TRANSCRIPTIONAL AND TRANSLATIONAL EXPRESSION OF A WILD TYPE BACTERIAL TOXIN GENE SEQUENCE IN TRANSGENIC SUGARCANE

TN CASSIM[1], BB MONOSI[1], GM MEYER[1], FC BOTHA[1,2], RS RUTHERFORD[1] AND BI HUCKETT[1,2]

[1] *South African Sugar Association Experiment Station, Private Bag X02, Mount Edgecombe, 4300, South Africa*
[2] *Institute for Plant Biotechnology, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa*

Abstract

Genes from bacterial sources often confer characteristics that are seen as highly desirable for introduction into crops by genetic engineering. However, bacterial gene sequences are not always compatible with the molecular mechanisms operating in plants. In this work, expression of a truncated but otherwise unmodified bacterial toxin gene was examined in transgenic sugarcane clones. Molecular analysis of specific RNA transcripts and protein products, by Northern and Western blotting respectively, showed that RNA transcripts were characterised by significant premature polyadenylation triggered at specific points in the gene sequence, while protein levels were undetectable.

Introduction

In the genetic engineering of higher plants, the chosen transgene of interest is often bacterial in origin. Bacteria have evolved a diverse array of metabolic pathways and products not found in eukaryotic organisms, and the genes encoding those characteristics have the potential to add novelty to plant phenotypes. In addition, bacterial characters of interest are often single gene traits encoded by simple genes, suitable for cloning into small DNA vectors for delivery to the plant and subsequent integration into the plant genome. Examples of bacterial genes used widely in plant transformation are those encoding enzymes that effect herbicide resistance and those producing insecticidal proteins such as the endotoxins from strains of *Bacillus thuringiensis* (*B.t.*). A truncated native *B.t.* gene from *Bacillus thuringiensis* strain 234, isolated at Mount Edgecombe (Herrera *et al.*, 1994), has been used to produce a number of sugarcane transformants of varieties NCo310 and NCo376. The *B.t.* 234 toxin, of the CryIA(c) type, is particularly effective against the sugarcane stalk borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae). A subset of NCo310 plants confirmed as transgenic for the *B.t.* 234 gene has been the subject of gene expression studies at transcriptional (mRNA) and translational (protein) levels. Results of these expression analyses are reported here.

Materials and Methods

Sugarcane material

Various individual *B.t.* transformant plants and non-transgenic NCo310 were micropropagated to produce a number of clones of each type. These were maintained in the containment glasshouse at the South African Sugar Association Experiment Station (SASEX) at Mount Edgecombe using conventional pot fertilisation and automated watering regimes. Experimental material consisted of plants ranging from 500 mm to 2.5 m in height.

RNA extraction, RT-PCR and Northern analysis

Leaf tissue was the source of all RNA extracts used in this study. Third youngest leaves were randomly sampled from three NCo310 control plants and from three individual plants within each transformant line at each daily time point. Sampling was done at the same time each day (between 11h00 and 12 noon) and the same portion of the leaf removed in each case. Samples were pooled for each line, immediately frozen in liquid nitrogen and stored at -80°C. For reverse transcription-polymerase chain reactions (RT-PCR), DNA-free RNA was extracted using the SV Total RNA Isolation System (Promega), while for Northern analysis the RNeasy Extraction kit (Qiagen) was used to prepare larger amounts of total RNA. In each RT-PCR reaction, 1 µg RNA was used in a final volume of 50 µl in a single step procedure (Titan System, Boehringer Mannheim). RT-PCR products were analysed by electrophoresis in agarose gels (1%, w/v) and visualised by conventional ethidium bromide staining. RNA for Northern analysis (15 µg per sample) was fractionated through agarose (1,2%, w/v) in the presence of formaldehyde, and the resultant profiles checked by ethidium bromide staining for equality of loading and presence of undegraded ribosomal RNA bands before transfer to positively charged nylon membrane (Amersham) using the downward capillary blotting method of Chomczynski and Mackey (1994). Probe DNA was generated by PCR amplification from plasmid vector of a 1 850 bp fragment of the *B.t.* 234 gene using specific primers. *B.t.* gene amplification products were purified by agarose gel electrophoresis followed by excision of bands and column extraction (QIAquick, Qiagen). Isotopic labelling was by random

primed deoxynucleoside triphosphate incorporation (Megaprime, Amersham) using ^{32}P-dATP (Amersham) as signal component. Hybridization of probe to membrane and subsequent washes were at 65°C. Visualization of signal was by autoradiography using x-ray film (Hyperfilm-MP, Amersham).

Protein extraction and Western analysis

Samples (2 g) of plant material (leaf roll) were frozen in liquid nitrogen and ground in 38 ml of cold extraction buffer (50 mM K_2CO_3, pH 9,5, 100 mM KCl, 0,05% (w/v) Triton X100, 0,05% (w/v) Tween 20, 1 mM phenyl methyl sulphonyl fluoride and 1 mM iodoacetamide). Following centrifugation, the soluble protein concentration in each sample was estimated using the method of Bradford (1976) (BioRad) with bovine serum albumin (BSA) as the protein standard. Fifty µg of soluble protein per sample was loaded on to each lane and subjected to electrophoresis at 200 V through a 15% (w/v) SDS-PAGE gel (Schagger and von Jagow, 1987). Electroblotting to Hybond™-P membrane (Amersham) was performed in transfer buffer consisting of 25 mM Tris base, 192 mM glycine, and 20% (v/v) methanol (Towbin *et al.*, 1979). Following transfer, the membranes were blocked with SuperBlock™ (Pierce) overnight and then probed for 2h with rabbit polyclonal CryIA(c) antiserum at a 1:4000 dilution in 20ml TBST (50mM Tris-HCl, pH 7,5, 200 mM NaCl, 0,05% (w/v) Tween-20) containing 0,6 g BSA. The filters were then washed for 5 minutes, 3 times, in TBST and then probed with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) at a 1:1000 dilution in TBST for 30 min. The membranes were developed following three 5 minute washes in TBST by addition of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (Boehringer-Mannheim) in alkaline phosphatase substrate buffer (100 mM Tris-HCl, pH 9,5, 100 mM NaCl, 50 mM $MgCl_2$). The reaction was stopped by washing the membrane in 100 mM EDTA.

Results and Discussion

Preliminary evidence for the production in the leaves, via transcription, of *B.t.* transgene mRNA was provided by RT-PCR analyses. Results (not shown) demonstrated that all transformants tested in this study were *B.t.* RNA positive, as

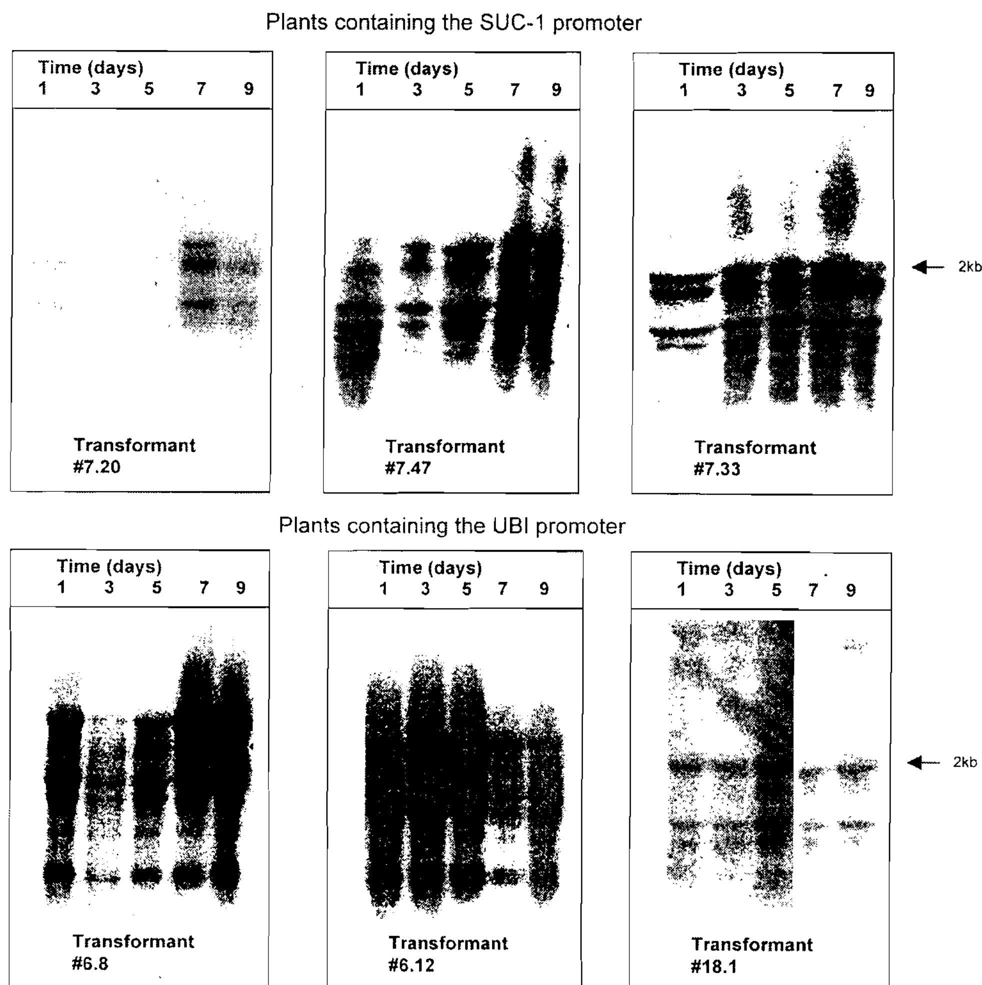

Figure 1. *B.t.* 234 gene transcripts in leaves of six different sugarcane transformants. Plant material was sampled for RNA extraction over a period of 9 days of normal glasshouse growth. Transcripts were characterised by Northern analysis using 15µg total RNA per sample (lane). The specific activity of the *B.t.* probe used was 1.9 x 10^9 cpm µg DNA^{-1}. Autoradiographic film was exposed to blots for 4 days at -80°C.

REFERENCES

Bradford, MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principles of protein-dye binding. *Analyt Biochem* 72: 248-254.

Chomczynski, P and Mackey, K (1994). One-hour downward capillary blotting of RNA at neutral pH. *Analyt Biochem* 221: 303-305.

De Rocher, EJ, Vargo-Gogola, TC, Diehn, SH and Green, PJ (1998). Direct evidence for rapid degradation of *Bacillus thuringiensis* toxin mRNA as a cause of poor expression in plants. *Plant Physiol* 117: 1445-1461

Diehn, SH, Chiu, W-L, De Rocher, EJ and Green, PJ (1998). Premature polyadenylation at multiple sites within a *Bacillus thuringiensis* toxin gene-coding region. *Plant Physiol* 117: 1433-1443.

Herrera, G, Snyman, SJ and Thomson, JA (1994). Construction of a bioinsecticidal strain of *Pseudomonas fluorescens* active against the sugarcane borer, *Eldana saccharina*. *Appl and Envtl Microbiol* 60: 682-690.

Höfte, H and Whiteley, HR (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Rev* 53: 242-255.

Perlak, FJ, Deaton, RW, Armstrong, TA, Fuchs, RL, Sims, SR, Greenplate, JT and Fischhoff, DA (1990). Insect resistant cotton plants. *BioTechnology* 8: 939-943.

Perlak, FJ, Fuchs, RL, Dean, DA, McPherson, SL and Fischhoff, DA (1991). Modification of the coding sequence enhances plant expression of insect control protein genes. *Proc Natl Acad Sci USA* 88: 3324-3328.

Schagger, H and von Jagow, G (1987). Tricine-SDS PAGE for the separation of proteins. *Analyt Biochem* 166: 368-379.

Towbin, H, Staehelin, T and Gordon, J (1979). Electrophoretic transfer of proteins from polyacryalmide gels. *Proc Natl Acad Sci USA*. 76: 4350-4354.

Weising, K, Schell, J and Kahl, G (1988). Foreign genes in plants: transfer, structure, expression, and applications. *Ann Rev Genet* 22: 421-477.

Table 1. Summarised results of plant and ratoon crops of self-trashing trials 1 (La Mercy) and 2 (Mount Edgecombe). Mean values are overall percentages of the trial mean (=100%) calculated from four variables measuring stalk damage and borer performance.

Treatment means & statistics	Trial 1 - Plant	Trial 1 - Ratoon	Trial 2 - Plant	Trial 2 - Ratoon
Means:				
89E0033	193	125	-	-
89E0042	287	152	-	-
89E0233	89	88	-	-
89E0259	71	74	-	-
89E0509	99	141	-	-
89E0795	83	67	-	-
89E1253	92	126	-	-
90W0074	-	-	57	106
90W1053	-	-	102	80
90W1207	-	-	40	40
90W1147	-	-	141	296
90E1297	-	-	7	20
Selections mean	131	116	110	108
N17	33	69	104	82
N17 Loose trash	-	-	46	56
N22	83	133	210	77
N22 Tight trash	-	-	165	183
NCo376	73	81	153	85
N11	131	129	187	115
N11 Tight trash	114	154	194	182
N11 Loose trash	199	155	114	150
N21	22	33	30	37
N21 Tight trash	30	39	30	56
N21 Loose trash	2	34	20	36
Tight trash effect	72	96	130	140
Loose trash effect	101	94	60	81
LSD (p=0,05)	78,6	44,4	69,9	69,8
CV	68,0	38,4	60,6	60,5
F values (* significant at p=0,05):				
Selections	8,32*	4,65*	4,50*	19,87*
Control varieties	2,44	7,30*	8,44*	1,26
Tight trash effect	0,03	1,36	0,38	10,06*
Loose trash effect	0,77	1,02	5,44*	0,02
Variety x T/T	0,20	0,36	0,64	1,56
Variety x L/T	2,53	0,65	0,88	0,76

for *E. saccharina* numbers and weight, where edge effects were pronounced in spite of the presence of guard rows on all sides. Normally, the lattice square trial design controls for variation in both rows and columns (Murdoch[1], personal communication).

Acknowledgements

The authors thank Moses Ramsamy and Andrew Govender for their invaluable assistance in the field. Murt Murdoch is thanked for his analysis of the results and useful discussions of the work. Des Conlong provided helpful comments on the manuscript. The Entomology Department Insect Unit at the SASA Experiment Station supplied the moths used in this study.

1 M. Murdoch, Head Biometry Dept, SASEX, Mt Edgecombe

REFERENCES

Carnegie, AJM and Smaill, RJ (1982). Pre-trashing of sugarcane as a means of combating the borer *Eldana saccharina* Walker. *Proc S Afr Sug Technol Ass* 56: 78-81.

Cock, JH, Amaya, A, Bohorquez, C and Munchmeyer, B (1997). Simulation of production potential of self-defoliating sugarcane cultivars. *Field Crops Res* 54: 1-8.

David, H and Joseph, KJ (1982). Role of rind hardness and leaf sheath tightness in sugarcane in varietal resistance to internode borer, *Chilo sacchariphagus indicus* (Kapur.). *Proc Sug Technol Ass India* 46: 7-15.

Leslie, GW (1989). Effects of pre-trashing sugarcane on oviposition, and on predation of eggs and moths of *Eldana saccharina* (Lepidoptera: Pyralidae). *Proc S Afr Sug Technol Ass* 63: 192-196.

Leslie, GW (1990). The influence of dead leaf material on the oviposition behaviour of *Eldana saccharina* (Lepidoptera: Pyralidae) in sugarcane. *Proc S Afr Sug Technol Ass* 64: 100-102.

Way, MJ (1995). Developmental biology of the immature stages of *Eldana saccharina* Walker (Lepidoptera: Pyralidae). *Proc S Afr Sug Technol Ass* 69: 83-86.

Table 1. Trial means and degree of genetic determination for variables measured in *Eldana saccharina* field Trial 1 (La Mercy) and Trial 2 (Mount Edgecombe).

Trial & crop	Borer numbers per plot (CV%)	Per cent stalks damaged (CV%)	Per cent internodes damaged (CV%)	Degree of Genetic Determination (DGD)*			
				Length damaged	Internodes damaged	Borer numbers	Borer mass
Trial 1:							
Plant	6,5 (64,3)	53,6 (21,9)	10,8 (30,2)	0,62	0,64	0,51	0,52
R1	12,2 (51,1)	78,0 (12,7)	17,8 (30,5)	0,89	0,86	0,64	0,56
R2	78,8 (45,5)	85,0 (10,4)	22,0 (28,6)	0,82	0,82	0,75	0,71
R3	6,6 (91,3)	45,1 (25,7)	5,8 (39,5)	-	0,71	0,56	-
Trial 2:							
Plant	7,4 (79,9)	26,0 (42,6)	2,8 (60,9)	0,83	0,88	0,85	0,82

*DGD (selections only) = (F-1)/F. The closer the DGD value is to 1,0, the higher the discrimination of heritable varietal differences.

thanked for his analysis of the results and useful discussions of the work. Graeme Leslie provided helpful comments on the manuscript. The Entomology Department Insect Unit at the SASA Experiment Station supplied the eggs for trial inoculation.

References

Nuss, KJ (1991). Screening sugarcane varieties for resistance to *Eldana* borer. *Proc S Afr Sug Technol Ass* 65: 92-95.

Nuss, KJ and Atkinson, PR (1983). Methods used to measure the susceptibility of sugarcane varieties to attack by *Eldana saccharina* Walker. *Proc S Afr Sug Technol Ass* 57: 1-3.

Sosa, O (1988). Pubescence in sugarcane as a plant resistance character affecting oviposition and mobility by the sugarcane borer (Lepidoptera: Pyralidae). *J Econ Entomol* 81 : 663-667.

Sosa, O (1990). Oviposition preference by the sugarcane borer (Lepidoptera: Pyralidae). *J Econ Entomol* 83 : 866-868.

White, WH and Legendre, BL (1991). Response of sugar cane germplasm clones to the sugar cane borer. *Sugar y Azucar* 87: 28-29.