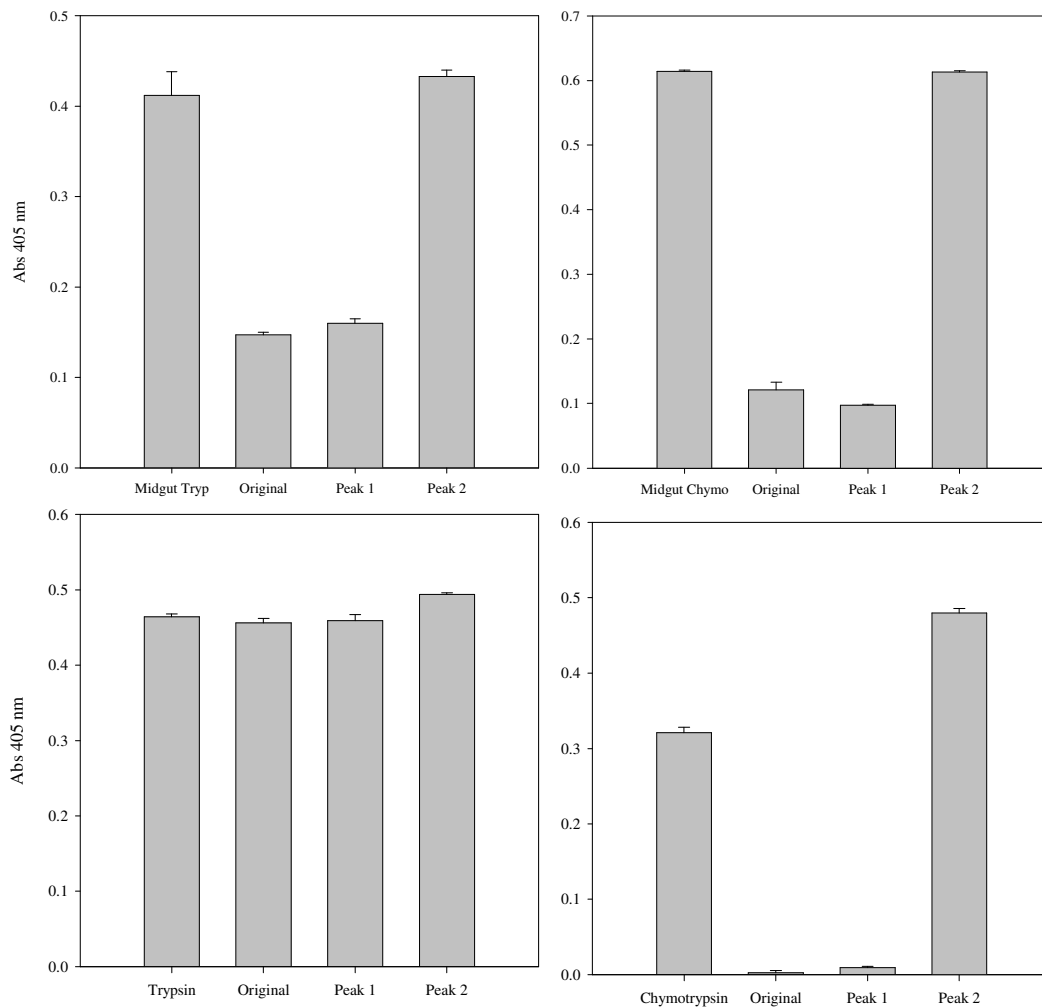
Figure 6 **Inhibitory activity of proteins purified using ion exchange chromatography. The proteolytic activity of each enzyme was measured and compared with the enzyme activity remaining after mixing with the partially purified protein (Original) prior to ion exchange chromatography, the flow through fraction from the column (Peak 1) and the purified DA10-12 (Peak 2). Enzymes: Midgut Tryp/Chymo – trypsin/chymotrypsin-like enzymes from *D. albohirtum* larval midgut; Trypsin and Chymotrypsin. Error bars indicate standard error of the mean.**

3.3.3   Conclusions

DA10-12 was purified from both a heterologous and native source and some biochemical properties were clarified. Purification of DA10-12 from the native source proved more fruitful than from the heterologous system, but a number of modifications to the bacterial system have been identified and application to the current system may improve yields substantially.

The failure of DA10-12, derived from either system, to demonstrate inhibitory activity may be due to a unique proline residue at P_1' which could cause steric and/or electrostatic interferences in the interaction of the reactive centre loop with proteases.

Proteins identified in the unbound fraction during purification of DA10-12 by ion exchange chromatography were found to inhibit not only the main digestive fluid proteases of *D. albohirtum*, but also chymotrypsin. However, whether a single inhibitor or multiple inhibitors are responsible for the inhibition of the three proteases is not clear.

3.4   Expression of DA10-12 in sugarcane

3.4.1   Methods

DA10-12 was introduced into sugarcane callus by microprojectile bombardment, along with an antibiotic resistance selectable marker plasmid. The presence of the DA10-12 gene in transgenic sugarcane lines was determined by PCR analysis. The level of DA10-12 protein in leaves harvested from regenerated plants was evaluated by western analysis using the antiserum described in section 3.3.2.3. Total RNA extracted from five DA10-12 PCR positive plants, and a 'no DNA' control plant, was also analysed by RT-PCR to evaluate transcription of the introduced DA10-12 DNA.

3.4.2   Results

More than 100 transgenic plants were recovered from three independent bombardment events after antibiotic selection, and 34 lines were randomly selected for biochemical characterisation. 16 plants from the 34 antibiotic-resistant lines tested were found to be PCR positive for the DA10-12 gene, giving a 47% success rate for co-transformation (Fig. 7).

The results of the western analysis (Fig. 8) indicate that either the DA10-12 protein is not being expressed, or the amount of DA10-12 was below the detection limit of the immunoblot.

An RT-PCR product of the appropriate size for the correctly processed DA10-12 transcript was detected in all but one of the tested lines (Fig. 9).

3.4.3   Conclusions

The DA10-12 cDNA originally isolated from *Dermolepida albohirtum* (greyback canegrub) fat bodies can not be expressed in transgenic sugarcane in the mature form.

The detection of mRNA, in at least four plants, suggests the most likely reasons for the absence of DA10-12 protein to be post-translational gene silencing, inefficient translation or inappropriate intracellular targeting leading to proteolytic degradation. The exact cause of the absence of DA10-12 protein remains to be determined.
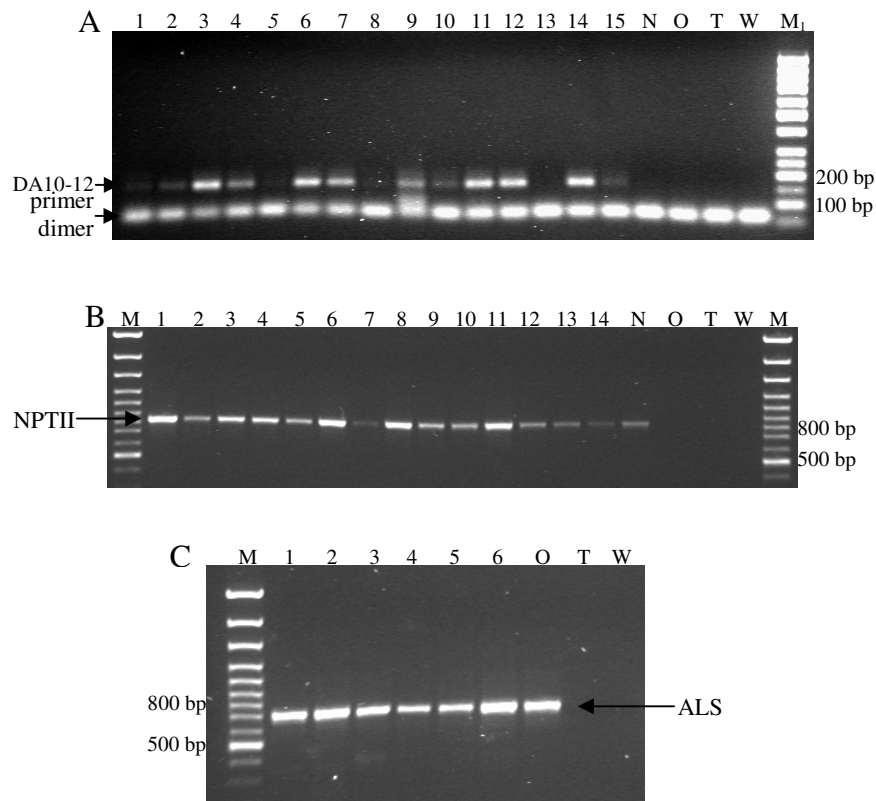
Figure 7   Examples of PCR analysis of DNA extracts from transformed and non-transformed sugarcane plants. Regenerated plantlets (lanes 1-15) were screened for the presence of both the DA10-12 transgene (A) and NPTII selectable marker gene (B). Screening with ALS primers (C) was performed as an amplification control. Plants transformed with only the selectable marker gene (N) and a 'no DNA' control plant (O) were included in the analysis as controls, along with an aliquot of the TPS (T) and water used as a diluent (W). The expected product sizes were 163 bp, 710 bp and 820 bp for DA10-12, NPTII and ALS respectively. DNA molecular weight markers: GeneRuler 100 bp DNA ladder plus (M) and GeneRuler 50 bp DNA ladder (M$_1$) (Fermentas).
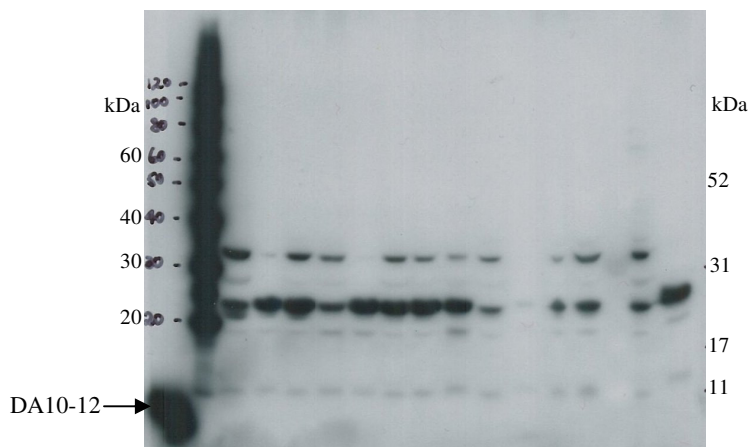
Figure 8   Western blot analysis of protein extracts from transformed and non-transformed sugarcane plants.   Total protein extracts from regenerated plantlets (lanes 1-13) were assayed for the expression of DA10-12 protein by western analysis.   Protein extracted from a 'no DNA' control plant was included on each blot (O) and semi-purified DA10-12 protein (D) was also present as a positive control. (M_1) NOVEX MagicMark markers; (M_2) NOVEX MultiMark markers.
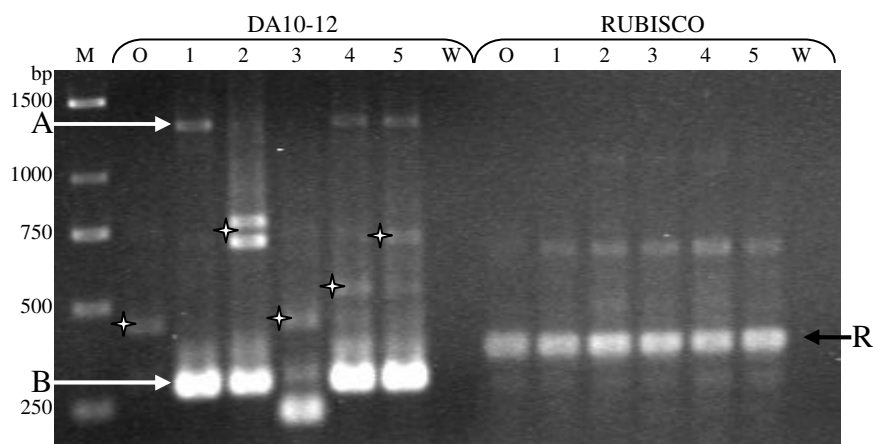
Figure 9   RT-PCR analysis of transformed and non-transformed sugarcane plants. Five DA10-12 PCR positive plants (1-5) were screened for the presence of DA10-12 mRNA. A 'no DNA' plant was included in the analysis as a control (O), along with an aliquot of water used as a diluent (W).   The expected size for correctly processed DA10-12 mRNA is 288 bp (arrow B), while amplification of contaminating DNA template would produce a product of 1298 bp (arrow A).   Bands marked with stars are believed to result from either non-specific amplification (e.g. lane O), or truncated/rearranged copies of the DA10-12 expression cassette. Screening with RUBISCO primers was performed as an amplification control, with an expected mRNA product at 365 bp (arrow R). GeneRuler 1 kb DNA ladder (M)(Fermentas).

4.0   OUTPUTS

- Preliminary data on inhibitors present in canegrub haemolymph that have activity against canegrub midgut enzymes.
- Methods for identification, purification, assay and cloning of potential inhibitors have been described.
- A cloned gene sequence with potential for modification into a valuable (IP-free) transgene.

5.0   EXPECTED OUTCOMES

The main benefit expected to arise from this project, was the acquisition of a novel transgene that would decrease sugar industry reliance on intellectual property from outside agencies. This outcome is still possible (discussed below), and is being pursued as part of the CRC SIIB project 1Bii: Environmentally sustainable control of canegrubs.

6.0   FUTURE NEEDS AND RECOMMENDATIONS

Although DA10-12 has homology to *Ascaris* family inhibitors, it does not inhibit the major midgut enzymes of *D. albohirtum*. The abundance of DA10-12 in the haemolymph suggests an important role for this protein, a role which could be elucidated by testing it against a wider range of proteases. Information about the tissue distribution of DA10-12, its expression at different developmental stages and possible induction of expression by invading pathogens, can now be obtained with the aid of the DA10-12 antiserum, generated during the course of this research.

The goal of this project was, however, to identify a novel transgene for insect resistance. Despite its obvious flaw, DA10-12 still has potential to fulfil this role. The conservative location of the reactive site in *Ascaris* family inhibitors suggests that the appropriate modification of amino acids within this region will transform DA10-12 into a functioning serine protease inhibitor.

(Thomas *et al.* 1995b) expanded the target range of the *Bombyx mori* anti-elastase ALAserpin, by using PCR-based oligonucleotide-directed mutagenesis to convert it to an anti-trypsin and an anti-chymotrypsin. This type of site directed mutagenesis could also be used to determine whether the P_1' proline residue is solely responsible for DA10-12's inability to inhibit trypsin and chymotrypsin.

The development of an efficient heterologous expression system will make possible the production of variant DA10-12 molecules, allowing examination of effects of amino-acid substitutions on folding, structure and function.

Production of a sufficient amount of modified DA10-12 for biochemical analysis could be achieved using the *E. coli* system described in Chapter 5 of my PhD thesis. Testing of a protein for efficacy against canegrubs *in vivo*, however, requires the purification of larger

amounts of protein than the current *E. coli* system could provide in soluble form. A number of options for improvements to the yield of soluble DA10-12 expression in *E. coli* are discussed in chapter 5, including alternative fusion proteins, periplasmic targeting and codon optimisation. Additionally, purification and refolding of recombinant protein from inclusion bodies remains to be tried, along with alternative host systems (e.g. yeast, mammalian or insect cells), or even the new Roche 'cell free' Rapid Translation System.

Expression of DA10-12 modified or not, remains to be seen in sugarcane cells. The main reasons for the absence of transprotein in transgenic sugarcane, discussed in detail in chapter 6 of my PhD thesis, are thought to be the lack of a signal peptide, leading to proteolytic degradation, or incompatible codon usage, resulting in inefficient translation. Once it has been established whether the absence of the signal peptide is the cause of non-expression, or whether it is a problem with translation, expression levels could be improved further by targeting the transprotein for retention in various subcellular locations, such as the endoplasmic reticulum, chloroplast or vacuole.

Although DA10-12 did not inhibit proteases tested in this study, it is produced at a level in *D. albohirtum* larval haemolymph, which suggests an important physiological role. The production of antiserum to DA10-12 allows further information about tissue distribution of DA10-12 and expression to be obtained. This information may offer some insight into the role of DA10-12 in *D. albohirtum* larvae.

Identification of an effective inhibitor isolated from canegrub haemolymph, which does not require modification, is still possible. A number of potential inhibitors were detected during the course of this research. The experience with DA10-12 highlights the need for a review of the inhibitor discovery method. Further purification and biochemical characterisation of candidate proteins from *D. albohirtum* haemolymph (described in chapters 4 and 5 of my PhD thesis) should be undertaken before any attempts to clone the cDNA are made. Reverse zymography results, described in chapter 2 of my PhD thesis, show several proteins in the haemolymph of the other three canegrub species surveyed, that may be worth pursuing.

7.0   PUBLICATIONS ARISING FROM THE PROJECT

Much of the material presented in my PhD thesis has been used to produce several draft manuscripts; these will be submitted for publication following acceptance of the thesis.

The results described in chapters 3 and 4 of the thesis were presented at the ISSCT Entomology Workshop, Montego Bay, Jamaica (2003), and also as an invited presentation at the Louisiana State University, Baton Rouge in the same year.

8.0   ACKNOWLEDGMENTS

I thank my PhD supervisors Peter Allsopp, Terry Walsh and Grant Smith, for their patience, encouragement and wisdom. Thank you to the BSES entomology team, Peter

9.0   REFERENCES

Babin, D. R., Peanasky, R. J. and Goos, S. M. (1984). The isoinhibitors of chymotrypsin/elastase from *Ascaris lumbricoides*: the primary structure. *Archives of Biochemistry and Biophysics* 232: 143-161.

Hejgaard, J. (2001). Inhibitory serpins from rye grain with glutamine as P_1 and P_2 residues in the reactive center. *FEBS Letters* 488: 149-153.

Irving, J. A., Pike, R. N., Lesk, A. M. and Whisstock, J. C. (2000). Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Research* 10: 1845-1864.

Laskowski, M., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., Schatzley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A. and Wieczorek, M. (1987). Ovomucoid third domains from 100 avian species: isolation, sequences, and hypervariability of enzyme-inhibitor contact residues. *Biochemistry* 26: 202-221.

McGhie, T. K., Christeller, J. T., Ford, R. and Allsopp, P. G. (1995). Characterization 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.

Thomas, J. C., Adams, D. G., Keppenne, V. D., Wasmann, C. C., Brown, J. K., Kanost, M. R. and Bohnert, H. J. (1995a). *Manduca sexta* encoded protease inhibitors expressed in *Nicotiana tabacum* provide protection against insects. *Plant Physiology and Biochemistry* 33: 611-614.

Thomas, J. C., Adams, D. G., Keppenne, V. D., Wasmann, C. C., Brown, J. K., Kanost, M. R. and Bohnert, H. J. (1995b). Protease inhibitors of *Manduca sexta* expressed in transgenic cotton. *Plant Cell Reports* 14: 758-762.

Thomas, J. C., Wasmann, C. C., Echt, C., Dunn, R. L., Bohnert, H. J. and McCoy, T. J. (1994). Introduction and expression of an insect proteinase inhibitor in alfalfa (*Medicago sativa* L.). *Plant Cell Reports* 14: 31-36.

APPENDIX 1 – Draft PhD Thesis

Electronic version only